Sexual reproduction in seed plants, ferns and mosses

Proceedings of the 8th international symposium on sexual reproduction in seed plants, ferns and mosses 20-24 August 1984, Wageningen, the Netherlands

M.T.M. Willemse and J.L. van Went (compilers)

Pudoc Wageningen 1985

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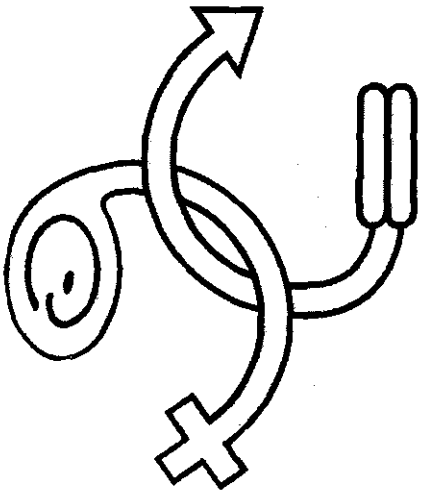

THE 8TH INTERNATIONAL SYMPOSIUM ON SEXUAL REPRODUCTION IN SEED PLANTS, FERNS AND MOSSES

The proceedings

These proceedings of the 8th International Symposium on Sexual reproduction in Seed Plants, Ferns and Mosses links up the previous seven editions.

In 1968 Prof.Dr. M. Favre Duchartre took the initiative to organize a symposium in Paris entitled: "Colloque sur les aspects cytologiques de la reproduction sexuele des plantes ovulées".

This was the beginning of a long row of scientific symposia: Reims 1970, Siena 1972, Nijmegen 1974, Reims 1976, Lublin 1980, High Tatra 1982, each with a growing interest from all countries of the world. Also in Wageningen new colleagues were present and many young scientists.

A symposium is a place of encounter, partly ordered in a programm, but for an important part running during the day and evening. Also at this symposium there were new and renewed acquintances. Both the Rector Magnificus of the Agricultural University, Prof.Dr. C.C. Oosterlee in his openingspeech, and Dr.Ir. H.N. Hasseloo at the reception of the Ministry of Agriculture and Fisheries, mentioned the value of fundamental research on sexual reproduction especially for plant breeding and crop sciences. The location of this symposium at Wageningen has promoted these practical aspects by visiting the Uniyersity Department of Plant Breeding and the Institute of Horticultural Plant Breeding.

Compared with the preceeding symposia there was increasing interest on male sterility, incompatibilty and apomixis; the number of presentations on mosses and ferns was small. During the poster sessions the presentation of specialized often adapted methods was manifest. Structural studies combined with histochemical, physiological or genetical data were very clarifying.

The programm covered the processes of sexual reproduction from sporogenesis to seed formation, a broad but essential scope. In this large field of research nearly all participants showed progress in their special field of interest by lectures, posters, discussions and the exchange of reprints. Altogether the presentations and contacts resulted in a stimulus to continue this interesting and important field of scientific research and applied aspects, not only by individual or cooperative research, but also through the next 9th symposium in 1986 at Reims, convenior Prof. Favre-Duchartre, and in Siena in 1988.

Congress organization

The scientific program comprised three lecture sessions with 29 lectures, three poster sessions and poster discussions with 49 posters and the film "Befruchtung und frühe Entwicklung von Embryo und Endosperm beim Schneeglöckchen (Galanthus nivalis)" by D.O. Erdelska.

The lectures and posters were placed in three groups:

I. Lectures: Microsporogenesis, anther and pollen development. This session was divided in three parts. Chairmen of these parts were respectively: Dr. E.G. Williams, Dr.Ir. H.J. Wilms and Prof.Dr. J.L. van Went.

I. Posters: Microsporogenesis, pollen development, progamic phase and incompatibility. The panel for the poster discussion were composed of Dr. H.G. Dickinson, Dr. W.V. Dashek, Dr. F.M. Engels and Prof.Dr. H.F. Linskens.

2. Megasporogenesis, ovary, embryo sac development, incompatibility, and fertilization. This session was divided in four parts. Chairmen of these parts were respectively: Dr. S.D. Russell, Prof.Dr. W.A. Jensen, Prof.Dr. L. Albertini and Dr. K. Larsen.

II. Posters: megasporogenesis, embryo sac development and embryogenesis. The panel of the poster discussion ws composed of Prof.Dr. D.D. Cass, Dr. J. Bednara and Dr. J.H.N. Schel.

3. Embryo and endosperm development. Apomixis and miscellaneous subjects. This session was divided in two parts. Chairmen of these parts were Prof.Dr. E. Pacini and Prof.Dr. P.R. Bell.

III. Posters: reproduction in mosses and ferns, hybridization and applications. The panel of the poster discussion was composed of Prof.Dr. P.R. Bell, Prof.Dr. R. Hagemann and Prof.Dr. M.T.M. Willemse.

In this proceedings volume both lectures and posters are compiled in three groups. I: microsporogenesis, microgametogenesis, anther and pollen germination in vitro; II: stigma incompatibility and pollen germination in vivo; III: megasporogenesis, ovary, embryosac development, fertilization, and embryo and endosperm development.

Some contributions of colleagues, unable to come, are added.

Members of the organizing committee were: A.C. van Aelst, F.M. Engels, C.J. Keijzer, A.A.M. van Lammeren, J.H.N. Schel, J.L. van Went, M.T.M. Willemse and H.J. Wilms.

Technical assistance was given by: I.B.A. van Brakel, J. Cobben-Molenaar, J. Drijver, Ir. A.J.M. Groenenwegen, A.B. Haasdijk, G.G. van de Hoef-van Espelo, E.J.L. Hotke-Staal, P.A. van Snippenburg, E.P. van de Wetering.

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This proceedings volume of the 8th meeting of plant embryologists links up the previous editions, to begin with:

Paris 1968: Colloque sur les aspects cytologiques de la reproduction sexuée des plantes ovuies. Revue de la Cytologie et Biologie Végétale 1969, 32: 1-404.

followed by:

Reims 1970: Colloque sur les aspects cytologiques des gametogénèses femelles et mâles chez les Cormophytes. Ann. de l' Université et de l'ARERS 1971, 9: 1-237.

Siena 1972: From ovule to seed: ultrastructural and biochemical aspects. Caryologia 1973, 25: 1-314.

Nijmegen 1974: Fertilization in higher plants. H.F. Linskens, ed., North Holland Publ. Comp. 1974, p. 1-373.

Reims 1976: Cytobiologie de la reproduction sexuée des plantes ovulées. Bulletin de la Société Botanique de France 1978, 125: 1-299.

Lublin 1980: Proceedings of the VIth International Symposium Advances in plant cytoembryology. Acta Societatis botanicorum Poloniae 1981, 50: 1-356.

High Tatra 1982: Fertilization and embryogenesis in ovulated plants. O. Erdelska, H. Ciamporova, A. Lux, A. Petrova, I. Tupy, Centre of Biological and Ecological sciences. Institute of Experimental Biology and Ecology, Slovak Academy of Sciences, Bratislava, 1983: 1-384.

P A R T I

MICROSPOROGENESIS, MICROGAMETOGENESIS ANTHER AND POLLEN GERMINATION IN VITRO

★★★

Part I: Microsporogenesis, microgametogenesis, anther and pollen germination in vitro

Introduction

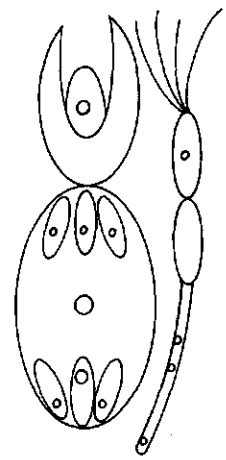
Spore and microspore development, male sterility, pollen tube formation in vitro and external influences on different developmental stages are the main topics of this part. Mainly ultrastructural and biochemical methods are used, in some cases a combination of structural and biochemical approach is offered. Some more or less new methods are described as the use of fluorochromes, immunological technics and quantitative measurement of pollen. For applications the study of male sterility and the use of hybridizing agents are important in plant breeding.

During the poster session the complex question about the genetic order and program arises related to the various developmental processes.. Till now only few complete studies of anther and pollen development, including tissue interaction are available. However, gradually progress is made by comparison, and the impression arises of a genetic order in the major steps, as induction of meiosis, isolation of the meiotic cell by a special wall, the spore formation, formation of storage products and a resting period. When such stages appear to be common steps in micro- and megasporogenesis, perhaps a common genetic base for the production of spores, pollen and seed as dispersal organs can be postulated. However, the development of spores, pollen and seed is also species-specific and strongly dependending on the surrounding tissues and environment. In such an approach a comparison between sporogenesis in mosses, ferns and higher plants is important too and can sustain a general view on spore development. In that aspect also links with animal cells should be included. Also the study of the normal process and the exact estimation of the anomalies will be very helpful to understand the study of spore development.

On her way to Holland Dr. Williams from Australia had a delay for 7 hours in a KLM airplane. This situation stimulated her to produce a number of limerics about KLM. During the symposium this poetical attitude was still present and so she shortly summarized the situation:

> The poster display it was fun.
> As from poster to poster we run
> Sixtythree, sixtyfour -
> and twentyfive more?
> But I still haven't seen number one

Therefore the proceedings will permit the reader to run on his own way in his own speed.



ULTRASTRUCTURE OF MEIOSIS IN THE MOSS ENTODON SEDUCTRIX

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Summary

The aim of this ultrastructural investigation of meiosis in the moss Entodon seductrix was to provide a comprehensive account of nuclear and plastid inheritance and to provide information on the control of division planes in plant sporogenesis. The eventual cleavage planes are predicted by aggregations of vesicles that mark the sites where, following meiosis, cell plates will fuse with the sporocyte wall. Vesicle complexes and precocious infurrowing of the cytoplasm predict the future plane of division as accurately as do preprophase bands of microtubules known from mitosis in parenchymatous plant tissues. Cytoplasmic microtubules of the prophase system are first associated with vesicles of the division site and later comprise an extensive system associated with prophasic distribution of the four plastids, one to each of the tetrad poles.

Keywords: meiosis, microtubules, moss, polarity, sporogenesis

Introduction

Previous studies on moss sporogenesis have emphasized that the marked polarity exhibited throughout sporogenesis stems from events of meiotic prophase (Brown & Lemmon, 1982a, b, c; 1983). Of particular importance is the presence of a mechanism for the quadripartitioning of the single plastid in the premeiotic sporocyte so that each haploid spore receives a single plastid in addition to a nucleus. Migration of daughter plastids to the future tetrad poles is a striking manifestation of the establishment of polarity in moss sporocytes. From our studies it is clear that the moss sporocyte approaches the process of meiosis as a single event rather than as two sequential divisions. The three polar axes necessary for the two nuclear divisions are established early in meiotic prophase.

Results and discussion

The nucleus migrates from a central position to a peripheral position in the synaptic stage and returns to a central position in pachynema. The single plastid undergoes two consecutive divisions early in meiotic prophase and the four resulting plastids move to the future telophase II poles prior to metaphase I. This pattern of regular distribution of plastids, one to each of the future telophase II poles, is associated with an elaborate system of microtubules. Typical preprophase bands of microtubules are not associated with the prediction of cleavage planes. Instead, numerous vesicles develop in the planes of cleavage furrows and there is reason to suspect that microtubules of the prophasic system are nucleated in this division site. By late prophase, chromatin decondenses and the diffuse nucleus is ensheathed by a system of microtubules that interconnects the four plastids. The cytoplasm has become deeply lobed around the tetrahedrally arranged plastids and planes of the future post-meiotic cytokinesis are clearly established as deep furrows between the lobes. It must be remembered that this cytoplasmic prepatterning anticipates the second nuclear division. In Entodon, as in some other mosses (Brown and Lemmon, 1982d) an organelle band incompletely separates the cytoplasm into two portions. Following a brief inframeiotic interphase, the second division spindles, with axes at right angles to each other, form between pairs of plastids located on either side of the organelle band. Cytokinesis along second division equatorial planes precedes septal formation in the persistant meiosis I organelle band. Cleavage along planes predetermined in early meiotic prophase results in four tetrahedrally arranged spores each with a plastid near the distal surface and a nucleus proximal to it.

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Brown, R. C. & B. E. Lemmon, 1982a. Ultrastructure of meiosis in the moss Rhynchostegium serrulatum I. Prophasic microtubules and spindle dynamics. Protoplasma 110:23-33.

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CYTOCHEMICAL AND ULTRASTRUCTURAL STUDIES ON SPOROGENESIS IN THE MOSS PHYSCOMITRIUM CYATHICARPUM MITT.

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Summary

Present investigations comprise a comprehensive cytochemical and ultrastructural study of various stages of differentiation in the moss Physcomitrium cyathicarpum Mitt., a member of Funariaceae. Presence of a special non-callosic PAS-positive sporocyte wall, monoplastidic condition of the sporocytes, indications of intense RNA and protein synthesis in the archesporium, and marked histochemical events in the cells of the spore-sac corresponding to the development of the sporogenous tissue are some of the interesting features recorded with the help of quantitative and qualitative histochemical techniques.

Electron microscopic studies have revealed monoplastidic condition as a transitory stage during sporogenesis. Also studied are some of important aspects of spore mother cell development, intersporal septum and spore wall formation, and the fine structural attributes of the spore-sac which are parallel and complimentary to the spore development.
Keywords: cytochemistry, moss, sporogenesis, ultrastructure.

Material and methods

Capsules of various ages of P. cyathicarpum were excised from the leafy plants and fixed according to conventional procedures for light and electron microscopy. Paraffin-embedded sections cut at 7 µm were stained with Pyronin Y for the localization of RNA and with MBB for total proteins. Cytophotometric observations were carried out using a Reichert Microphotometer. Measurements were made at 550 nm (for RNA) and 610 nm (for total proteins) peak transmission of the interference wedge filter. The mean absorption values were substituted in the plug-method to determine the relative amounts of chromophore per cell. Sections of 1.5 µm thickness cut from the Glut-Osmium fixed/ Durcupan embedded capsules were stained with PAS and TBO reactions, for pectins, and for callose. Ultrathin sections, stained with uranyl acetate and lead citrate were viewed and photographed in Philips EM 300 at 80 kV.

Results and Discussion

Early stages of differentiation of the sporogonium are characterized by very active RNA and protein synthesis in cells of the amphithecium and endothecium. There is a high content of these till the spore mother cell stage. The mature SMC's show a single, elongated plastid around the nucleus (monoplastidy). The SMC protoplast shows prominent cytoplasmic protrusions. The SMC wall is intensely PAS-positive and reveals toluidine blue metachromasia. The wall also stains with ruthenium red but the callose is absent. Thus, it seems that the isolation of the spore-mother cells by callose walls may not be a stringent prerequisite for normal sporogenesis (Chauhan, 1981; Lal & Chauhan, 1982). The SMC wall and the intersporal septum are a product of the protoplast itself. The protoplast reveals abundance of smooth ER and golgi along the SMC membranes.

Cytochemical and preliminary ultrastructural studies on the cells of the spore-sac suggest that these may be playing a role in making available precursors for the deposition of the perine coat on the young spores as well as metabolites for the development of the spores themselves. Fine structural attributes of cells of the spore-sac are indicative of their secretory nature, like those of the secretory tapetum in higher plants. The ornamentation on the spores seems to be exogenous, possibly of the tapetal (spore-sac) origin.

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DISTRIBUTION OF PLASTIDS AND MITOCHONDRIA DURING SPOROGENESIS IN EQUISETUM HYEMALE

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Summary

At the meiotic telophase I plastids and mitochondria of Equisetum aggregate in an equatorial layer. It persists until the telophase II, then it is reshaped and separates postmiotic cells. After formation of cellplates plastids take the position in a distal part of each spore while mitochondria are distributed randomly.

Introduction

There are conflicting data on the behaviour of cytoplasmic organoids in sporogenesis of Equisetum (Lenoir, 1934, Jungers, 1934). Using an electron microscope we followed localization of plastids and mitochondria in meiosis and in spore differentiation of Equisetum hyemale. Plastids of its meiocytes contain starch grains visible in squash preparations of sporangia after PAS-reaction.

Results and discussion

During meiosis plastids and mitochondria of Equisetum meiocytes change their localization in a regular pattern (fig. 1 a-p). In the early prophase they are randomly dispersed throughout cytoplasm. Somewhat later all plastids and mitochondria aggregate in a large group close to the nuclear envelope (fig. 2). Later in prophase they disperse again around the nucleus, but before the metaphase I they form two groups at the opposite sides of the nucleus. At metaphase and early telophase plastids and mitochondria move towards the equator of the meiocyte where they congregate in a ring. At the late telophase I all these organoids occupy the equatorial plane (fig. 3) separating the cell into two parts. This aggregation of organoids differentiates into three layers: the middle one of closely packed mitochondria and two others, mostly of plastids (fig. 4). The aggregation remains until the late telophase II, when it changes its shape and stretches between telophase nuclei. Later a cell plate is set up along the middle of the mitochondrial layer; plastids scatter in the cytoplasm, but aggregate again in the distal part of the spore. When the cell plate is completed, mitochondria are randomly distributed in the spore.

Equatorial aggregation of organoids has been observed in meiosis of a few plants - from mosses to Angiosperms (for references see Rodkiewicz et al., 1984). This type of arrangement, according to some authors, may ensure apportionment of organoids among postmeiotic cells. It may also play a different, not necessarily alternative, role. All meiocytes with organoid layers form tetrads after simultaneous cytokinesis. Therefore it seems possible that the layers substitute cell plates and, at a later stage, are related to cell plate formation.

Distal localization of plastids in young spores of Equisetum precedes the division into rhizoidal and prothalial cells.

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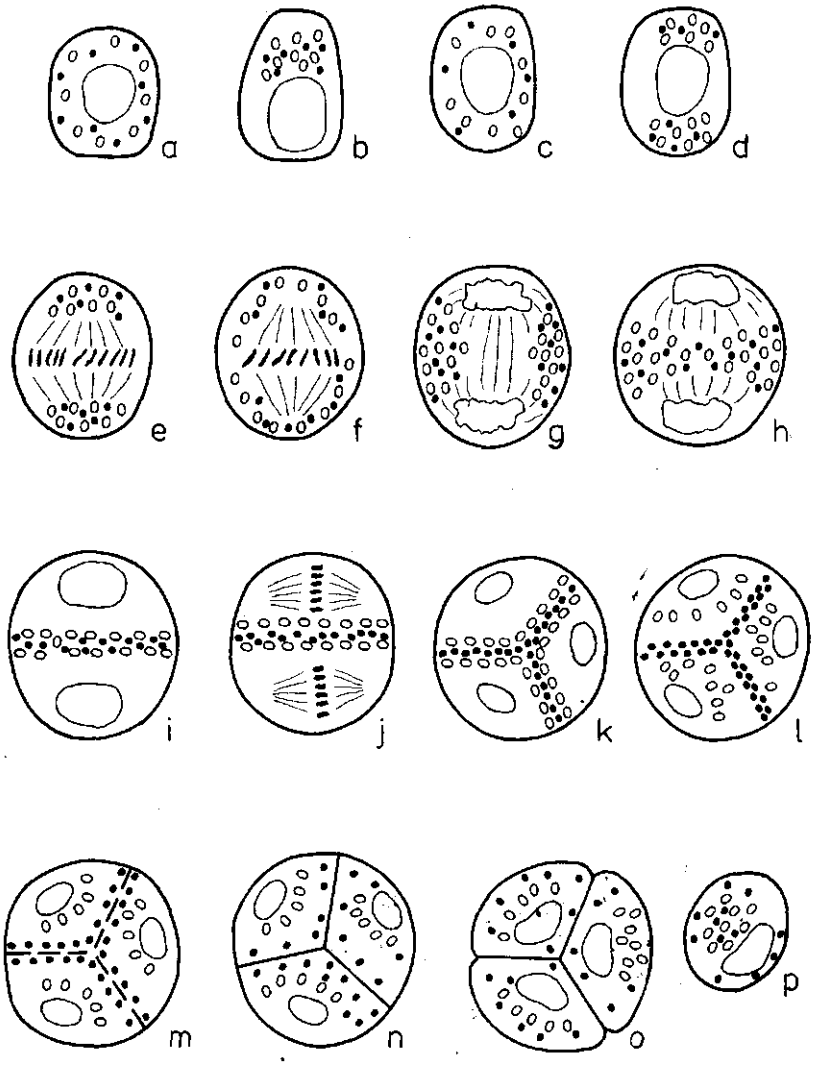
Acknowledgements: We thank I. Gielwanowska M. Sc. for her assistance.

Fig. 1. Equisetum sporogenesis

• - mitochondrion
o - plastid



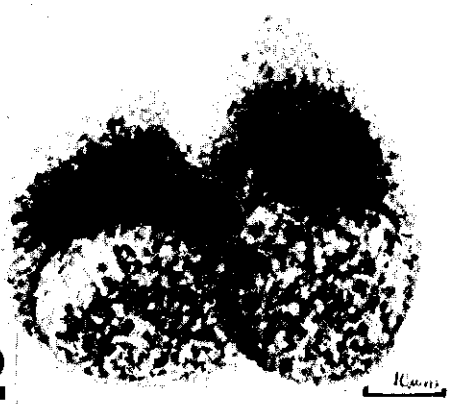


Fig. 2 Light micrograph of prophase I meiocytes after PAS reaction. Amyloplasts gathered in one dense group close to the nuclear envelope.

Fig. 3

Electron micrograph of sporocyte in telophase I stage. All plastids and mitochondria arranged in a band at the equatorial region of the cell.

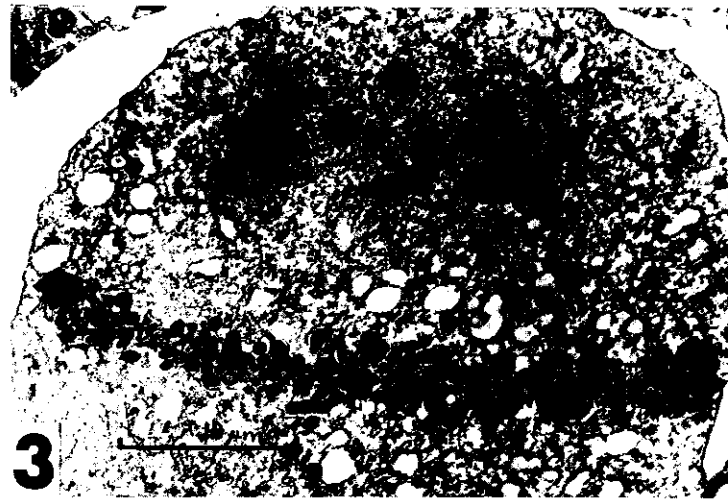
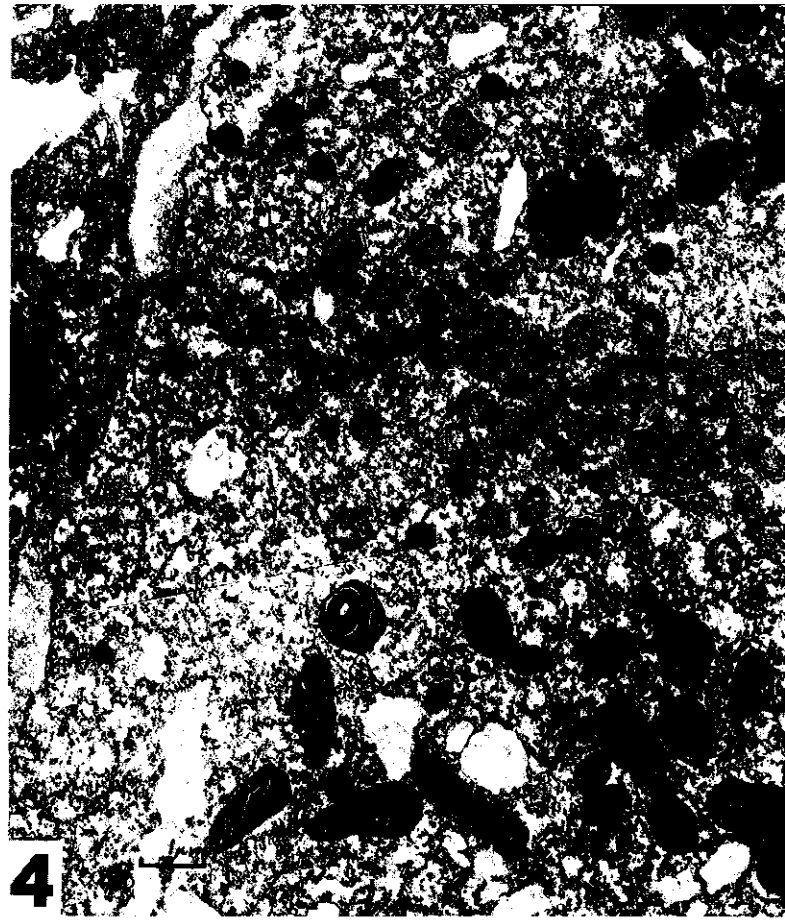


Fig. 4

Mitochondria closely packed and plastids in post telophase band.



ANTHER DEVELOPMENT OF GASTERIA

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The developing anther of Gasteria verrucosa (Mill.) Haw. was investigated by means of light and electronmicroscopy. Some aspects of the development and behaviour of living anthers in situ had been followed earlier by means of micromanipulation (Keijzer, 1983). These observations together lead to some functional relationships and can explain the development of the living anther.

The two main goals of a developing anther are the formation and the exposure of mature pollen. The young Gasteria locule consists of an epidermis, an endothecium, one middle layer, a tapetum and a multilayered sporogenous tissue. These strongly differing cell layers appear to develop on behalf of either the first or the second goal, or both. Moreover interactions between these tissues can be found besides the ones that are already known.

The epidermis and the endothecium develop somewhat comparable. Both layers are already vacuolated before meiosis and contain chloroamyloplasts. After the first pollen mitosis the endothecium cells lose most of this starch and form their characteristic wall thickenings. At the same time the cells of both layers swell by vacuolation, causing the inward bending of the locule wall, thanks to the inflexible inner tangential wall of the endothecium cells. This mechanism keeps the opened stomium closed and works opposite to the well known outward directed bending later on, at dehiscence. It prevents the loss of immature pollen into the closed flowerbud.

Shortly before the flower opens, the epidermis enlarges its cuticular surface by the formation of ridges on its outer tangential wall. This enlargement enables a very fast evaporation from epidermis and endothecium, by which the anther dehisces immediately after the flower opens.

The function of the single middle layer is more difficult to explain. During anther development its cells are stretched in tangential direction by which they are flattened. Although this phenomenon is often described as disruption, degeneration takes place only just before anther dehiscence. Next the degenerating fatty cell content mixes with the tapetal remnants, but keeps separated from the content of the bordering, swollen endothecium cells.

The tapetum and the microspores interact to form three final products: the mature pollengrains, the pollenkitt and the orbicule covered inside of the locule wall. The amount of cytoplasm in the tapetum cells increases during meiosis. The acetolysis resistant structures, being the orbicules, the tapetal membranes and the exine (on the microspores) arise gradually and synchronously. After the microspores are released from the callose wall, the tapetum starts to degenerate. The plastids lose their starch forming lipid-like drops and fuse to form a part of the pollenkitt. Next this substance fuses with the content of the ER to form the pollenkitt. Shortly before dehiscence the tapetal cells consist merely of pollenkitt and some cell remants, covered by the tapetal membranes with the orbicules.

The first visible spare substance of the future pollengrains is presumably the meiotic callose wall, as the main pollen storage product, starch, is formed immediately after the post-meiotic dissolution of this wall. After the first pollen mitosis the amount of cytoplasm in the vegetative cell increases, by which the cell expands strongly. This expansion is made possible by the stretching of the colporal region, less by the rest of the exine. Finally the swelling pollengrains press themselves into the tapetum and the middle layer remnants, by which the pollenkitt finds its way capillary into the intercellulary spaces between the pollengrains and even between the epidermis and the endothecium cells. In this way the exines stick to each other and to the orbicules and the tapetum membranes. So the pollengrains stay on the locule wall after dehiscence, waiting for a pollinator.

Reference

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ANTHER DEVELOPMENT AND POLLEN FORMATION IN CUCURBITACEAE

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Summary

Development of anther and formation of pollen grains were studied in 18 species covering 14 genera and six tribes of Cucurbitaceae. The number of anther lobes was variable and they showed cohesion to various degrees. The anther wall development is of "dicotyledons type". The mature wall compresis epidermis, fiberous endothecium, 2 ephemeral middle layers and the secretary tapetum. In Luffa cylindrica alone tapetal cells formed periplasmodicum in situ. Tapetal cells showed enlargement, division of their nuclei and fusion of resulting nuclei.

Microsporogenesis is normal and the microspores are arranged in tetrahedral and isobilateral fashion in tetrads. Usually the microspore nucleus is uninucleolate but occasionally multinucleolate condition occurred in L. cylindrica and Mukia scabrella. Enucleate cytoplasmic nodules were observed in developing vacuolated pollen grains of Cucurbita pepo, Trichosanthes bracteata and T. dioica, a feataure hitherto unknown to pollen grains.

Pollen grains are 2 celled at shedding. Usually uninucleolate but rarely multinucleolate vegetative nucleus was observed in Dactyliandra welwitschii, Edgaria darjeelingensis, L. cylindrica and Trichosanthes spp. Mature pollen grains were found to be colpate, colporate and porate and exine surface varied from reticulate to spinate.

Keywords: dicotyledons, fibrous, ephemeral, secretary, multinucleolate, cytoplasmic nodules.

Introduction

Tropical family Cucurbitaceae with 110 genera and 640 species (Willis, 1973) is characterized by many distinctive morphological features. Chakravary (1959) recorded 34 genera and 108 species from India. The embryological work on the family has been summerised by Kirkwood (1904), Johansen (1950), Davis (1966) and Singh (1970). The literature on the development of anther and pollen is scanty. Kirkwood (1907) reported, in addition to epidermis, three wall layers in anthers of Micrampelis and Cyclanthera and four in Cucumis and Fevillea. Heimlich (1927) observed four parietal layers in Cucmis sativus.

Dzevaltovsky et al. (1973) and Dzevaltovsky & Zhalalov (1976) have also reported 5-layered anther wall (including epidermis) in Cucumis anguria and Mukia scabrella, respectively.

The tapetum is secretary and the cells become multinucleate (Castetter, 1926; Passmore, 1930; Asana & Sutaria, 1932). The multinucleate nature of the tapetal cells in Cucurbitaceae has attracted the attention of many workers who tried to explain the mechanism by which this condition has been attained.

In India although a large number of investigations have appeared on the origin and development of female gametophyte (Banerjee & Das, 1937; Chakravorty, 1947; Singh, 1970) which cover a good number of plants from each tribe of the family, studies on the development and structure of anther and the male gametophyte are scanty (Asana & Sutaria, 1932; Banerjee & Das, 1937). The present study was therefore undertaken.

Materials and methods

Buds of all stages, youngest to open flower, of Momordica charantia, Citrullus colocynthis, C. lanatus, Coccinia grandis, Lagenaria siceraria, Cucurbita pepo, Luffa cylindrica, cyclanthera pedata, Sechium edule, Trichosanthes anguina, T. bracteata and T. dioica, T. lobata, Edgaria darjeelingensis, Ctenolepis garcinii, Dactyliandra welwitschii, Cucumis melo var. utilissimus and Mukia scabrella were studied. Usual microtome techniques were employed to prepare double stained slides for study.

Observations

Anther lobes in the members of Cucurbitaceae vary from 5 (Mukia scabrella) to 18 (Coccinia grandis). In Cyclanthera pedata, the whole androecium forms a circular disc with a central partition running throughout the disc.

Development and structure of anther wall

The young anther comprises one to three hypodermal male archesporial cells (Fig. 1) differentiate at each of the sites destined to form the pollen secs. The archesporial cell(s) divide periclinally forming an outer primary parietal layer and an inner sporogenous layer (Fig. 1). The parietal

cells undergo periclinal as well as anticlinaldivisions forming outer and inner secondary parietal layers (Fig. 2). The latter enlarges and differentiates to form the tapetum (Figs 2, 3, 4) whereas the former redivides periclinally (Figs 2, 3). Of the two resulting layers the outer undergoes one more periclinal division (Fig. 4). Thus the parietal layer forms in all four anther wall layers. The outermost derivative functions as the endothecium, the innermost as the tapetum and the remaining two as the middle layers (Fig. 4). The development of the anther wall conforms to the "Dicotyledonous type" of Davis (1966) and is summarised in the scheme given below:

p.p.l. → o.s.p.l. → endothecium (en)
middle layer (ml)
o.s.p.l. → tapetum (1)

The innermost wall layer forms the glandular secretary tapetum (periplasmodium in L. cylindrica) which is of dual origin. To begin with the tapetal cells are uninucleate but later on as the microspore mother cells enter meiosis, these cells enlarge radially and become usually binucleate, rarely polynucleate in M. charantia (Fig. 5).

Microsporogenesis

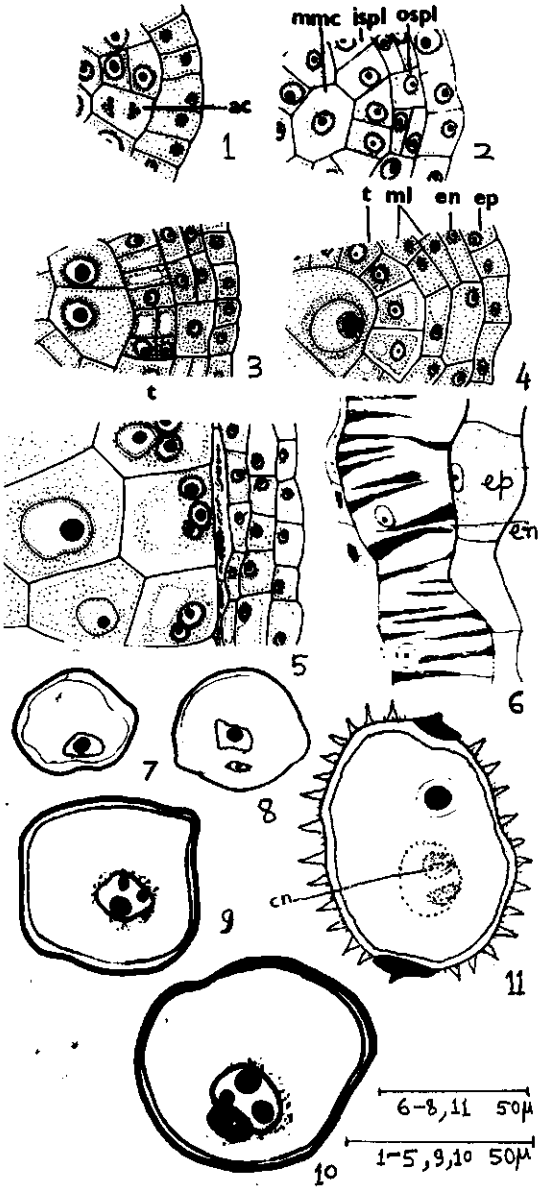
The microsporogenesis follows a common pattern. The sporogenous tissue is usually 1-layered (Fig. 3). The cells undergo a few mitotic divisions and a mucileginous wall is secreted around each cell. The microspore mother cells undergo normal meiotic division.

Microgametogenesis

The young microspore grows in size and its nucleus is usually uninucleolate (Fig. 7), rarely the multinucleolate condition is observed in L. cylindrica (Fig. 9) and M. scabrella.

An interesting phenomenon, hitherto unreported in pollen grains, is the formation of enucleate cytoplasmic nodules observed in the pollen grains of Cucurbita pepo, T. bracteata and T. dioica. The nodules are observed as small balloon-like outgrowths from the inner lining of the cytoplasm into the large central vacuole (Fig. 11).

The nucleus of the microspore divides to form a large vegetative and a small lenticular generative cell. The vegetative nucleus which is usually uninucleolate (Fig. 8) becomes multinucleolate during the maturation of pollen grains in L. cylindrica (Fig. 10) and in 4 other plants.



Figs 1-11: Figs 1-5: Momordica charantia. Fig. 1: T.s. of anther portion showing two archesporial cells; Figs 2-4: same showing formation of wall layers and microspore mother cell; Fig. 5: T.s. part of anther. Note the degeneration of the inner middle layer and 2 or 3 nucleate tapetal cells. Figs 6, 7, 8: Citrullus lanatus. Fig. 6: T.S. part of mature anther wall; Figs 7, 8: one and two celled pollen grain respectively. Figs 9, 10: Luffa cylindrica, pollen grains showing multinucleate condition in microspore nucleus and vegetative nucleus, respectively. Fig. 11: Cucurbita pepo: uninucleate pollen grain showing enucleate cytoplasmic nodules.

Discussion

The present study on 18 species of the Cucurbitaceae gives the first comprehensive account of the anther wall development and structure in the family. The development of the anther wall follows the "Dicotyledonous type" and the anther wall is of 4 layers. The inner middle layer degenerates soon after its formation. This may account for the observation of only 3 wall layers in Cylanthera and Micrampelis by Kirkwood (1907). The endothecium develops the characteristic fibrous thickenings.

Periaswamy & Swamy (1966) have reported that the tapetum is of dual origin in angiosperms. This is confiremd in the present study also. Initially the tapetal cells are uninucleate but they become binucleate in all the taxa investigated presently. The tapetum is secretary and in L. cylindrica alone it forms a periplasmodium which is absorbed while in situ. Turala (1958, 1963) has asserted that the tapetum remains uninucleate throughout in 7 members studied by her and that further differentiation is attained through endomitosis. This is in contrast to our observations which have clearly shown that the cells become usually 2-nucleate in all the taxa.

An interesting and hitherto unrecorded feature is the formation of enucleate cytoplasmic nodules in the uninucleate pollen grains in C. pepo and Trichosanthes spp. The nodules arise from the inner lining layer of the cytoplasm and buldge into the large central vacuole of the uninucleate pollen grains. Such cytoplasmic nodules have been recorded in the endosperm of many plants belonging to dicotyledons and monocotyledons (see Singh, 1964). The significance of the cytoplasmic nodules even in endosperm development remains disputed. Singh (1964) consider it an active method of endosperm growth helping in the obliteration of the central vacuole.

Another interesting feature in pollen grains is the multinucleolate condition of the microspore nucleus and vegetative nucleus in some of the investigated members.

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MICROSPORE AND POLLEN GRAIN FORMATION IN RHOEO DISCOLOR HANCE

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Introduction

The present report, ultrastructural, cytochemical and radioautographic study of microspore and pollen grain development in Rhoeo discolor (Commelinaceae) is a part of our team research devoted to the study of microsporocyte and tapetum (periplasmodial) development and the interrelation of these two tissues.

Observations

Microspore protoplasm

After the lysis of the special callosic wall surrounding the tetrad, the young microspores with a central nucleus (stage 1) are separated ; approximatively 20 % of microspores are fertile. During the maturation of microspores, the narrow zone of cytoplasm which is crushed between plasmalemma (close to the sporoderm) and vacuolar tonoplast contains an increasing density of ribosomes and polyribosomes. The mitochondria swell, the RER profiles with numerous ribosomes attached multiply and the diameter of the polysaccharidic granule increases from microspore stage 1 (1-2 µm) to premitotic stage (1,5-3,5 µm). But, the number of dictyosomes remains poor.

At the beginning of microspore maturation, its cytoplasm stains deeply with Fast Green FCF (basic proteins). During the period of maturation, there is a slow incorporation of several precursors (^{3}H-uridine and ^{3}H-orotic acid : RNA ; ^{3}H-arginine : histones ; ^{3}H-leucine : total proteins) and a low incorporation for the ^{3}H-tryptophane (non-histone proteins) into the nuclei and cytoplasm of microspores. The cytoplasmic labelling with ^{3}H-proline and ^{14}C-hydroxyproline (the amino-acids often lied with glycoproteins) is moderate in the young microspores and it decreases during further development. The ^{3}H-glucose is mainly incorporated into the insoluble polysaccharides, in relation to the amylogenesis as previously observed by TEM.

Pollen protoplasm

In the young pollen grain, the hemispheroidal wall that separating the vegetative cell (VC) and the generative cell (GC) is attached to intine and it is Thiery-positive. At the juvenile stage this fine wall contains callose, cellulose and pectic compounds but after there is a progressive loss of the pecto-callosic compounds. When the detachment of GC from intine proceeds , the VC and the GC are separated by their plasmalemma.

The nucleus of the VC is highly convoluted and there is a high density of organelles in the vegetative cytoplasm ; particularely, dictyosomes, mitochondria and lipidic globules (in the VC, the neutral lipids are strongly stained by Sudan black and acidic lipids by Nile blue). After the pre-mitotic amylolysis, amylogenesis occurs which renews the polysaccharidic granules of amyloplasts. During the early period of pollen, GC contains the same organelles as that of VC but the density is lower. At the mature stage of the two-celled pollen grains, the generative nucleus is horseshoe shaped and it occupies the major part of the GC.

A flash-labelling with ^{3}H-uridine (RNA) for isolated pollen grains marks firstly the generative and vegetative nuclei. For the long period of precursor supply, the VC is more labelled both in its cytoplasm and nucleus ; the incorporation of ^{3}H-uridine into vegetative cytoplasm can be associated with the multiplication of its RER profiles.

The vegetative cytoplasm strongly incorporates the ^{3}H-glucose, ^{3}H-acetate (neutral lipids) and ^{3}H-choline (phospholipids with choline), these activities correspond to the amylogenesis, the increase of lipidic globules and the cytomembrane multiplication, respectively.

Sporoderm

The main part of exine is built under the callosic wall. The presence of white-lines in the inner part of the protoexine indicates the role of plasmalemma in the exine development. After the breaking up of the tetrad, the exine reacts positively to the lipid reagents but only the exine of the fertile microspores incorporates the lipid precursors, particularely the ^{14}C-mevalonate (carotenoidic lipids).

Intine which is devoid of callose, is positive for PAS and Thiery tests. It contains a lot of acidic pectic compounds (whith free -COOH) in the outer layer and pecto-cellulosic compounds in the inner layer. When the thickness of intine increases, the sporoderm of the fertile microspores (from stage 3) and pollen grains incorporates the ^{3}H-myo-inositol.

POLLEN GRAIN DEVELOPMENT IN THE OLIVE (OLEA EUROPAEA L.): ULTRASTRUCTURE AND ANOMALIES.

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Summary

The ultrastructure of pollen grain development in the olive is described from archesporium differentiation to pollen shedding. A particular emphasis is given to the relationships between tapetum and meiocytes/microspores/pollen grains, and to some cytological events. Also the presence of anomalies is described from the ultrastructural point of view.
Key words: Olea europaea, pollen grain development, anther tapetum.

Introduction

The ultrastructural features of some aspects of pollen grain development in olive (Olea europaea L.), i.e. the tapetum development (Pacini & Juniper, 1979b; Pacini & Casadoro, 1981), the formation of sporophytic proteins in correspondence to the pores (Pacini & Juniper, 1979a), the sporoderm pattern in some cvs. (Pacini & Vosa, 1979), the main anomalies at the L.M. (Pacini et al., 1978), have already been described. In this paper we intend to complete the missing sections of the ultrastructural description, together with pollen anomalies.

Materials and Methods

Olive anthers were collected, fixed, embedded, cut, stained and observed as previously described (Pacini & Juniper, 1979a).

Results and Discussion

Pollen grain development is the result of an interaction between two tissues, which appear after archesporium differentiation, namely the microspore mother cells (mMC) and the tapetum. This interaction is explained in Fig. 1, which refers to the observations at the E.M. level. In this figure all the steps leading to pollen ripening are represented, starting from archesporium differentiation. This whole process occurs in about seventeen days.

Tapetum cooperates in the pollen grain development producing: 1) nutritive substances, 2) control substances (callase), 3) sporopollenin precursors and orbicles, 4) substances to be deposited on pollen grain surface e.g. tapetal prints, sporophytic proteins and pollenkitt. Tapetal protopast volume (detected by cross-sections) increases up to the early microspore stage, followed by tapetal degeneration in correspondence to pollen first haploid mitosis. Also volume and number of mitochondria and undifferentiated plastids (at last becoming elaioplasts) continuously increase up to pollen first haploid mitosis, whilst free and ER ribosomes, and ER cisterns, reach the maximum at the mid-microspore stage; then a little decrease is detectable, before degeneration occurs. As the result of their degeneration, tapetal cells are transformed in round lipidic masses, which before dehiscence will cover the grain surfaces (pollenkitt).

After archesporium differentiation, but before the first meiotic prophase, mMC are connected to one another and to tapetal cells by plasmodesmata. During leptotene plasmodesmata between mMC and tapetum disappear, whilst some connecting mMC between themselves seal and some others are transformed in cytomictic channels; these channels close during interphase.

The callosic wall starts developing around meiocytes during zygotene, and is completed at diplotene; at the mean time mMC, firstly polyhedral, become round, and the locular fluid is formed. During the first and the second meiotic prophases, some nucleoloids are formed: these are accessory nucleoli and are released in the cytoplasm during the first and the second karyokinesis (Dickinson & Heslop-Harrison, 1977). They are detectable in the microspore cytoplasm up to the mid microspore stage, and probably originate new ribosomes (Fig. 2).

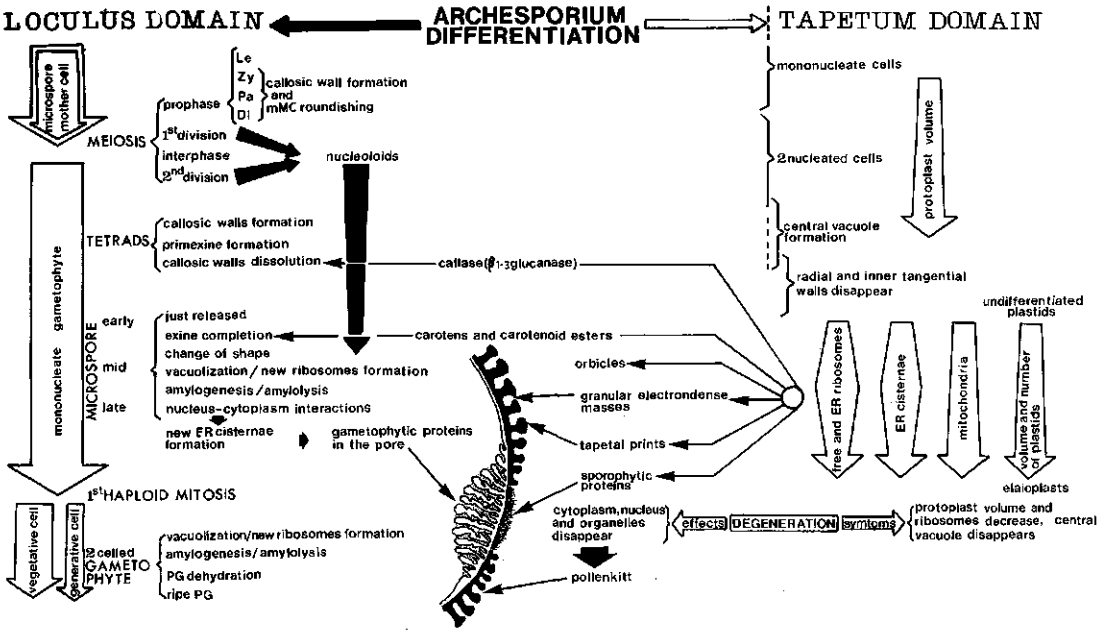

FIG. 1. Schematic representation of the main phenomena occurring in the tapetal cells and loculus during pollen grain development.

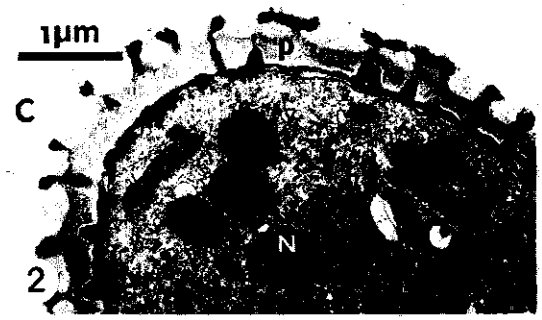

FIG.2. Microspore during callose dissolution. C: callose; P: primexine; N: nucleoloid releasing polyribosomes (arrow heads) in the adjacent cytoplasm.

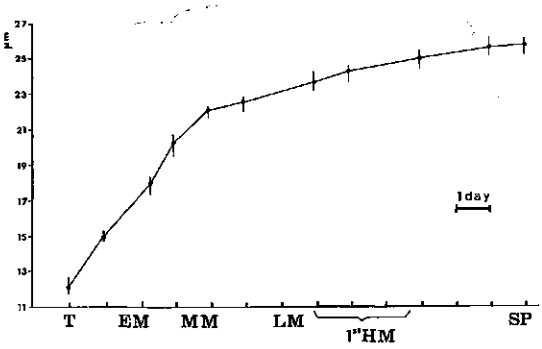

FIG. 3. Increase in the equatorial diameters of microspores and pollen grains in function of developmental stages. T: tetrads; EM: early microspores; MM: middle microspores; LM: late microspores; 1st HM: first haploid mitosis; SP: shedding pollen.

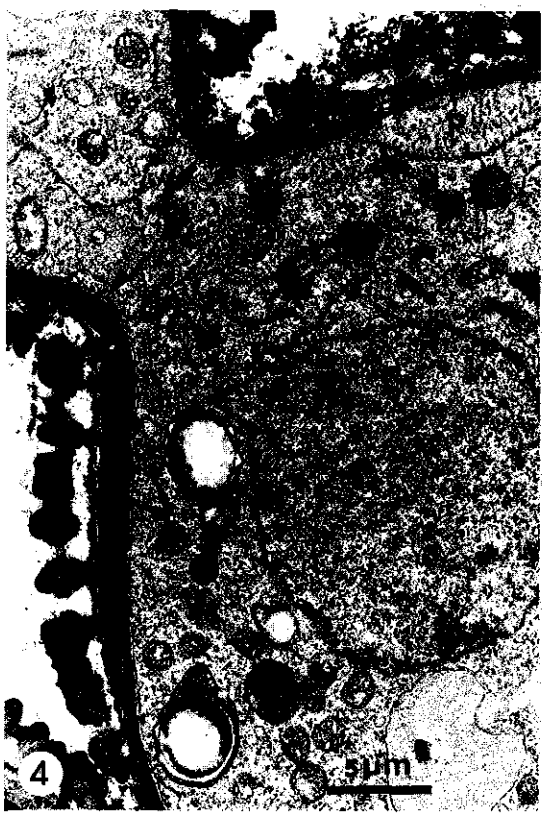

FIG. 4. Twin late microspores. Intine (arrow heads) and pores (P) are already formed.

Meiosis leads to the formation of tetrahedric tetrads and the callosic wall is formed centripetally after the second meiotic division. As soon as microspores are isolated from one another, primexine is laid down, showing all exine components, with the exception of endexine (Fig. 2). After the dissolution of the callosic wall, the free microspores rapidly change their shape and become round; a sharp increase in the equatorial diameter occurs during the early and middle microspore stages (Fig. 3), followed by a slight increase in the late microspore stage and the two celled stage: when pollen is shed, grain diameter is more or less the double than that of the single microspore inside the tetrads (Fig. 3). Olea europaea microspores do not increase very much , if compared with other species (Heslop-Harrison, 1972).

Exine is completed before the mid microspore stage, but the tapetum still produces sporopollenin precursors, up to the first haploid mitosis, when coreless orbicles start to appear. Other cytological events during the middle and the late microspore stages are: microspore vacuolization, followed by the formation of new cytoplasm and ribosomes; the first amylogenesis, with few small starch grains per plastid (these grains are hydrolized just before the first haploid mitosis); the formation of ER stacks from the nuclear membrane, which migrate towards the pores and, according to Pacini & Juniper (1979a), are probably responsible for the gametophytic proteins deposited into the tubules of poral intine.

The first haploid mitosis is asynchronous, lasting about three days, and is followed by a new vacuolization, and by a new wave of starch deposition in plastids. Starch in this second amylogenesis is more abundant than in the first one, and it is cumulated in one single big grain per plastid (often a composed grain). As reported by Pacini et al. (1978) in some cvs. this starch is hydrolized before pollen shedding, whilst in other pollen is shed starchy.

As far as morphological anomalies are concerned they have been classified (Pacini et al., 1978),in four groups:

1) macro and micro-grains;
2) pollen grains not separated but connected by a cytoplasmic bridge;
3) pollen grains with irregular pores or more than three pores;
4) pollen grains germinated inside anthers.

At the ultrastructural level, both micro and macro-grains may be empty or full of cytoplasm when shed, as they derive from meiotic anomalies. It is remarkable that sometimes macro-grains contain two generative cells. These anomalies are generally also linked to the presence of irregularly placed pores, and macro-grains pores are often four or more.

When pollen grains (two pollen grains, and sometimes four) are connected by cytoplasmic bridges (Fig. 4), they might derive either from two adjacent tetrads, where cytomictic channels between the mMC were not sealed off during the meiotic interphase, or from two microspores of the same tetrad, not completely separated after the second meiotic division (the callosic wall is incomplete and perforated - Pacini & Cresti, 1977). In these twin grains cytoplasms are continuous, and plastids behave in the same way, whilst in adjacent microspores/grains their development is slightly asynchronous in the amylogenesis/amylolysis.

Dehiscing anthers (in seven cvs. out of the forty-eight observed) often contain pre-germinated grains. The hypothetical causes and the ultrastructural features of this anomaly have already been discussed (Pacini & Franchi, 1982).

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THE EFFECT OF COLD (4°C) ON ^{3}H-PROLINE AND ^{14}C-HYDROXYPROLINE INCORPORATION IN MICROSPORES, TAPETUM AND CONNECTIVE OF RHOEO DISCOLOR HANCE. A RADIOAUTOGRAPHIC STUDY

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Summary

The effect of cold (4°C) on the level of the incorporation of ^{3}H-proline and ^{14}C-hydroxyproline into the nuclear, cytoplasmic and wall proteins of microsporocytes, tapetum and anther connective during microsporogenesis has been studied in Rhoeo discolor Hance, a plant sensitive to chilling stress. The cold treatment was applied to the excised inflorescences either during or after labelling whereas the controls were labelled at 22°C.
Keywords : cold treatment, ^{3}H-proline, ^{3}H-hydroxyproline, radioautography, microsporogenesis, Rhoeo discolor.

Introduction

Rhoeo discolor Hance, a plant which is characterized by a meiosis with a phenomenon of catenation and by a plasmodial tapetum, is sensitive to chilling stress. Recently, we have studied by radioautoradiography, the effect of cold (4°C) on protein synthesis in microsporocytes and tapetum of R. discolor by using ^{3}H-leucine (^{3}H-LEU), ^{3}H-arginine (^{3}H-ARG) and ^{3}H-tryptophane (^{3}H-TRP) (Albertini and Souvré, 1983).

It is known that pollens contain high levels of free proline and of proteins rich in proline (Zhang Hong-qi, Croes and Linskens, 1982). The glycoproteins of plant cell walls contain hydroxyproline (Lamport, 1977) produced by the hydroxylation of proline (Chrispeels, 1970). Furthermore, proline metabolism undergoes wide modifications when the plants are chilled (Dashek and Erickson, 1981).

The present paper reports the influence of cold on the ^{3}H-PRO and ^{14}C-HYP incorporation into the proteins of nucleus and cytoplasm of microsporocytes and tapetum and into the cell wall proteins of connective during microsporogenesis in R. discolor. The present data are discussed with some of the results of our previous work and of the literature.

Material and methods

The different stages of the microsporogenesis of R. discolor Hance have been previously described (Albertini, 1970). The incorporation of ^{3}H-PRO and ^{14}C-HYP into the excised inflorescences of R. discolor was monitored as previously described (Albertini, 1970). The effect of cold (4°C) on amino-acid incorporation in the microsporocytes, tapetum and connective of anther has been studied by comparing the incorporation data obtained from anther sets treated differently : 1st set : 24 h labelling at 22°C (control 1) ; 2nd set : 24 h labelling at 22°C followed by 48 h incubation without radiotracer at 4°C ; 3rd set : 24 h labellingat 22°C (^{14}C-HYP) followed by 48 h incubation without radiotracer at 22°C (control 2) or 24 h labelling at 4°C (^{3}H-PRO). The anthers fixed by ethanol-acetic acid 3/1, then embedded in paraffin, were sectionned (8 µm) and treated by Ficq's (1961) radioautographic technique (Ilford nuclear emulsion in gel form, K2 type for ^{3}H-PRO and G5 type for ^{14}C-HYP ; time of exposure in dark room: 1 month for ^{3}H-PRO and 15 days for ^{14}C-HYP). Before mounting, the sections were stained with methyl green-pyronin.

The total activity of a given cellular structure was determined as being the number of silver grains, counted over this structure. Each result in Table 1 is the average value obtained after counting the number of silver grains over 15-30 nuclei, cytoplasms or wall portions at a given stage.

Results (Tables 1 and 2)

a) 24 h labelling at 22°C (control 1)

Pollen mother cells (PMC) and microspores:

The determinations carried on control 1 corroborated earlier results obtained with ^{3}H-LEU, ^{3}H-ARG and ^{3}H-TRP (Albertini, 1970, 1975 ; Albertini and Souvré, 1983) : the incorporations of ^{3}H-PRO and ^{14}C-TYP into PMC nucleus and cytoplasm were high during the premeiotic resting stage (PMR), then decreased markedly during the meiotic prophase and remained moderate to low until the end of meiosis and through the tetrad stage and microspore stages 1-4. The nucleolar activity was very high during the PMR, then decreased markedly (40 %) at mid-synizesis before vanishing at the beginning of the zygotene stage. During PMR and synizesis, the primary wall proteins of PMC has not incorporated ^{3}H-PRO and ^{14}C-HYP but the PMC wall was labelled from zygotene/pachytene until the end of division I (during the formation of callosic special wall). The callosic wall surrounding the tetrads did not incorporate the

radiotracers but the penetration of these radiotracers into the tetrad cells occurred as there were silver grains found over the cytoplasm and nucleus. The activity of microspore sporoderm is significant from microspore stage 1 to stage 4.

Tapetum :

The incorporation of ^{3}H-PRO and ^{14}C-HYP into the nuclei of tapetum at different stages (meiosis, tetrad formation and microspore stages 1-2) was relatively moderate when compared to the incorporation of ^{3}H-LEU, ^{3}H-ARG, ^{3}H-TRP (Albertini, 1970 ; Albertini et al., 1983). During the maturation of microspores, the incorporation of precursors into tapetum nuclei progressively decreased and finally was close to zero just before the division of microspore. The cytoplasmic activity of the tapetum (periplasmodium) increased during meiosis, became high beyond the pachytene stage and reached a maximum at the tetrad stage (Tables 1 and 2).

Cell wall of anther connective :

From premeiosis until the end of microspore stage 2, the cell wall and the narrow cytoplasmic film bound to the wall incorporated more strongly the ^{3}H-PRO (104 to 120 silver grains per 100 µm length of cell wall section) than the ^{14}C-HYP in the anther connective. Then, the incorporation of these precursors into the cell wall progressively decreased and became zero at the stage of young pollen grain.

b) 24 h labelling at 22°C followed by 48 h incubation without radiotracer at 22°C (control 2 ; ^{14}C-HYP : Table 2)

The level of ^{14}C-HYP incorporation observed into the different cellular structures was more often equal or even superior (PMC nuclei during meiotic prophase ; connective cell wall) to the level of incorporation of the control 1 (24 h labelling at 22°C). This can be explained by the prolonged presence of the radiotracer in the anther tissues and more especially in the vascular tissue of connective.

c) Inflorescences cold treated at 4°C

When the inflorescences were treated by cold (4°C for 48 h) after the radioactive precursor supply, the incorporation of radiotracer into the nuclei and cytoplasms of PMC and tapetum and into the connective cell wall more often markedly decreased when compared to the controls 1 (Tables 1 and 2) and 2 (^{14}C-HYP) ; Table 2). The ^{3}H-PRO labelling of connective cell wall was very sensitive to cold as well during the meiosis as during the microspore development : the observed reduction of labelling was always above 73 % and even reached 100 % at microspore stage 2 (Table 1). A marked reduction of wall labelling by ^{3}H-PRO and ^{14}C-HYP was also observed in the PMC wall (from zygotene-pachytene until the end of division I) and in the sporoderm of microspores during the stages 1 to 4.

When inflorescences were treated by cold during the entire period (24 h) of ^{3}H-PRO

Table 1. Distribution of radioautographic silver grains over nucleus and cytoplasm (in brackets) of microsporocytes and tapetum and also over connective cell wall following ^{3}H-proline (20 µCi/ml) incorporation into excised and chilled inflorescence of Rhoeo discolor Hance.

		PMR	Syn.	D.M.$_1$	T	M_2	M_4
PMC	* (24 h at 22°C)	59(31)	29(28)	10 (8)	4 (8)	5(19)	2(16)
	* (id.) then 48 h°at 4°C	26(12)	10 (5)	4 (8)	1 (2)	0 (6)	1 (0)
	* during 24 h at 4°C	6 (8)	2 (2)	1 (1)	0 (2)	1 (2)	0 (3)
Tapetum	* (24 h at 22°C)			9(32)	15(62)	5 (5)	0 (0)
	* (id.) then 48 h°at 4°C			5(22)	4(25)	0 (0)	0 (0)
	* during 24 h at 4°C			1 (5)	2(18)	1 (5)	0 (2)
Connective (100 µm of cell wall)	* (24 h at 22°C)		115	-	104	120	60
	* (id.) then 48 h°at 4°C		11	-	27	0	11
	* during 24 h at 4°C		16	-	17	17	5

* = incorporation of radiotracer ; 48 h° = 48 h incubation without radiotracer.

Abbreviations : PMC : microsporocytes, PMR = premeiotic resting stage, Syn. = synizesis, D.M.$_1$ = diakinesis to metaphase I, T = Tetrad, M_2 = microspore stage 2, M_4 = microspore stage 4.

Table 2. Distribution of radioautographic silver grains over nucleus and cytoplasm (in brackets) of microsporocytes and tapetum and also over connective cell wall following ^{14}C-hydroxyproline (4 µCi/ml) incorporation into the excised and chilled (or not chilled) inflorescences of Rhoeo discolor Hance.

		PMR	Syn.	D.M.I	T	M_2	M_4
PMC	* (24 h at 22°C)	+++ (++)	++ (++)	+ (++)	+ (+)	++	++
	* (id.) then 48 h° at 22°C	+++ (++)	+++ (+++)	+/++ (++)	+/++ (+/++)	+/++	+
	* (id.) then 48 h° at 4°C	++ (++)	+ (+)	+/++ (+/++)	-/+ (+)	++/+	-/+
Tapetum	* (24 h at 22°C)			++ (++++)	++ (++++)	+ (+++)	+ (++)
	* (id.) then 48 h° at 22°C			++ (+++)	++ (++++)	+ (+++)	+ (+)
	* (id.) then 48 h° at 4°C			+ (++)	+ (+++)	+ (+/++)	+ (+/++)
Connective (cell wall)	* (24 h at 22°C)	+	+	+	+	+	+
	* (id.) then 48 h° at 22°C	+	+	+	+	+/++	+
	* (id.) then 48 h° at 4°C	-/+	+	+	-/+	-/+	-

* = incorporation of radiotracer ; 48 h° = 48 h incubation without radiotracer.

See Table 1 for abbreviations
- = no labelling, -/+ = very poor labelling (0 to 2 grains), + = poor labelling (2 to 10 grains), ++ = moderate labelling (10 to 20 grains), +++ average labelling (20 to 40 grains), ++++ = strong labelling.
The labelling of M_2 and M_4 (PMC) correspond to the whole microspores.

supply, the level of incorporation into the nuclei and cytoplasms of PMC and tapetum and into the connective cell wall was markedly decreased with respect to the control 1 (the activity loss most often ranged from 70 to 90 %). The activity of PMC wall (between the zygotene and the end of division I) and of sporoderm of microspores at stages 1 to 4 was low or zero.

Remarks and discussion

The present results are to be compared with the data of our earlier experiments on the incorporation of ^{3}H-LEU, ^{3}H-ARG and ^{3}H-TRP (Albertini and Souvré, 1983) and ^{3}H-orotic acid (Souvré and Albertini, 1978) into PMC and tapetum of Rhoeo discolor and to be discussed in the light of the data available from literature.

At 22°C, the general trend of incorporation of ^{3}H-PRO and ^{3}H-HYP into PMC and microspores runs parallel with that of ^{3}H-LEU and more especially with ^{3}H-ARG (the ^{3}H-PRO incorporation into PMC is moderate but it is significant from the beginning of zygotene to the young microspore stages : the PMC labelling then represents 13 to 26 % of the PMC activity at PMR). At 22°C, ^{3}H-PRO and ^{14}C-HYP were still significantly incorporated into the proteins of cytoplasm and sporoderm of old microspores ; this result is to be related with two facts : 1°/ pollen proteins contain PRO (Zhang Hong-qi et al., 1982), 2°/ tryphine which coats the pollen exine, contains antigenic proteins (Knox, 1971) that are rich in PRO (Underdown and Goodfriend, 1969). At 22°C, the pattern of incorporation of ^{3}H-PRO and ^{14}C-HYP into the increasing periplasmodium (from the zygotene to the tetrad stage) was similar to that of ^{3}H-ARG and ^{3}H-TRP (Albertini, 1970, 1975 ; Albertini et al., 1983) ; on the other hand, during the senescence phase (after the tetrad stage) the periplasmodium incorporated less actively ^{14}C-HYP and especially ^{3}H-PRO than ^{3}H-LEU, ^{3}H-ARG and ^{3}H-TRP (Albertini et al., 1983) into its proteins.

When the cold treatment (4°C) was applied during the ^{3}H-PRO supply, in PMC (at PMR or meiotic prophase) and in expanding or fully developed periplasmodium, only a small but

significant amount of the radiotracer (7 to 27 %) of the control 1 radioactivity) was incorporated in the nuclear and cytoplasmic proteins (Table 1). Under similar conditions, no RNA synthesis could be detected when ^{3}H--orotic acid was used as a precursor (Souvré et al., 1978). The latter two results suggest that the RNAs present in the cell before the action of cold (particularly the long-lived mRNAs detected for example in Tradescantia pollen by Mascarenhas (1966)) are able to induce at 4°C a low, but significant protein synthesis in the PMC and periplasmodium considered.

At 22°C, in the anther connective, the cell wall and the narrow cytoplasmic film bound to the wall have incorporated significant amount of ^{3}H-PRO and ^{14}C-HYP ; but a reduction in the level of incorporation was observed when the inflorescences were exposed to cold after the application of the radioisotopes. The wall proteins might have undergone the process of proteolysis, and the loss of proteins in partially compensated by a substitution synthesis as indicated by the results concerning the effect of cold during supply of ^{3}H-PRO (Table 1). Although R. discolor is a plant sensitive to chilling stress, the results presented here could be related to those of Kacperska-Palacz, Jasinska, Sobczyk and Wcislinska (1977) who noted, in the cold-tolerant winter rape, an increase of free PRO resulting from the proteolysis induced by cold and, more precisely, to those of Rochat and Therrien (1976) ; according to these two authors, there would be a lysis of the wall HYP-glycoproteins with the release of PRO as a result of dehydroxylation of wall HYP.

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BIOCHEMISTRY OF POLLEN PIGMENTATION

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Summary

Pollen pigmentation is highly variable and is generally due to the accumulation of carotenoids and/or flavonoids such as chalcones, anthocyanins and diverse flavonolglycosides. The anther tapetum plays a crucial role in the accumulation of these substances. This was demonstrated by enzymological and immunohistochemical studies.
Keywords: pollen pigmentation, phenylpropanoids, enzyme localization, anther tapetum.

Introduction

Pollen pigmentation, which is highly variable, is generally due to the accumulation of carotenoids and/or flavonoids such as chalcones, anthocyanins and diverse flavonolglycosides. (Stanley & Linskens, 1974).

As part of a study of the biochemistry of pollen pigmentation, the role of the anther tapetum in the accumulation process of phenylpropanoids was investigated. If the accumulation of these substances is regulated by the tapetum cells, enzymes involved in their biosynthesis should be located in the tapetum.

Results and discussion

Experiment 1

The contents of anthers were squeezed out in a buffer/sucrose medium and were seperated by filtration into pollen and tapetum fractions for determination of enzyme activities involved in phenylpropanoid biosynthesis.

The highest specific enzyme activities - with the exception of chalcone-flavanone isomerase - were obtained with the tapetum fraction. The pollen fraction exhibited only low activities (Herdt et al., 1978).

Experiment 2

In order to study the distribution of relevant enzymes within the anthers, specifically in the tapetum cells, the tapetum tissue was isolated by the use of hydrolytic enzymes and was seperately analysed for comparison with pollen and the other anther tissue.

The results demonstrate that a high specific PAL activity is located in the tapetum tissue. The pollen and other anther tissues contain only marginal activity (Table 1; Rittscher & Wiermann, 1983).

Experiment 3

The location of enzymes of phenylpropanoid metabolism was studied using antibodies against chalcone synthase (CHS) and phenylalanine ammonialyase (PAL) in immunohistochemical assays.

After incubation of cross sections of tulip anthers with antibodies directed against PAL and CHS the most intense fluorescence was seen in the tapetum cells (see Kehrel & Wiermann, in press).

Table 1. Distribution of PAL activity in different anther tissues (1: whole anther, 2: anther sections without pollen, 3: rest of the anther after squeezing the loculus material, 5: pollen, 6: tapetum cells.

PAL activity (µkat/kg protein)					
1.	2.	3.	4.	5.	6.
33,6	34,7	31,1	9,7	-	755,5

From these experiments one can conclude, that the enzyme of phenylpropanoid metabolism are predominately localized in the tapetum cells. The tapetum thus plays a crucial role in this metabolism in the loculus of the anther and so in the pigmentation of pollen.

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DEGRADATION OF TAPETAL CELL NUCLEI DURING THE FORMATION OF MICROSPORES

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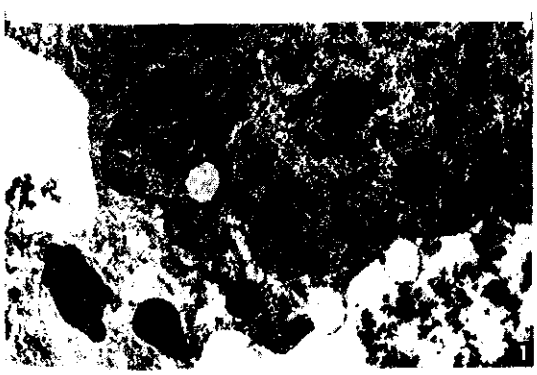
Degradation of tapetal cell nuclei and protoplast was followed in different species of family Liliaceae, taking place during the last stages of microspores formation. It was found, that the process is realized in differnt species by differing ways.

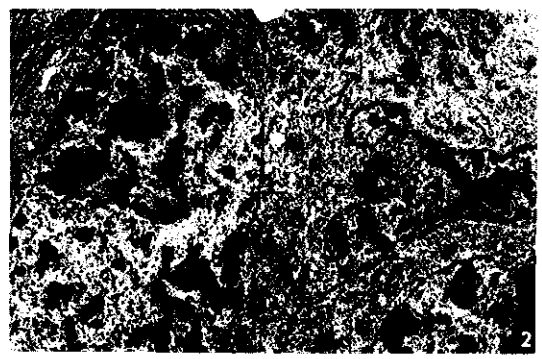
In Lilium henryi Bak. a gradual desintegration of nuclei occurs, nuclei become lobed, karyotheca desintegrates, light vacuole-like bodies appear in karyoplasm, passing to the cytoplasm /Fig.1/. A great amount of reserve material /lipids/ is formed in plastids. Plastids are filled with numerous plastoglobuli in later stages of ontogenesis, their envelope desintegrates.

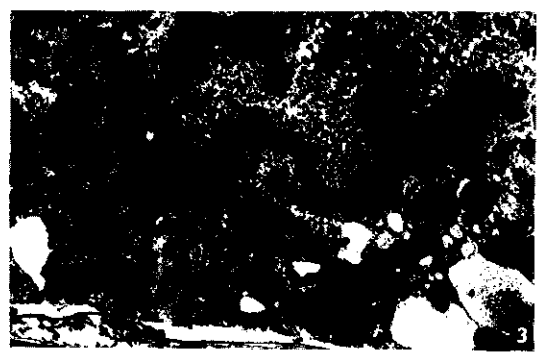
In Lilium davidii Duch. the nucleus changing in shape becomes deeply lobed. It is of interest, that the process does not take place in all nuclei simultaneously, compact globular nuclei are present together with intensively lobed nuclei /Fig. 2/. In that time the number of plastids is increasing, they are frequently constricted in shape. Plastids contain numerous small plastoglobuli. A great number of parallel membranes of ER is present in the cytoplasm.

Lobed nuclei are present in tapetal cells of Tulipa kaufmanniana Regel var.Shakespeare. Plastids are cup-shaped in those cells in later stages of ontogenesis. Their cavations can be filled with cytoplasm or a vacuole. Dilatation of inner membranes and vacuolar degradation of plastids occur.

The last stage of tapetal cell degradation in Lilium martagon L. occurs without the presence of vacuoles. Cytoplasm is filled with dark patches of reserve material. Nuclei are compact, globular, they does not form lobes. Chromatin is condensed. /Fig.3/.







THE EFFECT OF A CHEMICAL HYBRIDIYING AGENT ON THE DEVELOPMENT OF WHEAT POLLEN

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Cultivated variation of wheat were treated with a chemical hybridiying agent developed by Rohm and Haas Chemical Corporation. This compound caused the pollen to abort and this study was undertaken to determine the mechanism of action of the chemical. Plants were treated with the compound early in flower development. Such treated plants had normal development except that the pollen wall formed was much thinner than in the control. When the stage of pollen development is reached where the cell goes from a highly vacuolate stage to one where the cell is filled with cytoplasmic organelles, the cell collapse. Preliminary chemical studies indicate that carotene content of the anthers was normal or higher than in the control. Also, preliminary data indicate that a peroxydase with a pH optimum of 7.2 is strongly inhibited in the treated anthers. Thus, it appear that the mode of action of the compound is to prevent formation of the pollen wall by the inhibition of the deposition of the sporopollenlin.

GENETICAL APPROACH OF AUTOSTERILITY IN CAMELIA SINENSIS L.

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Summary

The tea plant has a trilocular ovary with two ovules per loculus. In a polyclonal seed garden, the majority of fruits contents one seed, exceptionally, five. A statistical study of the number of seed in upper and lower position per clone is given. Diallel crosses after hand emasculation and pollination were realized. The results demonstrate a marked difference between the reciprocal crosses, a total autosterility and a multiallelism.

Introduction

Clonal selection was the more important strategy in tea improvement giving a uniform plantation and a stability of organoleptic characteristics. But the seedage of synthetic populations is not without interest. Synthetics are easily produced in biclonal seed garden. The value of the synthetic can be estimated by a progeny-test; all progeny-testing procedures depend upon the estimation of combining abilities (a general and a specific). Diallel is a mating pattern for estimation of combining abilities and the relative importance of the two and so, authorizes a choice between synthetic or clonal selection. Often, the two occur together as in Cacao; many clones are also self-incompatible and synthetics show a "dramatic" interpopulation heterosis (Simmonds, 1979). A diallel is an excellent tool to understand the incompatibility system.

Results and discussion

Experiment 1

In order to understand the nature of the encountered sterility, fruits were collected at random in an openpollinated seed garden (polyclonal). Fruits were carefully dissected. Three categories are found : seed, unfertilized and aborted ovules (table 1). A distinction is made between the upper (style) and lower (pedicel) position of ovules. χ^2 analysis show a significantly difference between the number of seed in lower and upper position with all the clones and no difference with the aborted ovules. A comparison of the means of seed in upper and lower position show a significantly difference.

Clone	Seed		Unfertil.		Aborted	
	U	L	U	L	U	L
Tri 31/8	37	12	162	174	40	55
Tri 31/11	44	19	145	173	51	66
BB 35	44	17	157	211	32	38
IB 174	66	9	86	113	80	73
IB 241	12	3	60	70	2	1
IB 401	62	34	110	139	76	75
IB 108	73	20	97	135	67	84
IB 92	72	61	245	260	91	76
SHAN	116	62	84	107	98	132
IB 62	83	10	182	121	34	28

Experiment 2 : some results of diallel crosses, % of fruiting after 6 months

Male	Female	%
Tri 31/8	Tri 31/11	75
Tri 31/11	Tri 31/8	16
Tri 31/8	IB 174	65
IB 174	Tri 31/8	20
Tri 31/8	IB 108	20
IB 108	Tri 31/8	0

These results show a marked difference between the reciprocal crosses, a great importance of the female cytoplasm.

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HORMONAL AND TEMPERATURE CONTROL OF MALE-STERILITY IN A TOMATO MUTANT

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Summary

The male-sterile, single gene, stamenless-2 (sl_2/sl_2) mutant of tomato can be induced to form normal stamens and viable pollen either by the application of gibberellic acid (GA_3) or by growing plants under low temperatures. IAA treatment or high temperatures resulted in the production of carpel-like stamens. The mutant stamens contained lower levels of proteins and different banding pattern in SDS polyacrylamide gels than the normal (+/+) stamens. The reversion of mutant stamens to normal by GA_3 or low temperatures partly restored the differences in the protein content and the banding pattern.
Keywords: male-sterility, tomato mutant, GA_3, IAA, temperature, proteins.

Introduction

Male-sterility in plants is extremely valuable for the production of hybrid seeds and for increasing the yield of crops. Generally, cytoplasmic male-sterility (CMS) is used in producing hybrids, since CMS can be readily maintained in the F_1 generation. Alternatively, nuclear or genic male-sterile lines can also be useful for hybrid production, but the maintenance of such lines is tedious and involves the process of roguing (Frankel and Galun, 1977).

We propose that if male-sterile mutant plants can be reverted by chemical or environmental treatments to produce normal stamens, such pollen can be used to pollinate mutant flowers and the seeds thus produced will generate all mutant plants. This approach can therefore be useful in maintaining the pure male-sterile lines (Fig. 1).

In tomato (Lycopersicon esculentum Mill.) CMS is not commonly known, but several single gene male-sterile mutants exist which exhibit a varying degree of male-sterility (Rick, 1953, 1960, 1962; Hafen and Stevenson, 1955). These mutants have been shown to serve as female parents in hybrid seed production (Hafen and Stevenson, 1958; Lapushner and Frankel, 1967). As proposed in Fig. 1, if the fertility of these mutants can be restored by hormonal or environmental means, such mutants can be very useful in hybrid seed production.

In this article we report on the regulation of male-sterility in a male-sterile, single gene, stamenless-2 (sl_2/sl_2) mutant of tomato by plant hormones and temperature conditions and also provide information on the protein patterns associated with male-sterility/fertility.

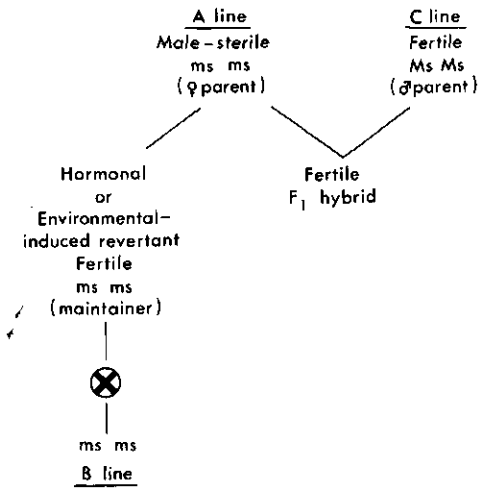

Fig. 1. A proposal for the maintenance of genic male-sterile line by the experimental induction of revertants and the production of mutant and hybrid lines (Adapted from Frankel & Galun, 1977)

Materials and Methods

Seeds of the stamenless-2 mutant were originally obtained from Dr. C.D. Clayberg of the Connecticut Agricultural Station, New Haven, CT, U.S.A. Initially, mutant plants were maintained through cuttings; later the gibberellic acid-reverted flowers were used for selfing and pure homozygous seeds were obtained. The normal line used was homozygous dominant (+/+).

Hormonal and temperature experiments were performed on clones of plants obtained through cuttings. Gibberellic acid (GA_3, 10^{-3}M) and indole-acetic-acid (IAA, 10^{-4}M) was applied with a micropipette (10 or 25 µl) to the axil of the youngest leaf immediately before floral initiation. For temperature experiments, plants were grown in growth chambers maintained in one of the following three regimes: Low - 18°C day/15°C night,

intermediate - 23°C/18°C night, and high - 28°C day/23°C night.

Proteins were extracted by homogenizing the tissue in 10 mM Tris-HCl buffer (pH 7.5) with mercaptoethanol. The homogenate was centrifuged at 10,000 x g for 10 min. and the supernatant was used for protein determinations and electrophoresis. Protein determinations were performed according to the method of Bradford (1976). The reaction mixture was read at 595 nm in Beckman DU-7 spectrophotometer.

For electrophoresis, the supernatant of plant extract was mixed with equal volume of a buffer containing 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS (sodium dodecyl-sulfate) and 0.0625 M tris-HCl (pH 6.8). The mixture was dipped in boiling water for 3 minutes and the cooled sample was loaded on a 12% polyacrylamide slab gel in the Bio-Rad Protean Cell. The gels were run at a constant electric current of 20 mA for each gel and were stained with coomassie blue (R-250).

Results and Discussion

The stamenless-2 mutant produced abnormal stamens which were pale yellow in color, bore naked external ovules (E.O.) on the adaxial surface, were laterally free, produced non-viable pollen and were shorter in length than the normal stamens (Sawhney and Greyson, 1973a). The normal stamens were orange-yellow in colour and pubescent (Y.P.), and were laterally fused to form a staminal cone.

Gibberellic acid at the concentration of 10^{-3}M induced the formation of normal stamens in flowers which were initiated after the treatment. The stamens were yellow and pubescent, produced normal pollen, formed a staminal cone, and were as long as the normal stamens (Table 1; see also Sawhney and Greyson, 1973b). The pollen produced were viable and when used to pollinate the sl_2/sl_2 flowers formed fruits with normal seeds, which upon germination produced all mutant plants.

Indole-acetic-acid induced the production of carpel-like stamens (C.S.). The C.S. showed no signs of microsporogenesis and possessed a basal ovary-like region which contained ovules inside (see Sawhney and Greyson, 1973b). The stamen length of such stamens was smaller than that of the GA_3-treated sl_2/sl_2 and +/+ flowers, and the stamens did not fuse to form a staminal cone (Table 1). More importantly, such stamens were functionally male-sterile.

These observations showed that the male-fertility of the sl_2/sl_2 mutant can be either restored by GA_3 treatment or the stamens can be induced to form a completely sterile structure by IAA. The timing of hormonal treatment was, however, a critical factor (Sawhney and Greyson, 1979). Since normal pollen and viable seeds were produced following GA_3 treatment, it is clear that this treatment can be used to maintain the male-sterile line, i.e. the B-line (Fig. 1).

Table 1. Effects of GA_3 (2 x 10 µl of 10^{-3}M) and IAA (2 x 25 µl of 10^{-4}M) on the development of stamens of the stamenless-2 (sl_2/sl_2) mutant of tomato. Y.P. = yellow and pubescent (normal), c.s. = carpel-like stamens. Values presented are the mean. n = 15.

Treatment	No. of Y.P. stamens/ flower	No. of c.s. stamens/ flower	Stamen length (mm)	Staminal cone (% flowers)
GA_3	6.60[a]	0.0[a]	9.30[a]	100.0
IAA	0.25[b]	4.25[b]	6.50[b]	0.0
sl_2/sl_2 (control)	2.25[c]	0.5[a]	7.80[c]	0.0
+/+ (control)	6.36[a]	0.0[a]	9.80[a]	100.0

Note: Means followed by the same letter in a vertical column not different at 5% level.

(Data from Sawhney and Greyson, 1973b).

Mutant plants grown under different temperature regimes also showed somewhat similar results. Plants grown under low temperatures produced normal stamens which were yellow and pubescent, were as long as +/+ stamens and were fused to form a staminal cone (Table 2, see also Sawhney, 1983a). The pollen produced in these stamens was normal and viable and if used to pollinate the mutant flowers, resulted in fruits with seeds which were all sl_2/sl_2. The high temperature conditions on the other hand caused the production of carpel-

Table 2. Temperature effects on the stamen development of *the stamenless-2* (sl_2/sl_2) *mutant of tomato. Temperature* regimes: LTR = low, ITR = intermediate, HTR = high. Y.P. = yellow and pubescent (normal), c.s. = carpel-like stamens. Values presented are the mean. n = 30.

Genotype/ Temperature regime[1]	No. of Y.P. stamens/ flower	No. of c.s. stamens/ flower	Stamen length (mm)	Staminal cone (% flowers)
sl_2/sl_2 (LTR)	6.16[a]	0.13[a]	9.92[a]	66.6
" (ITR)	0.50[b]	1.30[b]	7.61[b]	0.0
" (HTR)	0.00[b]	5.46[c]	5.72[c]	0.0
+/+ (ITR)	6.70[a]	0.00[a]	9.42[a]	100.0

Note: Means followed by the same letter in a column not different at 5% level.

[1]For details on different temperature regimes see the text.

(Data from Sawhney, 1983a).

like stamens (Table 2) in which microsporogenesis did not take place and instead ovules were produced at the basal end (See Sawhney, 1983a).

Thus the male-sterility of sl_2/sl_2 mutant can also be controlled by growing mutant plants under different temperature regimes. This can therefore be an alternative approach for the maintenance of pure sterile lines (Fig. 1). That temperature effects may be mediated through changes in endogenous hormones was discussed elsewhere (Sawhney, 1983b).

A protein analysis showed that the normal stamens contained nearly twice as much protein as the mutant stamens under the intermediate temperatures (12.26 for sl_2/sl_2 and 24.34 for +/+, µg/mg fresh wt. of protein). The sl_2/sl_2 stamens reverted to normal by low temperatures (15.86 µg/mg) or GA_3 (18.44 µg/mg) contained greater protein than the untreated sl_2/sl_2 stamens, but less than the +/+ stamens. The carpel-like stamens produced under high temperatures contained lowest levels of protein (10.1 µg/mg) than all the other conditions.

The lower level of protein in the mutant vs normal stamens, may be primarily related to the proteins associated with pollen development; the reversion to normal stamens and pollen by GA_3 and low temperatures restored some of these proteins.

An SDS-polyacryalamide gel analysis showed that mutant stamens contained 3 bands of approx. 33, 28 and 23 k daltons which were either faint or absent in the +/+ stamens. The low temperature reverted mutant stamens showed the absence of these bands, but they were present in the high temperature stamens. The +/+ stamens had a zone of protein bands ca. 35-45 k daltons which were also more distinct in the low temperature reverted mutant stamens, than in the intermediate or high temperature mutant stamens. In the GA_3- treated stamens also protein bands found in the mutant stamens were much fainter and the zone of protein bands found in +/+ stamens was much more distinct.

It appears therefore that the differences in the protein levels or protein bands in the mutant and normal stamens were partly recovered by the restoration of normality in the mutant stamens either by low temperature or by GA_3. The nature of such proteins, however, remains to be resolved.

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CHARACTERIZATION OF CYTOPLASMIC MALE STERILITY IN PETUNIA X HYBRIDA (HOOK) VILM.

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Summary

Cytoplasmic male sterility (CMS) in many species is accompanied by a number of cytological and biochemical deviations in the male sex organ. An inventory of all abnormalities in CMS Petunia x hybrida is underway. Callase activity appears to be strongly reduced in CMS types compared to normal male fertile plants. Also deviations have been observed in the esterase and acid phosphatase iso-enzyme patterns, whereas peroxidase composition did not show conspicuous differences.
Keywords: cytoplasmic male sterility (CMS), Petunia x hybrida, callase, isoelectric focussing, iso-enzyme pattern.

Male sterility

Male sterility (MS) can be defined as the incapacity of plants to produce or to release functional pollen (Van Marrewijk, 1979). Male sterility occurs in many forms: absence of the male sex organ, atrophy of anthers, reduction of the stamens into staminodia or conversion of the male sex organ into carpels or petals. The most common type, however, is pollen sterility. In this case of MS the anthers develop normally but irregularities in the development of the microspores cause abortion of the pollen grains. Pollen sterility is widely spread throughout the Plant Kingdom. It sometimes occurs spontaneously under extreme environmental conditions, but is usually controlled by hereditary factors. In most cases pollen sterility is governed by one nuclear gene: genic male sterility, GMS. Some species (tomato, barley) involve large numbers of GMS loci.

In a limited number of cases pollen sterility is brought about by extranuclear hereditary factors or by the joint action of both nuclear genes and extrachromosomal factors: cytoplasmic male sterility: CMS.

Significance of male sterility

Male sterility has been extensively adopted in plant breeding. Its main use is found in the production of hybrid varieties. Introduction of MS in one of the parental lines enables the breeder to produce pure hybrid seed.

A complicating factor is the inheritance of male sterility. Nearly all cases of GMS inherit monofactorially recessive. Such implies that the male sterile plants have to be maintained by crossing with a heterozygous fertile isogenic counterpart, resulting in a progeny consisting of only 50% male sterile plants. So the breeder is compelled to rogue half of the seed line plants to prevent undesired sibbing. Usually this is technically and economically unfeasible.

CMS, on the other hand, lends itself very well to hybrid breeding. Crossing between a CMS-plant and a male fertile maintainer line results in a completely male sterile progeny, since the plasmagenes responsible for sterility are transferred from the mother to all the offspring via the egg cells.

CMS can be restored by the introduction of fertility restoring genes (Rf-genes). The way in which the gene products of the sterilizing cytoplasm and nuclear Rf-genes interact has not yet been unravelled.

The causal agent of CMS

Several investigators have preoccupied themselves with the question about the causative factor of CMS. In the nineteen-sixties it was predominantly thought that this agent of CMS had a viroid or episomic nature (Atanasoff, 1964). This hypothesis was corroborated by the observation that CMS could be transferred to a normal fertile plant by means of grafting, such as with petunia (Frankel, 1956), sugarbeet (Leonova, 1974) and sunflower (Pimakhin & Agashev, 1978). On the strength of many negative results with the same crops as well as with others, the accuracy of these observations, however, has been questioned (see i.a. Van Marrewijk, 1970). Recent studies have revealed an involvement of the mitochondrial genome in the mechanism of CMS, especially in maize (Levings & Pring, 1979), sorghum (Levings, 1983) and most probably petunia (Kool et al, 1982).

Expression of CMS

Cytoplasmic male sterility expresses itself primarily by deterioration of the sporogenous tissue.The time of collapse ranges from premeiosis up to the maturing pollen stage. One particular CMS type can cause degradation at different stages in different genetic backgrounds or even in the same plant (Chauhan & Singh, 1966). Generally CMS is accompanied by other histological and biochemical abnormalities in the sex organ.

Schreur (1978) inventorizing the literature on CMS expression distinguishes six main categories of deviations from the normale fertile situation:

1. Deviations in the development of the tapetal layer.
2. Abnormalities in the vascular system of the filament and/or connective tissue.
3. Substantially modified free amino acid composition.
4. Decreased content of nucleic acids and their derivatives.
5. Changed activities of enzymes, especially of redox systems.
6. Deregulated callase activity.

Also agriculturally important characteristics may be affected, but it is often difficult to say whether this results from pleiotropic gene action or from closely linked genes.

The data in the literature, however, cause confusion, because the different aspects have been investigated separately in different stages of development for diverse crops under varying conditions and by means of non-standardized methods.

Motivation of the resarch subject

In the preceding part the importance of CMS for hybrid seed production has been mentioned. Practical application, however, is limited by the fact that CMS only occurs in a limited number of species. MS induction by chemical pollen killers or gametocides has offered so far no reliable alternative (Van Marrewijk, 1979). The present research is aimed at obtaining insight in the mechanism of CMS and to discover where the process of the deregulation in the sporogenous development takes a start.

The work is done with well-described material, grown under standard conditions. This material will be examined for all possible differences between male fertile and cytoplasmic male sterile plants. It is hoped that the data obtained in this way can be joined as pieces of a jigsaw puzzle and may lead to a fathoming of the CMS-system. By imitating this mechanism, it will then be tried to induce male sterility in fertile plants.

Some results obtained so far will be discussed briefly below. It is intended to publish the results with the separate subjects elsewhere in more detail.

Materials and methods

The plant material consists of a number of clones of Petunia hybrida, namely:

1. Two clones of cytoplasmic male sterile idiotypes of cvs. Snowball (SBS), Blue Bedder (BBS) and Rosy Morn (RMS) and the matching male fertile maintainers (SBF, BBF, RMF).
2. Two clones of restored fertile idiotypes (R1-2, R2-2).

This material was described earlier by Van Marrewijk (1969).

3. F_1-progenies from crosses between CMS- and R-idiotypes.

All clones are grown in climatic chambers under standard conditions of illumination and daylength at a temperature range of 13°-17°-21°-25°C. Not all conditions nor all plant types are used in each experiment. Since the composition and activity of enzymes and other components is dependent of the stage of development of the anther, all determinations are executed in 8 developmental stages. The proper stage is established cytologically after squashing one of the anthers or with the aid of an empirically determined relation between flower bud size and sporogenic stages. The last method is especially used for CMS types in which the sporogenous tissue deteriorates already during meiosis.

In several enzyme activity assessments use is made of ultrathin-layer isoelectric focussing (IEF) in acrylamide gels. The focussing is done at a temperature of 4°C in an LKB Ultrophor flat-bed container connected to a power supply (Pharmacia ECPS 3000/150 with Volthour integrator VH-1) at a constant input of 3 Watt. Samples are obtained by extraction of 10 mg ground anther tissue in 20 µl H_2O, centrifugation (10.000 g) for 10 min and dialyzation of the supernatant (25 µl) against 2 l H_2O.

The composition of the 100 µm gel is according to Radola (1980) adding 10% glycerol to prevent desiccation.

Callase activity

Callase activity plays an important part in the development of the microspore. Proper timing of the build-up and degradation of callase appears essential for the production of vital pollen. Frankel et al (1969) studying isogenic male fertile and cms lines of Petunia hybrida cv. Rosy Morn observed a remarkable difference in callase activity. In fertile plants callase activity was nil between prophase and tetradformation and increased sharply during the release of the microspores. Sterile anthers on the contrary displayed a strong activity of callase between prophase and the second meiotic division. After that the activity decreased. The authors concluded 'that the precocious rise of callase activity in CMS anthers is a cause rather than an effect of the breakdown of microsporocytes'. In a later article Izhar & Frankel (1971) demonstrate the relationship between callase activity and pH level.

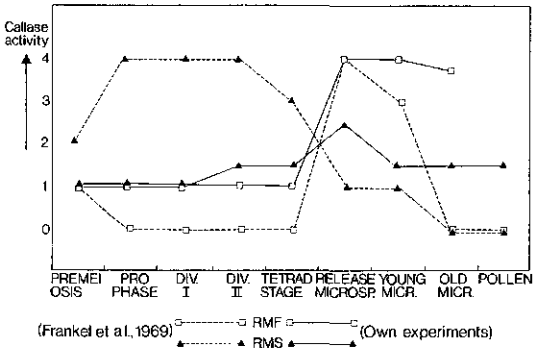
We have investigated whether we could verify the results of Frankel c.s. through our material. This has been done (i) by means of the semiquantitative bioassay used by Frankel c.s. and (ii) with a quantitative biochemical approach, the laminarin digest test.

The semi-quantitative lacmoid test

The lacmoid test is a semi-quantitative bioassay. The essential part of this test is that extracts are made of anthers at different developmental stages. These extracts are subsequently added to a standard substrate which consists of young tetrads of fertile anthers. The latter contains a great amount of callose, which can be digested by the callase of the extract. After incubation at 38°C lacmoid (resorcin blue in absolute ethanol) is added to stop the reaction and to stain the indigested callose. The callase activity in the extract is inversely proportionate to the staining intensity of the substrate. The method is described in Frankel et al (1969) and has been adopted by us after a few modifications.

All fertile idiotypes showed the same trend in activity: low callase activity from premeiosis up to the young tetrad stage and a sudden outburst of activity in the late tetrad stage just before the release of the microspores (Fig. 1).

Fig. 1. Callase activity in relation to microsporogenesis in male fertile (RMF1) and cytoplasmic male sterile (RMS1) Petunia hybrida cv. Rosy Morn, determined with the lacmoid staining test.



CMS idiotypes were characterized by an equal or slightly higher callase activity compared to the fertile counterparts in the early developmental stages and a less complete and inconsistent increase of activity in later stages. There is no clear point of turn and the level of activity is considerably lower than in the fertile plants.

Restored fertile plants act in complete accordance with the pattern followed by normal fertile plants.

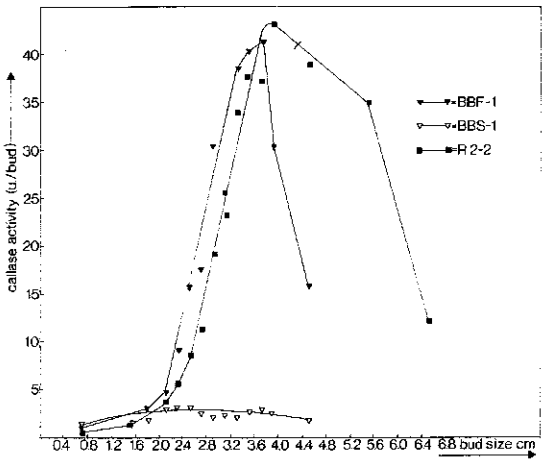
The laminarin-digest test

The laminarin-digest test makes it possible to quantitatively assess callase activity. Laminarin is a commercial preparation whose primary structure completely corresponds with callose.

In this test laminarin is added as a substrate to carbohydrate-free extracts of anther tissue. The laminarin is converted by the callase in the extract into D-glucose, which in its turn is oxidized to gluconic acid by glucose oxidase. The released hydrogen peroxide reacts with indicator reagents (4-amino phenazon, sodium hydroxybenzoate, and peroxidase) to become a stable red-stained quinoncomplex. The intensity of the staining of this complex is assessed spectophotometrically at 510 nm and is directly proportionate to the D-glucose concentration in the sample. Such assessment requires large samples (300 mg per assessment) and is therefore performed with only one normal fertile (BBF), one male sterile (BBS) and one restored fertile (R2-2) clone.

The results of the laminarin test are given in Fig. 2.

Fig. 2. Callase activity in relation to flower bud size in male fertile (BBF1) and cytoplasmic male sterile (BBS1) Petunia hybrida cv. Blue Bedder and in a restorer line (R2-2) determined with the laminarin-digest test.



The figure shows that the fertile anthers have very little callase activity in the first phases of development. At the flower-bud size of 2.3 mm, which correspondends with the second meiotic division, a rapid increase in activity occurs reaching its maximum at flower bud sizes between 3.4 and 3.8 mm (=microspore release). Sterile anthers appear to have a very low callase activity at all stages of development, whereas restored fertile anthers show almost the same trend as observed in normal fertile ones.

Although the results of both tests are certainly not identical, they do demonstrate a clear similarity. In any case, neither of the approaches lends support to the hypothesis of Frankel c.s. that the sporogenous cells degenerate because of a too early callase activity.

Iso-enzyme patterns

Esterase

Esterases are aspecific enzymes of common occurrence whose function it is to hydrolize esters. Esterase iso-enzyme patterns are in use as a tool for the identification of cultivars of a crop.
After IEF treatment esterase was stained with the aid of α-naphtalene acetate and Fast Blue RR at pH 7.2.
In male fertile plants a gradual increase both in number and activity of esterase iso-enzymes was observed, maximum intensity being reached at the early tetrad stage. CMS idiotypes, in contrast, showed low enzyme activity and few bands in all developmental stages.

No differences between cultivars on both the fertile and the CMS level were discerned. The restorer idiotypes produced zymogrammes which were nearly identical to those of the male fertile plants. Both the number of bands and the density of the particular bands were much alike those in the fertile zymogrammes.

We may conclude that CMS affects the iso-enzyme pattern both qualitatively and quantitatively. Also, the effects are in turn cancelled by the introduction of Rf-genes.

Peroxidase

Peroxidases occur in all plant tissues. Their activity and iso-enzyme number increases with the age of the plant. According to Parish (1968) it is an indicator for the physiological situation of a plant. Peroxidase is a very stable enzyme with a high water-solubility.
It does not denaturate when tissues are stored in deep-frozen conditions. The biochemical function of peroxidase is unclear: one recent hypothesis is that it has to be considered as a degradation product of catalase. Both anthers and leaves were investigated for peroxidase patterns.
Peroxidases were detected by incubating the gel after IEF-treatment in a staining solution according to Shaw & Prasad (1970).

It appeared that iso-enzyme patterns are organ-specific. The zymogramme of leaf samples was identical for all idiotypes involved. Slight differences could be discerned in the peroxidase composition of anthers between developmental stages, varieties and fertility types. So, a few bands which occurred in BBF could not be traced in the sterile version and vice versa. There also seemed to be an influence of the temperature in which the plants had been grown. A few bands which were clearly visible in the 17°C BBF sample disappeared or were less clear at 25°C, but also reverse situations occurred.

Differences between varieties were hardly more conspicuous than those between fertility types. It was concluded that the observed differences were of a quantitative rather than a qualitative nature.

Acid phosphatase

Acid phosphatase is detected after staining the gel in α-naphtyl acid phosphate and Fast Garmet GBC at pH 4.8.

With this enzyme the number of iso-enzyme bands and their density increase between the premeiotic stage and the tetrad stage, but to a considerably less high degree than with esterases. This process occurs in the fertile as well as in the CMS types. A most conspicuous thing however is that the zymogrammes of BBF and BBS differ qualitatively. BBS shows three clear bands that are absent in BBF, whereas BBF has a few indistinct bands which are absent in BBS.
In cv. Rosy Morn, though, these differences have not been found.

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CYTOCHEMICAL LOCALIZATION OF CYTOCHROME OXIDASE IN ANTHERS OF CYTOPLASMIC MALE STERILE PETUNIA HYBRIDA (HOOK.) VILM.

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Summary

In Petunia it was demonstrated that changes in mitochondrial DNA are associated with cytoplasmic male sterility (Kool et al., in preparation). Investigations of translational products from isolated mitochondria have revealed several unique polypeptides. This distinct polypeptide composition possibly influences sterile microsporogenesis and may ultimately result in abortion of the tapetal and sporogenous cells. In the present study, cytochrome oxidase is localized at the ultrastructural level, using the diaminobenzidine method. Cytochrome oxidase activity is demonstrated in the cristae and at the membranes of the mitochondria. We could not detect any difference in staining specificity, correlated with sterility.

Keywords: cytoplasmic male sterility, Petunia, cytochrome oxidase, cytochemical localization.

Introduction

Cytoplasmic male sterility (cms) is a maternally inherited trait which interferes with pollen production and is used to produce F1 hybrids by preventing self fertilization of the seed parent (for more details the reader is kindly referred to the contribution of Van Marrewijk).

Substantial evidence, especially in maize and Sorghum, suggests that the trait is encoded by the mitochondrial genome (Levings, 1983). A number of proteins are produced within the mitochondria. For example, three subunits of cytochrome c oxidase, two subunits of the cytochrome bc complex and mitochondrial ATPase are synthesized within this organelle (Dillon, 1981). Deviations in the synthesis of one of these proteins may ultimately result in abortion of the tapetal and sporogenous cells. In several studies, sterility appears to be associated with alterations in cytochrome oxidases. Mitochondria from a cms Sorghum, synthesize a cytochrome c oxidase subunit I, 4000 daltons larger than the form found in fertile cytoplasms (Dixon and Leaver, 1982). In maize, differences in the number of isozymes of cytochrome oxidase were observed during meiosis in the anther (Watson et al., 1977). Throughout sporogenesis, sterile anthers showed fewer isozymes than their fertile counterparts. In addition, the enzymatic activity of cytochrome oxidase appeared to be different. Sterile anthers of various types of cms maize plants, exhibited a significantly lower cytochrome oxidase activity than fertile ones (Watson et al., 1977; Ohmasa et al., 1976). During fertile meiosis, cytochrome oxidase activity increased rapidly, a phenomenon not found in sterile sporogenesis (Ohmasa et al., 1976). However, since the differences in enzyme activity occured rather late in sporogenesis, they may be evoked by the preceding process of degeneration. Cytochrome oxidase activity assayed from leaf tissue, was similar for fertile and sterile plants.

In Petunia, Kool et al. (in preparation), demonstrated that changes in mitochondrial DNA and its translational products are correlated with sterility. Investigations of translational products from isolated mitochondria have revealed several unique polypeptides associated with male sterile cytoplasms. Recently, specific sequence analysis of mitochondrial DNA affirmed changes in the genetic code for subunit II of cytochrome c oxidase, possibly related with cms (Hanson et al., 1984). In the present study, we attempt to establish whether these alterations in mitochondrial DNA do have implications for enzymatic activity in the anther at the ultrastructural level. The results demonstrate the localization of cytochrome oxidase in the cristae and at the membranes of the mitochondria. We could not detect any difference in staining specifity correlated with sterility.

Materials and methods

Two isogenic types of Petunia hybrida (Hook.) Vilm. were used in this study, i.e. male fertile cv. Blue Bedder (BBF), and the cms Blue Bedder (BBS), described by Van Marrewijk (1968).

The localization of cytochrome oxidase was based on the methods developed by Seligman et al. (1968) and Anderson et al. (1975). The medium contained 2.5 mM 3,3'-diaminobenzidine (DAB) in 0.05 M Na-phosphate buffer at pH 7.2.

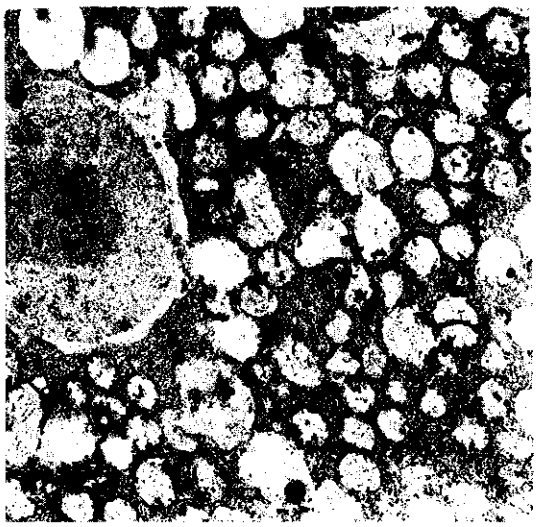

Fig. 1. Part of a microspore of BBF, stained for cytochrome oxidase activity. Enzyme reaction product appears in the mitochondria.
Tetrad stage, x 15,000.

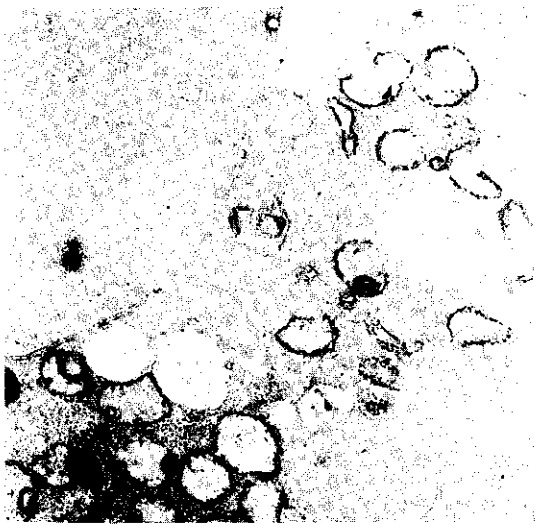

Fig. 2. Part of a meiocyte of BBS, stained for cytochrome oxidase activity. Enzyme reaction product appears in the mitochondria.
Prophase I, x 15,000.

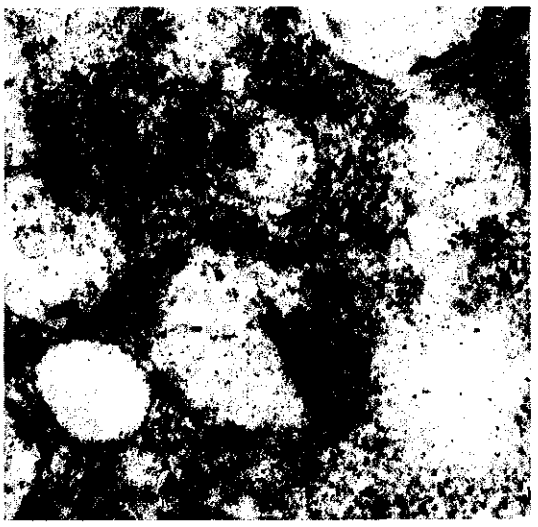

Fig. 3. Distribution of the reaction product of DAB within mitochondria of a microspore of BBF. A few cristae and the outer surface of the inner membrane of the envelope are stained.
Tetrad stage, x 52,500.

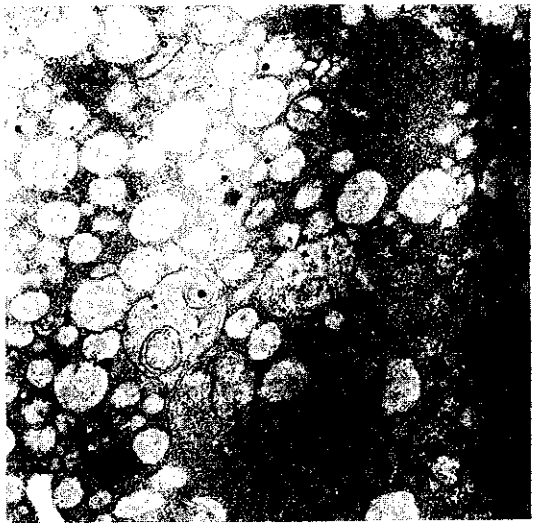

Fig. 4. Part of a microspore of BBF. Staining for cytochrome oxidase is inhibited with KCN. No formation of reaction product is observed.
Tetrad stage, x 15,000.

Results and discussion

The electron transport system regulates the flow of electrons during oxidative phosphorylation and is an integral part of the inner mitochondrial membrane (Dillon, 1981). Cytochrome oxidase, which catalyzes the oxidation of cytochrome c, is an essential component of this system and consists of a complex of several transmembrane polypeptides that are integrated into the inner membrane. Using DAB as an electron acceptor, the consistent morphologic visualization of an oxidase system can be demonstrated on the outer surface of the inner membrane (Seligman et al., 1968). The initial step in the mitochondrial oxidation of DAB relies upon cytochrome c, acting as the electron acceptor. The next step is the reoxidation of DAB by cytochrome oxidase. The oxidated DAB forms a nondroplet, amorphous reaction product at the enzyme site. The precipitate is osmiophilic and insoluble in the dehydration and embedding agents used in specimen preparation. If the reaction is conducted under controlled conditions, the intensity of the reaction product affords a dependable criterion for indicating cytochrome oxidase activity (Perotti et al., 1983).

In Petunia, the DAB reaction product is located in the cristae and at the mitochondrial envelope (Figs. 1, 2 and 3). Cytochrome oxidase reaction is presumed because formation of the reaction product is inhibited by short prefixation in glutaraldehyde and by cyanide (Fig. 4). Mitochondria from fertile and sterile sporogenous cells comprise only a small number of reacting cristae. The distribution of the reaction product is not influenced by the developmental stage. Staining specificity of the meiocytes of BBS, and the microspores of BBF appeared to be similar.

We could not detect any difference in enzyme localization correlated with sterility. The localization of the reaction product in fertile and sterile mitochondria, suggests a similar cytochrome oxidase activity. Nevertheless, it cannot be ruled out that differences in mitochondrial enzyme activity play a role in sterility. Viz., the specificity of the reaction for cytochrome oxidase is not completely certain. In plant tissues, other cytochromes may participate in the oxidation of DAB (Opik, 1975). Comparison of tissue specific localization of cytochrome oxidase and total biochemical activity should reveal whether the altered mitochondrial DNA correlates with differences in enzyme activity in the anther. However, preliminary results could not establish any biochemical difference in cytochrome oxidase activity between fertile and sterile anthers. Hence, the situation in maize appears to be not representable for cms species in general.

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STERILITY OF MICROSPORES IN OENOTHERA HOOKERI.VELANS

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Summary

During the non vacuolate stage dense material accumulates outside the precursor layer of the ektexine. Later the microspores vacuolate but no paracrystalline structure is formed in the ektexine. Enzyme digestion of the endexine is followed by dissolution of the abnormal dense deposits of the microspore surface. Lamellations are the last remains of the exine on the external surface of the intine which comes in direct contact with the pollen sac environment. After the microspores collapse without suffering mitosis, lamellar material as well as dense deposits and cytoplasm remains are finally contained in the degenerating anthers. Tapetal cells show concomitant modifications to these changes, affecting mainly the transpher phase, the E.R. and the plastids and mitochondria.

Introduction

The aim of this work is to compare the developmental stages in normal and sterile anthers after the release of the microspores from the tetrad. It seems important to state the developmental stages of microspores and pollen grains using electron microscopy methods.

Some work on pollen sterility in the genus Oenothera was first published using methods of light microscopy (Oehlkers, 1927). After this the work of Harte & Bissinger (1952) reported on the changes occuringin O. hookeri. velans using paraffin-embedded cuttings of normal and sterile anthers. The same hybrid is the material used in this work, which was kindly supplied by Prof. C. Harte of the University of Cologne. At electron microscopy level only the pollen sterility of O. erytrosepala BORBAS (lamarckiana DE VRIES) was studied before (Jean, 1971). Normal development is partially known through work done using electron microscopy (Afzelius, 1956; Lepousé & Romain, 1967; Skvarla et al., 1978; Skvarla, 1975; Skvarla et al., 1976). No work was done before with a developmental point of view.

Results and discussion

Four developmental stages were recognized after release of the microspores from the tetrad (Fig. 1): 1. Non vacuolate microspores; 2. Vacuolate microspores; 3. Two-celled vacuolate pollen grains; 4. Two-celled pollen grains with starch. These are coincident with those generally described by Laser & Lersten (1972).

After release from the tetrad the microspores are covered by an external fibrillar layer which can be considered the ektexine precursor layer. An internal, dark, homogeneous layer can be identified as the endexine. The apertural chamber has fibrillar contents and shows at the base lamelles which look like compressed vesicules (Fig.1 a). Later the paracrystalline structure of the ektexine appears and the beaded viscin threads become their final structure. Dark sporopollenine grains appear at the base of the apertural chamber. At this stage the tapetal cells reach the transpher phase.

The vacuolization of the microspores is concomitant to the deposition of the intine (Fig. 1 b). The tapetal cells at this stage are characterized by plastids which contain vesicules of light material surrounded by a dark halo. Deposits of dark material are seen between the mitochondrial membranes and the ammount of RER decreases.

After the first mitosis of the microspore is completed, the sporoderm arrives at the typical final structure. The anther tapetum shows some cells with degenerating cytoplasm still "in situ" but abundant cell remains are swimming in the pollen sac.

Shortly before anther dehiscence, the pollen grains reach the dormancy stage which is marked by the loading of abundant plastids with starch and at the same time the disappearance of the big central vacuole (Fig.1 c). No tapetal cells remain "in situ".

An accurate description of the modifications occuring in sterile anthers during development will be published elsewhere. Only the main differences with normal development are pointed out in this symposium book.

As Harte & Bissinger stated (1952), pollen sterility is monogenic. The ster gene in homozigous plants determines pollen sterility, causing a very deep modification of the metabolism in the flower. The maturation sequence of the anthers in the flowers of the inflorescence is altered, as well as the synchronism of developmental stages in the pollen sacs of the same anther.

Regarding the microspores no differences between normal and sterile development could

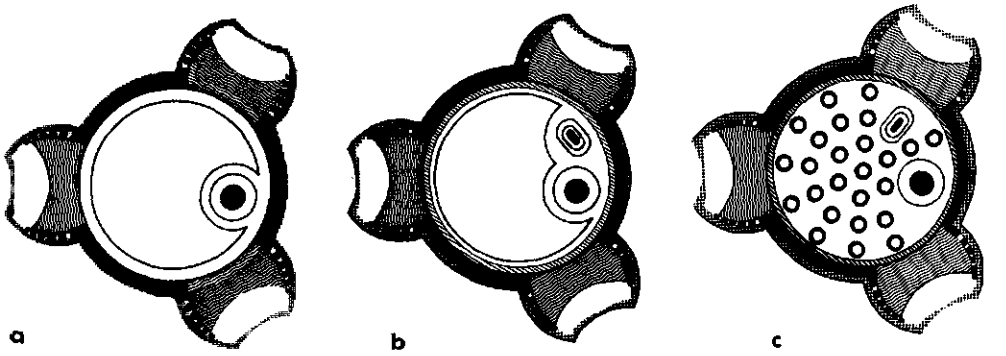

Fig. 1. Stages of normal development in O. hookeri. velans. a, vacuolate microspore. b, Two-celled vacuolate pollen grain. c, Two-celled pollen grain with starch. Symbols: ektexine precursor; ektexine; endexine; intine; apertural chamber; lamelles plastids with starch.

be verified during meiosis. This is coincident with findings in O. erytrosepala (Jean, 1971), where at a very late stage pollen die leaving empty grains with almost normal sporoderms.

In O. hookeri. velans differences between normal and sterile pollen development appear as the final consequence of deep metabolic alterations of the last stages of sporoderm morphogenesis, which fail to develop the paracrystalline structure of the ektexine (Fig. 2 a).

Two main changes could be stated in sterile anthers (Fig. 2 a-c):

1. Dark material deposited on the outer surface of the microspores is the first evidence of modifications during sterile development. It is produced in vesicules of the tapetal cytoplasm, passing on to the pollen sac and accumulating on the outer surface of the ektexine precursor without originating the paracrystalline structure.
2. Later on the material secreted by the tapetal cells becomes lamellated. Lamellations of the exine seem to be a common fact in modified pollen grains. They were observed in aborted pollen of several species (Rowley & Flynn, 1969).

On the base of my observations I assume that dark material accumulated in tapetal vesicules and on the microspore surface is probably a kind of lipid self-assembly (Sitte, 1981) of colourless sporopollenin precursors produced in the tapetal cells during the transpher phase, which fail to organize as definitive sporopollenin molecule in sterile anthers. Production of colourless precursors of sporopollenin was assumed before (Heslop-Harrison, 1968). Three alternative pathways can be postulated for these precursors: they originate either sporopollenin or β carotenoids in normal anthers and amorphous lipids in sterile pollen sacs. Plastid morphology in tapetal cells allow the assumption that they play a role during sporoderm development. This is also supported by the fact that they fail to develop normally in this hybrid. The tapetal cell mitochondria also look different, which is remarkable.

The dissolution of the endexine and later on the digestion of the dark deposits on the microspore surface can be a secondary consequence of the metabolic disturbance in the pollen sac. Enzymes could be responsible for the initiation of digestion of the endexine. These enzymes are acting from the inside of the layer towards the outside. Once the endexine becomes discontinuous the dark material of the microspore surface is also digested. In barley, esterases are shown to digest the exine (Ahorkas, 1976). Otherwise the endexine is not a continuous layer, as has been demonstrated in Epilobium angustifolium L. (Rowley, 1976). The endexine channels could be the sites of location of the inactive enzymes. The beginning of enzyme digestion is marked by suitable conditions arriving in the environment. This occurs either during germination on the stigmatic surface after pollen dormancy in normal compatible pollinations, or still in the pollen sac before pollen grains arrive at their maturity and enter dormancy in sterile anthers.

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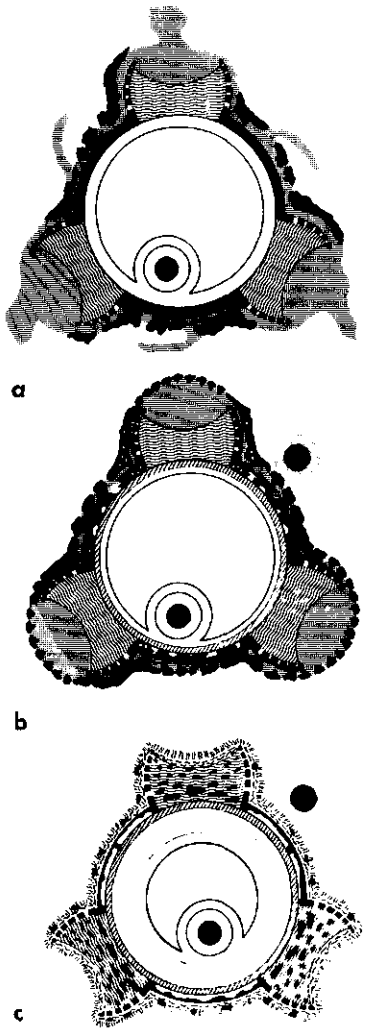

Fig. 2. Stages of sterile development in O. hookeri. velans. a, vacuolate microspore with dense deposits on the ektexine precursor. b, beginning of the endexine dissolution. c, last stages of exine dissolution. Symbols: as Fig. 1.

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STAGES OF TAPETAL CELL CELL DEVELOPMENT IN STERILE ANTHERS OF OENOTHERA HOOKERI. VELANS

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Summary

The tapetal cell stages were identified and their ultrastructure related to microspore morphology. During sterile development they show changes in their plastids and ER. Vesicles with dense contents and paramural bodies appear as main modifications.

Introduction

O.hookeri.velans is a hybrid with sporophytically controlled microspore sterility (Noher de Halac,1984).Developmental stages of tapetal cells had not been recognized before at electron microscopy level, either in normal or in degenerating anthers, in spite of the fact that pollen sterility has been known to be present in the genus Oenothera for a long time.

Results and discussion

After release from tetrads the microspores are at the non vacuolate stage. The secretory tapetal cells reach the transfer phase which is characterized by the digitations of the plasmalemma with a mitochondrion at the base of each digitation. At the vacuolate stage of microspores, the tapetal cells gradually loose the digitations of the plasmalemma and the amount of RER decreases while the plastids become full of light vesicules similar to those observed in Raphanus (Dickinson, 1973),Antirrhinum maius (Lombardo &Carraro, 1976) and Olea europaea (Pacini& Casadoro, 1981). Vacuolation of tapetal cells follows and the cell remains partially migrate into the pollen sac. Tapetal cells completelly desappear when pollen grains get charged with starch after mitosis.

During sterile development, tapetal cells show alterations in the transfer phase. Later on no plastids containing light vesicles develop. Instead, a hypertrophya of the ER is seen and dark materials accumulate first in vesicles and then as paramural bodies opposite the locular face of the cells(Fig.1). This demonstrates the relationship between the transfer phase of tapetal cells and the development of the paracristalline structure in the ektexine.

The main functional activity of the tapetum is associated with synthesis of lipids and carotenoids. This was assumed for the developing lily anther (Reznikova & Willemse, 1981). In the sterile anthers of O.hookeri.

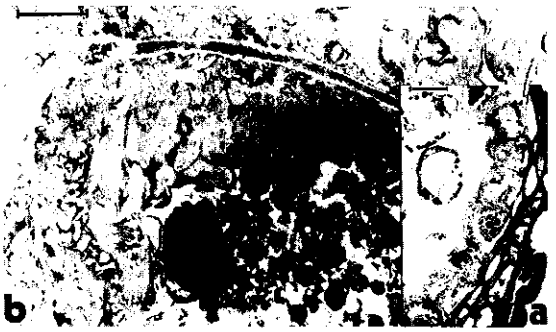

Fig.1. Autolytic tapetal cells. a, Tapetal layer with lamellar material at the locular face.Scale:25um. b, Lamellar material and dark droplets in the autolytic cytoplasm. Scale: 2 um.

velans a deep disturbance of lipid metabolism in tapetal cells conduces in the ektexine to the failure of the paracristalline organization of sporopollenin. It has been assumed that the tapetum plays a role in the synthesis of informational macromolecules (Raghavan,1981). Further work should provide more evidence on the kind of macromolecules involved in lipid metabolism which could be modified in these hybrids.

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ISOLATION OF PETUNIA HYBRIDA CYTOPLASTS

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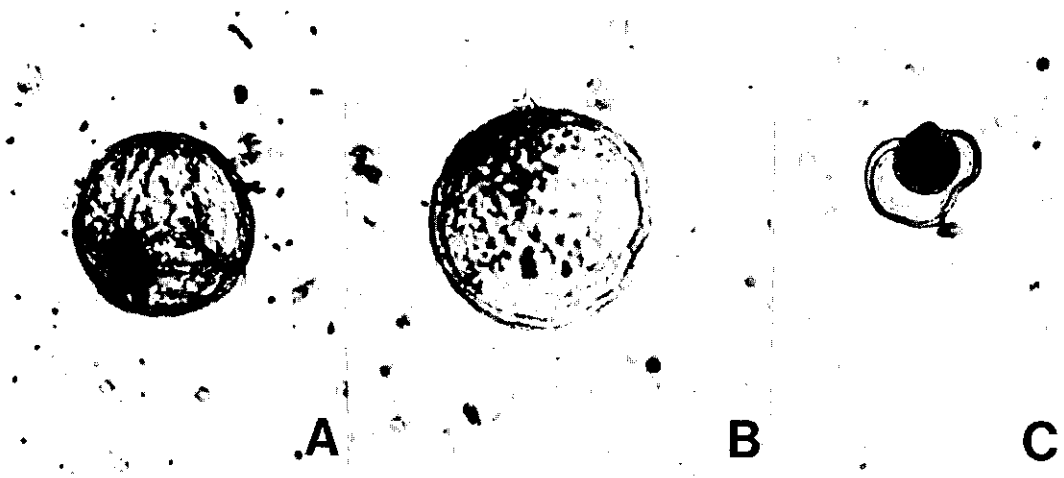
Somatic cell fusion of protoplasts with cytoplasts (enucleated protoplasts) represents an ideal tool for transfer of specific cytoplasmic genetic information from one plant into another, without changing the nuclear background of the acceptor protoplast.

By using a simple method of Percoll centrifugation, we have developed a procedure for cytoplast isolation of Petunia hybrida protoplasts, aiming at the transfer of cytoplasmic male sterility from P. hybrida to Lycopersicon species.

Freshly isolated Petunia protoplasts were centrifuged (50,000 g for one hour at 12 °C) on top of a discontinuous Percoll gradient. Fractions of protoplasts (A), cytoplasts (B) and nuclear fragments (C) were collected and analyzed microscopically after staining with 1% acetocarmine.



Plastid content of generative cells in Liliaceae

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Summary

The plastid content of generative cells in species of the Liliaceae has been studied using electron microscopy. Mature generative cells without plastids are formed either by the unequal distribution of plastids during first pollen mitosis (G.verrucosa & F.meleagris) or by the degeneration of plastids during the maturation of generative cells (F.imperialis & H. ventricosa). The genetic consequence is a maternal inheritance of plastids.

Generative cells of Lilium martagon contain plastids during the whole pollen development. The mode of the plastid inheritance in this species depends on the fate of plastids during sperm cell formation and fertilization; this has to be analyzed in future investigations.

Introduction

The mode of plastid inheritance depends on the mode of plastid distribution during the development of the male gametophyte and the fertlization in angiosperms. Accordingly, four types of plastid transmission have been described (Hagemann, 1983). The underlying cytological mechanisms are not yet fully understood. Further investigations on many species are necessary.

Results and discussion

Five species of Liliaceae have been investigated using electron microscopy. Three types of plants have been found:

1. Lilium martagon: The young microspore as well as the generative and the vegetative cell contain plastids. Degenerating plastids have not been observed. Therefore, we assume that the transmission of plastids into the sperm cells and later into the zygote may be possible. Further studies on the plastid distribution during the progamic phase and fertilization have to be performed.
2. Fritillaria imperialis and Hosta ventricosa: The young generative cell contains some plastids as a result of the first pollen mitosis. During the maturation of the generative cell these plastids seem to degenerate. Only mitochondria, dictyosomes, ribosomes, endoplasmic reticulum and myelin-like structures could be found within the generative cytoplasm at anthesis. The lack of plastids in mature generative cells leads to a maternal inheritance of plastids in these species.
3. Gasteria verrucosa and Fritillaria meleagris: The young generative cell as well as the mature generative cell do not contain plastids. Plastids are excluded from the generative cell during the first pollen mitosis. In Gasteria verrucosa we could observe a polarization of plastids far from the dividing microspore nucleus starting at the late prophase and ending at the early two-cellular stage. This plastid arrangement preventing the plastids from being transmitted into the generative cell may be controlled by a biochemical or a biophysical gradient (Schröder 1984). The genetic consequence of the absence of plastids in mature generative cells is a maternal inheritance of plastids.

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NEW RESULTS ABOUT THE PRESENCE OF PLASTIDS IN GENERATIVE AND SPERM CELLS OF GRAMINEAE

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Summary

Data in the literature seemed to indicate a striking heterogeneity of the cereals regarding the plastid content of their generative and sperm cells as well as the transmission of male plastids into the egg cell. However, electron microscopic studies in our lab and critical analyses of recent literature data make a revision necessary: The Gramineae seem to be more homogeneous than previously thought. All cereals, studied intensively, do contain plastids in the generative and sperm cells of their pollen grains and tubes resp. Most of them belong to the Triticum type: Presence of plastids in generative and sperm cells, but their stripping off the sperm nucleus during the fertilization process. Careful genetic studies are necessary to analyse the situation in Secale which may show an exceptional behaviour.

Introduction

On the previous Symposium in the High Tatra the first author has characterized 4 types of angiospermous species regarding the presence or absence of plastids in generative and sperm cells of the pollen and the transmission of male plastids into the egg cell (Hagemann 1983): Lycopersicon type, Solanum type Triticum type and Pelargonium type.

Data in the (older) literature led to the opinion that the Gramineae (= Poaceae) show a striking heterogeneity in that respect; different species seemed to belong to all 4 types. There are papers reporting that Hordeum, Agropyron and Zea belong to the Lycopersicon type, Oryza to the Solanum type, Triticum and Triticale to the Triticum type, and Secale to the Pelargonium type.

Electron microscopic studies in our laboratory and detailed critical analyses of several recent literature data seem to make a revision necessary: The cereals seem to be much more homogeneous regarding the plastid content of their generative and sperm cells than previously thought.

Results and discussion

Hordeum vulgare:

Cass & Karas (1975) reported that in barley 'there are no recognizable plastids in the generative cell'. Młodzianowski (person. comm. 1982) reported the observation of plastids in generative cells. Mogensen (1982) stated that the sperm cytoplasm of barley 'contains numerous organelles, most of which appear to be mitochondria, but some of which may be proplastids'. - We have, thus, to take into account the presence of (at least some) plastids in generative and sperm cells.

The genetic data of Arnason et al. (1946) show a uniparental maternal inheritance of chlorophyll deficient mutant plastids in barley.

Zea mays:

Larson (1965) reported the observation of sperm cells without starch suggesting an absence of plastids. But the paper of Chebotaru (1981) clearly shows the presence of plastids in sperm cells.

Genetic studies demonstrate a uniparental maternal inheritance of mutant plastids (Shumway and Bauman, 1967).

Oryza sativa:

Vaughn et al. (1980) discussed rice as an example for their hypothesis that 'chloroplasts may be physically altered in the process of microsporogenesis, preventing them from being transmitted' into the egg cell, but have not documented this statement by electron micrographs.

In crosses Pal and Ramanujam (1941)

found a uniparental maternal inheritance of plastid mutations.

Triticum aestivum:

In our lab in Halle the pollen development of wheat was studied. The generative and sperm cells regularly contain plastids (Schröder, Risch; Fig.1). Genetic studies showed a uniparental maternal inheritance of mutant plastids (Briggle, 1966). The uniparental inheritance of plastid characters was also demonstrated by restriction analyses after crosses (Vedel et al. 1981). From these facts we drew (1983) the conclusion that wheat represents a new type: In the Triticum type of plastid distribution both generative and sperm cells do contain plastids, but during the process of fertilization the plastids are stripped off the sperm nucleus and are not transmitted into the egg cell.

The mitochondria of wheat seem to behave in the same way: purely maternal transmission (as does cytoplasmic male sterility).

Triticale:

Our studies with Triticale demonstrated also the presence of plastids in generative and sperm cells (Schröder, 1983; Fig.2).

So far genetic results about the transmission of plastids in Triticale are not available.

Common characteristics of the species, dealt with in the preceding paragraphs:

Summarizing the main results of our studies and the critical analysis of the literature data regarding barley, maize, rice, wheat and triticale, we come to the conclusion:

- In all these species the generative and sperm cells contain plastids (The situation in rice is not clear in the moment.)
- The genetic results indicate a uniparental maternal inheritance of plastids. Thus it is to be concluded, that the plastids, still present in the sperm cells, are stripped off the sperm nucleus in the process of fertilization and are not transmitted into the egg cell.
- That is to say, that the species, mentioned above, all belong to the Triticum type of plastid distribution, described by Hagemann (1983).

Secale cereale:

Electron microscopic studies in our lab proved the presence of plastids in generative and sperm cells of rye (Laub, Schröder; Fig.3).

In contrast to the findings about the species, mentioned above, there is a paper by Frösť et al.(1970) reporting a biparental inheritance of an extranuclear chlorophyll deficiency. This is the only report about a biparental plastid inheritance in a monocot species.

However, the analysis of restriction patterns of organelle DNAs of eight Triticale lines with different wheat cytoplasms showed exclusively the presence of the maternal plastids and mitochondria, thus giving no hint at a transmission of paternal (Secale organelles into the hybrid zygotes (Vedel et al. 1981). - Studies of the restriction patterns of plastid DNA of primary triticales in our lab (Siemenroth) also revealed only the presence of wheat plastids, and no indication for a transmission of male rye plastids.

Therefore it is highly desirable to perform reciprocal crosses in rye with regard to genetic plastid differences in order to confirm or to disprove the report of Frösť et al.

Concluding remarks

The study of the distribution of plastids and mitochondria during male gametogenesis and the elucidation, whether male plastids and mitochondria are transmitted into the zygote (and thus the next generation) are not only interesting questions of the fertilization biology of higher plants. This problem is also of great practical value for plant breeders, because they wish to know with what types of cytoplasmic organelles a hybrid nucleus is combined, and whether other plastids or mitochondria might show a better interaction with a hybrid nucleus with regard to hybrid vigour and plant production.

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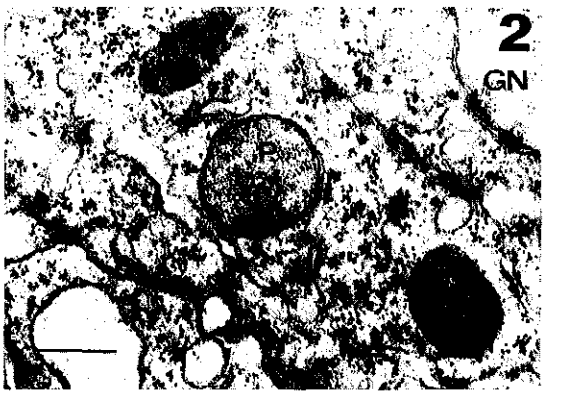

Fig.2. Triticale. Generative cell containing a proplastid (P) and a mitochondrion (M). Generative nucleus (GN). Electron micrograph. Bar represents 0.5 /um.

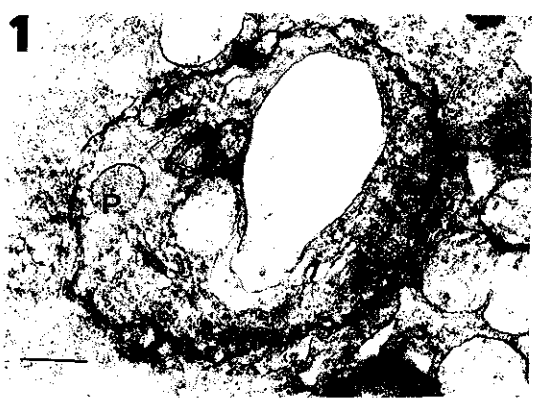

Fig.1. Triticum aestivum. Sperm cell containing a proplastid (P), a mitochondrion (M) and other cytoplasmic organelles. Electron micrograph S. Risch. Bar represents 0.5 /um.

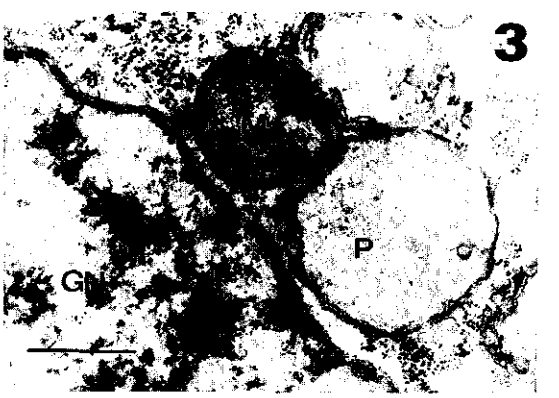

Fig.3. Secale cereale. Generative cell containing a proplastid (P) and a mitochondrion (M). Generative nucleus (GN). Electron micrograph H. Oldenburg. Bar represents 0.5 /um.

THE SURFACE COATING OF MICROSPORES AND MICROSPORE MOTHER CELLS IN PINUS SYLVESTRIS

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Summary

Components of the microspore surface coating are structurally distinctive soon after cytokinesis of the tetrad of microspores and before a recognizable preexinous pattern is discernible. The distinctive structures include a system of rod-like subunits and processes. This appears to be due to specific glycocalyx receptors on which sporopollenin accumulates.
Keywords: exine, pollen, glycocalyx receptors, sporopollenin.

Introduction

Surface coatings of microspore mother cells and of microspores both form as plasma membrane glycocalyces. Both are present when a sporopolleninous exine is initiated so that monomers are presumably available to both coatings. As is well known, an exine forms only on the microspore — the microspore mother cell coating eventually vanishes. This situation offers an opportunity for investigating systems having or not having the capability for sporopollenin polymerization.

Results and discussion

The microspore mother cell surface coating is structurally distinct from the surface coating of microspores. The mother cell coating has the appearance of an irregular filamentous mesh (Fig. 1) whereas the microspore surface coating has complex structures which include rod-like subunits ca 30 nm in diameter (Fig. 2). Rodlets appear to be linked and associated with processes that are ca 100 nm in width. These processes are prominent in the "air bladder" regions but are also present in generally radial orientation on all but the apertural area of microspores. They may be considered as units of an exine template since the outer portion of the exine forms directly on them. Both when it is initiated and again later when it is thickened the exine surface is rough because of the presence of the rodlet surface coating (Fig. 3). The processes are smoothed out as they increase in thickness due, presumably, to the addition of sporopollenin. The distinctive plasma membrane surface components are also observed on tapetal surfaces which likewise accumulate sporopollenin.

Sporopollenin accumulation not only requires the availability of monomers (carotenoids and carotenoid esters) but also surface receptors, according to the findings of Atkinson et al. (1972) with algal cells. We assume that the structure of the sporopolleninous coating is influenced by the specific chemical composition of the receptors.

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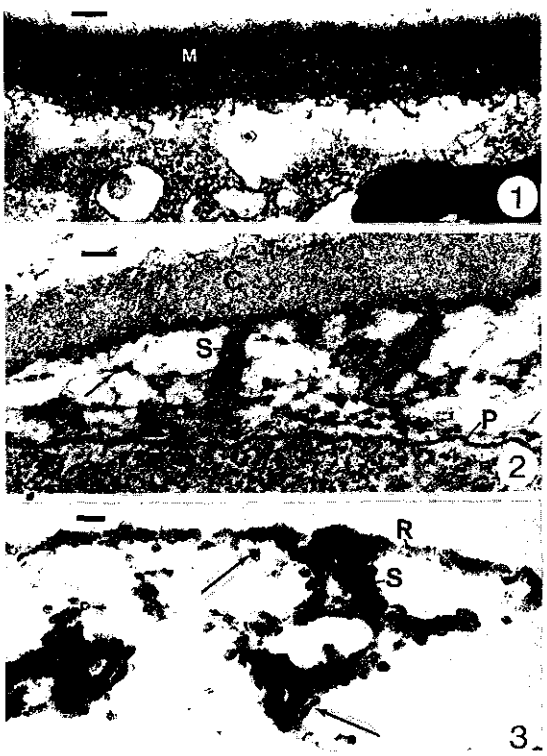

Figs. 1-3. Karnovsky fixative plus ruthenium red. Staining: U, Pb. Bars: 100 nm.
Fig. 1. Microspore mother cell surface coating (M). — Fig. 2. Microspore surface coating between plasma membrane (P) and callosic envelope (C). Dark rodlets (arrows) occur between and on processes of future exine (S). — Fig. 3. Exinous processes (S) and tectum (R) are studded with components of the microspore surface coating which are ca 30 nm in diameter and appear to be rod-shaped; end views are marked by arrow heads and rod-shaped side views by arrows.

LIGHT ABSORPTION OF POLLEN TUBE WALLS AND POLLEN WALLS

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Summary

It is feasible using a microscope to determine the light absorption of a selected small area of a pollengrain or pollen tube. Light absorption of developing pollen, different pollen species and the pollen tube can be related to the changes in wall thickness and composition and the cytoplasmic volume.

Methods

The pollen grain is suspended in water on a microscope slide and the absorption determined of a known fixed area using a 40x objective. A 75W xenon lamp and an automatically driven Ivon-Jobin monochromator provides a continuous spectrum from 400-750 nm. Transmitted light was measured using a RCA photomultiplier supplied with a selected voltage. Compared with a control without specimen (= 100%), it was possible to determine the optical density of a sample in mV, which was expressed as A%. If not otherwise mentioned the variance in the mean value of 3 measurements is 7%.

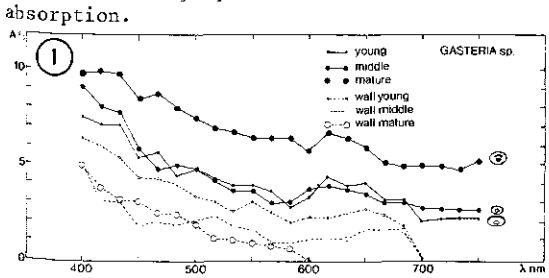
Results and conclusions

1. Developing and mature pollen.

During the development of Gasteria verrucosa pollen absorption remains nearly constant during the early stages. Increases at the mature stages are mainly due to changes in the composition and augumentation of the plasma. Absorption from 700-750 nm is primarily due to the cytoplasmic content of the pollen.

Absorption by pollen wall in aborted empty pollen is high in the young microspore due to the continuing wall formation. Thereafter the wall streches because of the increasing cell volume. See figure 1, measured volume was 20x20x10 µm at 1070 V; I_0 at 500 nm = 4,5 mV 100%.

Comparable results in Lilium and Physiostegia suggests that especially the thickness and composition of the pollen wall and the volume of the cytoplasm influence the rate of absorption.



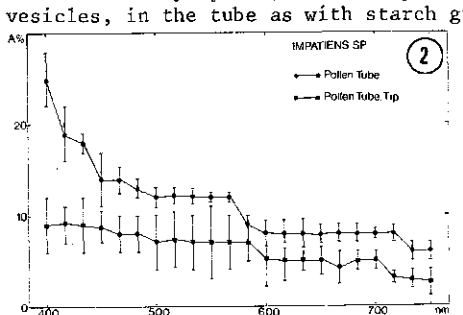
Measuring mature pollen of different species three types of spectral absorption curves can be distinguiwhed: a maximum present between 400-450 nm (Delphinium elatum hyb., Digitalis purpurea, Campanula bononiensis, Phyostegia virginiana); a maximum present between 450-550 nm (Chrysanthemum segetum, Potentilla pyrenaica, Lupinus mirabilis, Gasteria verrucosa) and no maximum present at all (Cedrus atlantica, Colchicum autumnale, Hibiscus syriacus, Lilium c.v. Enchantment).

The nature and concentration of pigments such as delphidine and carotenoids probably determine absorption between 400-450 nm. Flavonoids absorb in general between 200-400 nm. The specific absorption spectrum of each pollen is determined by their specific cell wall components including pigments and its cell contents.

2. Pollen tube and pollen tube tip.

The pollen tube and the tip of the pollen tube of Impatiens walleriana show distinct absorption curves. The ratio in absorption from 450 nm between pollen tube and its tip is about 1:0,75. See fig. 2, measured area 10x10 µm at 1170 V. I_0 at 500 nm = 6,4 mV = 100%,

From 400-450 nm the pollen tube shows a strong decrease in absorption. The tip has no slope and a lower percentage of absorption. The difference between the tip and the other parts of the tube can be related to the composition of the tube wall, the presence of callose and the cytoplasm, at the tip rich in vesicles, in the tube as with starch grains.



When stained with calcofluor white, mainly for polysaccharides, the intensity of a measured area of 10x10 µm for the tip is 24 ± 2 mV (n=5) and for the tube is 16 ± 1 mV (n=7) at 450 nm and 1000 V. This suggests a 1.5x higher level of cell wall components in the tip, partly present in the vesicles in the cytoplasm.

When stained with water blue, a stain mainly for callose, the intensity of a measured area of 10x10 µm for the tip is 20 ± 6 mV (n =7) and for the tube 30 ± 8 mV (n=5) at 500 nm and 1170 V (Willemse, 1981). There exsists a 0.6 x lower callose concentration in the tip.

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EFFECT OF WATER LOSS ON GERMINATION ABILITY OF MAIZE POLLEN

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Summary

The correlation between the water content and viability of maize pollen grains was studied on the basis os the germination ability of pollen from a single cross hybrid. There was found to be a close correlation between viability of the grains and their tolerance to desiccation.
Key words: maize, pollen viability, dehydration, pollen treatment.

Introduction

Knowlton /1922/ has ascribed the rapid loss of viability in cereal pollen to the desiccation of the grains. According to Shivanna Heslop-Harrison /1981/ structural changes take place in the membranes of the pollen grains during dehydration, and the permeability of the plasma membranes alters. No records appear yet to have been published with respect to the effect of various degrees of water loss on the viability of maize pollen.

Materials and methods

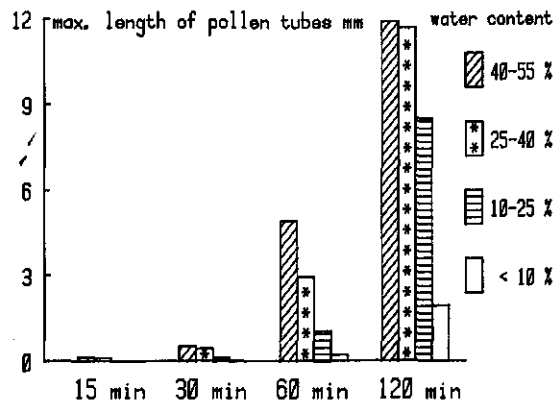
Pollen was collected from a single cross maize hybrid and was then dried to various water contents. 15-20 mm pieces of silk were pollinated with pollen under examination and were incubated for 15, 30, 60 and 120 min at 25 °C in petri dishes. After these periods the numbers of germinated pollen grains were counted and the maximum length of the pollen tubes was measured.

Results and discussion

A comparison of the germination of pollen grains with various water content showed that grains with high /40-55 per cent/ water content adhered to the stigma and germinated most rapidly and in greater numbers. Almost 60 min were required for germination of the pollen grains with low /10-25 per cent/ water contents.

With regard to the length of the pollen tubes, it was observed that pollen grains containing less than 25 per cent water developed considerably shorter pollen tubes during the period 60-120 min after pollination than those with higher water contents /Fig.1./.

Fig. 1.



It is reasonable to assume that is the most tolerant grains which survive the experimental treatment /drying/ without loss of their normal function.

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MICROTUBULAR CONFIGURATIONS DURING ENDOSPERM AND POLLEN DEVELOPMENT IN ZEA AND GASTERIA AS VISUALIZED BY IMMUNOFLUORESCENCE

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Summary

The arrangement of microtubular (MT) configurations was studied in differentiating cells to elucidate the role of the cytoskeleton during cytomorphogenesis. Endosperm from Zea mays and anthers from Gasteria verrucosa were fixed with aldehydes and embedded in polyethylene glycol (PEG) at various stages of development. Semi-thin sections were mounted on slides and processed for immunocytochemistry. The specificity of antisera was confirmed by proper control experiments.

In the developing maize endosperm a variety of MT arrangements was detected. During cell division microtubules were conspicuously labeled in spindles and phragmoplasts. At interphase, however, they were preferentially oriented along the cell membrane. In isodiametric cells the microtubules appeared in a criss-cross texture while in the elongated cells of the epidermal layer they were highly ordered into bundles arranged parallel to each other along the anticlinal cell walls.

Throughout the process of pollen development in Gasteria MTs were demonstrated in various concentrations and configurations. Their location at meiosis and pollen mitosis was well established. The shape of the generative cell proved to be determined and maintained by bundles of MTs.

Introduction

In recent years immunocytochemistry has been used frequently to study the arrangements and functions of microtubules (MTs) in various organisms (for surveys, see Osborn & Weber, 1982; Lloyd, 1982). Besides functioning during cell division in the formation of the spindle (McIntosh, 1984) their functioning in cell motility was stressed (Hyams, 1982). During interphase their main function has proved to be the forming of a cytoskeleton establishing and maintaining cell shape, both in animal and plant cells (see Brinkley et al., 1980; Marchant, 1982).

Plant tissues, however, often cause specific difficulties during preparation for immunocytochemistry as was discussed by Knox et al. (1980). Ensuring access of antibody to cellular sites of antigen seems to be the main problem. Indeed in maize endosperm the celluiose cell wall appeared to be a barrier to immunoglobulins (IgGs) and also during pollen development the callose cell wall and later on the sporopollenin cell wall impeded IgGs to penetrate protoplasts. This problem was initially overcome by enzymic digestion of the cell walls (Van Lammeren, 1982). The subcellular organization, however, changes during cell wall digestion because protoplasts round up and protease activity may interfere (Wick & Duniec, 1983).

To circumvent the problem, Wolosewick and De Mey (1982) embedded animal tissue in polyethylene glycol (PEG). This paper sets out to describe a modification of their method leading to a successful application for plant material. Microtubular arrangements will be shown using this technique in the study of developing maize endosperm and developing Gasteria pollen.

Materials and methods

Young caryopses of Zea mays L., strain A-188 (kindly provided by Dr. C.E. Green, Minnesota) were dissected in the greenhouse at various developmental stages. Sagittal sections of endosperm were fixed in a mixture of 3% depolymerised paraformaldehyde and 0.25% glutardialdehyde in phosphate buffered saline (PBS: 135 mM NaCl; 25 mM KCl; 1.5 mM KH_2PO_4; 8 mM Na_2HPO_4, pH 7.2) for 2 hours at room-temperature.

Anthers from immature flowers of the inflorescence of Gasteria verrucosa (Mill. H. Duval) were dissected and to improve penetration of the same fixative into the loculi the distal ends of the anthers were cut prior to fixation. The developmental stages of the cells in the loculi were determined for each flower by Nomarski optics.

After fixation tissues were subsequently rinsed in PBS for 30 minutes, treated with 15 mM $NaBH_4$ and 0.1 M NH_4Cl, both in PBS, for 15 minutes each and rinsed again in PBS for 30 minutes. Samples were washed in water and processed through a graded series of ethanol. Temperature was raised up to 55°C when ethanol 100% was replaced by a 1:1 mixture of ethanol : PEG 1500/4000 (w/w PEG 1500 : PEG 4000 = 2:1). After two hours of infiltration, the mixture was replaced by pure PEG 1500/-4000. After one hour samples were transferred into silicone moulds or gelatine capsules containing pure PEG 1500/4000 at 55°C. This temperature was maintained during another two hours. Then the heater was switched off to allow the PEG to cool down slowly. Semi-thin sections (5 µm) were prepared with a Kulzer Histoknife

(Kulzer, Friedrichsdorf) mounted on a LKB Ultrotome V. Serial sections were picked up with hanging drops of 40% PEG 6000 in water to permit wrinkles to disappear slowly. Then the sections were carefully tipped onto poly-L-lysine coated slides (poly-L-lysine, Sigma, MW > 70,000, 0.1% w/v in distilled water, pH 8.6) for adhesion. Slides were rinsed twice in PBS to remove the PEG prior to the application of antisera.

For immunocytochemistry, the first antiserum was raised in rabbit against tubulin purified from calf brain essentially according to Shelanski et al. (1973). The IgG fraction was isolated with column chromatography (DEAE Affi-gel Blue, Bio-rad). Sections were incubated at 37°C with 20 µl diluted antiserum (1:15 in PBS) or 20 µl IgG solution for one hour, subsequently rinsed in PBS and exposed to the secondary fluorochrome-labelled antibody (goat anti-rabbit IgG-FITC, Nordic, Tilburg) diluted 1:40 in PBS for one hour. All steps were at 37°C. Slides were rinsed in PBS for 1 hour and sections were embedded in Mowiol 4-88 (20% Mowiol 4-88, Hoechst, Frankfurt am Main, in 0.1 M Tris-HCl, pH 8.5).

Sections were observed with epifluorescence on a Leitz Ortholux microscope with Xenon 150 or HBO 100 W high-pressure mercury lamp illumination. The objectives used were Nikon Plan Achromate 50x oil (N.A. 0.50-0.85), Leitz Fluor 95x oil (N.A. 1.10-1.32) or Leitz 100x oil (N.A. 1.25).

Black and white images were recorded on Agfapan 400. Color slides were made on Kodac Ektachrome 400 and processed commercially.

Results

Technical aspects. Both the fixation and PEG embedding procedures appeared to be appropriate to detect MTs at various stages of the cell cycle. This not only holds for the endosperm and the developing pollen tested but for the embryo and carpels of maize and for root tips of maize, onion and radish as well (Van Lammeren, unpublished). With respect to immunolabeling both the first antiserum and the IgG fraction gave excellent visualization of MTs. However, serum dilutions less than 1:25 coincided with increasing aspecific labeling of the background. Two control experiments in which either the first antiserum was omitted or pre-immune serum was applied resulted in but a faint aspecific labeling of the cytoplasm while no MTs were to be detected. The first antiserum was stored for more than 2 years at -60°C without an apparent loss of binding capacity. The high temperature of 55°C during PEG embedding influenced neither the distribution of MTs nor the intensity of their labeling as was concluded from data obtained from unembedded FITC-labeled tissues.

The composition of the mixture of PEG with various molecular weights was determined experimentally and the combination used gave the best results during sectioning. A microtome was used to obtain reproducible section thickness which proved to be of particular importance for quantitative analysis and photography. Semi-thin sections did adhere well at poly-L-lysine coated slides and the cut single cells in the loculi of the anthers remained localized when PEG was washed away and slides were further processed. It should be taken into account that direct microscopic observation of the fluorescent cytoskeleton revealed far more details than can be shown on prints or slides because of the small depth of field and the limited resolving power of the photographical emulsion.

Microtubular configurations in the endosperm. Approximately nine days after pollination (DAP) several regions could be distinguished in the developing maize endosperm. At the base of the endosperm, near the chalaza, cells are elongated and densely cytoplasmic. The central region of the endosperm consists of isodiametric cells which often have large central vacuoles. The outer region of the endosperm consists of an epidermal layer with small, densely cytoplasmic cells.

Both in the epidermal layer and in the central region of the young endosperm, cell divisions were detected at low frequency. Mitotic spindles were observed and during cytokinesis various stages of phragmoplast development were seen. In interphase cells of the central region MTs were detected preferentially surrounding the nucleus and running criss-cross along the cell membrane (Fig. 1a).

When the caryopsis was about 15 DAP cells of the central region of the endosperm contained only few vacuoles but numerous plastids. Microtubules were still found running along the cell membrane but also throughout the central part of the cytoplasm. Interphase cells of the epidermis had a strikingly different configuration. Here, MTs were highly ordered into bundles which ran along the anticlinal cell walls parallel to the surface of the endosperm (Fig. 1b). In the nucellus tissue also two cell types were observed. The isodiametric cells showed a cortical mesh-work of MTs. The epidermal cells exhibited bundles of MTs as can be seen in Fig. 1c.

Microtubular configurations during pollen development. At the pre-meiotic stage of pollen development, cells of the sporogenous tissue

and young microspore mother cells of Gasteria are polyhedral and about isodiametric. The large nucleus occupies the central region and only few small vacuoles appear in the cytoplasm. Starting at this stage MTs were detected in various, but stage specific configurations. As is shown in Fig. 2a, microtubules in microspore mother cells are arranged in a criss-cross texture throughout the whole cytoplasm. At meiosis MTs were visualized in spindle and phragmoplast configurations (Figs. 2b and c).

Cell wall synthesis of pollen starts from early tetrad stage; when microspores are set free in the loculus they have a bilateral symmetry. Then microspores are elongated and the nucleus is situated near the cell wall opposite the colpus (Fig. 2d). Strands of MTs preferentially appear in between the nucleus and the colpus.

Cytoplasmic MTs disappear at pollen mitosis. The position of the fluorescent spindle and phragmoplast indicate the asymmetric plane of division (Fig. 2e). The newly formed generative cell is firstly attached to the intine, but as it differentiates, it becomes completely surrounded by the cytoplasm of the vegetative cell and subsequently elongates. Eventually the spindle-like cell merely consists of a nucleus surrounded by a thin layer of cytoplasm, which is enveloped by the plasma membrane. In the cytoplasm of the vegetative cell only a diffuse fluorescence was observed and MTs were rarely seen. However, in the generative cell striking fluorescent cables running from pole to pole indicate a highly ordered arrangement of MTs (Fig. 2f).

Discussion

Sectioning plant tissues embedded in a mixture of polyethylene glycol proved to be useful in overcoming the problem of the plant cell walls forming a barrier to IgGs in their penetration of the protoplasm. The antigenicity of tubulin structures appeared not to be altered compared to hand made sections. Moreover it was quite easy to study serial sections. Initial experiments directed towards the addition of PEG with a lower molecular weight which strongly improved the section quality. In pure PEG 4000 as used by Wolosewick and De Mey (1982) sections were too brittle. The use of the solidifying polyvinylalcohol Mowiol 4-88 (see Osborn & Weber, 1982) enabled us to manipulate the slides without risk of damaging the sections.

It is beyond the scope of this paper to describe and interpret the various MT configurations extensively. However, some striking features will be discussed.

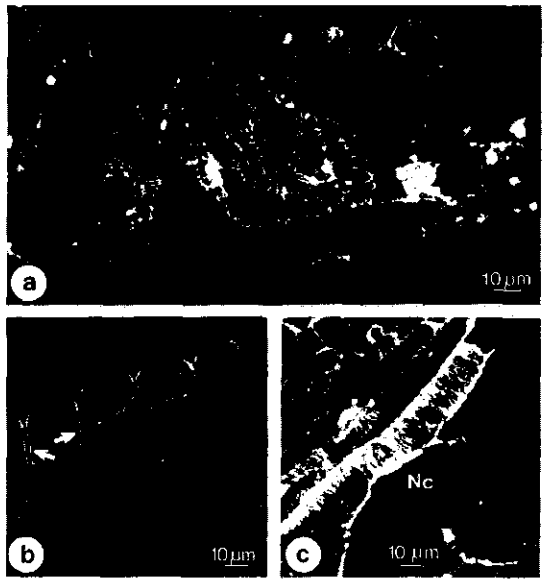

Fig. 1. Microtubular configurations in Zea mays.
a. Endosperm cells at the central region at interphase. Note the criss-cross texture of fluorescent MTs.
b. Epidermal endosperm cells with cortical bundles of MTs (arrows) at interphase.
c. Epidermal cells of the nucellus (Nc) with MTs along the anticlinal cell - walls. The MTs are oriented parallel to each other and perpendicular to the surface of the epidermis.

The cytoplasmic MTs of the interphase cells of maize endosperm appear to be organized in two different configurations, depending on the location of the cells. In the small epidermal cells they are mainly arranged into bundles which align the anticlinal cell walls whereas in isodiametric cells a cortical criss-cross texture of MTs appears to be most frequent. These findings are in agreement with the now widely accepted view that the asymmetric shape of plant cells is largely the result of the orientation in which cellulose microfibrils are deposited within their cell walls. During the establishment of the mature shape and size of cells, MTs appear to influence the pattern of cellulose deposition and thus MTs control indirectly the developing shape (Marchant, 1982; Traas et al., 1984). Spindle and phragmoplast MTs are not likely to interfere with the ultimate cell shape. The plane of division, however, is determined at mitosis. The position of the cell plate of epidermal endosperm cells is predominantly perpendicular to the outer surface, while the orientation is random in the central region.

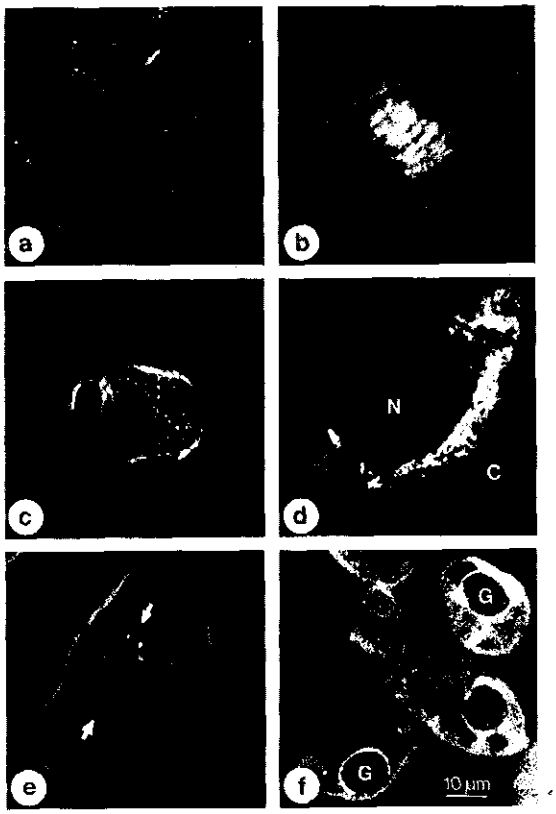

Fig. 2. Microtubular configurations in Gasteria verrucosa.

a. Microspore mother cells with fluorescent MTs in a criss-cross texture throughout the cytoplasm.
b. Diade stage with MTs radiating from the nuclei and forming the phragmoplast.
c. Tetrade stage. Note MTs radiating from the nuclei towards newly formed cell walls.
d. Microspores showing MTs preferentially in between the nucleus (N) and the colpus (C).
e. First pollen mitosis at metaphase with spindle MTs indicating the asymmetric plane of cell division (arrows).
f. Pollen grains with generative cells (G) in transverse section. Bundles of cytoplasmic MTs surround the unstained nucleus of the generative cell.

In Gasteria only the isodiametric microspore mother cells exhibited the criss-cross texture throughout the cytoplasm. All other cell types showed altered MT configurations.

In the diade (Fig. 2b) and tetrad (Fig. 2c) stage MTs were observed extending from the nuclear envelope to the plasma membrane as was also reported recently for very young microspores in Lilium by Dickinson and Sheldon (1984). These authors proposed that the MTs play part in orientation of the nucleus and in transport of materials to the cell surface. In our observations your microspores of Gasteria have a length axis while they are still encapsulated in the callose wall and the MTs that run throughout the cytoplasm sometimes have a pole-to-pole orientation. The same pole-to-pole orientation is most striking after pollen mitosis in the spindle-shaped generative cell in which several bundles of MTs appear. The morphogenetic function of these MT bundles was already stressed by Sanger and Jackson (1971) who reported that the elongation of generative cells of Haemanthus katherinae is accompanied by the development of a peripheral array of MTs which are oriented parallel to the long axis of the cell. When they destroyed MTs generative cells partially reverted to their former spheroidal shape. Elongated generative cells with bundles of MTs were shown in a variety of plant species (Cresti et al., 1984) and indeed for Gasteria their presence was also recorded by electron microscopy (Keijzer, this volume). In free microspores which have a colpus and a pollen wall, MTs were not equally distributed throughout the cytoplasm but merely restricted to the colpus region (Fig. 2d). This probably indicates the conservation of the cell shape at the colpus zone by the MTs.

The data obtained indicate a coincidence of a proper MT configuration with a certain cell shape. We suggest that MTs influence the cell shape as follows: MTs in a criss-cross texture only permit cells to differentiate isodiametrically while MTs in parallel arrays direct towards longitudinal cell growth or shape. In all it can be stated that the PEG embedding procedure as modified by us was suited to study MT configurations and their function in the endosperm cells of maize and in developing pollen of Gasteria. More experimental conditions interfering MT arrangements will be used in further studies.

Acknowledgements

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GROWTH OF TOBACCO POLLEN TUBES IN VITRO; EFFECTS OF DRUGS INTERFERING WITH THE CYTOSKELETON

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Abstract

The presence of the drugs colchicine, deuterium oxide, cytochalasin B and phalloïdin inhibited germination and tube growth of Nicotiana tabacum pollen in vitro. The pollen tubes did not loose their polarity in the presence of the drugs. The effect on the microtubule skeleton was examined using immunofluorescence. In the presence of colchicine no or almost no microtubule skeletons could be observed. If deuterium oxide, phalloïdin or cytochalasin B were present in the culture medium, microtubule skeletons, though distorted, remained present

Introduction

Pollen tubes are able to direct their growth, probably guided by environmental factors.

They are growing at their tip only and thus show a distinct polarity (Sievers and Schnepf, 1981). Therefore pollen tubes must have at their disposal a growth directing system that is probably located in or near the tip. The main structural element of plant cells, the cell wall, cannot be considered such a system since the wall is a passive element only. The pre-eminent morphogenetic element in plant cells is probably, like in animal cells, the cytoskeleton, a coherent network of various elements, mainly microtubules and microfilaments (for reviews see a.o.: Lloyd, 1984 and Robards, 1983). The presence of these elements in pollen tubes has been shown already in 1972 by Franke and coworkers. Microfilaments (Picton and Steer, 1982) and, due to their presumptive role in cell wall deposition, especially microtubules (Lloyd, 1984) have been supposed to be involved in plant cell morphogenesis. Various drugs are known to interfere with the cytoskeleton. We used phalloïdin and cytochalasin B, both known to interact with microfilaments and to inhibit plasma streaming and colchicine and deuterium oxide (D_2O) respectively depolymerizing and stabilizing microtubules (reviews: Robards, 1983, Sato et al., 1982). Here we present not only the effects on germination and pollen tube growth of these drugs, but also their effect on microtubule skeletons using immunofluorescence (Wick et al., 1981), recently adapted to pollen tubes (Derksen et al. manuscript in prep.).

Material and methods

Culture conditions

Pollen from Nicotiana tabacum (L) cv Samsun were collected and stored as described by Kroh and Knuiman (1982).

Pollen (10 mg) were germinated and grown in sucrose/borate medium (2 ml) for several hours under standardization conditions (Kroh and Knuiman, 1982). Samples were taken at 2 and 3 hours after start of the incubation. Representative area's were photographed. Germination and tube length were determined from micrographs with suitable magnifications Germination was scored at least 1000 pollen grains. Pollen tube length was determined from at least 200 germinated pollen using a Kontron videoplan computer. Drugs were added to the medium in various concentrations prior to the incubation. All experiments were carried out in duplo.

Immunofluorescence

Immunofluorescence was carried out principally as described by Wick et al. (1981) with some modifications (Derksen et al. manuscript in prep.). The primary antibody was a rat monoclonal anti-tubulin (Mas 077; Sera labs), the second antibody was a rabbit Fitc labelled anti-rat Igg (Nordic BV). Preprarations were examined in a Leitz orthoplan microscope equipped for immunofluorescence, using a Leitz 100x water immersion lens. Photographs were made with a Leitz vario-orthomat combination on either Fujichrome 400 or Agfapan 400 professional film.

Results

Germination

In preliminary experiments the curves representing germination were sigmoïd with a linear range between 1 and 3 hours after start of the incubation. Though experiments carried out in duplo showed almost identical results, larger differences occured between independent experiments. Therefore, experiments were not pooled. The effect of various concentrations of colchicine, D_2O, cytochalasin B and phalloïdin of a representative experiment is shown in fig. 1.

Figure 1. Inhibition of germination by colchichine, D_2O, cytochalasin B and phalloïdin.
explanation: see text.

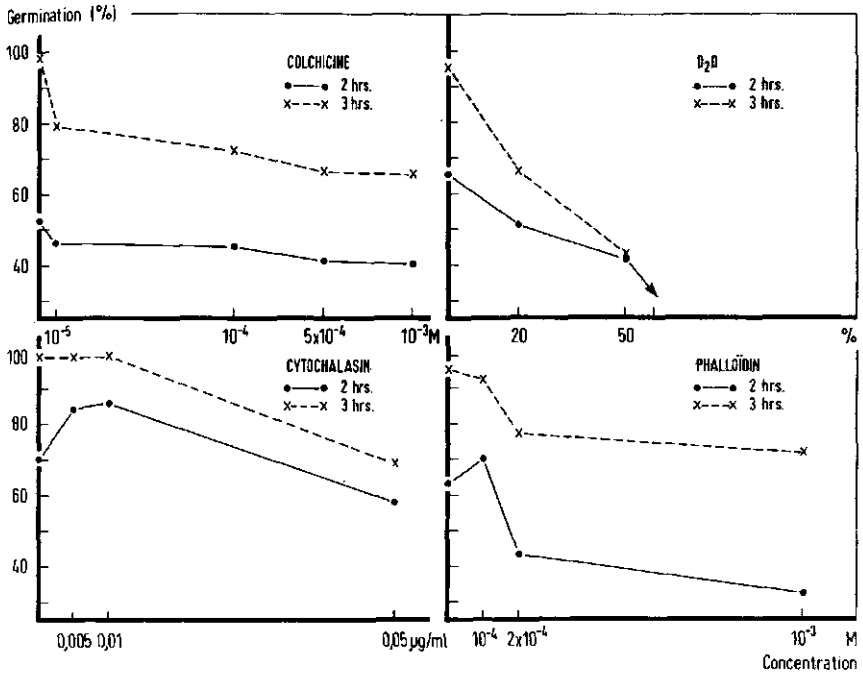
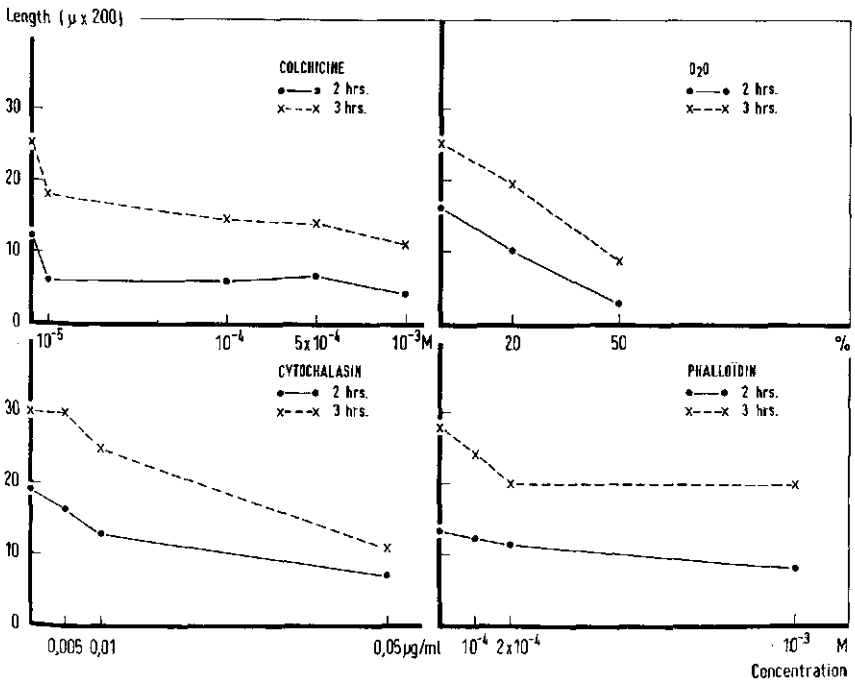


Figure 2. Inhibition of pollen tube growth by colchicine, D_2O, cytochalasin B and phalloïdin.
explanation: see text.



The effect of colchicine shown at a concentration of 10^{-5}M is hardly stronger at increasing concentrations. D_2O strongly inhibited germination and almost complete inhibition is reached at concentrations somewhat higher than 50%. Both phalloïdin and cytochalasin at low concentrations did not inhibit germination and even showed slight stimulation. However, this effect was not consistent and probably may not be significant. Higher concentrations of these drugs were clearly inhibiting. In control experiments the germination percentage was almost 100% after 3 hours. If an inhibitory effect of a drug was recorded also the maximum germination percentage became lower than 100%. The maximum germination percentage was still reached then after about 3 hours.

Pollen tube growth

In representing our results we chosed to use the mean value of the pollen tube lengths as an arbitrary measure. All drugs clearly inhibited pollen tube growth (fig. 2). Inhibition of pollen tube growth by colchicine and D_2O showed a similar course as the inhibition of germination (compare figs 1 and 2). The effects of pollen tube growth of cytochalasin and phalloïdin occurred at lower concentrations than the effects on pollen germination (compare figs 1 and 2). The effect of the drugs on tube growth seem to be more pronounced than on germination.

Effects of drugs on microtubule skeleton.

In immunofluoresence preparations parallel strands of microtubules were visible throughout the cell with mainly axial orientations (fig. 3A and table 1), but also helices with a right turn (S helix) occurred.
The presence of the drugs caused marked differences in microtubule organization (fig. 3) and are described below for the different drugs. The concentrations used were: 10^{-4}M colchicine, 50% D_2O, 10^{-3}M phalloïdin and 0.002 µgr/ml cytochalasin B.

In the presence of colchicine pollen tubes generally did not show numbers of parellel strands of microtubules anymore. Only few individual strands, mainly axially oriented were present (fig. 3B), but occasionally arrays of microtubules could be observed. The tubes showed a high background. The strands that remained were less strictly oriented and less straight than in the controls (compare figs 1A and B). The diameters of the pollen tubes were irregular and larger than in the control cells, sometimes a knob at the tip of the tube occurred. As judged from phase contrast microscopy, the organization of the plasma and the plasma streaming was disturbed.

D_2O cuased changes in the microtubule skeleton: more and shorter strands that were less strict oriented and less straight than in the control cells (figs 1A and C and table 1).

Figure 3. Immunofluorescence of microtubule

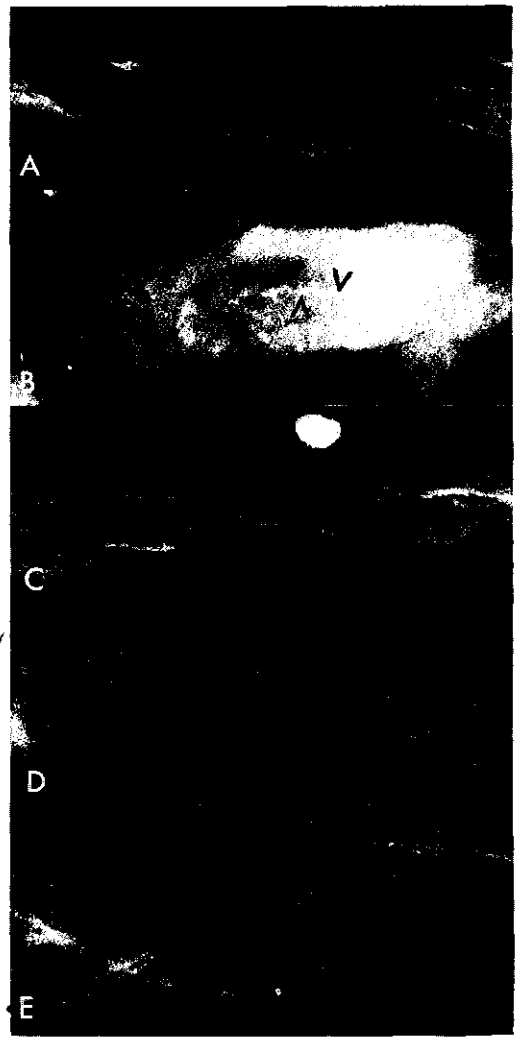


Fig. 3. Immunofluorescence of microtubules. A: untreated; B: colchicine, C: D_2O, D: phalloïdin, and E: cytochalasin treated. Pointer microtubules. All magnifications: 3000 x.

The main orientations remained axial but mor and steeper helices occurred (see table 1). Near the tip of the tube fewer and less strictly oriented microtubules strands were visible. Organization of cytoplasm and plasm streaming were disturbed. The diameter of th tubes was irregular, mostly larger than in the controls and endknobs occurred regularly Often, about 50%, the pollen tubes were twisted in a right turn (S helix; see figs 3A and C).

Phalloïdin did not clearly effect the number of microtubule strands, but they often appeared to occur in bundles. The strands we less strictly oriented, especially near the tip, but they remained straight (fig. 3D).

Crossing strands were observed (fig. 3D). More and steeper helices occurred (see: table 1). Organization of the cytoplasm and plasma streaming seemed to be normal.

The effects of cytochalasin were similar to those of phalloïdin but much more pronounced (fig 3E and table 1). Clear wavelike patterns with many crossing strands (fig. 3E) were seen. Also bundles of non wavelike strands and spirals were observed. The density of the microtubule strands is obviously higher than in the control cells. Near the tip the microtubule skeleton is less well organized and sometimes the helices in the tip are steeper than in the rest of the cell. Occasionally, pollen tubes appeared to be twisted in a S helix. No obvious disturbances in the organization of the cytoplasm could be observed out plasma streaming seemed to be irregular.

None of the drugs caused great changes in the microtubule strands of the generative cell. However, colchicine, but especially D_2O and cytochalasin B caused a somewhat wavelike pattern in the microtubule skeleton of the generative cells.

Table 1. Main direction of the microtubules.

	range	mode	mean	N
control	0^o–45^o	0^o(60%)	4^o	95(-1)
D_2O	0^o–85^o	0^o(20%)	21^o	118(-17)
cytochalasin	0^o–70^o	0^o(32%)	14^o	121(-16)
phalloïdin	0^o–75^o	0^o(23%)	12^o	129(-11)

Table 1. The angle of microtubule skeletons with the cell axis measured at intervals of 5^o. Between parenthesis in column 2: percentage of microtubule skeleton with an angle within the mode. N: number of cells measured; number of cells from which no main orientation could be determined in parenthesis.

Discussion

All drugs showed significant effects on germination, growth and microtubule organization of pollen. Cells growing in the presence of phalloïdin or cytochalasin still showed plasma streaming, indicating a resistance of the microfilaments or a failure of the cell to take up these drugs. The inhibitory effect of D_2O is striking and is probably related to its 'freezing' effect. However, the disturbance of growth and plasma organization indicates different effects on morphogenesis leaving their interpretation uncertain (see for discussion: Sato et al., 1982). Though colchicine inhibited germination and growth, still numerous pollen could grow in its presence. These pollen tubes hardly showed any coherent microtubule skeletons, although microtubules remained always present, may be protected by MAPs. It cannot be excluded that the fluorescence background in colchicine treated pollen tubes is caused by numerous small, non-cortical microtubules that persist in the cell. In various types of cells growing at their tip colchicine causes loss of polarity (for discussion see a.o.: Mizukami and Wada, 1983). However, for pollen tubes it was concluded (Franke et al., 1972) that microtubules would play only a minor role in morphogenesis. In our material no loss of polarity was observed.

Increased microtubule densities after cytochalasin treatment, as observed here have also been reported for root hairs (Seagull and Heath, 1980) and associations between microtubules and microfilaments are regularly observed (see also for refs.: Traas et al., manuscript submitted). Such observations indicate a narrow relationship between the microtubular and microfilamental system. Thus it might well be that both systems act together, one system taking over when the other system fails. Thus cells might still be able to maintain polarity when one of the systems is non-operational. As proposed recently by Lloyd (1984) the occurrence of steeper helices after inhibition may be related to a lower growth rate.

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FINE STRUCTURAL RESEARCH ON GERMINATING LILY POLLEN TUBES IN VITRO AND IN VIVO

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Summary

In vitro, the percentage of germination and tube length of the pollen grains in Lilium longiflorum are best when the grains are cultured in a medium containing boron. The thickness of the tube wall of the pollen grains cultured in a sucrose medium containing calcium is the greatest, and they are thinest in a medium containing boron. The pollen tube walls in compatible and incompatible styles are thin, while those in incompatible stylar exudate are very thick. Structural differences are observed on the surface of the stylar canal cells between compatible and incompatible styles. The cytoplasm of the pollen tubes in the incompatible style at 96h after pollination degenerates.
Keywords: pollen tube, compatible, incompatible, boron, calcium.

Introduction

Pollen germination and pollen tube elongation are greatly stimulated by calcium and boron contained in the culture media used. The presence of boron has been reported to help the translocation of sugar(Gauch & Dugger,1953), and play a definite role in pectic synthesis in germinating pollen-(Stanley & Loewus, 1964). In addition, the requirement for Ca^{2+} ions during membrane fusion and the release of the secretory product is well established(Morris & Northcote, 1977; Koter et al., 1978; Baydoun & Northcote,1980).

We report here the fine structural differences of the germinated pollen tubes in Lilium longiflorum, which have different physiological conditions depending on the presence or absence of sucrose in the medium and depending on the presence or absence of calcium and/or boron. In the incompatible lily style, pollen tube elongation ceases after growth has occured for half the length of the style(Ascher & Peloquin,1966). A comparison of the fine structure of pollen tubes and conductive tissues in the style between compatible and incompatible conditions was also made by the transmission and scanning electron microscopes.

For materials, pollen grains and pistils of Lilium longiflorum Georgia were used. The compatible pollen grains were obtained from another variety, and they were tested for their fertilitly on the flowers of Lilium longiflorum Georgia. For the test of incompatibility, a pistil, pollen grains and exudate were obtained from the same flower.

For the experiments in vitro, 5 mg of pollen grains were shaken at 28C for 3h in a small Erlenmeyer's flask with 1 ml of the medium, which was composed of distilled water, 10% sucrose, 10% sucrose and 0.03% $Ca(NO_3)_2$, 10% sucrose and 0.001% H_3BO_3, 0.03% $Ca(NO_3)_2$ and 0.001% H_3BO_3 in 10% sucrose, Dickinson's medium(1968) contained 0.03% $Ca(NO_3)_2$, 0.001% H_3BO_3 and 0.01%KNO_3 in 0.322M pentaerythritol, respectively. In addition, pollen grains were germinated in the compatible and incompatible exudates. Calcium ion(Picton & Steer,1983) and boric acid(Stanley & Lichtenberg,1963; Vasil,1964) concentrations are optimum. The elongated pollen tubes were collected from the canal of the compatible or incompatible styles 48, 72, 96 hours after pollination, and the germinated pollen grains cultured in vitro in the media described above, were fixed with 3% glutaraldehyde in cacodylate buffer(pH 7.0) for 3h, postfixed with 1% osmium tetroxide for 3h, dehydrated in an ethanol series at 4 C, embedded in low viscosity epoxy resin, sectioned with a diamond knife on an LKB-Ultratome and observed with a JEM-100B TEM. Uranyl acetate and Raynolds' lead citrate(1963) were used for staining. For scanning electron microscopy, pollinated compatible and incompatible styles were fixed with 3% glutaraldehyde for 3h, rinsed and post-fixed with 1% osmium tetroxide for 3h, dehydrated with alcohol, critical-point dried, coated with carbon and gold and examined in a JEM-15 SEM at 15kV.

Results and discussion

The pollen grains of Lilium longiflorum cultivated in the media which are described in Table 1, absorb water from the culture medium and germinate. The percentage of germination, pollen tube length and the thickness of the tube wall of germinated pollen grains in each culture medium are shown in Table 1.

The percentage of germination in the sucrose medium and in sucrose medium plus calcium and/or boron, is higher than that obtained in Dickinson's medium. The tube length in the sucrose medium plus boron, is longer than that in the medium lacking boron, and is similar to that in the exudate. In general, the percentage of germination and tube length of the pollen grains in the medium containning boron are

the best in vitro conditions.

All of the pollen tube walls showed in Table 1 have double layers. The thickness of the pollen tube wall in the sucrose plus calcium medium is the greatest among those in the artificial media. On the other hand, the tube wall is thin in the media containing boron. It was suggested that calcium combined with pectin substances made cell wall components(Ito & Fujiwara,1968), promoted pollen tube growth by stimulating vesicle fusion at the tip(Herth,1978), and that boron helped the translocation of sugar and pectic synthesis. From our results, it is assumed that calcium promotes the vesicle fusion at the tip more than boron.

In stigmatic exudates, germination is better in the incompatible stylar material, and growth is better in the compatible material. While the thickness of the pollen tube wall in the compatible stylar exudate, is not great, and resembles the results obtained in 10 % sucrose medium, the tube wall is very thick in the incompatible stylar exudate. It is supposed that the wall formation is affected by some aspect of the incompatiblity. There were no ultrastructural difference among the cell organelles in the pollen tubes in either the compatible or incompatible exudates, except for the thickness of the tube wall. On the other hand, the thickness of the tube wall in the style(in vivo) is very thin; the thickness of the walls in compatible styles is similar to the results obtained in the medium containing boron, and the thickness of the walls in incompatible styles is similar to the results obtained in Dickinson's medium.

It is supposed that the tissues of the style offer protection to the developing tube so that thick tube walls are not formed and in addition a high rate of elongation mitigates against thickness.

Ultrastructural observation of the pollen grain cultured in 10% sucrose medium, show that the Golgi bodies produce numerous Golgi vesicles, the number of amyloplasts increases, and starch grains in amyloplasts and lipid bodies are converted to polysaccharide particles(p-particles,Miki-Hirosige & Nakamura,1982) which sometimes enclose fibrous substances. The situations of the cell organelles in the pollen tubes cultured in the used media except water medium, are almost the same. The outer layer of the tube wall is formed at the pollen tube tip region by Golgi vesicles, and the inner layer is formed at the lateral tube wall region from p-particles(Miki-Hirosige & Nakamura,1982). Although uniform large vesicles(ca.400 nm in diameter) which make the pollen tube wall, can be seen in the tip region of the pollen tubes cultured in distilled water, small vesicles(ca. 150 nm in diameter) derived from the Golgi body and large vesicles(ca. 500-1000 nm in diameter) which are made by the fusion of small vesicles(these vesicles make the pollen tube wall), are seen in tubes cultured in other media. The number, size and distribution of vesicles in the pollen tubes in the medium containning calcium and those in the medium containning boron, appeared to be the same. Amyloplasts and lipid bodies which are the reserve substances in the pollen grain, are scarce in the pollen tube cultured in water

Table 1. Percentage of pollen germination, tube length, and tube wall thickness of the pollen grain of Lilium longiflorum in artificial culture media, exudates and styles.

	CULTURE MEDIUM	% of germination	Tube length(mm)	Tube wall thickness(nm)
ARTIFICIAL	Water	33.4-41.3	1.23±0.42	550
	10% sucrose	76.7-81.7	7.79±2.43	180
	10% sucrose 0.03% $Ca(NO_3)_2$	65.7-71.6	8.48±1.64	600
	10% sucrose 0.03% $Ca(NO_3)_2$ 0.001% H_3BO_3	71.8-77.6	12.04±1.98	150
	10% sucrose 0.001% H_3BO_3	82.9-87.8	19.04±2.04	110
	0.322M pentaerythritol 0.03% $Ca(NO_3)_2$ 0.001% H_3BO_3 0.01% KNO_3 (Dickinson's medium,1968)	49.4-57.9	8.38±1.97	190
EXUDATE	Compatible	87.1-91.2	17.06±2.13	170
	Incompatible	93.8-97.1	12.76±1.80	880
		after pollination	48h	96h
In compatible style			110	120
In incompatible style			120	250

and Dickinson's medium, but they are numerous in those cultured in media containing sucrose. In the pollen tube cultured in water, very thick tube wall, numerous rough ERs(rERs) and few Golgi bodies can be seen.

In the pollen tubes which develop in the compatible style, numerous vesicles and rERs are seen. Cresti et al(1979) made the same observation in Prunus avium. In our experiment, it is observed that the numbers of the vesicles which contain fibrous materials and form the pollen tube wall, and rERs are much greater in the pollen tubes grown in the compatible style than those found in tubes grown in the artificial media and exudates. Many rERs are associated with many vesicles in the in vivo situation, but it is not clear that the rERs are making the vesicles or secreting something into them. The cytoplasmic fine structure of the pollen tubes growing in incompatible styles is normal until 48h after pollination, but at 96h and after, degeneration occurs. Cresti et al.(1980) also reported that no differences were observed in vivo between pollen tubes of Lycopersicum peruvianum growing in compatible and incompatible styles during activation and organization, until 4h and 30 min after pollination, while Dickinson & Lawson(1975) reported that differences become evident from a very early stage in Oenothera organensis. In the style, secreted materials from the stylar canal cells are observed on the surface of the conductive canal tissue. The amount of the secreted materials from the incompatible tissue is less than those from the compatible one. Presumably the pollen tubes grown in the incompatible style are affected with some substances secreted from the canal tissue of the style, and degenerate. Most probably the entire pollen tube in the incompatible style is damaged uniformaly. Furthermore, a new layer(Miki-Hirosige & Nakamura,1982) has been found to form between the cytoplasmic membrane and the intine layer 30 min after water absorption. This layer protrudes from the aperture and develops into the pollen tube wall, therefore, we wish to propose the term "germinative layer" for this new layer.

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MEMBRANE-STRUCTURE AND EVENTS IN NON-PLASMOLYZED AND PLASMOLYZED TOBACCO POLLEN TUBES: A FREEZE-FRACTURE STUDY

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Introduction

The outer layer of the tobacco pollen tube wall (primary wall) is formed by the growing pollen tube tip, whereas the inner wall layer (secondary wall) and the plugs are deposited by the non-growing part of the tube. The secondary wall and the plugs contain callose in addition to pectin, hemicellulose and cellulose (Kroh & Knuiman, 1982).

Plasmolyzed tobacco pollen tubes form a cap of wall material (with cellulose microfibrils) between the retracted protoplast and the tube tip (Kudlicka et al., 1981). Membrane-bound vesicles and irregular membrane systems were observed just outside the plasma membrane but also within the cap material. The membraneous structures were assumed to play a role in exocytosis and reduction of the plasma membrane (Kroh et al., 1983). In order to test this assumption we carried out a freeze-fracture study on non-plasmolyzed and plasmolyzed tobacco pollen tubes.

Results and conclusions

Exocytosis

Polysaccharides for the matrix material of tube wall, plugs and wall cap may be extruded

A. by fusion of secretory vesicles with the plasma membrane and incorporation of the vesicle membrane into the extending plasma membrane,

B. by membrane-bound vesicles via invaginations of the plasma membrane; no extension of the plasma membrane occurs then.

The formation of the primary wall may occur predominantly in the first manner. The formation of the secondary wall, the plugs and the wall cap of plasmolyzed pollen tubes possibly occur in the second way.

Hexagonal arrays of particles

Hexagonal arrays of particles were observed in the plasmatic fracture faces of more than 50% of both non-plasmolyzed/pollen tubes. In few cases of non-plasmolyzed pollen tubes small hexagonal arrays of particles were observed together with imprints of microfibrils of the tube wall. For these cases it can be excluded that the hexagonal patterns and plasmolyzed are the result of distortions of the plasma membrane caused e.g. by a loss in turgidity of the tube.

Reduction of plasma membrane

During the formation of the secondary wall and the plugs in non-plasmolyzed pollen tubes and of the wall cap in plasmolyzed tubes a reduction in surface of the plasma membrane occurs. This reduction is apparently achieved by vesiculation and often also by strong invaginations of the plasma membrane with a subsequent outward detachment of vesicles and membrane pieces.

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ULTRASTRUCTURE OF NICOTIANA ALATA AND PETUNIA HYBRIDA POLLEN TUBES GROWN IN SEMI-VITRO CONDITIONS.

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Research on pollination and fertilization "in vitro" could be important for plant breeding as it could allow the functional characterization and selection of various types of pollen (Tilton & Russell, 1984). Similarly pollen tube growth in "semi-vitro" conditions could be used for the same purposes with the advantage of quick results and the possibility of relating tube growth rate and ultrastructural features. In order to test this hypothesis we have started a number of experiments especially in order to find the most suitable techniques.

Styles of N. alata and P. hybrida (self and cross-pollination) were cut in two equal parts 18 h after pollination and the samples with the stigmas were transferred to the culture medium (Brewbaker & Kowach, 1964) under controlled conditions.

After compatible pollination the pollen tubes of N. alata and P. hybrida grow out of the style and successively developed in the culture medium. After self-incompatible pollination no pollen tube grows out of the style. Both in N. alata and P. hybrida the same cytological zonation was observed as already described for pollen tubes of Nicotiana grown "in vitro" (Cresti et al., 1984) and Petunia grown "in vivo" (Cresti et al., 1979).

Nicotiana alata (Fig. 1): in comparison to the tubes grown "in vitro" the tubes in "semi vitro" show the presence, especially in the sub-apical zone and nuclear zone, of many lipidic bodies and concentric configurations of rough endoplasmic reticulum (CER); sometimes autophagic vacuoles are present and surround the lipidic bodies. These morphological aspects may be interpreted as cessation of protein synthesis (CER) or as the beginning of degeneration (lipid formation) and thereby arrest of tube growth.

In Petunia hybrida pollen tubes (Fig. 2) the cytoplasm shows the presence of numerous lipid bodies also, but RER concentric configurations were not observed while plastids containing a single starch granule were frequent. In comparison to what was described "in vivo" (Cresti et al., 1979), the callosic tube wall in "semi vitro" is slightly undulated.

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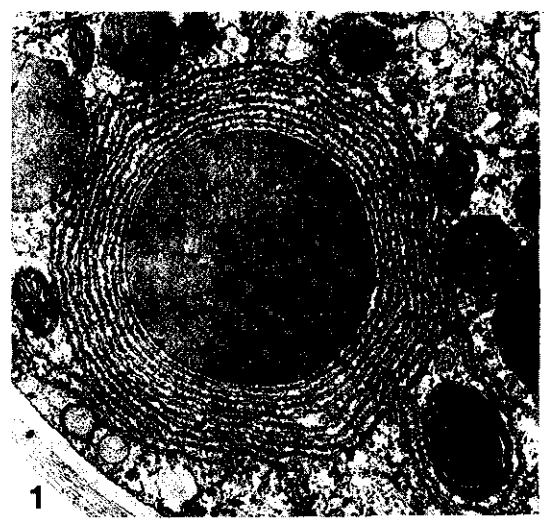

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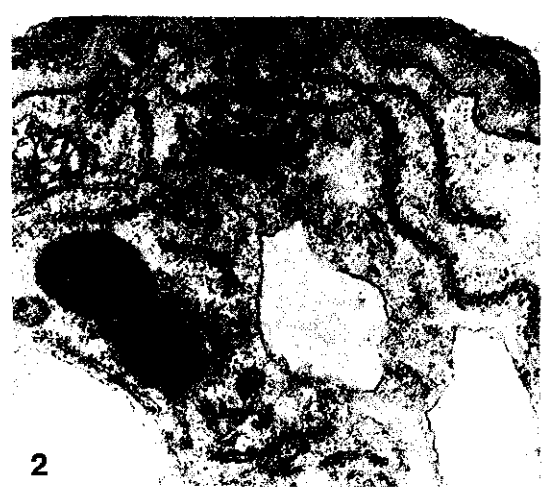

2

USE OF FLUOROCHROMES IN POLLEN BIOLOGY

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Summary

The fluorescent DNA probes ethidium bromide or Hoechst 33258, when used in conjunction with the aniline blue fluorochrome (sirofluor) which stains the callose component of pollen tube walls, allow location of nuclei within pollen tubes and permit discrimination between the tube nuclei and those of the background pistil tissue.

Introduction

Fertilization depends on precise behaviour and migration of pollen nuclei in the pollen tube as it traverses the pistil. Single-staining techniques used to locate nuclei have been of limited use for the study of pollination, owing to the length and narrow diameter of the tubes, together with both the obscuring density of the grain and tube walls and the similarity of pollen tubes to narrow, elongate cells in the transmitting tract of many types of pistil. Here we report on techniques for visualising pollen tube nuclei, and for selective double staining to contrast nuclei against a differentially stained pollen tube wall within the pistil.

Staining procedures

Ethidium bromide (EB) and Hoechst 33258 (H33258) were used as vital stains or applied after fixation with 1:3 acetic acid: ethanol. They were employed singly or with sirofluor (purified fluorochrome from aniline blue stain. Evans & Hoyne 1982. Commercial release pending). Pollen and *in vitro* grown tubes were treated with stains 5-10 min. on microscope slides and whole pistils for 1-7 hours in small vials. Osmotic adjustment of stains was made only for vital prestaining of pollen before pollination, where a substantial period of viability was required before observation. (For further details see Hough *et al.* 1984).

For single staining with EB the stain was applied as a 0.01% aqueous solution. For double staining with sirofluor the stains were applied together as an aqueous mixture of 3 parts 0.01% EB: 1 part sirofluor (0.25 mg/ml). For single staining with H33258 the stain was applied in aqueous solution (20 µg/ml). For double staining with sirofluor the stains were applied as a mixture of 1 part H33258 (40 µg/ml) : 1 part sirofluor (0.25 mg/ml). For vital pre-staining for *in vivo* pollination studies, fresh pollen was hydrated 5 minutes before pollination in either EB 0.01% in 18% sucrose or H33258 20 µg/ml in 18% sucrose. At various times after pollintion pistils were fixed, cleared and stained in sirofluor (0.25 mg/ml).

Observations were made with Zeiss epifluorescence optics on materials mounted in stain or water. Specimens stained with EB were viewed using blue-violet excitation (FITC system) and specimens stained with H33258 were viewed with UV excitation.

Results and discussion

Double staining with the callose stain, sirofluor, in combination with either of the fluorescent DNA probes, ethidium bromide (EB) or Hoechst 33258 (H33258), allowed visualisation of all pollen or pollen tube nuclei and discrimination not only between vegetative and generative or sperm nuclei, but also between nuclei within the pollen tube and those of the pistil tissue after pollination. Under blue-violet excitation with EB plus sirofluor, orange nuclei were enclosed by yellow green tube walls. Under UV excitation with H33258 plus sirofluor, blue nuclei were contrasted against light yellow tube walls. For ungerminated pollen grains and *in vitro* grown tubes, definition of nuclei was better after vital staining than after fixation. A major advantage of sirofluor over decolorised aniline blue is that it will stain at neutral pH and hence can be used for vital staining. Pre-pollination vital staining with EB or H33258 followed by sirofluor staining after pistil fixation, allowed visualisation of pollen tube nuclei against unstained pistil tissue at least in the early stages of tube growth within the pistil.

The techniques described are potentially suitable for monitoring nuclear behaviour during pollen tube growth, and possibly during the fertilization process itself with further development of pre-pollination vital staining procedures.

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STRESS RELATED STRUCTURAL MODIFICATIONS IN THE POLLEN OF DATURA

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(The described experiments have been done in the laboratories of Cytologie expérimentale et Morphogenèse végétale, Université de Paris VI and of Biologie de la reproduction des végétaux supérieurs, Université de Paris VII)

Under certain in vitro conditions, the microspores of a number of Angiosperm species can be induced to an embryogenic developmental pathway to produce sporophytes. The key to the formation of a greater number of microspore-derived sporophytes therefore lies in the means of modifying the normal microspore development so that embryos or calli differentiate into plants. A number of factors controlling the deviation from a gametophytic to a sporophytic pathway have already been elucidate: among these, cold treatment and centrifugation have been shown to increase androgenic potentiality to a large extend especially in Datura (1, 2, 3, 4). In the present study we have made observations on the ultrastructural changes occuring in Datura anthers (in both microspores and somatic tissues) caused by a cold treatment (3°C for 48h) or a centrifugation (40 g for 5 min or 150 g for 5 min).

Percentages of embryogenic microspores.

Dead pollen grains and young embryos were counted in the anthers after 11 days of culture. Statistical studies of the results show that the treatments did not increase the percentage of androgenic microspores when applied 1-2 days before the first haploid mitosis. However when applied 1-2 days after this division the same treatment greatly enhanced the embryogenic potentialities of the pollen grains. After mitosis chilling flower buds increased the number of androgenic pollen grains five fold; centrifugating the anthers for 5 min at 150 g before culture increased six fold the number of embryogenic pollen grains; but the best result was obtained by the association of the 2 treatments which increased 12 folds the yield of embryos (8.6 % androgenic pollen grains).

Structural changes..

The general pattern of ultrastructural changes by the above treatments are:
- a modification in the pollen wall. The midified exine shows sole slighly crumbled and split endexine.
- a rapid disorganization of the tapetum. This give rise to a mass of cytoplasm which infiltrated between the microspores.
- the formation of periodic structures in vacuoles and apertural intine of some pollen grains.
- in somatic tissues of the anthers, many cells seem damaged and starch grains in the plastids begin to dissappear or to reduce.

These results suggest that the efficiency of the treatments could be due to a delay in first haploid mitosis and/or an increase in the percentage of viability of the androgenic pollen grains.

On an other hand, the structural changes, in the pollen wall and in the tapetum, may modify the biological environment of the microspores and the exchanges between this environment and the pollen in a way favourable to androgenesis.

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LILIUM LONGIFLORUM POLLEN GERMINATION AND TUBE ELONGATION AS MARKERS FOR SULFUR DIOXIDE

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Summary

Atmospheric sulfur dioxide (SO_2) is an air-pollutant requiring surveillance as it impairs human health and promotes crop damage at certain levels. This surveillance has included attempts to utilize both pollen germination (G) and tube elongation (TE) as air-pollutant indicators. Here, we report SO_2-induced alterations of Lilium pollen G and TE.

Introduction

While a variety of plants has been surveyed for their assay of air-pollutants (Kellerher & Feder, 1978), few investigations have dealt with the possibility that pollen G and/or TE could serve to bioassay SO_2. Here, we report SO_2 -induced alterations in Lilium pollen G and TE.

Materials and Methods

Fumigation conditions - Whole anthers (stored 4 C, 1-2 months) with adhering Lilium longiflorum, cv. 'Ace' pollen were fumigated with either 2.5 (4 hr) or 10.0 (10 hr) ppm SO_2 within a Plexiglass chamber and the SO_2 quantified as in Erickson & Dashek (1982).

Germination conditions - Twenty mg fr wt lots of both fumigated and unfumigated pollen were sown in sterile Petri dishes containing 10 ml each of sterile Dickinson's (1965) medium except for tetracycline. Pollen was germinated either 2 or 4 hr at 24 ± 2 C in constant darkness. At either time medium aliquots were withdrawn to shell vials containing 100 µl 40% formaldehyde. The experiment was replicated three times.

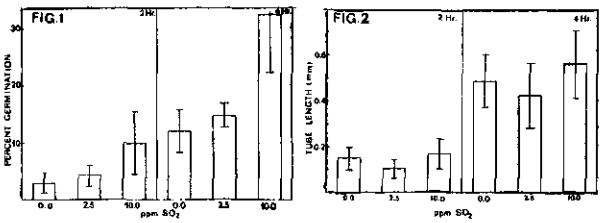
Quantification of both % G and TE - Germinated pollen was removed from the vials and transferred to microscope slides for scoring of both % G and TE. Sixteen hundred grains and tubes were scored for both the control and each treatment. Tube lengths were measured with a calibrated ocular micrometer.

Significant differences in either % G or TE between fumigated and nonfumigated pollen were assessed by a n ≥ 30 T-Test (Mendenhall & Ott, 1972).

Results

Germination - Fumigation of pollen upon the anthers with 2.5 ppm SO_2 followed by in vitro G resulted in 1.5 and 1.2-fold elevations in % G > the control at 2 and 4 hr, respectively. At 10 ppm, the enhancements were 3.4 and 2.7-folds (Fig. 1).

Tube elongation - Neither 2.5 nor 10.0 ppm SO_2 significantly ($p=0.05$) affected TE at either 2 or 4 hr (Fig. 2).



Discussion

The data within Figs. 1 and 2 demonstrate that only G could be a possible monitor since neither 2.5 nor 10.0 ppm SO_2 significantly affected TE at either 2 or 4 hr. These results differ markedly from investigations with other pollens.

Acknowlegements

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EARLY INDICATORS OF IN VITRO TRANSFORMATION FROM GAMETOPHYTE TO SPOROPHYTE IN DATURA

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Summary

At the verge of the first haploid mitosis, some of the microspores/pollen grains of Datura may be induced to follow a sporophytic development. At this stage, the microspore before culture in vitro, form three distincts size types having mean diameter of 32, 39, 45 um respectively. At the mature anther stage, only two major types are evident with a mean diameter of 48 um and of 58 um.

In vitro, the first manifestation of the androgenic induction is the development of a coating or deposit on the tonoplast, only in the embryogenic microspores. Cytochemical tests indicated the presence of tannins in the deposits. The early embryo globular stage is also characterized by the formation of ribosomal bodies. These bodies persists only at the globular stage and disorganize when the embryo enter the heart-shaped stage. Similar bodies are also observed in the young zygotic embryos of Datura. The presence of the above indicators, in relation to in vitro androgenesis, are discussed.

Keywords: in vitro culture, androgenesis, pollen, embryo, Datura, haploidy.

Introduction

Since Guha and Maheshwari (1964) obtained haploid plants from cultured anthers, works in our laboratory have been aimed to understand the mechanism of in vitro androgenic induction in view to increase the yield of haploid plants. We have been successful to increase theyields by using different traumatic shocks before the anthers are cultured (see Sangwan-Norreel, 1980). But, it is now evident that the percentages of microspores forming embryos can not be increased behond certain level (approxymately 5 % in Datura even by applying the best culture conditions (stress, media, hormones etc..). The above facts suggest that some species e.g. Solanaceae and Graminae family may have pollen grains which are genetically predisposed to develop in culture. Therefore, it is not the normal pollen grains which are deviated from their gametophytic pathway in vitro. In fact pollen dimorphism have been observed in many species (Sunderland, 1980; Maheshwari et al 1983). Our aims in this investigation have been: 1) to catalogue, depending on the diameter, the main types of pollen at the androgenic as well as at the mature pollen stage, 2) to evaluate the early cytological behaviour of in vitro pollen population, 3) to find a specific cytological marker in order to distinguish embryogenic from non embryogenic microspores at an early stage of androgenesis.

Material and methods

Anthers, for fixing directly and for culturing, were taken from the plants of Datura innoxia grown in the Phytotron (Gif sur Yvette) according to the methods previously described (Sangwan and Camefort, 1982; Sangwan, 1983).

For examination of the androgenic (uninucleate or early binucleate) and mature pollen grain stages, 5 anthers of each floral buds were pressed gently in 2 ml of distilledwater with 2 % sucrose. The pollen suspensions thus obtained were observed directly under the light microscope. A minimum of 1000 grains were counted for each stage

Results and discussions

a) In vivo characterization of different pollen populations

Although all stages of pollen development have been studied, here only two particular stages (i.e. androgenic and mature pollen stages) are described. Pollen of androgenic stage is characterize by a large central vacuole, thin layer cytoplasm and periphral nucleus/nuclei. As is evident from the Table I, Three principals types of pollen could be

Table I. Percentages of different types of pollen grains after spore-diameter at androgenic/uninucleate and mature pollen stage in Datura. MD: mean diameter, A: androgenic stage B: mature pollen stage.

Stage	Floral buds	Type 1 MD: 32um %	Type 2 MD: 39um %	Type 3 MD: 45um %
A	1	27.3	52.8	19.9
	2	30.4	56.6	13
	3	22.6	63	14.4
		Smaller type MD: 45 um %	Larger types MD: 58 um %	MD: 70 um %
B	1	46.10	45.65	8.38
	2	42.15	50.98	6.85
	3	33.55	60	6.44

recognized. Type 1 with a mean diameter of 32 um which represent about 30% of the total pollen population. Type 2 with a mean diameter of 39 um with more than 50 % and type 3 with a mean diameter of 45um which represent a low percentage of pollen population at the androgenic stage.

Although the majority of grains, in mature anthers were bicellular, there were two distinguish types: smaller (mean diameter 48 um) and larger (with mostly a mean diameter of 58 um and a few wih 70 um), (Table I). The frequencies of the above types vary largely from one floral bud to other and even from one anther to another in the same floral bud. Such types of variations in the pollen populationhas frquently been observed in many species (Maheshwari et al, 1983). When stained with acetocarmine, no clear cut differences could be observed within these three types at the androgenic stage. However, at the mature anther stage, pollen grains with small diameter (48 um)) were weakly stained as compared with the larger ones (58 um or 70 um). The larger size seems to correspond to regular normal mature pollen grains as is evident after the fluorescine diacetate staining.

b) In vitro observations

The early evolution of the microspores towards embryogenesis could be traced from Feulgen-squashes of material fixed at regular intervals of 48h for 16 days (Sangwan, 1983). Approxymately 3 to 5 % of the microspores formed embryos.

- Tonoplast feature during in vitro culture

Before culture the vacuolate androgenic microspore has a thin tonoplast (Fig. 1). Only about 50 % of the microspores remained viable after 48h in vitro and could be distinguished by electron microscopy into three types: 1) microspores with densely contrasted/coated tonoplast (Fig. 2; 4 to 6 %), 2) microspores in which tonoplast do not change (15 to 20 %) 3) microspores which develop into mature pollen as a result of which tonoplast rupture and finally disapear.

After 8 days in culture around 94 % of the microspores were dead, 1 % became mature pollen and the rest develop into embryos. These percentages agree with our light microscopic observations on the evolution of microspores in vitro and the origin of different pathways of embryo formation (Sangwan and Camefort, 1982; Sangwan, 1983)

All the microspore-derived embryos showed the coated tonoplast. This coating persisted only in the globular stage. By means of cytochemical testes (Sangwan and Camefort, 1983) we have confirmed the presence of tannins in the coating of vacuolar membrane/tonoplast. The forms and dimensions of the deposits/coatings were usually uniform at the beginning of the embryogenesis although it was occasionally more irregular in some microspores. Tannins were observed in the mature non androgenic pollen grains as discrete bodies or gloules in the remaiders of the central vacuole rather than as a coating. The origin and the role of this in vitro deposits are not clear, although there seems to be a correlation between its presence and the embryogenic pathway of the microspore in vitro.

- Ribosomal bodies formation.

The most important cytoplasmic feature of the early globular embryos (4 to 10 cells) was the formation of condensed pyroninophilic structures after Unna staining with positive Brachet test for RNA. About 3- 5 such structures could be seen per cell section, situated around or very near the nucleus. Thes cytoplasmic structures, called ribosomal bodies (Fig. 3; RB) lasted only during the globular stage and disorganized progressively as the embryo entered in the heart-shaped stage. These bodies consisted of dense masses of ribosomes and a rough endoplasmic reticulum. Similar bodies were also observed in the zygotic embryos of Datura. The RB are therefore characteristic of the early embryogenesis in whatever form. They are not observed during pollen maturation in vivo. Their role and function are not clear although in animals they are suggested to be an important site of enzyme formation necessary for induction and development of embryos (Van den Beggelaar, 1976).

Conclusions

1 -There are not two, as observed in other species but three types of pollen grains in Datura after spore size.

2 -No clear cut relationship could be observed between these types and in vitro androgenesis. i.e. which type will only forms embryo , instead of following the normal gametophytic development. The smaller pollen grains in the mature anther stage seems to correspond to the abnormal pollen population. It is quite possible that this type may develops embryo when cultured. However, the percentages of these grains is largely superior to the percentages of pollen grains undergoing in vitro androgenesis.

3 -Tannin-coated tonoplast was found to be specific for microspoes forming embryos.

4 -Ribosomal bodies were only present in the early embryogenesis in Datura.

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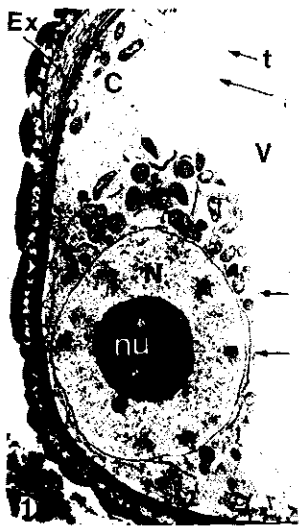
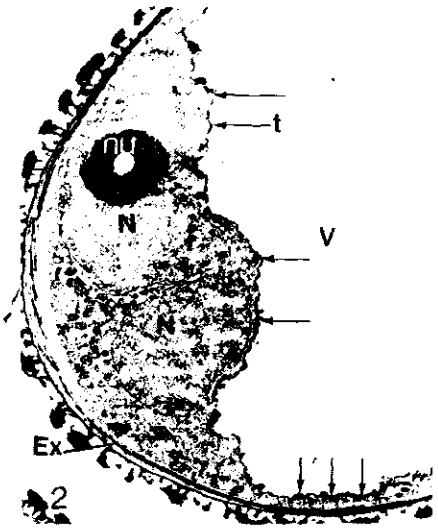
Explanation of figures

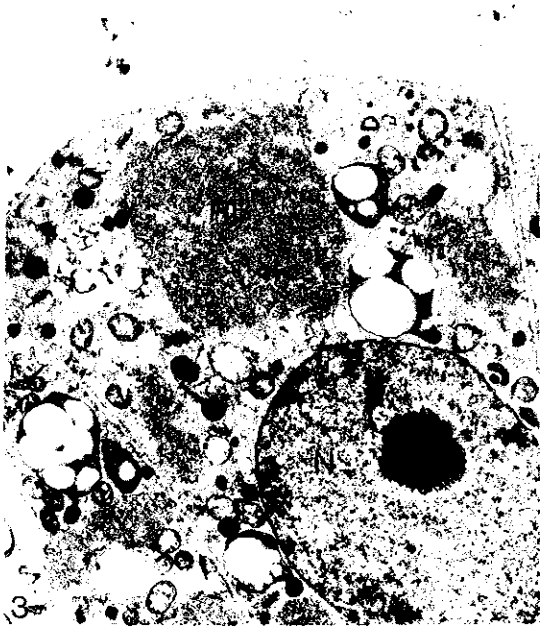
Fig. 1 - Electron micrograph of an uninucle te microspore before culture. Note tonoplast (t) without deposit. C: cytoplasm, Ex: exine, N: nucleus, nu: nucleolus, V: part of the large central vacuole. Arrows indicate the tonoplast (x 6 100).

Fig. 2 - Microspore after 48 h of in vitro culture. Note the densely contrasted tono plast (arrows) and the two-like nuclei , (x 4 950).

Fig. 3 - Ribosomic amas (RB) in a cell of a young pollen-embryo, (x 7 200).







P A R T II

STIGMA, INCOMPATIBILITY AND POLLEN GERMINATION IN VITRO

★★

Part II: Stigma, incompatibility and pollen germination in vivo

Introduction

The stigma surface, incompatibility and pollen germination in vivo are the topics of this part. Ultrastructural and biochemical methods are used and combined. A recent new technic is the thin layer chromatography - bio assay and the pollen tube test system. For application the study of incompatibility is of high importance in plant breeding.

The sporophytic incompatibility gets attention as an interaction which occurs at the place of contact between pollen coat and stigmatic surface.

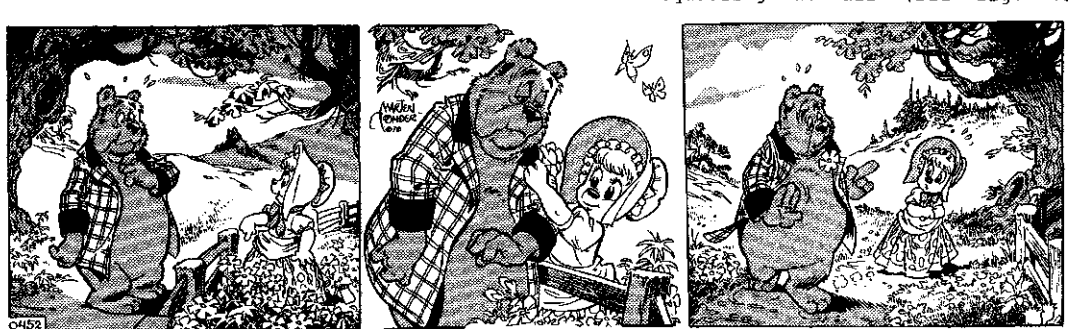
One of the major questions is still the way of recognition and nature of the signal or signals during this first contact. Different possibilities are proposed offered, but to fit them in the process of incompatibility is still difficult. The complexity of the pollination stimulus is also demonstrated in the wilting of the flower. Several other aspects of the flower behaviour, such as colour shift, changes in position, rate of opening or its general morphology could be studied in relation to pollination. Also the study of the differences in stigma morphology can offer more data about the conditions and tasks present in pollen or stigma.

Also the development of specific cytochemical methods is very important. Recognition is a very important mechanism, working on different moments from pollination till after fertilization, between sporophyte or gametophyte and gametophyte. This interaction is very dependent on timing and environmental factors. Constant comparable conditions of the flower and its way of acting in acceptation or rejection should be well known before conclusions can be made.

Because of the complexity in recognition, it is perhaps not by accident that Dr. Williams omits a word as "Incompatibility" or male sterility in the following verse:

> A poem you asked me for - please
> with a few words of "technicalese"
> I said "not to worry"
> But now I must hurry
> To get as many words like:
> apomixis
> gametophyte
> and sporogenesis
> into the last line as I can possibly
> squeeze!

To avoid all negative effects of squeezing in recognition systems a male sterile life form seems to be a simple solution, such as Lord Bommel, probably a male sterile boar, a well known cartoon hero (see fig. 1a, b). So he has to live in his own world of fairy tales, a situation of no squeezing at all (see fig. 1c).



This figure suggests an intensive meeting between individuals (a, b, c). In respect to a possible interbreeding, a lot of differences may be expected. However, contacts by hands and eyes show some incompatibility. Probably this also becomes clear in fig. c, shown by one person on which male sterility can be observed. Note the flower in c too. (From 'Den Andere Wereld'. Marten Toonder. Druk: Van Boekhoven Bosch B.V. Utrecht, 1982. With permission of Marten Toonder/De Bezige Bij, Amsterdam. Not permitted to copy the figure).

LOCALIZATION OF POLLEN SURFACE GLYCOPROTEINS USING MONOCLONAL ANTIBODIES

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Summary

The cellular sites of allergenic glycoproteins in mature ryegrass have been located by immunoelectronmicroscopy using monoclonal antibodies. Several chemical fixation procedures and two resins (Spurr's (S) and London resin white (LRW) were evaluated for their suitability in preserving both structure and antigenicity in mature pollen. Allergenic glycoproteins were localised in the pollen grain cytoplasm, wall layers and on the grain surface and anther orbicules.

Introduction

In ryegrass pollen, four groups of allergens (groups I-IV) have been described (Marsh, 1975). These allergens have no known role in the pollen grain, and thus the precise localization of these proteins and glycoproteins and their sites of synthesis are essential for an understanding of their function.

Recently, monoclonal antibodies to allergenic components of ryegrass pollen have been developed (Smart et al., 1983) and made available to us. In the present investigation, indirect post-embedding immunogold methods have been used together with different specific monoclonal antibodies to precisely localise some of the allergens.

Results and Discussion

Immunogold localization of pollen allergens

Overall, the periodate-lysine-paraformaldehyde (PLP) fixative (McLean and Nakane, 1974) produced superior structural preservation even though the pollen wall layers were well preserved using glutaraldehyde and cetylpyridinium chloride (GCC) fixative (Grote et al., 1983). Pollen grains embedded in Spurr's resin were less distorted and sections could be viewed for longer periods under the electron microscope than those in London Resin White. However, London Resin White is easier to use and requires less infiltration time. The distribution of colloidal gold particles (10 nm diameter) in different regions of the pollen grain showed considerable variation depending on the specific monoclonal antibody used (Table 1).

Table 1. Approximate molecular weight and specificities of FMC-A series of hybridoma antibodies (Smart et al. 1983).

Antibody	approx. mw (x1000 daltons)	specificity
FMC-A1	35	Group I
FMC-A5	16	Group II or III
FMC-A7	30,28	Group I
FMC-A9	71,87,49,47,18	Group IV

Negative Control: Supernatant from cultures of the mouse myeloma line X-63 which secretes an IgG molecule.

The binding of all four monoclonal antibodies in the cytoplasm of the pollen grain always occurred near ER membranes or membranes around vesicles and P-(polysaccharide) particles. In the intine, the gold particles bound mainly to the tubular structures of the matrix. In the exine the gold particles were localised in the arcades, the pores and the exine layers.

Although the different monoclonal antibodies show different densities of binding in the pollen grain sections, binding always occurred in similar regions (Table 2). Further information concerning the precise sites of synthesis of the allergens will depend on more detailed characterization of the monoclonal antibodies used.

Conclusions

Monoclonal antibodies bind to thin sections, and may be detected with immunogold labelling. Three interesting features of pollen allergens emerge:

- all the monoclonals bind to a similar range of cellular sites in the pollen grains.
- differences in number of gold particles bound may not reflect differences in glycoprotein concentration since the various antibodies had different titres, and the surface receptors may not always be accessible for antibody binding.
- antibody binding in the pollen cytoplasm is associated with ER and vesicles, and P-particles, suggesting that these organelles may be associated with the extracellular secretion of the glycoproteins into the pollen wall.

Table 2. Semi-quantitative distribution of gold label on thin sections of ryegrass pollen grains after different fixation and embedding protocols.

Fixation & Resin	FMC-Antibody & GAM Probe	Pollen Cytoplasm	Pollen Intine	Wall Exine Arcardes	Pollen Surface
PLP/S	A1	++	+	+	-
GCC/S	A1	++	+	+	+
GCC/LRW	A1	+	+	+	+
PLP/S	A5	++	+	+	+
GCC/S	A5	+	+	+	+
GCC/LRW	A5	+	+	+	+
PLP/S	A7	+	+	+	+
GCC/S	A7	-	-	-	-
GCC/LRW	A7	++	+	++	+
PLP/S	A9	+++	++	++	++
GCC/S	A9	+++	+++	++	++
GCC/LRW	A9	+++	++	++	++
PLP/S GCC/S GCC/LRW	X-63	-	-	-	-
PLP/S GCC/S GCC/LRW	GAM	-	-	-	-

- : no labelling; + : < 10 grains/section; ++: < 100 grains/section; +++: > 100 grains/section; GAM: colloidal gold goat-anti-mouse IgG.

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CYTOCHEMISTRY OF THE STIGMA SURFACE COMPONENTS USING THE PRINT TECHNIQUE

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Summary

Dry and wet stigmas possess surface components, easily diffusible. Stigma-print technique allows to visualize and identify these surface products, with light and scanning electron microscopy, without any inclusion procedure. We demonstrated that polysaccharides, proteins, lipids , enzymatic activity and mineral ions, especially Ca^{++} can be revealed by this method. Dry stigmas (Brassica oleracea L., Populus alba L.) or wet stigmas with small exudate (Forsythia intermedia Zab.) gave best results than wet stigmas with copious exudate (Petunia hybrida L.).

Keywords: cytochemistry, stigma print, print technique.

Introduction

In order to gain a better understanding of pollen-pistil interactions in Angiosperms, it is necessary to determine the nature of the interacting surface components. These components diffuse easily in an aqueous medium: this property has been used by some authors to develop the print method (Heslop-Harrison et al., 1973, 1975). We have adapted and improved the print method for both light and scanning electron microscopy.

Material and method

The study was performed on several species with dry type stigmas: Brassica napus L., Populus alba L. and wet type stigmas: Forsythia intermedia Zab., and Petunia hybrida L. Prints were realized on glass slides and fixed with glutaraldehyde or OsO_4 vapour in a hermetically sealed box.

For scanning electron microscopy, prints were coated with gold palladium and observed in a Cambridge 600, at 15 kv.

For light microscopy, different cytochemical tests summarized in the following table have been used.

Results

Cytochemical data from light and U.V. microscopy for Brassica napus, Populus alba and Forsythia intermedia.
(+ = positive reaction, - = negative reaction, ± = weak reaction, nt = not tested).

Fixation	Cytochemical test*					
	none	CTC	SB	PAS	CB	EA
none	-	+	+	+	+	nt
OsO_4	+	-	+	+	±	nt
Glutaral-dehyde	-	+	+	+	+	+

* CTC: chlorotetracycline for Ca^{++}, SB: Sudan Black for lipids, PAS: PAS reaction for polysaccharides, CB: Coomassie Blue for proteins, EA: esterase activity (review in Knox, 1984).

Petunia hybrida does not provide a clear print; just generally remains on the slide a diffuse lipophilic exudate. The shape of the stigma cells cannot be identified.

Discussion

Hydrophilic and adhesive compounds of the stigma surface have been demonstrated by the print technique. However, if the time of contact of the stigma with the slide is more than 30 min., protoplasmic components may be released from the stigma cells inducing false reactions.

Stigma prints are better after glutaraldehyde vapour fixation than after osmic vapour.

The results differ with the species tested: dry stigma types or those with a small amount of exudate give the best results.

In conclusion, the stigma print method provides a simple method for the characterization cell surface components which may be involved in pollen-pistil recognition.

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STIGMA STRUCTURE AND THE POLLEN-STIGMA INTERACTION IN CAESALPINIOIDEAE - LEGUMINOSAE

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Summary

Stigmatic surfaces of 6 genera in the Caesalpinioideae are briefly described. They can be divided into 2 groups, WP and WN, which show morphological affinities to the more advanced sub-families Papilionoideae (WP) and Mimosoideae (WN). The germination response of self pollen appears further to endorse these differences.

Cassia floribunda (WN) is examined in detail and data of self, interspecific and intergeneric pollinations presented. Stigmatic exudates in WN type stigmas are considered to be efficient germination media and the stigma to lack specific recognition molecules.

Introduction

The family Leguminosae is generally considered to comprise 3 sub-families; the more primitive genera and species are classified in the Caesalpinioideae. 152 genera are recognised (see the taxonomic analysis in Polhill & Raven 1981) and these can be assigned to 5 tribes.

Data on stigmatic surfaces are available from the survey of Heslop-Harrison & Shivanna (1977). Two genera of the Caesalpinioideae, Brownea and Cassia, were examined and were classified into the WP category, the same category as all papilionoid genera examined.

The breeding systems and pollination biology have recently been reviewed by Arroyo (1981). Ten species out of sixteen examined in the Caesalpinioideae were found to be self-incompatible; dioecy and andromonoecy were more widespread than in the other sub-families and apomixis was well documented in the Australian Cassia series Subverrucosae of section Psilorhegma. A wide spectrum of pollinating agents was also recorded including bees, wasps, butterflies, birds and bats.

Materials and Methods

Representatives of all tribes in the Caesalpinioideae are grown at Kew although at present only species of 3 tribes flower regularly. 6 genera (Cassia, Caesalpinia, Cercis, Bauhinia, Brownea, Gleditsia) and 16 species have been used in some part or all of this study. A further 45 genera and approximately 92 species were available for study of stigmatic morphology in the spirit collection of the Kew herbarium. These form the basis of another publication (Owens & McGrath in preparation).

SEM

Stigmas were removed directly from flowers, stuck to a stub using conducting 'Dotite' paint, examined and photographed fresh at 10 KV in a Jeol S35.

Light Microscopy

Morphology

Observations on fresh and spirit material were also made using dissecting microscopes and microscopes fitted with epi-objectives. Further examination of stigma structure was made from semi-thin (1µm) sections, fixed, dehydrated and embedded using conventional TEM techniques.

Non-specific Esterase

Esterase was localised on stigmas using the method of Pearse (1972).

Cutin

The presence of cuticle on the stigmatic surface was tested on fresh stigmas using Auramine O (Heslop-Harrison 1977). Examination was made with a Vickers Photoplan using transmitted UV illumination.

Pollen viability

Pollen viability was estimated for all species in this study using the technique of Shivanna & Heslop-Harrison (1981). With the exception of Cassia obtusa (13%) and Cercis species (25-80%), all pollens were >80% viable.

Pollen tube growth

Pollen tubes were examined in transmitted UV illumination (as above for Auramine O) after mounting freshly dissected stigmas into aniline blue (O'Brien & McCully 1981) on a slide.

Pollinations

All pollinations were carried out in the laboratory on virgin stigmas. Laboratory

temperatures ranged from 21-27° C and relative humidity between 36-56%.

Whole individual flowers were used in pollinations of Bauhinia, Brownea (these were also emasculated before removal from the inflorescence) and Caesalpinia and either individual flowers or whole inflorescences in Cassia and Cercis. Pollen was removed directly from freshly dehisced anthers using either a single paintbrush hair or a custom-made glass micro-needle. Grains were placed on the stigmatic surface and in solid, papillate stigmas were pushed down to make good contact using a custom-made micro-scalpel. For Cassia stigmas pollen was pushed into the stigma pore using the glass micro-needle and large numbers of Cassia pollen grains could be introduced into the pore in a single pollination attempt. Two or three grains (Bauhinia and Caesalpinia) were used at each attempt in intergeneric crosses because of greater pollen size.

Results

1. Stigma structure

Species can be separated into 2 groups based on stigma structure. The classification of Heslop-Harrison (1981) is used.

WP The stigma is solid, papillate and appears wet at maturity. Secretion, which is generated by the papillae and cells beneath the papillae (Owens & McGrath in prep.), is more copious in Cercis stigmas prior to pollination than in Bauhinia, Gleditsia or Brownea.

WN The stigma is either funnel-shaped and the inner receptive surface rugose (Caesalpinia) or flask-shaped with a narrow entrance pore and not papillate (Cassia). Both types of stigma are wet, the secretion being produced by the cell layers lining the receptive surface. Among many morphological differences which exist between stigmas of Caesalpinia and Cassia a major distinction is the lack of a cuticle over the stigmatic surface of Caesalpinia. The stigma of Caesalpinia appears to lack an epidermal surface layer, leaving the stylar transmitting tissue cells as the layer onto which pollen lands.

A large secretion droplet which covers the whole stigmatic surface at receptivity and into which the pollen lands is regularly produced by the stigmas of Cassia marylandica, C. mimosoides, C. nemophila and Caesalpinia pulcherrima. It is present irregularly on the stigma in Cassia obtusa and apparently very rare in Cassia floribunda. It is not produced at all by Caesalpinia gilliesii which does have a copious secretion on the surface of the stigma but it is not enough to generate a droplet. The stigma of C. gilliesii is larger than C. pulcherrima. The secretion is a clear, colourless, watery liquid which appears to increase in viscosity on exposure to the atmosphere.

2. The pollen-stigma interaction; pollen germination

A General

The two stigma types differed in their requirements for successful experimental pollination. All stigmas of the WP type needed pollen to make good contact with the surface before there was any response. In Bauhinia and Brownea species the pollen grains had to be pushed down onto the surface between the stigma papillae and in both species one pollen grain was not enough to provoke a response. In Brownea 5 grains were the minimum used and of these a maximum of 4 hydrated. The esterase test did not appear to indicate that there were any specific receptive sites on the papilla surface, however since the Brownea stigma is coloured red-brown localisation of the reaction produced is difficult.

For WN type stigmas contact with the secretion was enough to ensure germination even when only a single grain was used.

Table 1. The minimum time to observed germination after self-pollination.

Species	Minimum time to observable pollen germination (Mins.)
WP	
Bauhinia tomentosa	60(16)
Cercis siliquastrum	55(25)
WN	
Caesalpinia gilliesii	30(7)
C. pulcherrima	43(3)
Cassia floribunda	20(40)

() number of stigmas tested

B Cassia

Because of its abundant flower production a more detailed examination could be made of the pollen-stigma interaction in Cassia floribunda. Table 2 shows that the receptive stigma is wet even though stigmas rarely produce a secretion droplet. Examination of hand cut sections of the stigma clearly showed that the pollen is received into a large chamber (it can hold at least 100 grains). The chamber is lined with a cuticle which may rupture before pollination or may be ruptured following pollination. Rupture

releases the secretion and this is often accompanied by the release into the secretion of hundreds of the cells lining the chamber. The response to the esterase test (Pearse 1972) is variable, a positive reaction generally occurring at a site just below the porate entrance to the chamber in intact stigmas. Pollen germination takes place between 20-30 minutes after pollination for most self- and cross-(intraspecific) pollinations.

Table 2. The response of pollen grains on stigmas of different states (wet or dry) after either self- or cross-(intraspecific) pollination in Cassia floribunda.

Stigma state	Pollen response (individual flowers)	
	Germination	*No germination*
Wet	31	19
Dry	1	21

The results of interspecific and intergeneric crosses are shown in Table 3. All pollens germinated although the responses were slower or the same as for self-pollination. The one notable exception was in the cross involving Bauhinia pollen where a much more rapid response was observed.

Table 3. The response of foreign (interspecific & intergeneric) pollen on the stigmas of Cassia floribunda, C. obtusa and Caesalpinia gilliesii.

Cross	Min. time to germination (Mins.)
C. obtusa x C. floribunda	35(9)
C. obtusa x Caesalpinia pulcherrima	45(9)
C. floribunda x Caesalpinia pulcherrima	45(10)
C. floribunda x Caesalpinia gilliesii	45(19)
Caesalpinia gilliesii x C. floribunda	30(4)
C. floribunda x Bauhinia tomentosa	30(8)

() number of flowers tested

3. Self-incompatibility

Preliminary data on self-incompatibility suggests that the two Brownea hybrids (both highly pollen fertile) are self-incompatible while Cassia floribunda, Caesalpinia gilliesi and C. pulcherrima are self-compatible.

Discussion

The stigmas of Caesalpinioid legumes can be easily classified into two types which possess similar characteristics to either stigmas of the Papilionoideae or the Mimosoideae. A good example appears to be the similar morphology of stigmas of Caesalpinia (WN) and those in Acacia and Mimoseae (Kenrick & Knox 1981, 1982). Similarities between the Papilionoid legumes are less easy when comparisons are made with Trifolium (Heslop-Harrison & Heslop-Harrison 1983) and Phaseolus (Heslop-Harrison & Heslop-Harrison 1984) but recent unpublished work in our laboratory on the primitive Papilionoid genera Sophora and Swartzia suggest a closer relationship with those Caesalpinioideae in the WP group. Cuticle rupturing, a characteristic feature of many Papilionoideae (Heslop-Harrison & Heslop-Harrison 1983, Owens unpub.), appears to be required for the successful pollination of Bauhinia and Brownea (WP). However, in the Caesalpiniodeae, as with the primitive Papilionoideae this is not associated with a sophisticated tripping mechanism.

It would be inappropriate at this stage to overgeneralize about the evolution of stigma types within the family for it is certain that a substantial number of morphological types exist hitherto undiscovered. However there are clear indications (Owens & McGrath unpub.) that both in morphology and structure stigmas in the Caesalpinioideae can be divided into groups which appear to link closely with either one or other of the two, more advanced, sub-families.

The stigmas of all species are wet (see however the discussion of Heslop-Harrison & Heslop-Harrison 1983) and contact with secretion is a necessary pre-requisite to germination. The presence of secretion in Cassia floribunda is not stimulated by pollination. Manual pollination may rupture the intact cuticle of some stigmas and thus bring the pollen into contact with the secretion but pollination itself does not appear to influence the generation of secretion.

The high rate of germination of a wide selection of pollen in the stigmatic secretion of the two Cassia species used and of Cassia pollen on the Caesalpinia stigma may be related to the evolution of controlli systems in the style rather than at the stigmatic surface i.e. self-incompatibility in Leguminosae is apparently a stylar

reaction (De Nettancourt 1977). It appears that the exudates of Cassia and Caesalpinia stigmas are just efficient germination media. This is also interesting in relation to the non-specific exudation response of Acacia stigmas to various pollens, a situation which was considered to be a general pollination signal initiated at the commencement of pollen-stigma interactions (Kenrick & Knox 1981).

Acknowledgements

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ACACIA BREEDING SYSTEMS

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Summary

Breeding experiments have shown that several Australian species are self incompatible: A. pycnantha, A. retinodes, A. terminalis; partially self compatible: A. myrtifolia; and self compatible: A. ulicifolia. Many insects, especially 5 families of bees, forage for Acacia polyads and serve as pollen vectors. Two genera are wide-spectrum pollinators, while the others show narrower specificities. Acacia terminalis is also visited by small birds that forage for the hexose-rich nectar in extra-floral nectaries that appear to be an adaptation for bird pollination.

Introduction

Acacia is a nitrogen-fixing tree genus of considerable economic importance and there is evidence for self-incompatibility (SI). Experiments at the Wattle Research Institute in South Africa showed that selfing results in reduced seed set in A. decurrens and A. mearnsii. We present results of our breeding experiments in natural populations in south-eastern Australia of a summer-flowering species, A. retinodes; an autumn-flowering species, A. terminalis; a winter-flowering species, A. ulicifolia; and two spring-flowering species, A. myrtifolia and A. pycnantha. We have also investigated the pollen vectors of these species, with a view to establishing the modes of pollen transfer between trees.

Results and Discussion

These species of Acacia are protogynous. The stigma cup is just large enough to accept a sinlge polyad and 80% of pollinations are of this kind in A. retinodes. The unique 16 grain polyads provide a system for pollen transfer in which all the meiotic products of an anther loculus are retained as a single unit. This means that in most pollinations all the seeds in a pod may have the same father.

SI has been analysed in terms of fruit set, using the Index of Self Incompatibility (ISI) ie. yield of fruit following self/cross pollination (see Bernhardt et al., 1984). The results with 5 species of Acacia show a range of SI levels (Fig. 1).

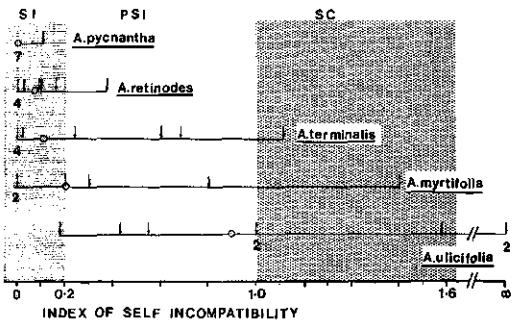

Fig. 1. SI relationships in five species of Acacia. Open circle: mean ISI; horizontal line: range of ISI values; arrows: individual parent values; numbers beneath arrows show the number of parents with indicated ISI value.

In most of these species, pollen transfer from one tree to another is essential for seed set.

Insects were attracted to the nectarless flowers by a combination of the massive yellow display and strong fragrances that varied between four of these species (A. ulicifolia not yet studied). Beetles and flies ate the polyads. Solitary bees, th most commonly collected insects, gathered polyads to feed to their larvae. We have added two bee families (Colletidae and Megachilidae) to the three families previousl known to pollinate Australian Acacia. The most constant pollinators, regardless of season or habitat, were Leioproctus spp. (Colletidae) and Lasioglossum spp. (Halictidae), which are widely distributed and pollinated all four Acacia spp. Conversely, some bees showed narrow specificities to one or two Acacia spp. For example, species of Euhesma, Hylaeus (Colletidae), Megachile (Megachilae) and five out of six Homalictus spp. (Halictidae) were restricted to A. retinodes.

In A. terminalis bees shared pollination duties with small birds like thornbills (Acanthiza), silvereyes (Zosterops) and spinebills (Acanthorhynchus). A. terminalis bears large, scarlet extrafloral nectaries on its leaf petioles. At flowering time these glands secrete drops of nectar rich in three sugars and 18 amino acids

(collaboration with Drs I. and H.G. Baker, U. of California, Berkeley). The flowering branches are strategically located arising in pairs from the nodes. As birds search for the glands, and drink the nectar, their bodies contact the flowering branches transferring polyads to their plumage. Observations of the birds, and analyses of the pollen loads of birds and bees, confirmed that both may act as pollen vectors in this species of Acacia.

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INCOMPATIBILITY BETWEEN PINUS NIGRA ARN. AND PINUS SYLVESTRIS L. AND OVERCOMING IT WITH CHEMOMUTAGENIC SUBSTANCES

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Summary

Using the pollen grains of Scots pine /P.sylvestris L./ mutagenically treated with the two derivatives of nitrosourea and sodium azide it was possible to obtain the hybrid seeds of interspecific combination P.nigra x P.sylvestris which under normal conditions is incompatible.

Introduction

Hybridological status of European black pine /P.nigra Arn./ and Scots pine /P.sylvestris L./ has in the past been the subject of certain controversy which to some extent persists until now. The spontaneous origin of P.nigra x P.sylvestris hybrids first postulated in the year 1867 in Austria /Vidaković, 1974/ and later assumed by Wettstein /1951/ and Vidaković /1958/ was primarily based on the finding of several individuals of intermediate morphology. However, according to the same author /Vidaković, 1977/, the hybrid nature of these putative hybrids has never been convincibly demonstrated. Also, the attempts to hybridize European black pine with Scots pine artificially, though previously reported as successful /Johnson, 1939; Wright and Gabriel, 1958/, have not been confirmed by the extensive experiments carried out by Vidaković and Borzan /1973/ over a span of 15 years.

Cytological examination revealed that inhibition of the pollen tube growth in the nucellar tissue of the ovules is the primary cause of their abortive development /Vidaković, Jurković-Bevilacqua, 1970/. In order to overcome this hybridization barrier, the application of irradiated pollen of Scots pine as well as the mentor pollen technique are recommended /Vidaković et al., 1975/. In their attempts with disturbing the mechanism of incompatibility of this crossing, the authors treated the pollen of Scots pine as well as the conelets of European black pine with seven types of chemical compounds involving hormones, growth substances and sugars. Aqueous solution of yeast was used as well. None of these treatments has however been proved to disturb this mechanism. Our approach based on application of nitrosomethyl-, nitrosoethylurea and sodium azide on the developing pollen buds of Scots pine seems to be more efficient in this respect as indicate the results of our investigation presented in this paper.

Material and methods

Two attempts with overcoming incompatible barrier between the European black pine /P. nigra Arn./ and the Scots pine /P.sylvestris L./ have been made in the years 1978 and 1981. In the first case one individual of the European black pine was used as female parent, while in the experiment performed in 1981 three individuals of the same species were involved. The Scots pine tested as a male species was represented by the two individuals separately used in both the above experiments.

As a preparatory step to the artificial hybridization, the application of N-nitroso-N-methylurea /NMU/, N-nitroso-N-ethylurea /NEU/ and sodium azide /Na_3N/ on pollen buds of Scots pine was performed in the year preceding pollination. The mixtures of these mutagens prepared with a vaseline paste have been applied separately at the bases of buds during their initiation in the first half of July. In the year 1977 only 25 and 50 mM concentrations of NEU were tested, whereas the treatments in 1980 were extended of the variants with 10, 30 and 50 mM concentrations of Na_3N as well as with 10 and 50 mM concentrations of both NMU and NEU /Table 1/. On average 300 buds of each variant were treated in this way. As a control served the non-treated buds of the same tree.

Viability of the pollen grains collected in the spring of the next year was tested by their germination in vitro. The pollen originating from the above treatments has been separately used in artificial pollination of the ovulate strobili of European black pine. The conventional technique of artificial pollination was applied in which the isolating bags and pollinator of own construction were used. Totally 100 ovulate strobili were pollinated in 1978, while the amount of strobili pollinated in 1981 reached the number 183. The exact proportions of the ovulate strobili pollinated by the pollen grains treated by the individual types of mutagens are given in table 1. The quality of mature seeds obtained was examined

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by the X-ray radiography /Simak, 1980/.

Results and discussion

The results of viability test indicate that applied chemomutagens had differentially affected the ability of mature pollen to germinate but in general adversely influenced the growth of pollen tubes. As it indicates Fig. 1, the lowest proportion of germinating pollen grains was registered in the samples treated by NMU. The extent of germinating pollen grains ascertained in its variants with 10 and 50 mM concentrations has as a rule been maintained at the level of 44 and 47 % what represents the figure only a little worse than a control with the 49 % fraction of germinating pollen revealed. The two other variants of treatment, i.e. with NEU and Na_3N have on the other hand yielded the pollen samples which consistently differed from a control in containing the larger populations of germinating pollen grains. The corresponding parameters ranged between 54 and 59 % in the former case and between 57 and 65 % in the latter one.

With the outlined variation pattern has only partially correlated the pollen tube growth. In comparison with a control, achieving under experimental conditions the mean length of 103 microns, was the growth of pollen tubes in all the variants of mutagenic treatment retarded. The degree of inhibition varied considerably, depending on the concentration of the applied mutagens which had generally reduced the pollen tube length to the level of 76,1-93,9 microns what in relation to control represents statistically highly significant deviation. However, in spite of such a wide variation in length of pollen tubes, the results obtained from the individual types of treatments were comparable, irrespective of the chemical nature of the mutagenic compounds utilized. Remarkable was the tendency displayed by the derivatives of nitrosourea, i.e. NMU and NEU which being tested in two concentrations exerted their strongest retardant effects in the variants with 10 mM concentrations differing thus from the treatments with the corresponding mutagens in 50 mM concentrations. No such tendency has on the other hand been observed in the case of Na_3N.

The pollen grains of Scots pine originating from the above described treatments have subsequently been utilized in artificial pollination of the European black pine performed in the year 1981. The results of this attempt are summarized in the lower part of table 1, while those referring to the hybridization experiment in 1978 are tabularized in the upper part of the table. The self- and cross-pollinations of the maternal trees stand for the control at the

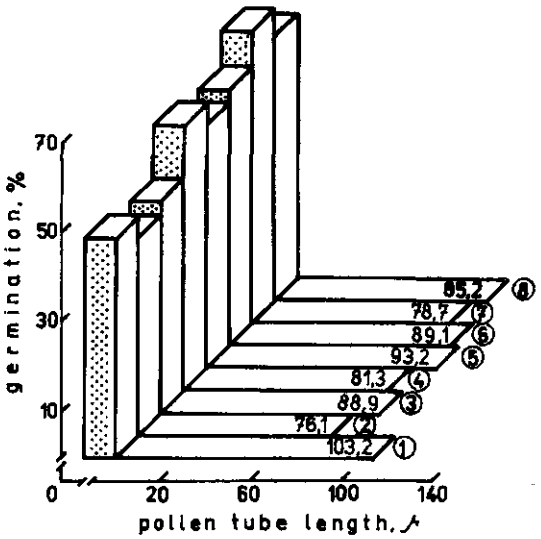

Fig. 1. Germination percentage and pollen tube length in pollen grains of Scots pine affected by 10 mM /2/ and 50 mM /3/ NMU, 10 mM /4/ and 50 mM /5/ NEU, 10 mM /6/, 30 mM /7/ and 50 mM /8/ Na_3N; 1-control

intraspecific level, whereas the species combination P.nigra x P.sylvestris-control serves as a control variant at the interspecific level. It is apparent from the table that both these controls differ strikingly in quality of the seed progenies produced. The intraspecific crossings of P.nigra involving self-pollinations of the maternal trees and cross-pollination of different individuals of the species have as a rule yielded the viable seed progenies. The efficiency of selfing differed annually but the presence of sound seeds was a constant feature of its progenies in both years tested. Particularly remarkable was the reduced amount of sound seeds in this category of progeny harvested in the year 1981 with only 24 % of fully developed seeds as compared with the 84 % proportion of seeds of the same category resulting from a cross-pollination as well as with the 91,5 % of filled seeds obtained from selfing in 1978. With the above data contrasted the results of interspecific hybridization of P.nigra x P.sylvestris in which the non-treated pollen of Scots pine was used /control/. As it follows from table 1, in the 1978 experiment this combination yielded 0,39 % of filled seeds what represents only four fully developed seeds of the total population of 1023 seeds obtained, while in the year 1981 no filled seeds were revealed at all. Taken together, these data support incompatible nature of P.nigra x P.sylvestris combination.

In an attempt to overcome incompatibility of this crossing, the pollination of fe-

Table 1. The results of attempts with overcoming the incompatible barrier between Pinus nigra Arn. and Pinus sylvestris L. species

Combinations tested		Number of pollinated female strobili	Number of collected mature cones	Number of cones with full seeds	Total number of seeds obtained	Number of sound seeds	Percentage of sound seeds
fem.	male parents						
P.nigra	P.nigra-selfing	24	16	16	983	899	91,50
	P.sylvestris-control	34	22	4	1023	4	0,39
	P.sylvestris-25 mM NEU	42	20	11	793	24	3,00
	P.sylvestris-50 mM NEU	80	42	19	1507	43	2,90
P.nigra	P.nigra-selfing	8	6	6	129	31	24,00
	P.nigra-cross-pollination	42	26	26	1096	920	84,00
	P.sylvestris-control	17	13	-	269	-	-
	P.sylvestris-10 mM Na_3N	18	4	1	149	1	0,60
	P.sylvestris-30 mM Na_3N	8	4	2	173	2	1,10
	P.sylvestris-50 mM Na_3N	5	4	2	121	3	2,40
	P.sylvestris-10 mM NMU	14	4	1	177	1	0,50
	P.sylvestris-50 mM NMU	12	3	-	69	-	-
	P.sylvestris-10 mM NEU	41	26	4	739	11	1,40
	P.sylvestris-50 mM NEU	18	12	3	146	3	2,00

male strobili of the maternal species was carried out using mutagenically treated pollen of Scots pine. Particularly efficient was in this respect the pollination in 1978 during which the pollen grains originating from treatments with 25 and 50 mM NEU were used. In such a way it was possible to raise the percentage of fully developed hybrid seeds to 3 and 2,9 %, respectively. On the other hand, the attempt repeated in 1981 nad based on the involvement of two additional mutagens /Na_3N and NMU/ was less successful. Thehighest proportion of filled hybrid seeds resulted from the pollination with the pollen influenced by 50 mM Na_3N and reached the value of 2,4 %. Comparable results had also been achieved by the pollen grains treated with 50mM NEU where 2,% of filled seeds were obtained.The variant with application of pollen grains derived from the treatment with NMU has on the other hand been less efficient in disturbing the mechanism of incompatibility between both the pine species. Among 177 seeds of this class only 1 seed was revealed to be fully developed /0,5 %/.

Summarizing we can state that among 2.300 seeds collected from the interspecific crossing P.nigra x P.sylvestris in 1978, 67 seeds were detected to be filled, whereas the experiment repeated in 1981 resulted in 21 hybrid seeds. Expressed in the relative values, these data represent 2,91 and 1,33 % of filled seeds, respectively, obtained with a help of mutagenically treated pollen grains of Scots pine.

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IMMEDIATE DISTINCTION BETWEEN SELF-POLLINATION AND CROSS-POLLINATION AND BETWEEN SELF-COMPATIBILITY AND SELF-INCOMPATIBILITY IN PAPILIONACEAE - PRELIMINARY REPORT

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Summary

Suitability of "new" morphological traits as indices of pollination mode was tested on several species of Papilionaceae
Keywords: Papilionaceae, self-pollination, cross-pollination, self-compatibility, self-incompatibility.

Introduction

The mode of pollination has the fundamental influence on the next generations. Progenies of full self-pollinated plants are genetically uniform while the progenies of full cross-pollinated plants are genetically very different. Full self-pollination appears in case of cleistogamy and - contrary to it - the absolutely cross-pollination appears in monoecious, bisexual plants population in case of full self-incompatibility. But we exactly know there are many modes of pollination between full self-pollination and full cross-pollination. It must be also noted that the mode of pollination may vary depending on external conditions and - like other traits - undergoes evolutional changes. Therefore certain adaptations to cross-pollination may be noted in actually self-pollinated plants - e. g., brush mechanism in Pisum - and vice versa. Thus at least several easily recognized traits have to be considered in plants populations for the accurate determination of the pollination mode. In many botanical and agricultural manuals there are the pieces of advice how to recognize a mode of pollination e. g., such well-known books as written by Allard or Elliot. To recognize more precisely a mode of pollination all authors advise to do the special experiments. Now I want to show that "new" morphological traits which have not been used till now for this purpose help us that in many cases these experiments could be omitted.

Results and discussion

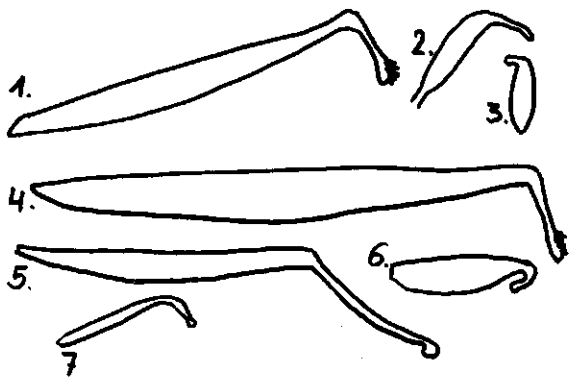
For easier presentation of the results, investigated plants have been divided into the groups according to their modes of florescence, pollination and fructification. "Typically self-pollinated plants" have been included into the first group. It consists of such self-pollinated plants which often bloom rather poorly and therefore they are pollinated rather rarely or in case of cleistogamy are not pollinated by insects at all. Nearly every flower is pollinated in these plants, because own pollen always fits to own stigma and it always matures in the most suitable time. Poorly blooming causes there are rather enough available resources to mature every pollinated flower into fruit /Table 1/.

Table 1. Fructification in "typically self-pollinated plants".

Species	Mode of pollination	Percentage of pods set	Percentage of seeds set
Vicia angustifolia L.	open	91.9	87.7
Vicia hirsuta /L./ S. F. Gray	open	95.7	90.0
	isolated	80. 4	95.0
Ornithopus sativus L.	isolated	88.7	82.5

So such plants, as we can see on the Table 1, in sufficient environmental conditions can produce in infructescence nearly such many pods as there were the flowers and nearly such many seeds in pods as there was the number of ovules. I have also assumed that in these typically self-pollinated plants the strong tendency should appear to form short styles of pistil. But why do I hope to see such a tendency? The style of pistil - in gametophyte system of self-incompatibility in Papilionaceae - is like a "strainer" which inhibits the inadeqate pollen tubes from the process of growing. Of course, such a strainer in the plants which have mostly "decided" for self-pollination is unnecessary. There is one more tendency in typically self-pollinated plants. I have noticed that the petals of these plants come out slowly from the calyces and therefore, in adult flowers the calyces are rather big. /W. Wojciechowska 1983/. The petals of the typically self-pollinated flowers do not have to grow very fast because they do not need to attract the insects. Slow growing

of petals which are together with generative organs deeply hidden is the calyx, is useful in this case because it protects these organs from damaging, diseases and so on. When we see the plants with the aforesaid traits we might assume that there is typically self-pollinated population. In most "typically self-pollinated plants" examined by me the short styles and long calyces have been found but more research works should be made /Figs. 1-7/.



Figs. 1-7. Pistils from "typically" self-pollinated plants with short styles - 1. Vicia angustifolia, 2. V. tetrasperma, 3. V. hirsuta, 4. V. sativa, 5. Tetragonalobus purpureus, 6. Glicyne hipsida, 7. Ornithopus sativus x 5,3.

The second group consists of such self-pollinated plants which bloom rather plentifully and therefore there are also often pollinated by insects. They produce, in contrary to the first group, distinctly less pods there were flowers. But the number of seeds in pods is nearly the same as the number of ovules similarly to the first group. It is called "self-pollinated and partly entomophilous plants" /Table 2/

Table 2. Fructification in self-pollinated and partly entomophilous plants.

Species	Mode of pollination	Percentage of pods set	seeds set
Lupinus luteus L.	open	27.8	83.6
Lupinus albus L.	open	52.3	96.2

"Entomophilous but self-compatible plants" belong to the third group. It is similar to the second group but it blooms usually more plentiful and contrary to the second group it must be pollinated by insects. The percentage of the pods set is distinctly lower similarly to the second group than the number of flowers and the percentage of seeds in pods is also high as in the previous group /Table 3/.

Table 3. Fructification in entomophilous but self-compatible plants.

Species	Mode of pollination	Percentage of pods set	seeds set
Astragalus glycyphyllos L.	open	42.9	82.6
	isolated	1.5	-
	artificial	47.5	77.3
Lupinus polyphyllus Ldl.	open	25.0	80.5
	artificial	inflorescences set pods with seeds	

According to me such a good seeds set in pods in entomophilous plants indicates their self-compatibility. Self-compatibility like as a self-pollination increases the chance of pollination and fertilization. As we can see on the table 3, the legitymacy of this assumption has been proved at two species, but of course it is not sufficient. I foresee that very good seeds set in entomophilous species can be as a rule connected with their self-compatibility. On the other hand poor set need not always indicate self-incompatibility. As we know, besides self-incompatibility there are other mechanisms as - e. g., dichogamy which prevent from self- pollination.

It is just impossible, having only the table data, to distinguish self-pollinated but partly entomophilous plants from entomophilous but self-compatible ones. According to my hypothesis both aforesaid groups could be easily distinguished by accurate observation of at least several infructescences. The main factor which limits the pods set in the second group is availability of resources. Besides it there is also another important factor in the third group which can form the infructescence. This is the "disposition" of insects. Their "indisposition" caused by the bad weather conditions during blooming brings about the gaps between the pods. Of course as the "indisposition" of insects is a random event, at least several infructescences must be looked through because

it need not be any gap in each. Photo 1 shows the appearance of entomophilous and autogamous lupins infructescence /Photo 1/

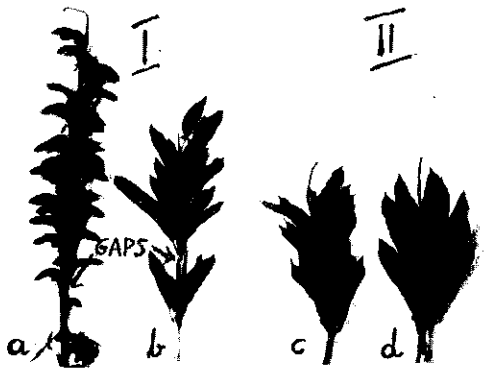


Photo 1. Appearance of lupins infructescence - I - entomophilous species with the gaps between the pods: a - Lupinus polyphyllus, b - L. mutabilis, II - autogamous species without the gaps: c - L. luteus, d - L. albus.

Table 4 presents fructification in entomophilous full self-incompatible plants.

Table 4. Fructification in self-incompatible plants.

Species	Mode of pollination	Percentage of pods set	Percentage of seeds set
Coronilla varia L.	open	34.5	32.5
	isolated	0.0	0.0
Lathyrus pratensis L.	open	62.5	34.5
	isolated	0.0	0.0
	artificial	3.3	5.0
		aphides	

The attention can be put to the low percentage of seeds set in these plants. This percentage should be always lower than in the previous groups but it may differ depending on the external and internal conditions from year to year and from population to population. Besides enthomophilous full self-incompatible plants there are only partly self-incompatible ones. In such plants the mechanisms of self-incompatibility are not quite efficient. In these cases some viable seeds could be set after self-pollination. Such events are very well documented especially in Polish literature /Barcikowska 1966, Młyniec 1962, Spiss 1972, Wojciechowska 1963 and many others/. Of course in such cases a percentage of seeds set should be higher than in full self-incompatible plants but lower than in self-compatible ones.

At the end I present shortly the "new" traits or tendencies which should be examined using more species:

1. The length of the style of pistil - in typically self-pollinated plants we expect the tendency to form the short styles.
2. The size of the calyx in the ratio to the petals - in typically self-pollinated plants we expect the tendency to form the long calyces, but these traits are only used in cases when the fundamental unit which attracts the insects is a single flower.
3. The position of pods in infructescence - the gaps between the pods indicate cross-pollination.
4. The very good seeds set in entomophilous plants indicates their self-compatibility.

Acknowledgment

I wish to express my thanks to Professor Stanisław Sulinowski for valuable discussion of the results.

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CYTOLOGICAL APPROACH OF STERILITY AND INCOMPATIBILITY IN TEA (CAMELIA SINENSIS)

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Summary

Microscope observations of self-incompatibility and sterility in the tea plant show that the time necessary to reach the ovules is twice as long after self-pollination than after crossing. A low proportion of the embryo-sacs fail during the differentiation of the gametophyte or show some irregularities. The seed sterility of the tea occurs at or after fertilization of the ovules and could be attributed to genic or physiological factors.

Introduction

Tea is propagated by cutting in order to secure the uniformity of the cultures. The shortcoming of that form of propagation is a lack of genetic diversity in the population that could have serious consequences in the case of epidemic disease or drought. Seed multiplication could be favourable to now, sexual reproduction of the tea plant has little been investigated. The sterility and self-incompatibility may make difficult the crossing and breeding of some cultivars.

The present work is a cytological study of the causes of sterility and incompatibility in the tea plant after artifical pollination.

Results

1. Observations on incompatibility

1.1. In vitro germination of pollen

Pollen grain is binucleate and shows three pores. In vitro germination has been achieved on a culture medium containing only 8 % sucrose and 1 % agar, at pH = 5. A few minutes after sowing the pollen germinates and nearly 90 % of normally differentiated pollen grains have produced a tube after about 3 hours; 85 % of the pollen is apparently fertile.

1.2. In vivo pollen germination and pollen tube growth

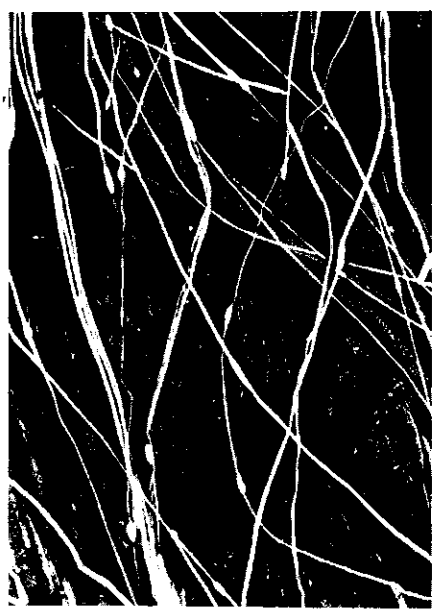
One hour after pollination, the germination of the self-pollen on the stigma is as good as after cross-pollination, but the elongation of the pollen tubes is slower. The level of the ovules is reached after about 40 hours for crossing and 72 hours for selfing. In that later case, the number of pollen tubes reaching the ovules is distinctly lower.

2. Observations on sterility

The ovary is trilocular with 4 ovules per loculus and axile placentation. The ovule is anatrope, the embryo-sac is bisporic (Allium type) and embryo is of the Solanum type.

Ovules showing abnormal development are not frequent. About 11% fail before the maturation of embryo-sac. Some embryo-sac (9 %) contain one or several supernumerary nuclei, differentiated or not. The most frequent case is a duplication of the whole content of the embryo-sac. One ovule showed 24 nuclei. Sometimes, only the sexual apparatus is duplicated. We have observed pollen tubes reaching the micropyle of ovules with duplicated embryo-sacs, but the ultimate fate of these abnormal embryo-sacs is not yet known.

The abortion rate before the maturity of embryo-sacs is not sufficient to explain the actual level of seed sterility. That level is high and could be due to genic or physiological phenomenon occuring at the fertilization time or soon after



Squash of style showing pollen tubes and their callose plugs (fluorescence).

INCOMPATIBILITY IN CAMELIA SINENSIS; A GENERAL APPROACH

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Summary

In spite of its economic importance, the tea plant has little been investigated on its reproductive biology; the crop production is vegetative and clonal selection is the most important of breeding strategies. However, a good knowledge of its incompatibility system is not without interest; since stump from seed garden (biclonal) is necessary in some dry areas, furthermore, attention is focused on the hybridization of materials of widely different geographical provenance for hybrid vigour and a novel objective is appeared : oil extraction from seeds. The sterility is high; one, two or three seeds for 12 ovules per fruit and fruiting rate is 20-30 %. After several investigations such as diallel crosses, pollen germination, cytological examinations, it seems to be a new incompatibility system. A like in the two major systems, autosterility is the rule, but the pollen tubes inhibition does not occur in the style nor in the ovary. A like in Theobroma cacao which is partly autofertile, incompatibility reaction is localized in the embryo-sac after gametophytic fusion; the system is thus primitive; other species which discriminate against incompatible pollen tubes in the style have the advantage of conserving their female gametes until such time as compatible pollen arises. Such a system where autopollen is always the first on the stigma could explain high sterility encountered in a polyclonal seed garden.

Keywords : tea, incompatibility, sterility.

Introduction

There are several kinds of self-incompatibility systems in plant kingdom, some characterized by morphological differentiation of the mating types (heteromorphic such as in Cinchona), some not (homomorphic). In the majority of the reported cases of incompatibility, a reaction of the pollen or pollen tube with the stigma or the style is involved. In incompatible pollinations, the germination of the pollen, when applied to the stigma, may be totally inhibited; or if germination does take place, the pollen tubes carrying male gametes which are incompatible with the female plant are arrested in their growth in the style and so, fail to reach the embryo-sac. The reaction of the pollen towards the diploid tissues may in some cases be determined by the haploid genotype of individual pollen grains, in such case, the incompatibility reaction is said to be gametophytic. In other instances, the pollen's reaction is determined by the diploid genotype, they all behave phenotypically similarly towards the style, such cases are said to be sporophytic.

Theobroma cacao is an anomaly in the incompatibility world; the incompatibility system is not established in all the trees (Cope, 1967-1958; Bouharmont, 1960). The incompatibility reaction is delayed until male and female gametes have met in the embryo-sac or in some cases, during the early development of the embryogenesis (Bouharmont, 1960). Once penetrated, the ovules are not receptive towards any other pollen tubes, so that an enormus waste of female material is realized when some trees are selfed, 50 % non-fusion or abortion appear in the ovary, due to encounter of gametes carrying the same allele. The control is doubly gametophytic (Bouharmont, 1960). Only the heterozygous combination of S-genes is viable.

Knight and Rogers (1953- 1955) suggested that a single locus was concerned which showed multiple allelomorphism, with dominance or equality relationships between alleles. For Cope (Cope, 1957-1958), two other loci are required besides the S-locus; these have been called A and B and are thought to be concerned with the production of a non-specific incompatibility precursor upon wich the S-alleles act to give specific incompatibility reactions. This system is primitive and seems to be alike that found in some Oenothera where the maintenance of heterozygosity is assured by a balanced lethal system at zygotic level which involves a 50 % reduction in seed set. For Cleland (1972), in these Oenothera, the non-fusion or the abortion of fertilized ovules is not due to a gene, but to the absence of necessary genes to gametes fusion or coordinated development of embryo

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and endosperm. A complementation between the male and female gametes secures the fertilization process or the development of the seed and maintains the heterozygosity.

Results and discussion

One compatible fecondation assures the fruit development.

The embryo-sac is bisporic (Allium type) and not as Polygonum type (Simura and Oosona, 1956). The bisporic nature of the embryo-sac may complicate the understanding of the incompatibility system. In such a case, the reaction at the zygotic and endospermic level will be different and may explain the abortion of ovules after some days of development.

Abnormal gametophytogenesis may explain a partial sterility; 11 % of the embryo-sac are degenerated at anthesis and some 9 % contain surnumerical nuclei. This ovular sterility is probably variable from clone to clone; in annual crops produced by seed, there is intensive selection for reproductive normality but in clonal crops, a degree of reproductive derangement is present and sometimes so highly developed as to forbid normal sexual reproduction (Tilquin and al, 1983).

The autopollen grows more slowly than allopollen when applied separately on the stigma but we know nothing about the growth of the mixed pollen. The two types reach the embryo-sac. The rate of seed is significantly different between the basal - and upper ovules while the abortion during the development is at random, suggesting that autopollen is the first to visit the basal ovules.

From the diallel crosses, some features must be pointed out :

- a total autosterility; the incompatibility mechanism is thus different from the cacao system. It must be sporophytic. The marked difference between reciprocal crosses argues in this meaning. A strong effect of the female cytoplasm (Tri 31/8 x Tri 31/11 - 75 % of fruiting and the reciprocal, Tri 31/11 x Tri 31/8,16 % - a sibcross). A great deletion is more decisive in the embryo-sac than in the male gamete.

Some observations show dominance relationships, for example, in the biclonal seed garden of Kayanza, only one clone (IB 92) set fruit, the other (Tri 31/11) is totally sterile. This latter is fertile in the polyclonal seed garden of Nyakagezi (see above). The dominance relationships could be explained by the importance of the delection carried by female or male gametes; the absence of one gene is recessive to the absence of two genes and so on.

The incompatibility system in Camelia sinensis is unknown in plant kingdom; to get the fundamental knowledge on the breeding system and seed production of the tea plant, several points must be investigated; the growth of mixed auto-allopollen tubes in the style, the nature of the abortion in the developing ovules.

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POLLEN TUBE BEHAVIOR BY CARBON DIOXIDE IN RELATION TO OVERCOMING SELF-INCOMPATIBILITY IN BRASSICA

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Summary

Studies of selfed-pollen behavior following application with high concentration of CO_2 gas, which has been noted to overcome the self-incompatibility reaction in Cruciferous species revealed that significant changes in the pollen tube growth of germinating grain on stigma occurred prior to its penetration into the papilla cell. Remarkable increase in pollen tube breadth was found to depend on the concentrations of CO_2 which enables the pollen to germinate and protrude the tube into the stigmatic papillae against the self-incompatible response. Observation of these pollen tubes using SEM also revealed that most of the pollen tubes enlarged at their tips of connecting region to the papilla cell. Significance of the response of pollen tubes to high CO_2 concentration is discussed in relation to their effect upon subsequent penetration and overcoming self-incompatibility.

Introduction

Carbon dioxide application to self-incompatible Brassica species has a great advantage in producing selfed seeds which are necessary to obtain inbred lines for F_1 seed production (Nakanishi & Hinata 1974). Previous work (Nakanishi et al. 1969) showed that high CO_2 ranging from 3 to 5 % in air enables the self-incompatible pollen to penetrate into the stigmatic papilla cell. Further studies revealed the effective time of CO_2 application for self-pollination (Nakanishi & Hinata 1973). The objective of this study was to demonstrate the pollen behavior on stigma following CO_2 treatment.

Materials & methods

Self-incompatible strains of B.oleracea, D.ercoides and B.campestris were used. Flowers were pollinated and then treated with 1-20 % of CO_2 using air-tight flask, for 6 hr at 23°C. Stigmas were collected and stained with water blue for fluorescent observation. Each pollen tube growth on stigma was evaluated by measuring the width of its narrow, median and wide portion.

Stigmas were also submitted to the scanning electron microscopy to examine the effect of CO_2 application on the profile of pollen tubes adhering to the papilla cells.

Results and discussion

Most of the self-incompatible pollen grains produced snaked or radially coiled pollen tubes onto the stigmatic papilla cells. These tubes were not uniform in width. Pollen tubes treated by 5 % of CO_2 grew fat, whether the tubes spread over the papilla cell or penetrated into them. The thickest region of the pollen tube grown in high conc. of CO_2 gas was approximately 10 μm as compared to 5 μm in the control.

Callose deposition which looked like lense always appeared on the tip of papilla cells where self-incompatible reaction occurred. The symptom of these callosic reaction still appeared on the papilla, when self-incompatibility had been broken down by CO_2 gas.

Pollen tube behavior was also studied in response to 1,3.10 and 20 % of CO_2 in air. The distribution of the pollen tube width showed that the pollen tube growth was affected by the concentrations of CO_2 gas. About 30 % of the tubes treated with 3 % CO_2 was over 3 times thicker than the control. Pollen tubes penetrated into the papilla cells when the gas concentrations were 3 to 10 %.

Pollen tube behavior on stigma were also observed by the use of SEM. The pollen tube which was enlarged by CO_2 showed simillar behavior to that of self-incompatible ones. The tubes often severely ridged both at the contact region and the inner wall of the papilla cell.

The present study indicated that increased atmospheric CO_2 gas to overcome self-incompatibility could have affected pollen by changing the metabolic pathway of the tube growth.

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CARBON DIOXIDE BLOCKS THE STIGMA CALLOSE RESPONSE FOLLOWING INCOMPATIBLE POLLINATIONS IN BRASSICA

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Summary

Carbon dioxide enrichment is known to overcome sporophytic self-incompatibility in Brassica. Elevated CO_2 (30 mmol CO_2 mol^{-1} air), supplied via a flow-through gas system, was shown to block the formation of rejection callose in the surface stigmatic papillae of Brassica campestris following self-pollination. Possible mechanisms by which CO_2 may affect callose formation are discussed.

Keywords: Brassica campestris, callose, self-incompatibility, carbon dioxide.

Introduction

The induction of self seed set with carbon dioxide in self-incompatible plants has been investigated extensively in Brassica. The CO_2 response was observed cytologically as the extent of pollen tube growth (Nakanishi et al., 1969) or by seed-set following CO_2 treatment of self-pollinated Brassica pistils (Nakanishi and Hinata 1975). Callose, a 1-3-β - linked glucan, has been shown to accumulate in the papillae of Brassica stigmas in response to self-incompatible pollinations (Heslop-Harrison, et al., 1974). Our study was undertaken to observe the effect of CO_2 on this callose rejection response.

Materials and Methods

Mature flower buds of Brassica campestris var. T15 were emasculated and branches bearing these buds were placed in distilled water overnight. The following morning, open flowers were excised and supported in agar. Pollen contaminated stigmas were discarded. Clean stigmas were self-pollinated, and several replicates including unpollinated controls were placed in each of two identical perspex containers A and B (1.5dm^3 volume) at 75% R.H.

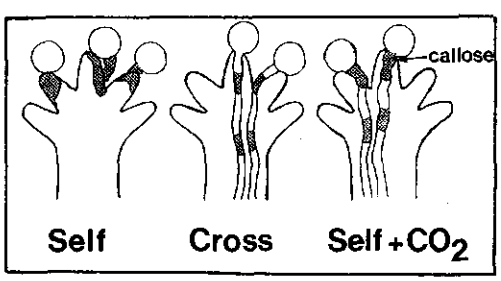
Two different gas mixtures were prepared by drawing ambient air from the laboratory roof. This air was cleaned through a filter of activated charcoal and humidified. Half of this air was pumped directly into container A. The rest was bubbled through KOH and passed through two soda lime towers before this now CO_2-free air was mixed with pure CO_2 to a concentration of 3% (v/v) and fed into container B. Both containers A (ambient air) and B (3% CO_2) were placed in the light at 25°C. After 22 hours the pistils were fixed in acetic acid: ethanol (1:3), washed in 70% (v/v) ethanol and stained with decolorised aniline blue. Fixed pistils were then lightly squashed and observed by incident fluorescence microscopy.

Results and Discussion

Unpollinated pistils from both ambient air and 3% CO_2 treatments showed no fluorescence in stigmatic papillae, although a strong callose rejection response was observed in self-pollinated stigmas exposed to ambient air. On self-pollinated stigmas, aniline blue fluorescent material could be seen in stigmatic papillae and a few pollen grains had germinated but no penetration of the stigma had occurred (Fig. 1).

However, in the 3% CO_2 treatment most pollen grains had germinated and many had penetrated the stigma. This bundle of pollen tubes could be traced down the style to the ovules. There was no accumulation of rejection callose in stigmatic papillae other than that in the walls of penetrating pollen tubes. The appearance of these CO_2 treated stigmas was similar to that observed in compatible cross pollinations.

The primary action of CO_2 in breaking incompatibility may be to directly inhibit the formation of callose and thus remove the physical barrier to self pollen entry. Alternatively, CO_2 may disturb molecular recognition between pollen and stigmatic papillae such that the callose reaction is not induced against self pollen. The Brassica pollen-stigma interaction offers an attractive system for further exploration of the role of CO_2 in breaking self-incompatibility.



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MOLECULAR INTERPRETATION OF OVERCOMING SELF-INCOMPATIBILITY IN BRASSICA

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Summary

In plant breeding, several methods have been developed to temporary overcome self-incompatibility in flowering plants in order to obtain parental pure lines. Such methods are based on the use of treatments which are applicated either on pollen or stigma alone, either on pollinated flowers. In this paper, we reviewed these different techniques and give some new data obtained in Brassica oleracea. Treatments with controlled concentrations of carbon dioxide and controlled relative humidity (rH) have effects on pollen quality, pollen adhesion and germination, stigma callose response and pollen tube entrance in the style. At the light of these results, a molecular interpretation of overcoming self-incompatibility in Brassica is proposed.
Keywords: Self-incompatibility, carbon dioxide, Brassica oleracea.

Introduction

In sporophytic self-incompatibility systems, the recognition reaction between male and female partners occurs at the stigma level (see Heslop-Harrison et al., 1975). A characteristic of this incompatibility system is the occurence of callose lenticules in stigmatic papillae cells in response to self-pollen or pollen tubes. The specificity of this callose rejection response has been recently examined in Brassica (Kerhoas et al., 1983). The callose reaction is thought to be due to specific molecules associated with expression of S-alleles. The existence of self-incompatibility constitutes an obstacle in producing inbred lines used to make F1 hybrid cultivars in breeding programs. However, several methods have been developed to overcome these barriers to selfed seed production. In this paper, we briefly reviewed these methods and discuss some new data obtained in cauliflower by the use of CO_2 treatments associated with high relative humidity (Palloix et al., 1984).

Classical methods used to overcome self-incompatibility

The flower age has early been reported to affect the partial breakdown of self-incompatibility in Brassica. Selfed seed can be obtained after self-pollination of aged flowers (Kakizaki, 1930) or after bud self-pollination (Kakizaki & Kasai, 1933). In bud pollination, the pseudo-compatibility appears related to the immature stage of stigma where recognition factors contained in the pellicle would be absent (Shivanna et al., 1978). This method is certainly the more often used by plant breeders to multiply highly self-incompatible lines. However, bud-selfing is time-consuming and very expensive because of the hand labour involved. Then, more practical methods have been investigated, based on the use of treatments applicated either on pollen or stigma alone, either on pollinated flowers.

Self-incompatible pollen grains can turn into compatible when mixed with killed compatible pollen or their extract (mentor effect). The washing of self-pollen with organic solvents has also been shown to overcome incompatibility in Brassica (Roggen, 1974). The efficiency of these treatments seems to come from diffusion of substances from compatible pollen. These compounds would act either directly on the stigma surface either on the pollen or pollen tube in altering their recognition potentialities. Though the precise nature of these substances is still unknown, Howlett et al. (1975) found that some purified proteins of the pollen wall in Cosmos bipinnatus were able to overcome self-incompatibility.

On mature stigma, physical and chemical treatments have been tried with success in Crucifers: mechanical damage, high temperature, local heating, electric shocks, organic solvents, high relative humidity and high concentrations of carbon dioxide... (see Ockendon, 1978, for example). Such treatments induce a partial breakdown of the self-incompatibility which is more or less pronounced according to the species or the line tested. The effects of these various treatments on the incompatibility reaction remain difficult to determine since the cellular or molecular alterations brought to the stigma are not precisely known. Nevertheless, these data demonstrate that the efficiency of the stigmatic barrier depends on the physical and chemical integrity of the stigma surface.

The effect of high concentrations of CO_2 in overcoming self-incompatibility in Brassica has been extensively investigated since the first work of Nakanishi et al. (1969). Their method consisted in incubating self-pollinated flowers with 3 to 5% CO_2 levels for 6 hours. Recently, O'Neill et al. (1984) found that the stigma callose response in Brassica campestris was blocked by exposure to a 3% level of CO_2.

In order to clarify the effects of carbon dioxide on pollen-pistil interaction in Brassica, a study was attempted in our laboratory based on an in vitro system of pollination (Palloix et al., 1984). Some of these results are now discussed.

Effects of increasing concentrations of CO_2 on a self-incompatible mating in Brassica oleracea var. Botrytis

The various CO_2 treatments were applicated to excised flowers which were placed on agar medium in a hermetic glass container. At the end of treatment, 4 events of fertilization process were examined: the adhesion, the germination, the penetration of pollen tubes in the style and the callose rejection response of stigma. The following conclusions have been reached for the 3 different high self-incompatible lines tested:

1- As firstly demonstrated in cabbage by Nakanishi et al. (1969), low concentrations of CO_2 applied during the progamic phase will overcome the self-incompatibility mechanism. The most efficient concentrations (4 to 6%) are similar to those reported for B. napus by Dhaliwal et al. (1979) and for B. campestris by Dhaliwal et al. (1981) and O'Neill et al. (1984). However, a longer period of application (8, 16 or 24h) proved to produce the most effective result in cauliflower.

2- Higher concentrations of CO_2 (above 8%) have apparently a toxic effect.

3- As found in B. campestris (O'Neill et al. 1984), CO_2 levels efficient in overcoming incompatibility block the stigma callose response following self-incompatible pollinations.

4- rH treatment alone did not overcome the incompatibility reaction in cauliflower.

5- CO_2 acts at the pollen-stigma interface rather than on the pollen or stigma separately since no effect on the self-incompatibility response was observed when CO_2 was applied prior to pollination.

6- CO_2 acts early in the interaction since the first event in fertilization, the adhesion step, is modified.

If the effects of CO_2 applications have been described, how can act the gas at a molecular level to modify the incompatibility reaction?

Interpretation of the CO_2 effects in overcoming self-incompatibility: molecular model of pollen-stigma interaction

Recently, we observed by transmission electron microscopy that the first contact of pollen grain on stigma papillae was followed by a reorganization of stigma surface components (Gaude & Dumas, in preparation). This event may play a preponderant role in pollen adhesion and recognition processes in assuring control of the water flow from stigma to pollen. As shown by the work of Roberts et al. (1980) in Brassica, the pollen hydration level after pollination seems directly correlated to the acceptance or rejection of the grain. The fact that high relative humidity may overcome self-incompatibility confirms the importance of the hydration step in the recognition mechanism.

These observations led us to recently propose a molecular model to explain pollen-stigma recognition in sporophytic self-incompatibility systems (see Dumas et al., 1984). This model is based on the control of water flow from stigma to pollen by the reorganization of cell surface components of both interacting partners . In a compatible mating the specific interaction between the recognition factors (S-products) would permit the arrangement of lipids and proteins in a macromolecular edifice which present facilitated ways for water flow and thus, would ensure a full pollen hydration (Fig. a). In an incompatible mating, the S-product interaction would induce the formation of a hydrophobic macromolecular structure which would constitute a barrier to efficient hydration of pollen grain (Fig. b).

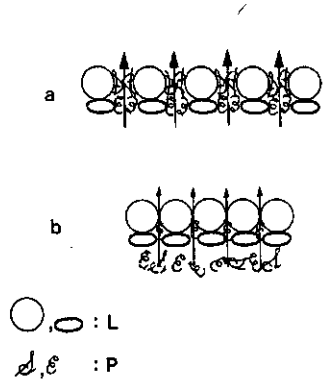

Fig.a & b: Model of molecular interaction between pollen and stigma surface components in Brassica (from Dumas et al., 1984). L=lipids, P=proteins, arrows indicate water flows from stigma to pollen.

This model for pollen-stigma interaction is in agreement with the various techniques used to overcome self-incompatibility. Treatments, in modifying either the pollen surface or the stigma pellicle would prevent the formation of the hydrophobic edifice or altere its hydrophobic character.

The action of CO_2 may be to disturb the dialogue between male and female surfaces at the moment of pollination. The gas may act on the various components of the pollen-stigma interface or specifically on the interaction between putative stigma and pollen S-gene products. The precise mechanism of this action of CO_2 is not yet clear.

Carbon dioxide has been shown to be a meta-

bolic regulator in animal and plant cells (review of Mitz,1979). CO_2 more particularly affects processes such as membrane permeability and molecular interactions. At the membrane level, carbon dioxide interferes as well as on proteins that on the lipid bilayer. CO_2 interacts with uncharged amino-groups of proteins to form carbamates which could lead to conformational changes in the molecule. On the lipid bilayer, the gas appears to change the physical state of lipids in increasing solubility.

During pollen-stigma interaction, it seems possible that conformational changes in proteins and modification of the interface lipid fluidity could affect the S-product interaction. This would result in a more permeable interface which would allow full hydration of self-pollen grains and growth of self-pollen tubes in the style, in the presence of elevated CO_2 levels.

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EARLY EVENTS ON THE BRASSICA STIGMA FOLLOWING COMPATIBLE AND INCOMPATIBLE INTRASPECIFIC MATINGS

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Summary

A combination of new electron microscopic (EM) techniques and simple physiological experiments have identified a superficial layer lying on the pollen grain coating. The behaviour of this layer after compatible and incompatible matings strongly suggests that it plays an important part in the control of pollen development. These findings are discussed in the light of models currently proposed for the operation of the self-incompatibility (SI) system in Brassica.

Introduction

From the first microscopical investigations it became evident that the sporophytically-controlled SI system of Brassica operated at the surface of the stigma for, if it germinated at all, incompatible pollen formed contorted tubes apparently incapable of penetrating the papillae. Although the use of the EM (Kanno and Hinata, 1969) confirmed that tubes from self-pollen failed to enter the stigma cuticle, and Mattsson et al.(1973) reported the presence of a 'pellicle' on the surface of the papillae, recent investigations into the SI mechanism in Brassica have indicated it to be a highly complex process and not analagous, for example, with the antigen-antibody system of animals. Further, the development of self-pollen may range from lack of germination to production of a long tube which, on attempting to enter the female papillae, often appears to stimulate the production of callose. This range of development is thus not easily reconciled with a simple interaction between S(incompatibility) gene products on initial contact between the sporophytically-synthesised pollen grain coating and stigma surface.

Investigation into the SI mechanism operating in Brassica has also been hindered by its extreme genetic and physiological lability. A number of Brassica S-genotypes are available ranging from very 'weak' to very 'strong', and it has long been known to plant breeders that the SI system can be overridden either by raising humidity (Carter and McNeilly 1975) or by increasing atmospheric CO_2. While recent work has succeeded in identifying some of the female molecules involved (Roberts, et al. 1979, Ferrari et al. 1981) and has underlined the dynamic nature of the pellicle (Roberts, et al. 1984b), no details are available of the pollen-held components of this system.

The surface of the stigmatic papillae

The only evidence that self-incompatibility factors are contained in the pellicle is provided by the adhesion assays of Stead et al. 1980 which demonstrated not only that adhesion of pollen to the stigma was related to SI, but also that enzymic treatment of the pellicle reduced 'compatible' adhesion to an 'incompatible' level. Interestingly, the source of the molecules implicated in SI was shown to be the stigmatic cytoplasm, and full adhesive capability was restored to the stigmatic surface within 2h, indicating either a very rapid repair system, or that molecules are 'turned over'. A clue to the nature of these factors was provided by the observation that acquisition of the SI system by buds was accompanied by the synthesis of a glycoprotein (pI 5.6) present in the stigmatic papillae. Subsequent elegant experiments have demonstrated this glycoprotein to be S-gene specific (Nishio and Hinata 1979) and involved in controlling pollen development on the stigma (Ferrari et al. 1981). While it is possible to use the stigma surface as a probe to b ind molecules extracted from the pollen-grain coating, and vice versa, this binding has proved variable and not S-gene specific in the aqueous environments used. Most recently an autoradiographic technique has confirmed that cycling of proteins does indeed occur in the stigmatic wall, and maybe even in the pellicle (Roberts, et al. 1984a) and tests with inhibitors indicate that maintenance of protein synthesis is required for the continued operation of the SI system (Roberts et al. 1984b). Thus a picture emerges of the pellicle as a dynamic fluid mosaic containing at least one cycling protein involved in the SI system.

The pollen grain and its coating

There is considerable circumstantial evidence that the pollen grain coating, or tryphine, is actively involved in the self incompatibility response. Extracts from pollen coatings have been shown to stimulate the production of callose in 'incompatible' stigmatic papillae (Dickinson and Lewis 1975) while exchange of coatings between pollen grains can alter their behaviour on self-stigmas (Dickinson, H.G.,unpublished).

Nevertheless the presence of S-specific molecules within the pollen coating remains to be proven.

The main barrier to progress in this field is that experiments are normally carried out in an aqueous environment, while the surface of the stigma is comparatively dry. The pollen itself is so sensitive to moisture that it can be shown to adsorb moisture immediately following anthesis. Similarly, attempts to examine the structure of germinating pollen are fraught by the fact that the grains will hydrate during the fixation process. We have now developed a range of fixation techniques which do not involve aqueous solutions (Elleman, et al.1984). Further, since fixation is nearly instantaneous, it has proved possible to chart the dehydration of the pollen in the anther and its rehydration on the stigma surface on a minute-by-minute basis.

In the anther the pollen coating assumes its final form just prior to the dehydration of the grain itself. Instead of the electron-opaque image offered by normal fixitives, the coating now appears mottled and electron-lucent bounded by a 'membrane'. This 'membrane', which is some 10nm in depth invests both coating and the exine (Fig. 1).

Fig.1. Superficial layer (L) bounding the pollen coating (C) and exine (E).
Scale bar = 0.1μm

Since the coating is held to be lipidic in nature, this 'membrane' clearly cannot be regarded as a true biological membrane and we shall therefore refer to it as the coating superficial layer (CSL). It is already known that the CSL has some membrane-like properties, for it will not only exclude lanthanum, but can also bind it.

In the final stages of pollen dehydration in the anther the periphery of the cell assumes an electron-opaque aspect, containing only small fibrillar spheres some 200 nm in diameter. It would also seem that even in this most dehydrated form a plasma membrane does invest the pollen protoplast.

Post pollination development and its modification by the SI system

The first contact between compatible pollen and the stigmatic surface involves fusion of the CSL with the pellicle. This occurs in a matter of seconds and is followed by a conspicuous reaction of the pollen coating adjacent to the region of contact. Here, the mottled appearance of the coat is replaced by an electron-opaque aspect and the emergence of some structure. The structure visible may vary from simple electron-lucent lines of membrane-like dimension, to profiles reminiscent of vesicles and organelles (Fig. 2).

Fig.2 Exine (E) held pollen coating,'converted' following a cross pollination.
Scale bar= 1μm

While this change is first detectable after 30s, it takes a matter of 2-3min for all the coating between the grain and the area of contact to become converted. The physico-chemical basis of this coat conversion is not known, but it may well reflect a change from a primarily covalent to an ionic environment induced by the flow of water into the coat.

Once complete, coating conversion is followed by a change in the organisation of the pollen protoplast from that characteristic of dehydration to a stratified condition(Fig.3).

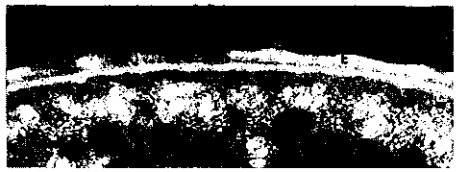

Fig.3 'Stratified' pollen cytoplasm. Note the coating (C), exine (E), vesicular layer (V) and mitochondrion (M). Scale bar = 1μm

These changes in the pollen cytoplasm are considered in detail elsewhere (Elleman, et al. 1984). Between 5 min and 1h after pollination the stratified aspect is lost and the pollen cytoplasm reorganises in an assymetrical manner, concentrating large accumulations of vesicles at the germinal pore from which the tube will eventually emerge. Further development of the tube is described in Dickinson & Lewis 1973.

Development is very different following selfing for fusion does not immediately occur between the CSL and the pellicle. Indeed, material sampled up to 5 min after pollination shows that the CSL remains intact. Events over the next 45 min suggest that contact is established between pollen and stigma, for some coating conversion does occur,albeit at a much reduced pace and only on the 'stigmatic' side of the layer. Development continues with a change to the stratified type of cytoplasmic organisation in some, but not all grains. In the presence of a 'strong' S-gene, pollen development ceases at this juncture.

Although the difference in the behaviour of the CSL goes some way to explain the adhesion results of Stead et al (1980), it does

not indicate a mechanism by which development of incompatible pollen may be arrested. There is, however, a large body of evidence pointing to the involvement of inhibitors in at least some part of the SI response (e.g. Hodgkin & Lyon 1984).

All the data currently available indicate that the SI system operates during the earliest stages of pollen development and it is thus not clear why some incompatible grains develop tubes, and why these tubes apparently are arrested by the deposition of callose. The first question is probably the easier to answer in that atmospheric water either bypasses any barrier to flow between pollen and stigma or, depending upon the hypothesis in favour, dilutes the level of inhibitor to below a threshold necessary for grain development. The formation of stigmatic callose is not so simply explained, it is possible that sporophytic coating material accompanies the tube tip and engenders a reaction once the cuticle has been eroded (Dickinson & Lewis 1975), but, equally, it may be that the callosic response is stimulated by a second, gametophytically-controlled gene, now known to be active in these plants. Nevertheless, since the formation of callose may also be stimulated by mechanical damage and fungal infection, it remains possible that a disruption in pollen tube organisation by the activities of the S-genes during the germination stages so disorganises the pollen tubes, that the stigma 'recognises' them as foreign organisms. Such an hypothesis is not easily tested, but the turgor of growing compatible tubes can be raised by supplying high levels of atmospheric water, and it is very striking that this treatment is accompanied by the development of long, tortuous tubes and the formation of stigmatic callose.

In conclusion, what can we now say of the SI system operating in Brassica? With the pellicle, the CSL clearly emerges as playing a central rôle in the control of adhesion and the subsequent development of pollen. Further, the lack of full fusion in incompatible pollination seems to result in slow coat-conversion, and the pollen cytoplasm taking longer to assume a stratified organisation. While the suppression of pollen development can be explained so far in terms of the availability of water, there is some evidence that, in the stratified condition, the pollen protoplast secretes protein into the intine and, perhaps, the coating. It remains possible that this interaction forms the basis of a second phase of the SI system - a phase involving the production of inhibitors. Whether S-gene action is directly or indirectly responsible for callose synthesis following incompatible tube growth seems unclear but, in view of our increasing knowledge of factors stimulating callose formation in plants, the latter alternative appears the more attractive.

Acknowledgements

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POLLEN GERMINATION INHIBITORS ASSOCIATED WITH THE SELF-INCOMPATIBLE RESPONSE OF BRASSICA OLERACEA L.

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Summary

Pollen germination inhibitors in Brassica oleracea L. tissue extracts were detected using a thin-layer chromatographic bioassay. Extracts of leaf, seeds, pollen and styles all contained inhibitory compounds, and stigma extracts had the greatest amounts and largest number of inhibitors. Some inhibitors were common to extracts of unpollinated, self-pollinated and cross-pollinated stigmas whilst others were only detected in stigma extracts obtained after incompatible self-pollinations. The inhibitors detected uniquely from self-pollinations were at their highest concentration approx. 2 h after pollination. The significance of these findings with respect to possible parallels between pollen-stigma interactions and plant-pathogen recognition is discussed.
Keywords: Brassica oleracea, pollen germination, thin layer chromatography, germination inhibitors, self-incompatibility, bioassay, phytoalexin.

Introduction

The sporophytic self-incompatibility system of Brassica oleracea (Thompson 1957) provides a useful model for studies on the nature of pollen-stigma recognition and for more general investigations of recognition reactions in flowering plants (Roberts et al., 1984).

Recently Hodgkin and Lyon (1979), Bushnell (1979), Lewis (1980) and Keen (1981) have drawn attention to the similarities between the interactions of plant and pathogen and stigma and pollen. We have developed a bioassay that can be used to investigate some aspects of the stigma's response following an incompatible pollination in B. oleracea (Hodgkin and Lyon, 1983; 1984). We here summarise the results obtained using this bioassay and discuss their significance in relation to possible parallels between the reaction of plant and pathogen and stigma and pollen.

Materials and Methods

Inhibitors of pollen germination were detected in B. oleracea tissue extracts using the techniques described by Hodgkin and Lyon (1983). Ethyl acetate soluble extracts were prepared from the required plant tissues and loaded on to cleaned thin layer chromatography (TLC) plates which were then developed, dried, sprayed with Petunia hybrida or Lilium lankongense pollen in appropriate germination media and incubated overnight to permit pollen germination. To obtain germination of B. oleracea pollen the procedure was modified by soaking TLC plates for 0.75 h in pH8 TAPS (Sigma Ltd) prior to loading the extracts and using the germination medium of Hodgkin (1983). Zones of inhibition were revealed by spraying the incubated plate with aniline blue to stain germinated pollen and examining under UV light or by incident light fluorescence microscopy. The effect of incompatible pollination on the inhibitors in the stigma was examined using extracts prepared from stigmas collected 2, 4 or 24 h after self- or cross-pollination or after being left unpollinated for 24 h (Hodgkin and Lyon, 1984).

Results

Fig. 1 summarises the results obtained for various tissue extracts using P. hybrida or

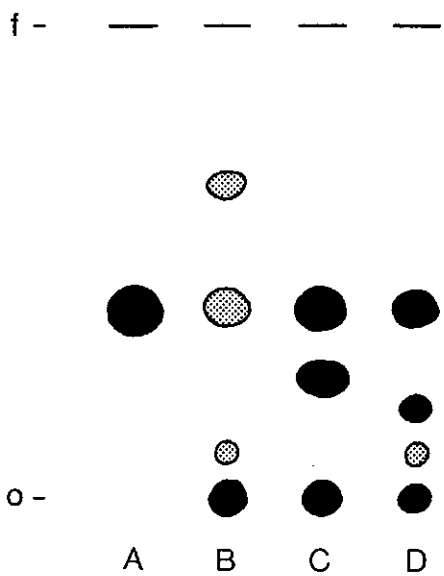
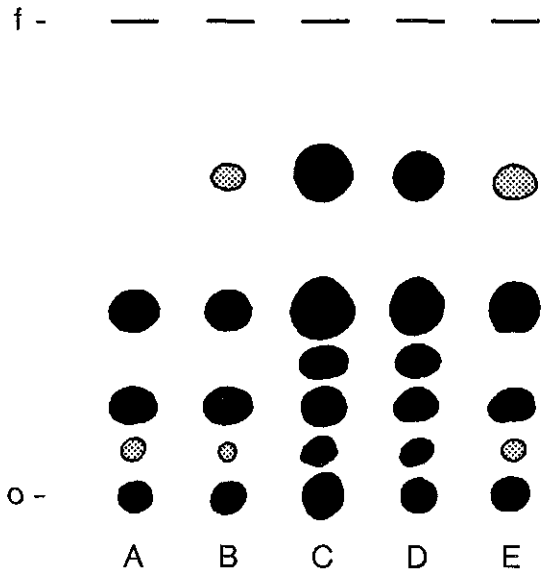


Fig. 1. Diagram of the major zones of P. hybrida pollen germination inhibition on TLC plates following chromatography of extracts of different B. oleracea tissues: A, seed; B, Leaf; C, pollen; D, style. Zones with a variable degree of inhibition are stippled.

L. lankongense pollen to detect inhibitory zones. One zone of inhibition (Rf 0.5) was common to all tissues tested although leaf and pollen extracts often gave less inhibition in this zone than other tissue extracts. Pollen extracts gave a zone of inhibition (Rf 0.25) not found in other extracts and extracts of unpollinated stigma tissue gave the largest number (up to 5) of inhibitory zones and these had larger dimensions than those from other extracts.

When extracts of stigma tissue collected 2, 4 or 24 h after self- or cross-pollination were bioassayed with P. hybrida pollen, zones of inhibition were detected from the 2 h self-pollinated extract which were not detected from the cross-pollinated or unpollinated stigma extracts (Fig. 2). The size of these additional zones was reduced in 4 h extracts and they were virtually absent from 24 h extracts. The inhibitory zones from extracts of self-pollinated stigmas tended to be larger than those from cross-pollinated or unpollinated extracts.

Fig. 2. Diagram of the major zones of P. hybrida pollen germination inhibition on TLC plates following chromatography of extracts of B. oleracea stigmas: A, unpollinated; B, cross-pollinated for 2 h; C, self-pollinated for 2 h; D, self-pollinated for 4 h; E, self-pollinated for 24 h.



At least two of the zones (Rf 0.12, 0.67) detected from the bioassay of 2 h self-pollinated stigmas were noted in 24 h self- and cross-pollinated stigmas of different inbred lines indicating that quantitative differences may exist between plants in the amounts of the inhibitors present. The clarification of such differences must await the development of improved quantitative techniques.

B. oleracea pollen germinated less well than P. hybrida or L. lankongense pollen on TLC plates. Percentage germination of 50%-65% were obtained and tube growth was poor (20-50 um). In contrast, B. oleracea pollen in hanging drops gave 80-90% germination and tube lengths of up to 1 mm. In the bioassay B. oleracea pollen germination was inhibited in clearly defined zones although they had different Rf values from those obtained using P. hybrida pollen. Zones of inhibition were detected for unpollinated stigma extracts at Rf 0 (zone diameter 3 mm) Rf 0.11 (diameter 10 mm) and Rf 0.25 (diameter 10 mm). No other zones of complete inhibition were detected although several areas of partial inhibition were seen. Extracts from stigmas self- or cross-pollinated for 2, 4 or 24 h possessed the same 3 zones but additional zones of partial inhibition, which have yet to be fully characterised, were also detected. Differences in number and Rf values of the inhibitory zones may result from the TLC plate pretreatment and high pH needed for B. oleracea pollen germination. When individual extracts were separated by HPLC, to avoid the need to use pretreated plates for chromatography, the numbers, Rf values and sizes of the inhibitory zones were similar to P. hybrida and B. oleracea pollen (Hodgkin and Lyon, unpublished).

Discussion

Several workers have drawn attention to similarities between the pollen-stigma interactions in interspecific and intraspecific incompatibility and host-pathogen interactions in plant disease. In particular, they have noted genetic parallels (Bushnell, 1979; Hogenboom, 1983), drawn attention to the significance of callose deposition (Bushnell, 1979; Lewis, 1980) and suggested a common role for cell wall components, particularly glycoproteins, in both recognition systems (Keen, 1981; Roberts, 1984). However, much of the discussion on the similarities has been speculative, reflecting key gaps in our knowledge. This is particularly true of the events that follow the primary pollen-stigma recognition reaction and result in the failure of incompatible pollen to penetrate the stigma surface.

We have described results from a bioassay technique analogous to those used to investigate host-pathogen interactions (Smith, 1982) and have shown that stigma extracts are particularly rich in pollen germination inhibitors that are effective in vitro against pollen from different species. The compounds involved would appear to be potent germination inhibitors because an amount of the extract corresponding to 10-15 stigmas

gave inhibitory zones up to 20 mm in diameter. Furthermore, following incompatible self-pollinations, the amount of pre-existing inhibitors increased and additional inhibitors were produced in the stigma that were not found in cross-pollinated stigmas. The chemical identity of the inhibitors is unknown but their solubility in organic solvents and mobility on TLC plates indicate that they have a low molecular weight. Their presence only 2 h after self-pollination shows that they are produced rapidly following the appropriate induction and their decline after 4 h indicates a rapid breakdown.

Phytoalexins are low molecular weight compounds capable of inhibiting fungal growth and are produced by plants in response to challenge by incompatible fungi (Keen, 1981). We have suggested (Hodgkin and Lyon, 1979) that compounds analogous to phytoalexins might play a part in pollen-tube inhibition in incompatible pollinations. The compounds detected by our bioassay have some of the properties of such inhibitors and some of them may therefore be involved in pollen-stigma interactions in vivo.

However, several factors must be borne in mind when examining this hypothesis. Pollen-stigma recognition and response are parts of a complex series of events which involve extreme morphological specialisation and ensure successful fusion of male and female gametes. The intraspecific incompatibility response involves inhibition of "self", in contrast to host pathogen interactions (or animal systems) where "non-self" is rejected. Incompatible pollinations may be recognised rapidly (10-15 minutes after pollination) and it is not clear whether inhibitors of the kind detected could be mobilised so quickly. Current theories of the induction and operation of phytoalexins involve the transmission of a 'stimulus' by a secondary messenger to the cell's nucleus and to neighbouring cells followed by de novo synthesis of protein. In fact, the ability of neighbouring cells to respond is central those theories whereas each pollen grain-stigma papilla cell interaction is usually considered to be an independent event (but see Hodgkin, 1977, for evidence to the contrary).

All recognition reactions involve detection, discrimination and response. At least one of the participating organisms or cells must detect the other and there must then be a process of discrimination followed by a discernible response dependent on the results of the discrimination processes. Host-pathogen and pollen-stigma interactions may share other features in that cell surface glycoproteins appear to be involved in the initial recognition events (detection and discrimination) and low molecular weight growth inhibitors can be detected in response to incompatible recognition in both systems. The extent and significance of these common features has yet to be defined but our work has shown how techniques developed for investigating one class of recognition systems can assist in investigating others. It has also opened up new avenues to be explored in pollen-stigma interactions and enabled us to detect a possible new class of biologically active compounds.

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SELF-INCOMPATIBILITY IN THE NITROGEN-FIXING TREE LEGUME, ACACIA RETINODES: PRE- OR POST-ZYGOTIC MECHANISM?

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Summary

The cytology of pollen tube growth has been followed in the self incompatible species Acacia retinodes. In both self and cross pollinations, the pollen tubes grow through the style to the ovules at approximately the same rate reaching the ovules within 11 hours. Though pollen tubes adhered to the nucellus, there was only 1.5% of ovules fertilized after self pollination compared with 14.1% after cross pollination. A pre-zygotic mechanism of self incompatibility is suggested.

Introduction

In our studies on natural populations of Acacia retinodes the plants were found to be highly self-incompatible (SI) (Bernhardt et al., 1984). Here, we present a brief account of the cytology of SI in A. retinodes.

Results and Discussion

Using the aniline blue fluorescence (ABF) method, we find that there is no inhibition of self pollen tube growth on the stigma surface or in the style. Pollen tubes enter ovules within 11 to 24 h after pollination. There is no significant difference in the rate of pollen tube growth in the style between self and cross pollinations (Table 1). During the first 6 h, tube growth rates were higher than during the final 5 h.

Table 1. Rate of growth of pollen tubes through the style of the same female parent of Acacia retinodes.

Pollination	Leading Pollen Tube Growth Rate um/min	
	(a) 0-6 h	(b) 6-11 h
Self	3.3	1.2
Cross	4.5	2.0

Differences < 10% were detected in the pollen tube growth rate between different pollen parents.

Unpollinated and most self pollinated flowers underwent abscision 6 to 8 days after pollination. We examined the cytological events in the ovules during this critical period. Fertilized and unfertilized embryo sacs differed in the appearance of key structures.

-- in fertilized embryo sacs one of the two synergids, with its filiform apparatus, nucleus and cytoplasm breaks down during pollen tube entry;

-- cell and nuclear size of the zygote are larger than that of the unfertilized egg;

-- in the central cell, one large fusion nucleus is present after fertilization of the embryo sac which may have one or three nucleoli present compared with two smaller polar nuclei in unfertilized sacs. Pollen tubes penetrating the nucellus are difficult to detect, as they did not show ABF.

Based on these criteria, the number of fertilized ovules were compared in cross and self pollinations, 72 hours after pollination (Table 2).

Table 2. Incidence of fertilization in ovules of two parents of A. retinodes fixed 72 hours after self or reciprocal cross pollination. Data obtained from 75 to 160 ovules per treatment.

Female Parent	% fertilized ovules
SELF POLLINATION	
1	1.6
2	1.3
CROSS POLLINATION	
1	14.7
2	12.8

In the self pollinated group, there were 1.5% of fertilizedovules and 14.1% after cross pollination between the same two parents. The difference is highly significant ($P < 0.001$). The Index of Self-Incompatibility (ISI), computed on the basis of yield of zygotes following self/cross pollination, 0.10, is in good agreement with the ISI computed from fruit set data (0.08).

We can conclude that the site of pollen tube arrest is in the ovary, probably in the nucellus and is likely to be a pre- zygotic mechanism. The question of whether SI is a result of pollen tube/ovule or gamete/gamete interactions is yet to be determined.

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CELL SURFACE RECOGNITION GLYCOPROTEINS AND THEIR POSSIBLE ROLE IN ANGIOSPERM SELF-INCOMPATIBILITY

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Summary

Ability to distinguish "self" or "kin" from "not self" or "not kin" is a prerequisite for proper contact, co-operation or even fusion between cells. Examples of cell surface recognition glycoproteins span from mere adhesion systems to the utmost ingenious major histocompatibility complex (MHC) of higher vertebrates. I propose that angiosperm self-incompatibility is caused by a mechanism similar to the MHC-restricted antigen recognition by cytotoxic T-lymphocytes: Pistil/pollen is recognized through *identical* S-glycoproteins in association with *complementary* sex-specific glycoproteins to trigger an *active* inhibition of self pollen tube growth. I further propose that the extreme S-gene polymorphism is caused and maintained by *gene conversion*, utilizing silent S-genes as reservoirs of diversity. This also explains the generation of new S-alleles occasionally seen after inbreeding.

Keywords: Self-incompatibility, recognition, glycoprotein, cell surface, fertilization, pollen, pistil, histocompatibility, gene polymorphism, gene conversion, mating type.

Introduction

Early in evolution, cell discrimination must have become an essential condition for cell-cell interactions. Certain cell surface molecules evolved into monitors, triggering co-operative or defensive responses, dependent on the signal received. *Speciation* must be followed (or preceded) by differentiation of such recognition molecules to establish stable barriers to sexual and/or somatic intermixing of species. *Cell surface glycoproteins* seem to be involved in most of these mechanisms.

Sexual recognition systems

It has been suggested that cell recognition systems originated with the appearance of sexuality (Monroy & Rosati, 1979), thus giving rise to other cellular communication systems in the evolving multicellular organisms.

In bacteria, "sexual" conjugation is best known from *Escherichia coli*. Here, the F (or F-like) plasmid of F^+ cells codes for pilin, a glycoprotein with a molecular weight (MW) of 11,000-12,000 daltons, polymerizing into the sex-pili (extracellular filaments) which attach to receptors on F^- cells (Willetts & Skurray, 1980). This specific recognition triggers adhesion and subsequent DNA transfer from donor to recipient cell.

In haploid yeast cells of opposite mating types, fusion is mediated by complementary sex-specific glycoproteins in the cell walls, through recognition/agglutination. The two glycoproteins are of different size: A smaller one with MW between 23,000 and 60,000, and a larger one between 130,000 and 500,000, dependent on the species (Tohoyama et al., 1979; Pierce & Ballou, 1983; Burke et al., 1980; Mendonca-Previato et al., 1982). The larger of these glyproteins is composed of monovalent subunits attached to a core molecule through disulfide links.

Among the ascomycetes, the hermaphroditic *Neurospora crassa* has one locus for its two mating types, A and a. These determine the homogenic sexual incompatibility, i.e. only gametes of opposite mating types will fuse (Carlile & Gooday, 1978). This is called heterothallism.

In the unicellular green alga *Chlamydomonas* gametes from opposite mating types (+ and -) recognize each other by their flagella tips. The flagellar membranes adhere to each other, triggering wall lysis and subsequent fusion (Matsuda et al., 1982). Sex-specific glycoproteins of high MW have been detected in *C. eugametos* (Musgrave et al., 1981) and *C. Reinhardi* (Cooper et al, 1983). - Homan et al. (1982) found indications that at least the (-) agglutination factor is externally anchored to an intrinsic membrane glycoprotein (a receptor), which on agglutination forwards an intracellular signal.

Ascidians (sea-squirts) are hermaphrodites and usually self-sterile. In the simple ascidian *Ciona intestinalis*, the glycoprotein egg envelope "chorion" prevents self-fertilization, polyspermy and heterospecific fertilization (Dale et al., 1978).

Oka & Watanabe (1957) observed that fertilization in the compound (colonial) ascidian *Botryllus primigenus* is only possible between different colonies. Scofield et al. (1982) in *Botryllus schlosseri* found this quality to be governed by one gene locus with multiple alleles. The haploid sperm is incompatible with the diploid, maternally derived egg envelopes if its allele in this locus is matched by the latter. This self-incompatibility is closely similar to the "gametophytic" type of angio-

sperm self-incompatibility.

Kinsey & Lenharz (1981) found an egg surface glycoprotein fraction from the sea urchin *Arbacia punctulata* that interfered with both sperm binding and fertilization, but in a *non*-specific manner. This led them to think that binding and species-specificity need not reside neither in the same site nor in the same molecule, contrary to prevailing ideas regarding fertilization specificity. - Lopo & Vacquier (1980) purified an antiserum against sperm surface of the sea urchin *Strongylocentrotus purpuratus*, and found it to cross-react with sperm from *all* 28 tested species from 7 phyla, including amphibia, birds and mammals.

The mammalian egg is sorrounded by the glycoprotein layer "zona pellucida" (produced by the oocyte itself) which is responsible for species-specific fertilization and subsequent block to polyspermy. One of the glycoproteins, "ZP3" (MW 83,000) is identified as the specific sperm receptor (Bleil & Wassarman, 1983). Likewise, a sperm glycoprotein seems to be involved in the sperm-egg recognition (Moore & Bedford, 1983).

Angiosperm self-incompatibility (SI, or S, when used as a prefix) must result from recognition events, but though thoroughly investigated for decades, definitive conclusions as to the biochemical basis has not been achieved. Recently, it has even been suggested that SI, at least the gametophytic type (pollen behaviour governed by the pollen S-genotype) might not be due to any qualitative mechanism, rather being the quantitative result of deleterious recessives (Mulcahy & Mulcahy, 1983). The authors enumerate several examples, some of which may well be plausibly explained by this alternative hypothesis. However, the evidence of *qualitative* SI mechanisms, though some with additional quantitative traits (e.g. *Beta vulgaris*, Larsen 1983) seem so overwhelming, and concordant with other recognition systems, that purely quantitative models may at the highest apply to exceptions from the rule.

There is now growing evidence of *glycoproteins* as being the specific products of angiosperm S-genes: In the stigma of *Brassica oleracea* (sporophytic SI, i.e. pollen behaviour governed maternally), several authors have found S-genotype-specific glycoproteins with MW between 54,000 and 66,000 (Nishio & Hinata, 1982; Nasrallah & Nasrallah, 1984; Ferrari et al., 1981). - From styles of *Prunus avium* (gametophytic SI), Mau et al. (1982) isolated two glycoproteins, MW 37,000 and 39,000, associated with the genotype S_3S_4.

Glycoproteins are common angiosperm pollen constituents (e.g. Vithanage et al., 1982), but up to the present a clear association with SI has not been demonstrated. - However, already in 1954, Linskens was able to detect glycoproteins as products of mutual reaction between *Petunia* pollen and style. Further, in 1960 he provided evidence of *like* S-specific antigens in pollen and style from the same S-genotype of *Petunia hybrida*.

Somatic recognition systems

Somatically, intercellular recognition is as essential for proper cell interactions as in the sexual mechanisms above. - A few examples should be mentioned:

In *Neurospora crassa*, somatic fusion is controlled by genes in about ten loci, all mediating heterogenic incompatibility. One of these is the mating locus mentioned above. I.e., A fuses somatically only with A, so the reaction is completely reversed compared to the gametes (Carlile & Gooday,1978)

Also in *Botryllus schlosseri*, the above mentioned ascidian, the locus for sexual self-incompatibility is involved in somatic incompatibility. Scofield et al. concluded this multiallelic locus to be solely responsible for both phenomenons, again determining homogenic incompatiblity between gametes, contrary to heterogenic discrimination between somatic cells (Scofield et al., 1982).

In plants, host-pathogen and symbiotic interactions with microorganisms attract attention. Though thoroughly investigated, the detailed mechanism of compatibility remains elusive. It has been discussed whether the basic reactions are similar to those of self- (or interspecific) incompatibility or not (e.g. Hogenboom, 1982). It might suffice here to mention that there are now reports of a glycoprotein receptor (MW 40,000) on legume roots for *Rhizobium* cells (Apte & Modi, 1983), and that *Phytophthora* glycoproteins elicit soybean production of phytoalexin (Keen & Legrand, 1980). - Phytoalexins are plant-produced, nonspecific antibiotics, which are also reported to be autophytotoxic (Boydston et al., 1983).

The major histocompatibility complex

In higher vertebrates, histocompatibility is governed by a complex of closely linked loci (MHC), which codes for transmembraneous glycoproteins (MW 44,000) (for a brief review, see e.g. Klein et al., 1981). It does not appear immediately logical for such organisms to maintain a complicated mechanism for rejection of foreign tissue. There are speculations that its primary aim is surveillance of modifications of cell surface molecules within the individual, and, as quoted earlier, the ancient origin may have been the rise of sexuality. In 1974, Zinkernagel & Doherty revealed the phenomenon now known as "MHC restriction" of cellular immunity: Cytotoxic T-cells only kill infected cells if they exhibit a specificity coded by the individual's own MHC-genotype. In other words, to be effective, the T-cell must associatively recognize "identity + difference" (MHC-molecule + foreign antigen).

Another peculiarity of the MHC is its extreme gene polymorphism, possibly only surpassed by that of the angiosperm S-genes. This variability, which as a rule involves blocks of nucleotides, rather than single base substitutions (as expected if generated though point mutations) now seems prooved to be caused by *gene conversion*, i.e. non-reciprocal transfer of information from a donor (presumably a pseudogene) to a recipient DNA duplex (Weiss et al., 1983).

Conclusion

The examples above were deliberately selected to illustrate what to the author appear overwhelmingly indicative of almost universal principles in intercellular recognition, due to common origin. Particularly, it is inferred that SI in angiosperms (as well as in *Botryllus*, *Neurospora* and others) may be caused by a mechanism parallel to the MHC restriction phenomenon: Associative recognition of identity (S-glycoprotein) and difference (♀ versus ♂ glycoproteins), triggering an *active* rejection response, possibly through release of phytoalexin (or phytoalexin-like substance), strictly localized on the individual pollen tube surface, or within it. Considering the relations in *Neurospora* and *Botryllus*, this apparently does not exclude the possibility that (some of) the S-genes also have functions in interspecific, *hetero*genic incompatibility.

Finally, it is proposed that the extreme S-gene polymorphism in angiosperms is caused and maintained by *gene conversion*. This also explains several reports of high-frequency generation of new (or known) S-alleles following inbreeding (reviewed by de Nettancourt, 1977). Silent S-genes (pseudogenes) may serve as reservoirs of diversity (and/or sometimes be called into action *in extenso*).

Looked upon in this way, angiosperm SI is no more than a special case of a general cell-cell recognition mechanism. Accordingly, it may rise and fall from time to time, without affecting its basic elements: Ubiquitous cellular recognition glycoproteins.

Acknowledgments

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UNILATERAL HYBRIDIZATION IN RHODODENDRON

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Summary

Hybridization between Vireya Rhododendrons and species in the Azalea compex is unilateral, occurring only with Vireya species as female parents. Hybridity has been confirmed by the intermediate morphology of leaf hairs on hybrid seedlings. By monitoring pollen tube growth me have also determined the arrest points associated with incompatibility of Vireya pollen on Azalea pistils.

Introduction

To our knowledge no previous confirmed successful crosses have been recorded between Vireya Rhododendrons which have flat scales on their first true leaves, and species in the Azalea complex which have simple or glandular hairs. As part of an extensive survey of interspecific compatibility in the genus Rhododendron we have made a number of controlled hand pollinations between these groups and here report on the occurrence of a general unilateral compatibility, and the recognition of hybrid seedlings by morphology of the juvenile indumentum.

Materials and methods

Pollinations involved the following materials:
Subgenus Rhododendron, section Vireya, subsection Euvireya, a number of species and fertile hybrids including R. sessilifolium, (R.laetum x R. aurigeranum), (R. intranervatum x R. 'Souvenir de J.H. Mangles'), (R. 'Dr Hermann Sleumer' x R. herzogii) & [(R. macgregoriae x R. lochae) x R. macgregoriae]; subsection Pseudovireya, R. retusum, R. quadrasianum (tropical) and R. kawakamii (temperate). Azalea complex, subgenus Pentanthera (deciduous Azaleas), R. periclymenoides, R. japonicum, R. bakeri, subgenus Tsutsusi, section Tsutsusi (evergreen Azaleas), R. indicum, R. simsii, section Tsusiopsis, R. tashiroi; subgenus Azaleastrum, section Choniastrum, R. ellipticum.

Pollen tube arrest points in incompatible crosses were determined by fluorescence microscopy of pistil squashes stained with decolorised aniline blue (Williams et al. 1982).

Results and discussion

In pollinations with Vireya pollen on Azalea pistils no capsules developed and no seed was obtained. In all but two crosses pollen tubes were arrested within the style or at the style base. In the two exceptional crosses pollen tubes entered the ovary but did not enter the ovules.

In pollinations with Azalea pollen on Vireya pistils ovules were entered by pollen tubes for all except those involving pistils of R. kawakamii in which arrest occurred within the ovary or at the style base. Viable intersubgeneric seed was produced on Vireya seed parents in a total of eight crosses involving pollen parents in sections Tsutsusi, Tsutsusiopsis, Pentanthera and Choniastrum: (R. laetum x R. aurigeranum) x R. indicum, R. retusum x R. simsii, (R. intranervatum x R. 'Souvenir de J.H. Mangles') x R. periclymenoides, (R. intranervatum x R. 'Souvenir de J.H. Mangles') x R. japonicum, (R. 'Dr Hermann Sleumer' x R. herzogii) x R. bakeri, R. retusum x R. periclymenoides, R. retusum x R. ellipticum, and [(R. macgregoriae x R. lochae) x R. macgregoriae] x R. tashiroi.

Hairs on the first true leaves of surviving hybrid seedlings were intermediate in morphology between the Vireya flat multicellular scales on a short stalk and the longer simpler hairs of the Azalea parents. Typically they were stalked with a bulbous glandular tip. The morphological differences in the juvenile indumentum of parent species and hybrids are suficiently marked to be used as an indicator of hybridity. Many of the intersubgeneric seedlings were weak, a number have died, and of the survivors none have yet flowered.

All Vireya Rhododendrons behaved similarly in crosses on Azalea pistils, showing arrest of pollen tubes before entry to ovules. However, among Vireya Rhododendrons used as seed parents, only R. kawakamii showed rejection of Azalea pollen before entry to ovules. We therefore conclude that the barrier to interbreeding of Vireya and Azalea Rhododendrons is, in general terms, unilateral, being more easily overcome when Vireya Rhododendrons are used as female parents.

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EARLY POST-FERTILIZATION DEVELOPMENT IN RHODODENDRON

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Summary

Early post-fertilization development has been studied in several species of Rhododendron (Ericaceae). Fertilization is marked by rapid development of a callose wall around the zygote. The first proembryonal division is delayed for 10 days or more after fertilization, but the primary endosperm nucleus begins development immediately after triple fusion. The endosperm is cellular from the first division. Apparent self-incompatibility in three species has been shown to be a post-zygotic mechanism. Interspecific incompatibility may also, in certain crosses, be expressed after entry of pollen tubes into the ovules.

Introduction

In an extensive study of pollen-pistil interactions and interspecific pollinations in Rhododendron we have observed a number of instances where foreign or occasionally self pollen tubes penetrate the ovules but produce little or no viable seed. Accordingly, we have set out to describe the course of early post-fertilization events leading to normal seed development, and to observe the deviations from this pattern which result in interspecific incompatibility or apparent self-incompatibility. Observations have been made either by bright field microscopy of semi-thin plastic sections of ovaries stained with PAS (periodic acid/Schiff) - toluidine blue, or fluorescence microscopy of whole pistil squashes or ovary sections stained with decolorised aniline blue (Williams et al. 1982a,b, 1984a,b).

Compatible Development

Ovule development has been studied in compatible hand-pollinations of seven Rhododendron species (Williams et al. 1984a). Pollen tubes reach the ovules in about 4-7 days after pollination and discharge their contents into one synergid cell. The endosperm begins development immediately after fertilization, and is cellular from the first division. The zygote shrinks slightly relative to the unfertilized egg, and lays down a special callose wall in the first two days after fertilization. The PAS-postive, aniline blue fluorescence-positive callosic wall is initiated adjacent to the degenerating synergid, extends to cover the entire zygote surface, and remains visible as the zygote elongates prior to the first proembryonal division. This division is delayed 10-15 days or more after fertilization. (We have observed undivided zygotes at 60 days after pollination in R. konori developing at winter temperatures in Melbourne). Prior to the first proembryonal division the zygote elongates at the end distant from the micropyle, and the first division separates a small cap cell from a larger basal cell. The distal portion of the embryo sac, which is lined with a single endothelial layer, enlarges and becomes filled with cellular endosperm before the first division of the zygote.

In ovulo expression of incompatibility

Interspecific incomptibility in Rhododendron may be expressed at any one of at least 7 different levels in the pistil, from the stigma surface to the ovules (Williams et al. 1982a,b). In species combinations where ovules are entered but no viable seeds produced we have observed two basic outcomes: In a few instances (e.g. R. kawakamii x Kalmia latifolia (Ericaceae) normal fertilization is not achieved, and there is an abnormal coiled overgrowth of the undischarged pollen tube within the embryo sac. More commonly, achievement of fertilization is marked by formation of a normal zygote callose wall and the initiation of endosperm development, with incompatibility being expressed as failure of zygote or early proembryonal divisions together with endosperm degeneration (eg. R. kawakamii x R. retusum and R. retusum x R. kawakamii). Some crosses (eg. R. retusum x R. santapaui, R. retusum x R. ovatum and R. retusum x R. tashiroi) show later abortion, producing matured seeds with partially developed, inviable embryos, or small seeds giving rise to weak, abortive seedlings.

Self-incompatibility in R. ellipticum, R. championae and R. amamiense is controlled by a post-zygotic barrier to development of selfed embryos (Williams et al., 1984b). Fertilization is apparently normal. The zygote contracts slightly and lays down a callose wall as seen after compatible pollination. Some pre-divisional zygote elongation occurs but no divisions have been observed. The endosperm begins development

normally, but within two weeks after fertilization degeneration becomes apparent (eg. at the 12-16 celled stage in R. amamiense). Collapse of the embryo sac and ovule wall follow rapidly. Although endosperm degeneration is the first visible sign of abnormality, this is not necessarily causal to embryo abortion, and may be a parallel expression of the same underlying factors. Self-incompatibility is presumably controlled by a system of multiple lethal genes functioning to produce severe "inbreeding depression" in early embryogeny.

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POLLEN TUBE PENETRATION IN THE STYLE OF GASTERIA

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Summary

The central part of the style is filled with a material of changing consistency. In the first part beyond the stigma the material is solid, in the direction of the ovule it is liquid.
The course of the pollen tube through the style seems to go through material mainly consisting of mono- and oligosaccharides.

Introduction

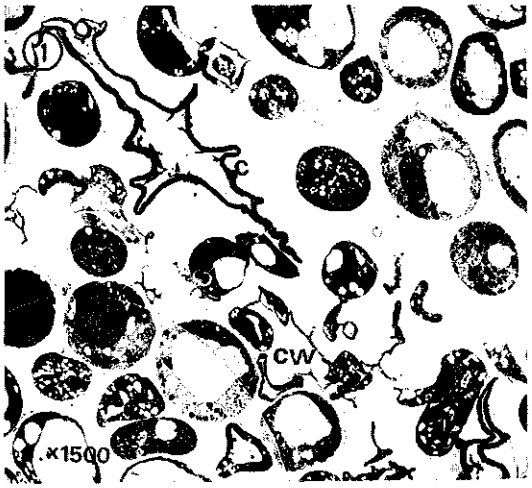
As in other Liliaceae, Gasteria verrucosa has a hollow style (Sears, 1937) with a wet stigma (Heslop-Harrison & Shivanna, 1977). However a submorphological study reveals a special type of a stylar canal in Gasteria, especially at the tip of the style.

Methods

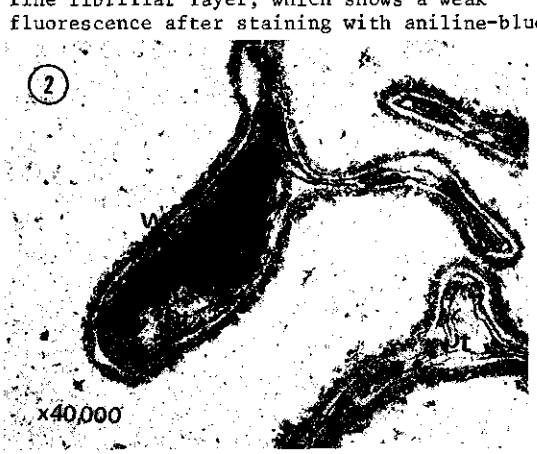
Parts of mature styles of Gasteria verrucosa were fixed in 3% glutaraldehyde in phosphate buffer 0.1 M at 7.2 pH for 18 hrs at 4°C. After washing the specimens were stained in 1% OsO_4 in buffer during 2 hrs at 20°C. After dehydration and embedding in Epon, sections were poststained with leadcitrate and uranylacetate, and examined in a Philips EM 301.

Results

The stigma of Gasteria has papillar cells of medium length. The cells are elongated and surrounded by a wall consisting of a small layer around the cell and a thick layer which is bordered by an undulating cuticle. Beyond the stigma the thick part of the papillar cell wall is continuous with the central part of the style (cw). In this part remnants of the cuticle (c) of the three carpels, which border three very small flat canals, are present. The central part beyond the stigma contains elongated cells. At the cortical part of the style the cells lay more close together. See fig. 1.
The material of the thick wall layer of the papillar cells and its continuation in the central part of the style is electron transparent. Histochemical tests on this material for pectine, cellulose and callose are negative. Staining of the liquid with naphtol-H_2SO_4 is positive and suggests the presence of carbohydrates as mono- (glucose test strips are positive) and oligosaccharides.



After pollination of a receptive stigma the growing pollen tubes (pt) penetrate the thick part of the papillar cell wall and grow into the solid central part of the style. A part of the pollen tubes are flatened (fig. 2). The pollen tube wall (w) consists of a thin fine fibrillar layer, which shows a weak fluorescence after staining with aniline-blue.



Conclusion

Among the different stylar types, Gasteria shows a central stylar part with changing consistency and a star-shaped canal tapered off to three points near the stigma. The pollen tubes penetrate the papillar cell wall, and follow a way rich in carbohydrates. Because of the high osmotic rate and the imbition capacity of the surrounding material in the style beyond the stigma probably the pollen tubes become flattened.

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THE POLLEN TUBE AS AN EXPERIMENTAL GROWTH SYSTEM

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Summary

The effects of various factors on pollen tube growth in vivo can be quantified by counting numbers of pollen tubes passing through optical transects of styles at fixed intervals after pollination. Pollen tube growth in vitro can be quantified by measurement of pollen tube lengths in hanging drop cultures or cultures on the surface of a semi-solid medium. These two in vitro techniques have been developed as bioassays for the effects on pollen tube growth of microgram amounts of test substances.

Introduction

To study the control of pollen-pistil interactions, pollen tubes are grown either in association with the living pistil in vivo, or separately in vitro. The in vivo system is typically used to examine effects on the interaction, of factors such as temperature, flower age, plant genotype etc. However, to identify signal molecules which pass between the pistil and pollen tube, a system is required with a less complex metabolism than that of the whole pollinated pistil. The obvious simplification is to remove the pistil and grow pollen tubes in vitro on a simple defined medium. Pistil fractions or other known molecules can then be introduced into the cultures to determine their effects. Here we report a method for quantification of pollen tube growth in vivo, and two techniques for in vitro bioassay of microgram amounts of substances affecting pollen tube growth.

Quantification of growth in vivo

Pollinated pistils are fixed after a known interval and squashed in decolorised aniline blue for visualisation of pollen tubes by fluorescence microscopy (Williams et al. 1982a, Willams & Knox 1982). Counts are made of numbers of pollen tubes passing through a series of optical transects across the style and pollen tube numbers are then plotted against distance down the style. This technique has been used for Lycopersicon peruvianum, to determine rates of compatible pollen tube growth, and to examine the effects on self-incompatible tube arrest of flower age, nutrient status, temperature and plant genotype (Williams & Knox 1982, Williams et al., 1982b).

Qualitative in vitro bioassay

Pollen is applied with the edge of a coverslip as a thin line across a microscope slide coated with an agarose-solidified germination medium. Two 2mm wide filter paper strips containing a test substance in solution are then placed 2 mm out on each side of the line of pollen (Williams et al. 1982c), or a single test strip directly above the pollen (Williams et al. 1982b). The slide is placed on moist filter paper in a closed petri dish, incubated 18 hours at 20-23°C, and then fixed with formaldehyde vapour by adding 0.5 ml formaldehyde solution to the filter paper and reclosing the dish for 10 min. Mean pollen tube length for each slide is determined using a Carl Zeiss Videoplan Computerized Image Analysis system attached to a compound microscope. This method has been used to survey stylar proteins for fractions affecting pollen tube growth (Williams et al. 1982c). The method cannot be used for precise quantification of the effects of these molecules since the diffusion gap between filter paper test strips and pollen tubes variably reduces the time of treatment and the concentration of test molecule reaching the tubes.

Quantification of growth in vitro

Quantification of the effects of substances on pollen tube growth in vitro can be achieved using multiple 200 µl hanging drop cultures in the wells of a 96-well PVC disposable microtitre tray (Hough et al. 1984). Cultures are set up using a constant amount of pollen suspended in germination medium, and a varying concentration of the test substance. The tray is carefully inverted so that the cultures form hanging drops suspended in the wells by surface tension. The inverted tray is then laid across two shallow supports in a closed container lined with moist filter papers, and incubated at 20-23°C. For sampling the tray is reverted and a 15-25 µl aliquot withdrawn from each well. Each sample is placed on a microscope slide, fixed by addition of a small drop of formaldehyde solution, and seated under a coverslip for microscopic measurement of pollen tube lengths as in the previous method above. After sampling the tray can be reinverted and incubation continued.

This method has been used to quantify the effects of inhibitor and promoter molecules on pollen tube growth (Hough *et al.* 1984, Hewitt *et al.* 1984).

Conclusion

Quantification of pollen tube growth in the pistil can provide data on pollen-pistil interactions, particularly the gametophytic self-incompatibility response. *In vitro* pollen tube cultures can be used to identify stylar molecules which may be active in pollen-pistil interactions, and to investigate the effects on pollen tubes of defined molecules. In the future, these methods may also be useful for selection of superior pollen sources and screening pollen for physiological mutants.

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STYLE-CONTROLLED COROLLA WILTING IN PETUNIA HYBRIDA L.

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Summary

In this poster a review is given of the results of research on style-controlled corolla wilting in Petunia hybrida L. Pollination followed by pollen tube penetration into the stigma, or injury of the stigma or style led to the generation of a wilting-inducing factor and its transfer to the corolla within 4 h. The wilting inducing factor is different from 1-aminocyclopropane-1-carboxylic acid (ACC).
Keywords: pollination, stigma wounding, corolla wilting, ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC).

Introduction

In some plant species corolla wilting or abscission is initiated by pollination (orchids, Dianthus, Petunia, Cyclamen, Digitalis etc.). In Digitalis the stimulus for accelerated abscission reaches the corolla basis well before pollen tubes have traversed the style (Stead and Moore, 1979). Ethylene biosynthesis is generally involved in these post-pollination phenomena.

This poster reports on the effects of pollination and wounding of the stigma in Petunia flowers and on a possible role of 1-aminocyclopropane-1-carboxylic acid (ACC) or ethylene in the transfer of the wilting-inducing factor from the stigma to the corolla.

Results and conclusions

The time required for 50% of the flowers of P. hybrida to wilt (W50) was considerably reduced after pollen tube penetration into the stigma. Pollination without tube penetration did not affect corolla longevity. The W50, due to pollen tube penetration, was higher after self-pollination than after cross-pollination. Wilting was accelerated in proportion to the number of penetrating tubes. Injury of the stigma or style also strongly accelerated wilting. Style-induced accelerated wilting was prevented by excision of the entire style (Gilissen, 1976; 1977). Although this treatment caused some decrease of W50 as compared with untreated flowers, it allowed for kinetic analyses of the transfer of the wilting-inducing factor. This transfer through the style to the corolla occurred within 4 h after pollination or wounding of the stigma. Ethylene evolution also increased immediately upon these treatments. Because of the similarity in effects of pollen tube penetration and stigma wounding on corolla wilting, a similar mode of action of the wilting-inducing factor is proposed. Eluates collected from the ovarian ends of styles contained a wilting-inducing factor, particularly in the case of previous pollination or wounding. The content of the ethylene precursor ACC in the eluates was below detection (Gilissen & Hoekstra, 1984).

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P A R T III

MEGASPORGENESIS, OVARY, EMBRYO SAC DEVELOPMENT EMBRYO AND ENDOSPERM DEVELOPMENT

★★★

Part III: Megasporogenesis, ovary, embryo sac development, fertilization and embryo an endosperm development.

Introduction

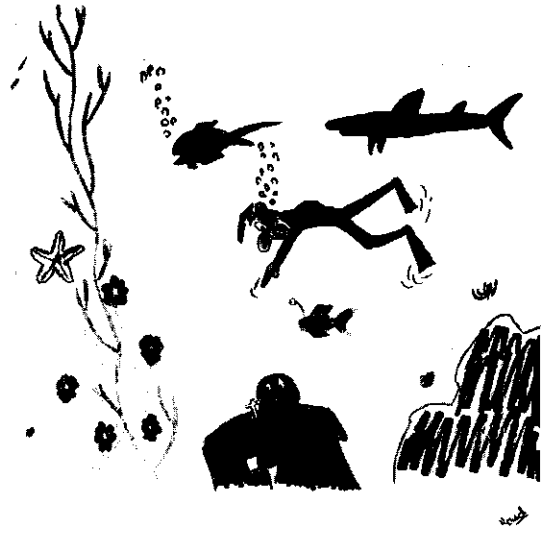
Megasporogenesis, sperm cells, embryo sac structure, fertilization, apomixis and embryogenesis are the main topics in this part, but to begin with some data on ferns. Most studies are done using the electron microscope, some combined with quantitative data and biochemistry. The use of image analysis systems and ultracryotomy is new. For application in breeding the study of apomixis, the in vitro embryoid formation and the data on sperm cell composition are of interest.

Ultrastructural studies on plant reproduction today become more detailed because the general phenomena are well known. In spite of the studies on the developing embryo sac and fertilization untill now too little attention has been payed to ultrastructural studies on embryogenesis and seed coat formation. The resolving of problems in keeping seed qualities can use some submorphological information. More comparisons could be made with somatic embryogenesis.

In experiments with ovules, but also in general on sexual reproduction, the reproductive calendar of the plant under study is very important, such as in relation to possible aberrations as apomixis.

In this part different aspects of the reproductive processes from megasporogenesis to embryogenesis are considered. Compared with studies on pollen more attention should be payed to the very complicated process of ovular development. Many contacts were made during the congress. The excursions focussed on the problem how to keep Holland dry. This stimulated Dr. Williams again to compile a poem linking this problem with an idea for research on sexual reproduction:

> Well, Prof. Willemse, I declare,
> Is that the Delta Project there?
> They tell us that the Dutchmen try
> To keep their little country dry.
> But surely that is hard to do,
> And costs a lot of guilders too.
> Why fight to keep the water out -
> If it came in, you could farm trout.
> And we could study something new,
> Like algal sex and gametes too.
> And scuba diving we could go
> To take our samples from below.
> To analyse the submarine
> Would open up our research scene.
> So we invite you, if you like,
> To pull your finger from the dyke.



As is illustrated by Noud Schel and can be find on the end of this volume, Prof. Willemse has followed this advise and pulled his finger from the dyke. He is liberated with all his fingers free in the sea. From this position he is stimulated to think how sexual reproduction started in the sea. Plants using the power of sexual reproduction have ultimately colonized land. In this way, pulling a finger from a dyke , it would stimulate the study of sexual reproduction in the sea, and it would again ultimately lead up to the study of seed plants on land, especially the flower.

CRYO SCANNING ELECTRON MICROSCOPY OF REPRODUCTION IN CRYPTOGAMS

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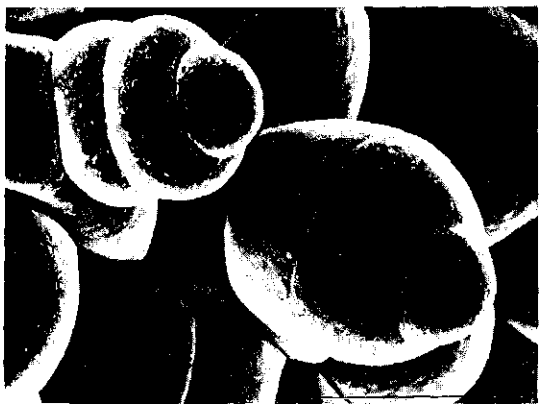
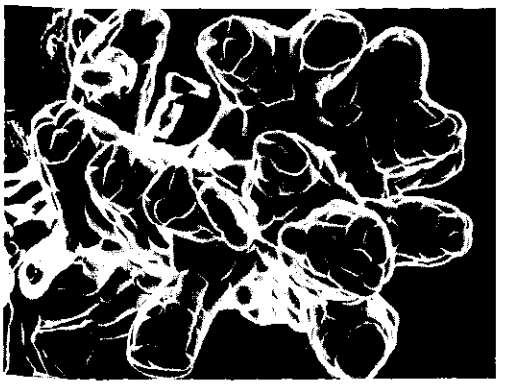
The scanning electron microscope (SEM) study of reproduction in pteridophytes and bryophytes has previously relied upon preparative techniques employing fixation, then critical-point-drying or freeze-drying. These techniques, however, yield variable results and frequently cause distortion, shrinkage and/or collapse of the fragile structures which effect reproduction in cryptogams. The new technique of cryo SEM avoids artefacts and damage attributable to fixation and dehydration since the tissue is simply frozen and observed directly. The method is therefore much quicker to perform than conventional procedures and can yield specimens of vastly superior appearance (hence informational content), as illustrated by these micrographs.



Antheridia of Pteridium aquilinum, each has two ring-like jacket cells and a single opercular cell. Bar = 50μm



Antheridia of Equisetum arvense, most have four opercular cells. Bar = 100μm

Preparative procedure

1. Generate plants in aseptic culture where possible.
2. Remove material, attach to SEM stub with silver paint.
3. Plunge stub into slushed liquid nitrogen.
4. Evacuate chamber, transfer stub (under vacuum) to SEM.
5. Observe specimen in SEM on cryo stage (Hexland) at -190°C.
6. Sublime any superficial ice by raising to -80°C.
7. Withdraw specimen to pre-chamber (-190°C), sputter coat.
8. Return to microscope (Cambridge S 150) for observation at -190°C.

Marchantia alpestris. Part of an archegoniophore, bisected prior to freezing, showing mature pendulous archegonia. These sit on a cup-shaped pedestal. Bar = 100μm

GAMETANGIA IN CEYLONESE SPECIES OF THE FERN FAMILY THELYPTERIDACEAE

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Summary

Variation in gametophyte anatomy in Thelypteridaceae is under investigation and includes studies of the structure of sex organs. In general archegonia consist externally of four rows of cells and one row has enlarged cells forming a curved "chord". Variation is found in number and size of top cells. Antheridia usually consist of three cells surrounding the spermatozoids. Different mechanisms of opening occur.

Introduction

The previous studies of gametophyte anatomy in Thelypteridaceae have been made with light microscope. Antheridia and archegonia are located on the central cushion in fully developed prothallia. Dehiscens of antheridia is reported to generally occur by a pore (Atkinson & Stokey 1973, Atkinson 1975) but in one species the cap cell is lifted off (Atkinson & Stokey 1973). The archegonial neck is elongated, 4-6 cells long and curved away from the notch region (Nayar & Kaur 1971, Atkinson 1975).

As the knowledge about gametophyte morphology in Thelypteridaceae is scarce it seemed important to undertake a detailed study making use of the resolution power of the SEM. The results about sex organs reported here is part of a more extensive investigation of the gametophyte generation in Thelypteridaceae.

Material and methods

Fertile sporophytes of 27 species of the family Thelypteridaceae were collected in Ceylon. Plants originating from this material are kept in cultivation. Gametophytes for structural investigation were grown from spores in sterile culture on agar. The research material was critical point dryed and studied in a Cambridge stereoscan 600.

Results

Archegonia (Fig 1) were found to consist of four rows of cells that are 5-6 cells high. In all species studied the mature archegonia are bent away from the notch and leaning somewhat to one side. One of the four rows of cells that build up the archegonium are enlarged forming a curved "chord". In one species, Amauropelta hakgalensis, there are more than four cells in the basal layer so that it looks like the archegonium is standing on a widened foundation. In most species the four cell rows terminate in a triangular cell on top of the archegonium, where there consequently are four triangular cells meeting. Sometimes only three cells meet on the top of the archegonium, the fourth row ending below. In Macrothelypteris torresiana the top cells are not triangular but differ from each other in shape and size.

Viewed from the outside the antheridium (Fig 2) generally consists of three cells, one basal cell, one ring cell that is wider than the basal cell and an undivided cap cell. In Metathelypteris flaccida the basal cell is wider than the ring cell. In some species the cap cell is occasionally divided into two, of which one is luneate and partly surrounds the other circular cell. Very often the opening of the antheridium is by detachment and lifting of the cap cell. Sometimes there is also a pore in the outer cell wall of the cap cell when it is lifted off. In most cases the cap cell is totally lost. The condition with a pore in the cap cell which remained attached to the antheridium was also found.

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Fig.1 Archegonium.Amauropelta hakgalensis.440
Fig.2 Antheridium.Christella hispidula.560x

WATER FERN (*MARSILEA QUADRIFOLIA*) REPRODUCTION AS A BIOASSAY FOR AFLATOXIN B_1 AND T-2 TRICOTHECENE TOXIN

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Summary

Both aflatoxin B_1 (AFB_1), a secretion of Aspergillus spp., and T-2 trichothecene toxin, a by-product of Fusarium spp., are subjects of intense research interests. This stems from the carcinogenic, mutagenic and teratogenic properties of AFB_1 and the hemorrhagic attributes of T-2 toxin. The latter has been used (allegedly) as a warfare agent thereby mandating the development of quantitative, rapid, reliable and simple assays for these mycotoxins. Here, we report the possible use of water fern reproduction as a mycotoxin bioassay.

Introduction

Whereas aflatoxins (AFTs) are secretions of Aspergillus flavus and A. parasiticus (Goldblatt, 1969), trichothecenes are products of 17 Fusarium species (Joffe, 1965). Under proper environmental conditions, these fungi can grow upon crops (Mirocha et al., 1976; Dashek & Llewellyn, 1982) thereby posing a health risk to both man and his domesticated animals since AFTs are carcinogenic, teratogenic and mutagenic (Wogan, 1965) and trichothecenes are hemorrahgic (Schoental et al., 1979). Recently, evidence has accumulated for the alleged use of certain trichothecenes as chemical/biological warfare agents (Rosen & Rosen, 1982; Mirocha et al., 1983). Furthermore, AFTs could serve as such agents. Thus, it is important to develop rapid, accurate, reliable and simple quantitative assays for the two toxins. The currently-available, highly-sensitive analytical methodologies are: non-transportable to the field, expensive and require highly-trained personnel. Thus, we are generating bioassays for both AFB_1 and T-2 toxin.

Here, we report whether water fern reproduction can be a bioassay for both toxins.

Materials and Methods

T-2 Toxin bioassay

Sporocarps - For germination, the tips of Marsilea quadrifolia L. sporocarps (Carolina Biological Supply Inc., Burlington, NC) were excised and then placed into a Petri dish containing 10 ml of sterile distilled H_2O. One sporocarp was used per treatment which occurred within an environmental chamber at 14 hr daylight (6:00 am to 8:00 pm), 21 C, 550 lumens fluorescent light. At 8:30 am one sporocarp was prepared as above and by 1:00 pm megaspores isolated and by 2:00 pm treated.

Megaspores - At megaspore release from the sorus, each megaspore displayed a small papilla projecting from one end. This indicated the megaspore nucleus which underwent division resulting in archegonium formation. The latter consists of both neck cells and a single egg cell, external evidences for archegonial formation (Bierhorst, 1971). Approximately 100 megaspores were produced by one sporocarp germination. Following their collection via a 50 µl capillary pipet, 3-5 megaspores per well were added to a tissue culture multi-well plate (Linbro Division Flow Laboratories, Inc., Hamden, CT). Each megaspore was developed to the papillary stage within covered well plates and the environmental chamber. Each well contained 2 ml sterile, distilled H_2O. Archegonial formation, fertilization and resultant sporophyte development were observed at least twice daily for 5-7 days at 7-30X magnification.

T-2 toxin treatment of megaspores - Using a 50 µl capillary pipet, the H_2O within each well was removed via an automatic vacuum pipettor connected to a series of traps. The T-2 toxin within 0.5 ml H_2O (Calbiochem, San Diego, CA) was added to each well. For toxin stock construction, 1 ml acetone was added to 5000 µg T-2 toxin with the dissolved toxin being added to 198 ml of sterile, distilled H_2O. A second ml of acetone washed out any residual vial toxin. The stock, 25 µg toxin/ml, was heated with stirring for 3 hr to both mix and drive off the acetone. Following cooling, 0.5 ml aliquots were added to wells following a 30 sec aeration involving vigorous shaking of 200 ml within a flask. The acetone-water control (AC) received 2 ml of acetone in 198 ml of water concurrently with the T-2 toxin stock. Another control (C) without either toxin or acetone was used also. Toxin (T-2), AC and C solutions were maintained at 22 C prior to addition to the wells containing the megaspores.

Megaspores were treated with 0.5 ml of T-2 toxin, AC or C solutions having 3-5 megaspores per well. The plate's first eight wells contained T-2 toxin, the next eight AC and the last eight C. Megaspores were treated for one hr when solutions were withdrawn using the automatic pipettes and traps. A new, sterile 50 µl pipet was used

for each evacuated treatment group. Immediately after removal of the solutions from the wells, two ml of sterile H_2O was added as the first wash. There were three washes which required about 10 min. Generally, the total megaspore "n" number for each treatment was >90 with an attempt to have 100. The T-2 toxin concentrations were as in Table 1.

Microspore addition to treated megaspores in the wells - Following washes, treated megaspores were maintained in 2 ml sterile H_2O. To each well were added numerous gelatinous clumps of microspores to ensure an excess of sperm for fertilization. From this time microscopic examinations were performed routinely at least twice daily.

AFB_1 bioassay

Marsilea sporocarps were scarified prior to their placement into various AFB_1 concentrations. A 20 µg/ml AFB_1 stock was prepared by combining 10 mg AFB_1 (Lot no. 387032, Calbiochem, LaJolla, CA) with 440 ml of sterile H_2O and 60 ml of acetone. From this stock, AFB_1 dilutions of 16.0, 12.0, 8.0, 4.0, 2.0, 1.0 and 0.5 µg/ml were prepared.

Seven replicates containing 20 ml of each dilution were prepared and placed into sterile Petri dishes. Seven controls (sterile H_2O and acetone) were made also. A single sporocarp was placed into each dish which were sealed with petroleum jelly to retard evaporation. Dishes were stacked 14 high within an environmental chamber with a photoperiod of 12 hr light and 12 hr dark at 23 ± 2 C. Cultures were examined daily for sorophore appearance, microspore release, sperm release, fertilization, sporophyte production and sporophyte occurrence.

Results

Tables 1 & 2 summarize T-2 toxin (1) and AFB_1 (2) data related to sporocarp germination percentage, fertilization percentage for those germinating, sporophyte production for those fertilized and sporophyte occurrence for those replica showing fertilization.

Sorophore appearance, microspore and sperm releases

AFB_1 - Sorophore appearance and both microspore and sperm releases were not inhibited by any AFB_1 level. Megaspore release was reduced to 66.67% at 1 ppm and 85.71% at 2 ppm, a response not observed at either higher or lower concentrations.

Sporocarp production

AFB_1 - Germination was increased from 85.71 (C) to 100% at 2.0, 4.0, 8.0, 12.0 and 16.0 ppm but was reduced to 57.14% at 0.5 ppm. Whereas the 1.0 ppm-treated sporocarps exhibited a germination of 85.71%, the 20.0 ppm-treated group showed a percentage of 71.43.

Fertilization

AFB_1 - A 50% fertilization level for the sporocarps that had germinated was observed for control cultures. In contrast, fertilization ranged from 8.0% for the 0.5 ppm replicas to 71.43% in the 2 ppm cultures. The highest AFB_1 concentration (20.0 ppm) possessed a germination percentage of 17% > the controls.

Sporophyte production

T-2 toxin - Table 1 summarizes sporophyte development for T-2 toxin-treated megaspores five days after treatment. Percent megaspores developing normal sporophytes was linear between 0.314 and 0.039 µg/ml. Further refinement of this potential bioassay might be accomplished by increasing the concentrations between the extremes.

AFB_1 - Both the control and 20.0 ppm treatment showed sporophyte occurrences of 100%. For sporophyte production originating from a single sporocarp, means of 81 sporophytes for controls and 87 for 20.0 ppm were observed. Sporophyte production in seven of eight remaining toxin levels was < that of both control and 20.0 ppm groups.

Discussion

Comparisons of Marsilea T-2 and AFB_1 toxin responses with those for other systems ((Tables 3, 4 and 5, the latter two in Llewellyn et al. (1982))

T-2 toxin - When the data within Tables 1 and 3 are compared, it is apparent that except for tobacco callus, water fern reproduction appears to be the most T-2 toxin sensitive plant system thus far examined yielding a linear dose-response curve (Fig. 1).

AFB_1 - The data of Table 2 do not lend themselves to construction of dose-response curves and thus prohibit the utilization of water fern reproduction as an AFB_1 bioassay. Perhaps 'fine-tuning' of the concentrations between 0.000 and 0.625 µg/ml may yield a curve.

Comparison of the responses of Marsilea sporocarp germination and sporophyte production (Table 2) with the germination of various seeds and pollen (Table 4) and the elongation of plant parts (Table 5) reveals Marsilea reproduction to be the most AFB_1 sensitive (0.5 µg/ml) system employed to date as most are affected by 100 and even 1000 µg/ml. Of interest is Onoclea sensibilis where a 43.8% inhibition of spore germination was obtained at 3.90 µg/ml.

In conclusion, ferns appear to be quite sensitive to mycotoxins and thus warrant further investigation as possible bioassay tools.

Acknowledgements

The AFB_1 results were completed by M. Meredith and G. Alexander Stephenson. Assays for AFB_1 were by Thomas Eadie, Virginia Division of Consolidated Laboratory Service, Richmond, VA. We thank Ms. Mary Wesley for thoughtful clerical assistance.

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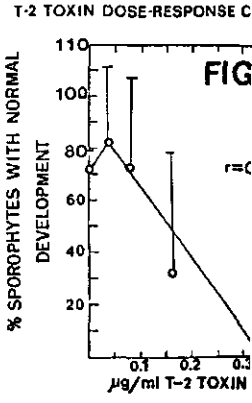
Table 1. Sporophyte Development For T-2 Toxin-Treated Marsilea Five Days After Treatment*

T-2 Toxin Concentration µg/mℓ	Total Megaspores Tested	Percent of Megaspores Showing Potential For Development	Percent of Megaspores Actually Attaining Normal Sporophyte Status
1.250	69	10.3 ± 17.9	0.0 ± 0.0
0.625	63	17.3 ± 30.0	0.0 ± 0.0
0.314	51	11.0 ± 15.5	0.0 ± 0.0
0.156	59	66.0 ± 33.7	31.7 ± 38.1
0.078	55	72.3 ± 32.4	72.3 ± 34.2
0.039	51	85.3 ± 25.4	83.3 ± 28.9
0.020	66	95.3 ± 4.5	82.7 ± 22.7
0.009	63	87.6 ± 6.9	81.0 ± 2.6
0.005	64	68.3 ± 8.3	63.0 ± 14.1
0.002	60	78.3 ± 6.7	71.3 ± 8.5
0.001	66	83.0 ± 9.8	69.3 ± 14.4
0.000	70	84.7 ± 3.8	71.7 ± 13.8

*Data are means and standard deviations for three replicate experiments.

Table 2. Response of Marsilea Germination, Fertilization and Sporophyte Production to Aflatoxin B_1 Treatments

AFB_1	Germination Percentage	Fertilization Percentage For Those Germinating	Sporophyte Production For Those Fertilized (Mean No./Sporocarp)	Sporophyte Occurence For Those Replica Showing Fertilization (% of cultures)
0.0	85.71	50.00	81	100.0 (3/3)
0.5	57.14	0.00	--	----
1.0	85.71	33.33	56	100.0 (2/2)
2.0	100.00	71.43	89	100.0 (5/5)
4.0	100.00	28.57	50	100.0 (2/2)
8.0	100.00	42.86	58	66.7 (2/3)
12.0	100.00	57.14	68	100.0 (4/4)
16.0	100.00	57.14	69	100.0 (4/4)
20.0	71.43	66.67	87	100.0 (4/4)



Data are means for seven replicates.

Table 3. Summary of Some Plant Systems Used as Possible Bioassays of Trichothecenes

System	Concentration	Effect	Investigator
Lesion development upon tobacco necrosis virus on beans	20 to 100 mg trichothecin/1	Inhibits infection when sprayed over leaves or day after they have been incubated with viruses, but ineffective when applied 2 days before	Bawden and Freeman (1952)
Pea seed germination	0.5µg T-2 Toxine/ml	Germination reduced by more than 50%	Burmeister and Hesseltine (1970)
Rhodotorula rubia NRRL Y-7222 and Penicillium digitatum NRRL 1202	4.0 µg T-2/ toxin assay disk	Growth retardation	Burmeister and Hesseltine (1970)
Tobacco callus upon agar	6.0×10^{-3} µM T-2 toxin	50% growth inhibitor	Helgeson et all.(1974); Stahl et al.(1973)

*Full referecnece citations can be found in Protection against trichothecene mycotoxins, 1983. National Academy of Sciences, Washington, DC, USA.

FERTILIZATION IN TAXUS BACCATA L.

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Summary

The development and nature of the gametes of the English Yew, Taxus baccata L., have been studied in depth. Mitosis of the body (spermatogenous) cell within the pollen tube is rapidly followed by the dissolution of the boundary cell, degeneration of the cytoplasm, and liberation of the sperm nuclei in to the tube lumen. The sperms lack any identifiable motile apparatus.

The mature egg measures 20-30 x 50 µm, the micropylar surface of the nucleus lying 10-15 µm below the neck cells. Insemination is brought about shortly after the pollen tube penetrates the archegonium and the wall is lysed at its tip. The cause of the movement of a single sperm nucleus towards the egg nucleus is not evident. The egg nucleus becomes cup-shaped as the sperm draws near, and the male gamete becomes partially enclosed by the depression so formed. Karyogamy then takes place as a number of local adhesions are formed between the parallel surfaces of the two gametic nuclei. Pore formation at these sites is followed by the enlargement of the areas of fusion and the complete comingling of the genomes.
Keywords: fertilization, Taxus.

Introduction

Fertilization in the so-called siphonogamous higher vascular plants is brought about by non-motile sperms. After the formation by lysis of a small pore near the tip of the pollen the two sperms are liberated either into a single or adjacent archegonium (Singh, 1978), or into an embryosac (Jensen & Fisher, 1968; Russell, 1982). In genera in which the male gametes are cells, discharge of the sperms is followed by the apposition of the plasmalemma of the sperm to that of either the synergid (Jensen & Fisher, 1968) or the egg (Russell, 1983). Many local adhesions then develop between the two membranes and, at the site of each of these, a pore develops. The subsequent enlargement and fusion of the pores ultimately removes the barrier between the two gametes. The means by which the male nucleus subsequently moves through the egg cytoplasm are not clear however. The final events in the sexual process have been observed in a number of instances, in Pinus (Haupt, 1941; Camefort, 1969) karyogamy takes place after the sperm nucleus has entered a cup-shaped depression in the surface of the egg nucleus and the envelopes of the pair become closely parallel. The formation of local adhesions and pores at the sites of contact then ocurs in a manner essentially similar to that which takes place between sperm cell and synergid in Gossypium (Jensen & Fisher, 1967). The inner membranes of each envelope fuse in turn. Similar events also take place during karyogamy in Plumbago (Russell, 1983), but in Gossypium nuclear contact comes about not by direct adhesion, but via the fusion of cisternae of endoplasmic reticulum midway between the two surfaces (Jensen, 1964; Jensen & Fisher, 1967; Schultz & Jensen, 1977). When several such contacts have been established the length of each cisterna decreases and the interposing membranes of the envelopes fuse over their entire surfaces.

Materials and Methods

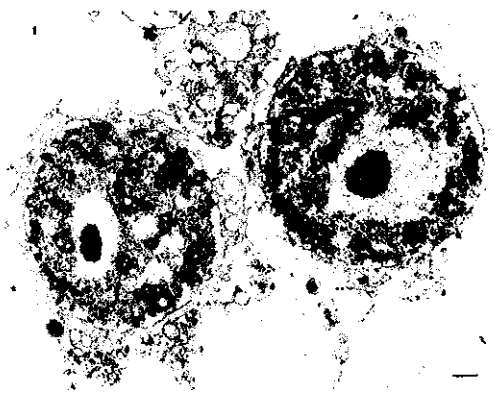
Thin median slices of well-pollinated ovules were fixed for 2-3 hours at room temperature in 2.5% glutaraldehyde (Taab, Reading) in 0.05 M Sorensen's phosphate buffer. After washing in buffer the slices were post-fixed for 2 hours in 2% aqueous osmium tetroxide and dehydrated in acetone. Embedding was in Epon 812 (Taab, Reading). 4 µm sections were searched for evidence of gametogenesis and fertilization. Sections containing appropriate structures were remounted according to the technique of Woodcock & Bell (1967) and resectioned in the thickness range 50-80 nm. Post-staining in uranyl acetate and lead citrate was followed by critical examination in a Siemens Elmiskop 102 Transmission Electron microscope, running at 60 kV.

Results and discussion

Although it has been widely reported that two sperms of Taxus are entire cells (Strasburger, 1879; Rohr, 1973; Gianordoli, 1974), this study demonstrates that the cytoplasm is lost as they mature (Fig. 1). Sister sperms appear to differ from one another only in respect of the extent to which the chromatin is condensed. The mis-identification of the body cell and stalk nucleus (Gianordoli, 1974) is in part responsible for the belief

that the sperms are cells of different sizes.

Fig. 1 The sperms shortly after liberation into the tube appear to differ from one another only in the degree of condensation of chromatin.
Scale: 1 µm.

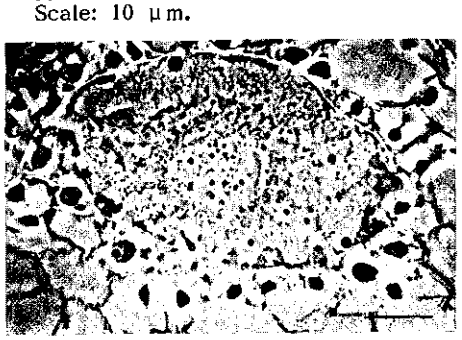


In Taxus the eccentric position of the body cell nucleus, and the consequent lateral displacement of the cell plate following division of this nucleus, have also given rise to the notion that the sperms differ in size. However, following degeneration of the boundary of the body cell, and of the cytoplasm, the nuclei alone are liberated into the tube. The situation is probably similar in Athrotaxis (Brennan & Doyle, 1956), although in this instance the later stages of spermatogenesis were not observed.

The sperms lack any visible motile apparatus, but bodies which may be relict blepharoplasts (Pennell, 1984) are conspicuous within the cytoplasm of the body cell, and are often associated with microtubules.

The egg cell occupies the whole archegonial chamber (Fig. 2). The nucleus of the egg lies deep within the protoplast, its micropylar surface approximately 15 µm from the neck cells. Groups of microtubules are occasionally seen radiating from egg nuclei.

Fig. 2 The mature egg cell. The micropylar pole of the egg is to the right.
Scale: 10 µm.



The microtubules within each group appear to originate from the nuclear envelope. Microtubules are also conspicuous features of the cytoplasm of the young zygote of Pinus (Camefort, 1969).

Insemination of the archegonium takes place following the penetration of the tip of the pollen tube between the archegonial neck cells and the lysis of its wall. In the electron microscope lysis is seen to be correlated with the passage of vesicles from the surface of the plasmalemma into the wall. This may indicate the secretion of hydrolases into the wall, leading to weakening and ultimately dissolution.

One or both sperms, sometimes accompanied by the stalk nucleus and some pollen tube cytoplasm, then move from the pollen tube into the egg. It is not clear how the sperm penetrates the egg cell cytoplasm, but in view of the known behaviour of the pollen tubes of some angiosperms (e.g. Cass & Jensen, 1970), it is possible that the plasmalemma of the female gamete is interrupted by the tube itself.

The cause of the movement of the sperm nucleus within the egg is not evident. Microtubules within each of the groups that arise from the surface of the egg nucleus extend for distances of up to 20 µm into the cytoplasm. Were they to attach to the envelope of the sperm they could, by means of sliding against one another, as is known in ciliary motion (Gibbons, 1981), draw the male gamete towards the nucleus of the egg. Indeed, microtubules are sometimes evident in the narrow region of cytoplasm which subsequently separates the two apposed gametic nuclei. Other proteins, possibly contractile, may be involved in the movement, and these are currently being searched for by immunocytochemical means. Conversely, the formation of the cytoplasm of the micropylar region of the egg into radially elongated channels may suggest that cytoplasmic streaming of the 'reverse-fountain' type is responsible. This has been suggested for flowering plants (Jensen, 1974).

The egg nucleus becomes cup-shaped as the sperm approaches. The sperm itself enters the depression so formed and becomes partially enclosed by the egg nucleus (Fig. 3a). The envelopes of the two gametic nuclei come to lie parallel to each other separated by a region of cytoplasm no more than 2 µm wide. At this stage, the adjacent membranes develop undulations, those of the egg nucleus being in register with those of the sperm nucleus (Fig. 3b). The outer membranes of the undulations ultimately make contact (Fig. 3c) and fuse. This fusion of the inner membranes then gives rise to pores which provide contact between the two genomes. As each pore widens it merges with others so that the parallel envelopes become wholly fused. Fusion events

of this kind are well described features of fertilization in both gymnosperms (e.g. Camefort, 1969) and angiosperms (e.g. Russell, 1982).

Fig. 3. S, sperm; EN; egg nucleus.
a. Nuclear apposition at the time of karyogamy. Scale: 10 µm.
b. Local adhesions (arrow) of the parallel nuclei are visible in the electron microscope. Scale: 1 µm.
c. Membrane adhesion is followed by the dissolution of the interposing inner membranes of the nuclear envelopes (arrow). Scale: 0.5 µm.

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PISTILLATE FLOWER DIFFERENTIATION IN JUGLANS REGIA: A DEVELOPMENTAL BASIS FOR HETERODICHOGAMY

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Walnuts (Juglans spp.) have a heterodichogamous mating system with some individuals being protandrous and others protogynous (Forde & Griggs, 1975; Gleeson, 1982). We investigated the phenology of pistillate flower differentiation in several protogynous and protandrous clones of J. regia in order to determine the relationship between the timing of floral organogenesis and the mode of dichogamy.

Because previous work on walnut pistillate flower development (Lin et al, 1977) provided data on protandrous cultivars, which are more common, we chose to emphasize protogynous individuals and examined each of the six protogynous clones available to us. These included early ('Chico', 'Sharkey'), midseason ('Amigo', 63-378) and late ('Meylan', 'XXX Mayette') individuals. Three protandrous cultivars ('Ashley', 'Chandler', 'Howard') not previously examined were included for comparison.

Buds were dissected to reveal floral primordia and prepared for optical or scanning electron microscopy using standard methods.

Results indicated that initiation of pistillate flowers in J. regia begins within six to eight weeks following pistillate anthesis in spring. By eight to eleven weeks organogenesis stops and does not resume until shortly before bloom the following spring. Cessation of organogenesis corresponds to the time that the growing fruits attain their full size and undergo marked changes in development and in their requirements for photosynthate (Pinney & Polito, 1983). Prior to this time there is rapid growth in fruit size characterized by increases mainly in endosperm and involucral tissues. As the fruits reach full size they begin a series of developmental events that include shell lignification, rapid embryo growth and, ultimately, accumulation of sugars and lipids in the embryo. This sequence suggests that nutrient partitioning phenomena may be an important factor leading to the cessation of organ initiation in the primordial flowers with the developing pistillate flowers becoming less favored sinks as nutritional requirements of developing fruits increase.

This abrupt termination of pistillate flower organogenesis as the growing fruits attained full size was noted in each of the clones examined, regardless of the mode of dichogamy characteristic for that clone. However, differences were evident in the extent of organ initiation that had occurred at the time organogenesis stopped. In each of the six protogynous clones perianth primordia were initiated before the cessation of organ initiation in the spring. In protandrous clones tepals did not form until growth resumed following the winter dormancy period. Thus, protogynous individuals enter the next growing season with pistillate flowers having attained a developmental stage that allows gynoecial differentiation to proceed immediately. By contrast, the protandrous cultivars require more organogenetic activity during the weeks prior to anthesis as perianth parts must be initiated in addition to the gynoecium. As the time required for this additional developmental activity is sufficient to account for the relative differences in pistillate bloom times between protandrous and protogynous walnuts, it appears that a fundamental basis for heterodichogamy in walnut is in the extent of pistillate flower differentiation that occurs during the post-anthesis period.

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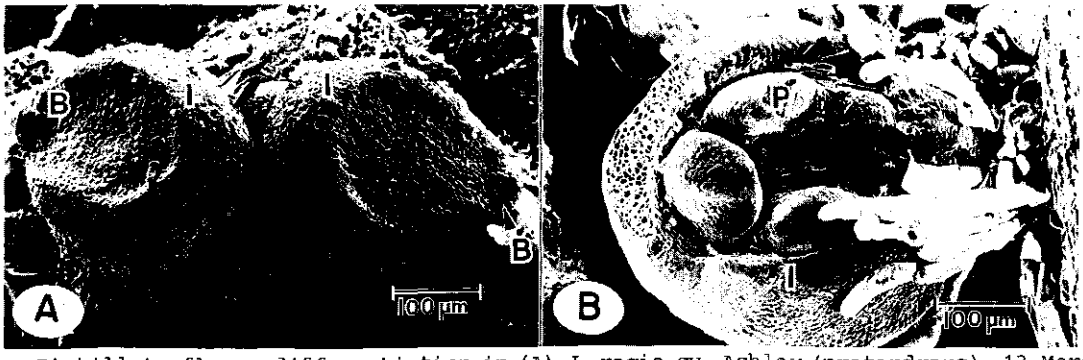

Pistillate flower differentiation in (A) J. regia cv. Ashley (protandrous), 13 March, approximately one month prior to pistillate anthesis, and (B) J. regia cv. XXX Mayette (protogynous), 26 October. B, bract; I, involucre; P, perianth.

OVULE DEVELOPMENT IN RELATION TO OVULE POSITION AND FLOWER DEVELOPMENT IN LILIUM

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Summary

Lilium ovules does not develop synchronously. Ovule development is quickest in segment 2 and 3 (central half of ovule), slower in segment 1 (apical quarter), and slowest in segment 4 (basal quarter). The used cultivars show considerable variation in development, which may partly result from differences in growth conditions. At one day before anthesis no ovule has reached maturity. At anthesis approximately 50% of ovules of cultivars 'White Europe' and 'White American' has reached maturity. In cultivar 'Gelria' at anthesis only 20% of ovules has reached maturity.
Keywords: lily, Lilium longiflorum, ovule development.

Introduction

For breeding purposes information on ovule development is essential to establish the optimum pollination time, the possible seedsetting, and the effect of manipulation as e.g. bud pollination.

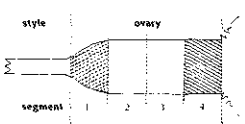
Therefore a quick and reliable method for the study of sporogenesis and gametogenesis was developed, based on staining with haemalum and clearing with methyl salicylate.

Results and discussion

For the study of megasporogenesis and gametogenesis a method developed by D. Stelly, Dept. of Genetics, Wisconsin University, U.S.A., and E. Jongendijk, Dept. of Plant Breeding, Agricultural University, Wageningen, The Netherlands, was modified.

The method eventually employed comprises fixation in a mixture of formaldehyde, propionic acid, and alcohol 50% (5,5,90 v/v); staining in haemalum (Sass' modification of Mayer's haemalum); and clearing in methyl salicylate.

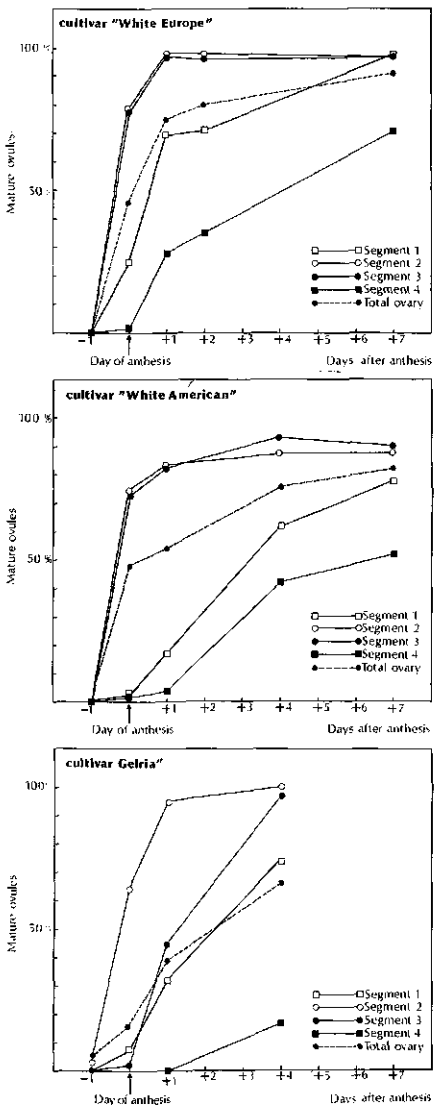
Three cultivars of Lilium longiflorum has been studied: 'White Europe', 'White American', and 'Gelria'. Ovule development appears to be not synchronous.



The ovules of the apical and basal quarters of the ovary are considerably slower in development (segment 1 and 4). At one day before anthesis no ovule has reached maturity. At anthesis approximately 50% of the ovules of 'White Europe' and 'White American', and 20% of the ovules of 'Gelria' have reached maturity. In all cultivars ultimately 80-90% of the ovules reaches maturity.

The observed differences in development of the various cultivars may partly be due to differences in growth conditions.

% mature ovules per ovary segment in relation to flower development in three different cultivars.



Conclusions

The results indicate that in Lilium breeding experiments the seedsetting can be influenced by the time and method of pollination and the stage of development of the ovary.

GENETIC BASIS OF CALLOSE PATTERN, MEGASPORE DEGENERATION AND POLARITY IN THE OVULES OF OENOTHERA

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Summary

The independence of the polarity in the ovule and the callose pattern of the meiotic cells in our material contradicts the assumption of a general causal relation between the two phenomena.

The segregation for different types of the course of megasporogenesis in the F_2-generation gives proof for a genetic basis of more than one but not many genes for each of the characters included in this investigation.

Introduction

In Oenothera a causal relationship between the deviations from mendelian segregation ratios and development of the megaspore tetrad is supposed. As polarity phenomena in the ovule are important, the relation between the development and degeneration of the megaspores and the formation of callose deposits in the ovule has to be investigated.

In spite of recent investigations concerning the relation between the megaspores competing in the ovule and polarity of the tetrad it is still open, if on a quantitative basis an agreement between development of the megaspore tetrad in Oenothera hybrids and genetic data from their progenies can be established.

Two species of Oenothera were used as parents. They differ in the tetrad polarity and competition between the megaspores. The pattern of the callose deposits in the walls of the meiotic cells is different in the two species. If there is a genetic basis for these phenomena, than in the F_2-generation of hybrids a segregation for the type of the embryo sac development can be expected.

Results and discussion

Species of Oenothera

Oe. hookeri shows a stable pattern of polarity in the ovules. In the megaspore tetrad the degeneration starts in the chalazal part of the tetrad. The embryo sac develops always from the micropylar megaspore.

Oe.suaveolens forms hetero- and equipolar tetrads of megaspores. Degeneration can start from various positions in the tetrad. The embryo sac develops mainly from the micropylar megaspore, but can develop from the chalazal spore as well.

F_1-hybrids

Similar types of megaspore tetrad polarity and embryo sac development as in Oe. suaveolens are observed. The quantitative analysis indicates, that the plasmon and the genom effect the polarity in the hybrid ovules.

F_2-hybrids

A considerable variability of phenotypes and embryological processes are observed, including the hookeri-type. The pattern of callose deposits in the walls of meiotic cells is variable and independent from the type of megaspore degeneration in the later stages. A correlation between the pattern of embryo sac development and other phenotypic characters of F_2-plants is established.

The pattern of callose formation in the walls of meiotic cells and the order of degeneration of the megaspores are variable between the ovules of any ovary in the F_1- and F_2-hybrids.

The differences between the F_2-plants are quantitative rather than qualitative. Differences are seen in the types of callose pattern and the polarity of the megaspore tetrad.

In the ovules of F_2-plants with equipolar tetrads two embryo sacs can develop. They differ in the genom of Their nuclei and compete for becoming the functioning embryo sac. This is the morphological basis for the genetic RENNER-effect.

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ULTRASTRUCTURE AND ULTRACYTOCHEMICAL LOCALIZATION OF ACID PHOSPHATASE IN THE DYAD, TETRAD AND DEVELOPING MEGASPORE OF CAPSELLA BURSA-PASTORIS

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Summary

Ovules of Capsella bursa-pastoris (monosporic Polygonum type of development) at the dyad and tetrad stages of development and at the megaspore and 2-nucleate stages of the gametophyte were fixed routinely or incubated in a modified Gomori medium for acid phosphatase localization using beta-glycerophosphate as a substrate. Organelle distribution and development were studied as well as the fate of autophagic vacuoles that localize acid phosphatase inherited from the megaspore mother cell.

Some plastids and mitochondria are destroyed and absorbed into the large vacuoles of the megaspore and young gametophyte. Other plastids and mitochondria survive meiosis and give rise to plastids and mitochondria of the gametophyte generation. There is no evidence for the de novo origin of these organelles. The rapidly growing functional megaspore synthesizes large numbers of ribosomes and produces microvillus-like extensions into the cell wall. The megaspore and 2-nucleate gametophyte maintain plasmodesmata at the chalazal end of the cell.

Keywords: ultrastructure, acid phosphatase, ovule, megasporogenesis, Capsella.

Introduction

The ultrastructure and ultracytochemical localization of acid phosphatase in the megasporocyte of Capsella have been described (Schulz and Jensen, 1981). Dramatic changes occur in the meiocyte at the beginning of meiosis that include the formation of acid phosphatase-rich autophagic vacuoles that encapsulate portions of cytoplasm containing ribosomes and organelles, the blebbing of double-membrane bound vesicles from the nuclear envelope, distinctive changes in plastids and mitochondria, and the appearance of aniline blue fluorescent substances in the cell wall. This is a continuation of the study of ultrastructural and ultracytochemical changes that occur during female meiosis in Capsella and describes the dyad, the tetrad, the developing megaspore and the 2-nucleate gametophyte.

Materials and methods

Field grown plants of Capsella bursa-pastoris (L.) Medic. were harvested at the University of California Botanical Garden. The procedures for the preparation of tissues for electron microscopy and fluorescence microscopy and for acid phosphatase localization have been previously described (Schulz and Jensen, 1981). Tissue prepared for histochemical localizations was fixed in glutaraldehyde, embedded in Epon and sectioned at 1½ μm. The procedure for the periodic acid-Schiff (PAS) reaction for insoluble carbohydrates was taken from Jensen(1962) and for the aniline blue black stain for general protein from Fisher (1968).

Results

The dyad. A transverse wall divides the meiocyte into two unequal cells. The chalazal cell receives the greater share of cytoplasm but the micropylar cell gets a proportionate share of all types of organelles present in the meiocyte. The transverse wall contains membranous vesicles and shows strong aniline blue fluorescence. Both cells have autophagic vacuoles (AVs) that contain ribosomes and organelles. Many plastids in the micropylar cell are enclosed in AVs, while those in the chalazal cell usually are not. Mitochondria in both cells look like those in the meiocyte. Both cells contain microbodies, small vacuoles and long, single RER cisternae that are closely associated with the transverse wall.

The tetrad. Meiosis II produces a linear tetrad of haploid megaspores with the chalazal most cell being somewhat larger. The wall separating the micropylar two cells of the tetrad is often incomplete or irregular. The transverse walls show strong aniline

blue fluorescence. The chalazal end wall and the lateral walls of the functional megaspore contain plasmodesmata but there are no plasmodesmata in the non-functional megaspores. Both the functional megaspore and the non-functional megaspores inherit AVs containing ribosomes and organelles that are rich in acid phosphatase. In the functional megaspore some AVs appear to be moving into the large micropylar vacuole of the cell.

Many plastids and mitochondria in the degenerating megaspores, but not in the functional megaspore, localize acid phosphatase. The plastids of the functional megaspore lose their internal membranes and some show a dissolved plastid envelope. Plastids in the functional megaspore do not accumulate starch but nucellar cells do. Mitochondria begin to enlarge and lose electron density in the functional megaspore. The nucleolus is small and granular and ribosomes are dense in the cell.

The developing megaspore. The expanding functional megaspore crushes the nucellus surrounding it while the degenerating megaspores are crushed by encroaching nucellar cells. The nucleolus of the functional megaspore becomes very large and the cytoplasm of the cell becomes extremely electron dense with ribosomes. Two prominent vacuoles fill with debris. AVs, plastids and lipid globules are seen in the vacuoles. Other plastids in the cytoplasm appear to be healthy and developing and possess a few thylakoids and in some cases starch. Some mitochondria appear to be destroyed at this stage while others appear to be quite healthy. Some plastids and mitochondria look like they are dividing and dictyosomes are active.

The cell wall is thickened and shows strong aniline blue fluorescence but does not stain with PAS or with aniline blue black. Plasmodesmata occur in this wall and microvillus-like projections at the megaspore surface extend into this wall to increase the absorptive surface area of the rapidly expanding cell.

The 2-nucleate gametophyte. This cell is very broad at the micropylar end. The two haploid nuclei are separated by a large central vacuole which contains cellular debris including degenerating plastids and mitochondria and AVs that localize acid phosphatase. The thickened cell wall and the microvillus-like projections of the megaspore have disappeared but plasmodesmata are still present at the chalazal end of the cell. Plastids and mitochondria in the cytoplasm appear to be healthy and dividing. Long single RER cisternae begin to develop and ribosome density remains high.

Discussion

This study shows that AVs that localize acid phosphatase play a major role in the destruction of ribosomes, organelles and other cytoplasmic constituents during megasporogenesis in Capsella. Many of the AVs that segregate into the functional megaspore are absorbed into the enlarging vacuoles of the maturing megaspore and 2-nucleate gametophyte. Concentric membrane-bound organelles similar to the AVs in Capsella have been observed and assigned various functions during female meiosis in several species of Lilium (Rodkiewicz and Nikulska, 1965; Dickinson and Andrews, 1977; De-Boer-De-Jeu, 1978). The present study which shows the specific localization of acid phosphatase in these structures and follows their fate during meiosis and early gametophyte development confirms that in Capsella they function as AVs that mediate the turnover of macromolecules and organelles of the sporophyte generation during the transition to the gametophyte phase of the life cycle.

A decrease in the concentration of cytoplasmic ribosomes has been observed during female meiosis in Pisum sativum (Medina et al.,1981), during female and male meiosis in Lilium longiflorum (Dickinson and Andrews, 1977, Dickinson and Heslop-Harrison, 1977), and in other species. In Capsella this reduction in ribosomes is correlated with their destruction inside of AVs. While the synthesis of new ribosomes is probably initiated in the functional megaspore of the tetrad there is a dramatic increase in the size of the nucleolus and a massive outpouring of new ribosomes in the enlarging megaspore. Ribosome restoration in the tetrad and maturing megaspore has also been reported in Pisum sativum (Medina et al., 1981), Zea mays (Russell, 1979) and Lilium longiflorum (Dickinson and Potter, 1978; Dickinson and Heslop-Harrison, 1977).

The fate of plastids and mitochondria during female meiosis is difficult to follow. Some plastids inherited by the functional megaspore are encased in AVs and are eventually absorbed into the vacuoles of the growing megaspore and 2-nucleate gameto-

phyte. Other plastids in the cytosol appear to dedifferentiate by losing internal membranes and in some cases the plastid envelope. Plastids never disappear altogether and some apparantly survive to proliferate in the enlarging megaspore. There is no evidence from this study that plastids are regenerated de novo from another membrane system in the cell. Plastid dedifferentiation and redifferentiation during male and female meiosis has been reported in Lilium longiflorum (Dickinson and Potter, 1978; Dickinson and Heslop-Harrison, 1977) and during female meiosis in Pisum sativum (Medina et al., 1981). A sizable portion of the plastid population is thought to be eliminated in Lilium (Dickinson and Potter, 1978).

The mitochondrial cycle during female meiosis in Capsella is also complex. The functional megaspore inherits its dense, contracted mitochondria characteristic of the megasporocyte (Schulz and Jensen, 1981). Some of these organelles localize acid phosphatase. It is not known if these are destined to degenerate or are only undergoing molecular reorganization. Hill (1977) observed the localization of significant levels of acid phosphatase in mitochondria of post-anthesis synergids of cotton ovules just prior to their degeneration. Some of the mitochondria of the functional megaspore of Capsella enlarge and begin to proliferate. Then a large number of mitochondria appear to be destroyed and absorbed by the large vacuoles of the maturing megaspore and the 2-nucleate gametophyte. Surviving mitochondria proliferate in the 2- and 4-nucleate gametophyte stages. As with plastids there is no evidence from this study that at any time all of the mitochondria of the dyad, functional megaspore or 2-nucleate gametophyte are destroyed or formed de novo. Mitochondria are reported to go through stages of dedifferentiation and redifferentiation during female meiosis in Myosurus minimum (Woodcock and Bell, 1968), Lilium longiflorum (Dickinson and Heslop-Harrison, 1977) and Pisum sativum (Medina et al., 1981). A portion of the mitochondrial population in Lilium is thought to be eliminated (Dickinson and Potter, 1978). Russell (1979) observed a postmeiotic redifferentiation and subsequent destruction of mitochondria in Zea mays. In Zea mitochondria apparantly dedifferentiate during meiosis, increase in complexity in the functional megaspore and 2-nucleate gametophyte and then degenerate in the 4-nucleate gametophyte.

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ASPECTS OF MEGAGAMETOGENESIS IN HORDEUM VULGARE

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Summary

Megagametogenesis in Hordeum vulgare (barley) has been studied using 1.5 µm sections photographed with Nomarski-interference optics and then re-embedded for transmission electron microscopy. Stages in megagametogenesis to be considered will be from the 1-nucleate megaspore stage to the early 8-nucleate, 7-celled embryo sac. By using a gynoecial staging technique modified after Waddington et al. (1983) we were able to obtain a fairly complete developmental sequence, particularly with respect to stages in the process of embryo sac cellularization.

Introduction

The study of megagametogenesis in flowering plants is difficult, particularly in the cellularization phase, because of the absence of a reliable external morphological marker for staging and the apparent rapidity of changes occurring once mitosis is complete. In this paper we will present some observations on megagametogenesis and on cell wall formation particularly with respect to the egg apparatus of barley using a staging technique modified after Waddington et al. (1983).

Results and discussion

Staging technique

Waddington et al. (1983) assigned numbers of 4 to 10 to gynoecial development stages from appearance of the pistil primordium (4) to pollination (10). We found a very rough correlation between their numbered stages and megasporogenesis or megagametogenesis stages in which we were interested. Their stage 7.5, for example, produced megasporocytes for us. Stage 8.5 gave us 2- or 4-nucleate embryo sacs. Stage 8.75 gave us cellularization with low numbers of embryo sac cells. Stage 9 gave us high numbers of embryo sac cells (barley has up to 100 antipodal cells). Although this staging technique was not precise, it allowed us to make a number of observations on this process.

Megagametogenesis

Barley is monosporic with degeneration occurring in the 3 micropylar megaspores. The functional megaspore is a large cell with its long axis oriented toward the ovular micropyle. Its cytoplasm has a distinctly polar distribution of plastids; the micropylar portion of the functional megaspore has more plastids than the chalazal portion. This plastid distribution difference is maintained through the 4-nucleate stage during which major vacuoles begin to appear. An additional mitosis occurs in both the micropylar and chalazal pair of nuclei at the 4-nucleate stage prior to cell wall formation.

Wall formation among both micropylar and chalazal quartets of nuclei is accompanied by changes in cytoplasmic components, specifically dictyosomes, endoplasmic reticulum and microtubules. Dictyosome activity during the wall formation process results in the production of both dense and rather clear vesicles. The endoplasmic reticulum during wall formation has dilated cisternae and is distributed both in a branched fashion and in circular arrays. In many cases growing walls and the branched endoplasmic reticulum are nearly congruous (Figs. 1 & 2). Microtubules are common near the free ends of growing walls and in areas of discontinuities (Figs. 1 & 2). Many such microtubules are parallel to each other and perpendicular to the growing cell wall (Figs. 1 & 2).

After wall formation in the egg apparatus is nearly complete, the 3 cells exhibit rather flat profiles with beaded cell walls (Fig. 1). Somewhat later cells of the egg apparatus develop more rounded profiles but retain the fairly uniform wall beading visible earlier. Cell wall beading becomes less pronounced during chalazal expansion of the egg apparatus. This is apparent in Fig. 3 as well as a marked thinning of the chalazal cell wall. The cell in Fig. 3 is a synergid, identifiable by the appearance of wall ingrowths of the filiform apparatus at its base. The ultrastructural appearance of the filiform apparatus is similar to that of transfer cell wall ingrowths. The adjacent

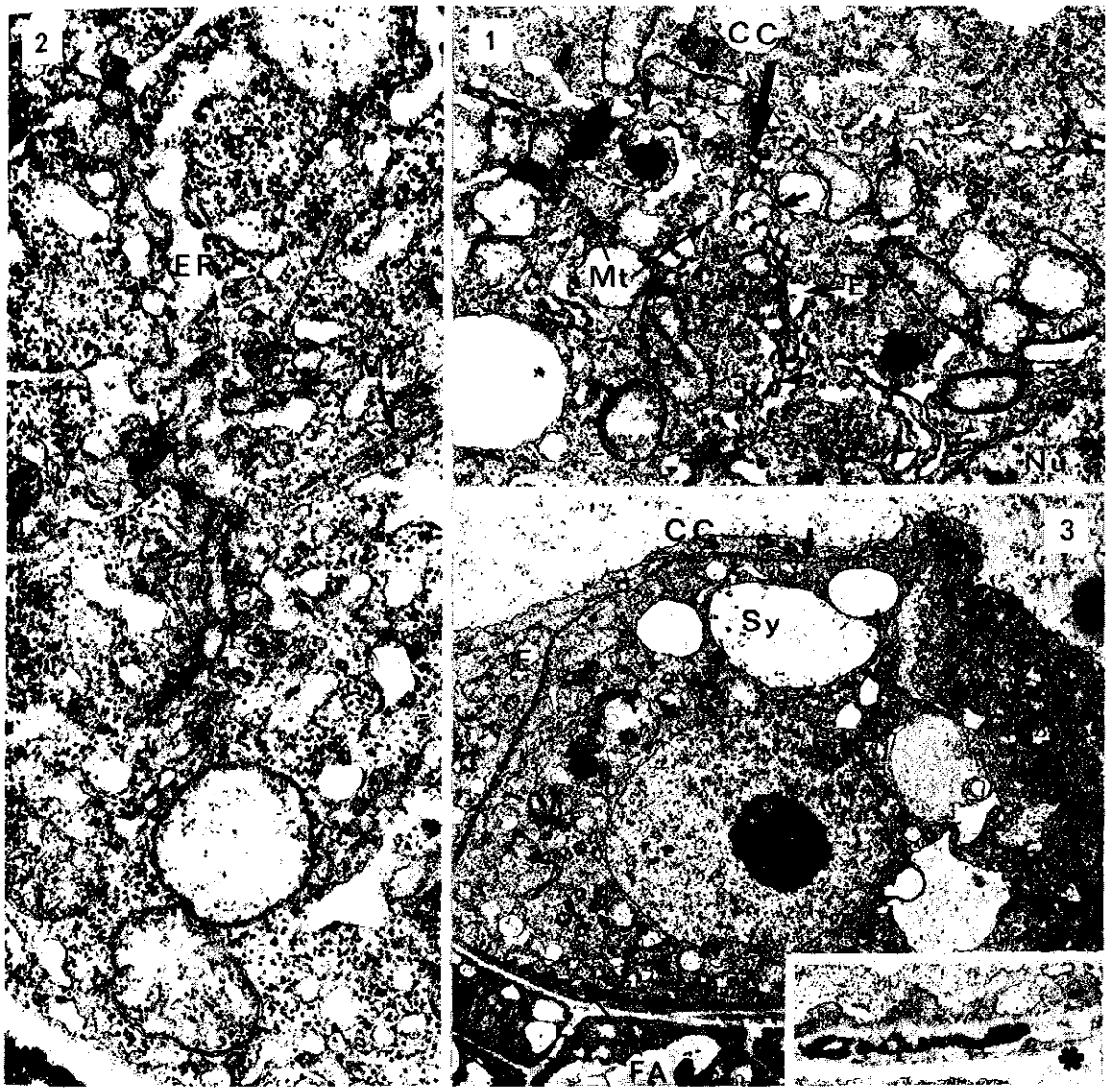

Fig. 1. Two egg apparatus cells and portion of central cell (CC) from early cellular barley embryo sac. The egg apparatus cells have low profiles and are partially separated by a 'T-shaped', beaded, discontinuous wall. Microtubule clusters (Mt) mark 2 discontinuities. Small, unlabelled arrows denote vertical and horizontal members of 'T'; a large, unlabelled arrow indicates intersection of 'T'. Dilated endoplasmic reticulum (ER) shown adjacent to vertical wall member. Part of the nucleus (Nu) of 1 egg apparatus cell at lower right (X17,000). Micropyle is downward in all 3 figures. Fig. 2. Growing end (large, unlabelled arrow) of another section of vertical wall from Fig. 1. A microtubule cluster is indicated (Mt) as well as endoplasmic reticulum (ER) adjacent to growing wall (X34,000). Fig. 3. A portion of central cell (CC), portion of egg (E), and 1 of 2 synergids from older embryo sac. Synergid differentiation is indicated by appearance of wall ingrowths of filiform apparatus (FA) and by thinning of its wall (small, unlabelled arrows) as it expands chalazally. (X5,500). Insert (*) shows portion of filiform apparatus (X14,500).

egg apparatus cell is the egg based on identification of the second synergid cell in later sections. This is the earliest stage at which we have observed morphological differentiation of egg apparatus cells; this embryo sac had about 10 cells (3 egg apparatus cells, central cell, about 6 antipodal cells) and would fit within stage 8.75 of Waddington et al. (1983). Transfer cell wall ingrowths also appeared in antipodal cells immediately bordering the embryo sac wall at this stage.

Discussion

Initial wall development in barley embryo sacs is in 2 stages. First is a beaded wall (Figs. 1 & 2) seen in all incomplete walls and in young complete walls. Second is a complete wall (Fig. 3) with a more regular appearance but which may vary in thickness. The beaded nature of incomplete walls suggests that their initial growth is by vesicle accretion. Vesicles similar in appearance to the beads but of variable sizes have been seen near growing walls. Active dictyosomes occurring near growing walls have vesicles with similar morphology to that of the wall beads. Microtubule clusters (Fig. 1 & 2) occur at wall ends and discontinuities but these microtubules do not appear to be consistently associated with any organelle. The ER may contribute to the transformation of young beaded walls to more uniform walls. Dilated ER (Figs. 1 & 2) is closely appressed to beaded walls whereas regular walls are associated with slender ER segments with only approximate alignment typical of mature walls.

Our observations of microtubule clusters in close association with free ends of growing walls segments and with intercalary wall discontinuities suggests the involvement of phragmoplasts in the process of wall formation in the barley embryo sac. The question of whether these phragmoplasts arise from mitotic spindles as would be suggested by the recent work of Fineran et al. (1982) on wheat endosperm or de novo, an alternative origin proposed by Bajer & Mole-Bajer (1972), must await further work.

The occurrence of transfer cell wall ingrowths in antipodal cells of barley at the same stage that similar ingrowths are forming in synergids suggests that both poles of the embryo sac may be involved with nutrient influx. Such an influx of nutrients would be compatible with the probable high metabolic demands of the embryo sac at this time.

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BEHAVIOUR OF SPINACH SPERM CELLS IN POLLEN TUBES PRIOR TO FERTILIZATION

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Summary

Cytological sperm cell changes are investigated during pollen tube growth in vivo, starting at the pollen germination and ending at the tube penetration in the degenerating synergid. The number of organelles and the amount of male cytoplasm in the sperm cells gradually decrease, while the contact between the two sperm cells remains intact.
Keywords: spinach, Spinacia oleracea, pollen tube growth, sperm cell(s).

Introduction

In order to investigate the fusion of male and female gametes and the influence of the male cytoplasm in the inheritance, the sperm cytology is important to know. Not only at the moment of fusion but already from the moment of formation of the sperm cells in the pollen grain. Priviously the situation in the mature pollen grain has been described (Wilms & Van Aelst, 1983). A consistent association of the sperm cells and the vegetative nucleus suggests a structurally connection. How this connection behaves during pollen tube growth is presented in this report.

Results and discussion

In the mature pollen grain stage the sperm cells are linked with each other (fig. 1). Their long, slender projections are found around the irregular shaped vegetative nucleus. The sperm

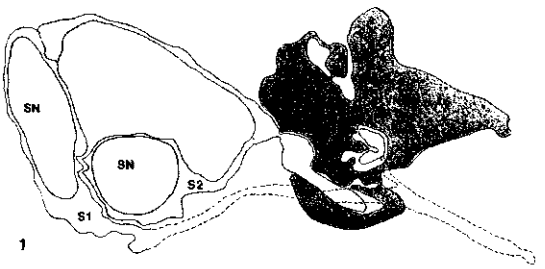
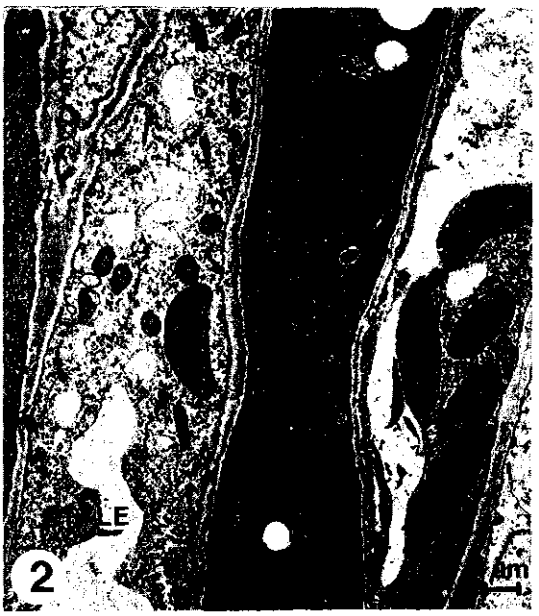
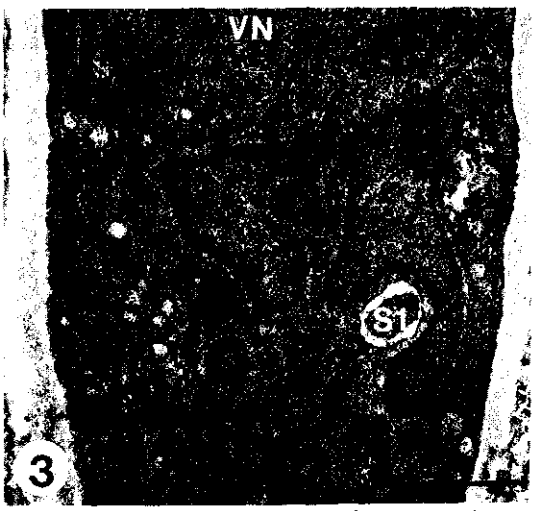


Fig. 1. Sperm cells-vegetative nucleus association in the pollen grain. Schematic reconstruction from serial sections. S1, S2 = sperm cell; SN = sperm nucleus; VN = vegetative nucleus.

cells - vegetative nucleus association becomes less evident in the style (figs. 2,3). Later on the shape of the vegetative nucleus becomes more elongated and less irregular (fig. 6); also degeneration of this nucleus starts (fig. 6). The sperm cells with the long slender runners follow the vegetative nucleus through the pllen tube. In the stylar tissue these runners are still in close vicinity





Figs. 2,3. Irregular shaped vegetative nucleus (VN) and sperm cell (S1) in pollen tube in stylar tissue.

of the vegetative nucleus. In the sperm cell cytoplasm mitochondria, dictyosomes, rough endoplasmic reticulum next to free ribosomes can be observed (fig. 3). Later degeneration of mitochondria is clearly present (fig. 5). The number of organelles and the amount of male

cytoplasm in the sperm cells gradually decrease. The sperm cell runners also decrease in length, while the contact between the two sperm cells remains intact (figs. 4,5,7). The process of deminishing of the total amount of sperm cell cytoplasm is not clear, but it finally leads to almost naked sperm nuclei which were observed earlier in close vicinity of the female gametes (Wilms, 1981).

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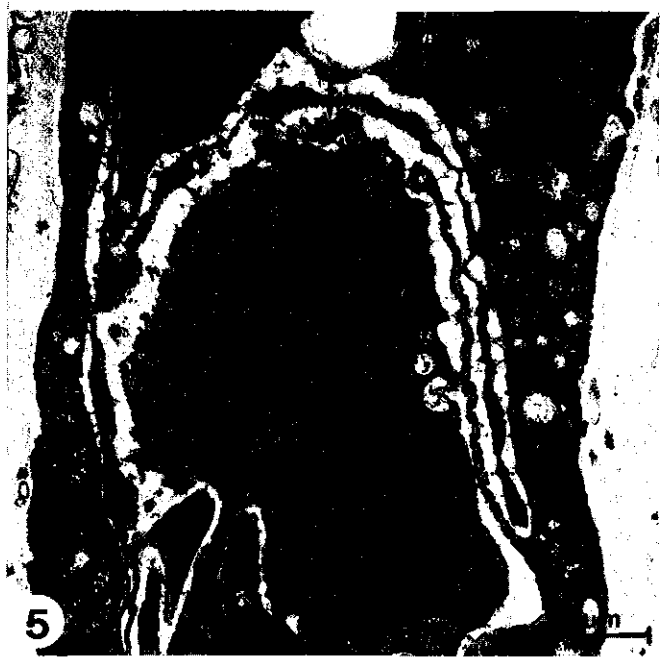
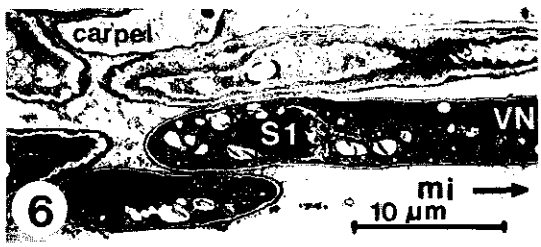
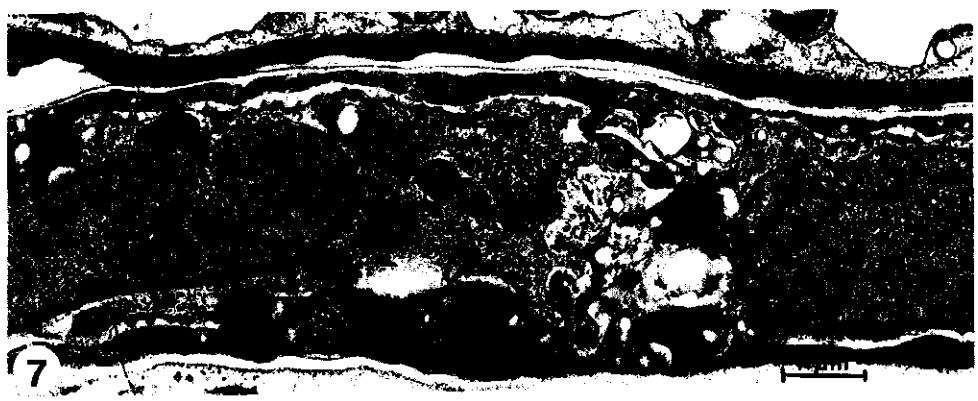




Figs. 4,5. Sperm cells in pollen tube in carpellar tissue near the ovule. Sperm cells (S1, S2) are still connected with each other (dotted lines). Mitochondrium degenerates (arrow).

Figs. 6,7. Degenerating vegetative nucleus, sperm cells in pollen tube between carpel and ovule near micropyle. D = dictyosome; M = mitochondrium; RER = rough endoplasmatic reticulum.



SPERM SPECIFICITY IN PLUMBAGO ZEYLANICA

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Summary

In the angiosperm Plumbago zeylanica the role of individual sperm cells during gametic fusion can be determined using paternal organelles observed within maternal cytoplasm to trace patterns of fusion after fertilization. The two sperm cells are distinguished by significant differences in content of mitochondria and plastids. In >94% of the cases observed, the plastid-rich sperm cell unassociated with the vegetative nucleus fuses with the egg to form the zygote. In only one instance did the opposite pattern occur. Since the possibility of this result occurring as the consequence of chance alone is less than 1 in 7,000, this represents strong evidence for the presence of a final putative recognition event occurring at the gametic level.

Introduction

Gametic fusion is the cellular process by which the sperm nucleus enters the female gamete, permitting nuclear fusion and the formation of the embryo. Presumably the means by which gametic fusion occurs entails cellular fusion, and consequently at least some surrounding male cytoplasm may be transmitted in the process (Russell, 1983). Since male cytoplasmic organelles are inherited in a number of angiosperms (Kirk and Tilney-Bassett, 1978), the recent report of dimorphism in sperm cell organization and organelle content (Russell, 1984) raises questions about whether the sperm cells, so produced, are equally capable of fertilizing the egg, or whether preferential patterns of fusion will occur.

In the angiosperm Plumbago zeylanica L., the two sperm cells differ significantly in both mitochondrion and plastid content (Russell, 1984). Furthermore, it has been demonstrated that the paternal and maternal organelles may be observed and distinguished by ultrastructural differences in both the zygote and endosperm of this plant following gametic fusion (Russell, 1980, 1982, 1983). The present study uses these characteristics as the basis for examining the fate of individual sperm cells during gametic fusion.

Materials and Methods

Plants of Plumbago zeylanica L. were grown at 18 to 23°C with 16 hour days in growth chambers at the University of Oklahoma. Ovaries were collected 8-1/4 to 9 hours after pollination, dissected and fixed at room temperature in 3% glutaraldehyde in 0.15M phosphate buffer (pH 6.8) for 6 to 8 hours. Tissue was rinsed briefly, fixed in 2% buffered osmium tetroxide, dehydrated in ethanol, and embedded in low viscosity resin. For further details of preparation see Russell (1983).

Results and discussion

The tricellular microgametophyte of P. zeylanica contains two dimorphic sperm cells which have been designated Svn and Sua and are distinguished by whether or not the sperm cell is physically associated with the vegetative nucleus (Russell, 1984). The sperm cell that is not associated with the vegetative nucleus is designated Sua; the other sperm cell, which is physically associated with the vegetative nucleus, is designated Svn. The two sperm cells differ significantly in organelle content. The Sua has the majority of the plastids, an average of 24.18 plastids and few mitochondria (average: 39.81; Russell, 1984). In contrast, the Svn contains an average of 0.45 plastids and the majority of mitochondria, an average of >256 (Russell, 1984). Thus, organelle differences alone may also be used to distinguish the two sperm cells. Interestingly, the Svn usually does not contain any plastids (Russell, 1984).

Paternal plastids can be used to trace which of the two male gametes fuses with the egg and central cell since their ultrastructure differs from that of maternal plastids. Assuming that sperm cytoplasm is transmitted, an assumption that has been verified in P. zeylanica (Russell, 1983), the presence and location of paternal plastids in cells of the female gametophyte should indicate which of the two male gametes fused with the egg. Provided that numerous paternal plastids are seen, the fate of the plastid-rich Sua during fertilization can be clearly deduced.

The probability of error in identifying whether a particular sperm cell type fertilized the egg reduces with each paternal plastid seen in a given female gametophyte. Since the statistical probability of the Svn

containing as many as three plastids is less than 1%, the presence of three or more paternal plastids in the maternal cytoplasm of either the zygote or endosperm should clearly establish the fate of the plastid-rich sperm cell over 99% of the time. The actual margin of error is probably significantly less than 1%, if one considers how likely it is that all paternal plastids will be observed without serially reconstructing the female gametophyte.

In the ovules used in this study, median sections were prepared through both the zygote and primary endosperm nucleus in order to examine the perinuclear cytoplasm in which plastids preferentially appear. This approach permits the fate of each sperm cell to be confirmed in both female reproductive cells. In some cases, numerous mitochondria similar in size to those normally observed in the sperm cell (Russell and Cass, 1981; Russell, 1983) were observed in one of the two female reproductive cells. In each circumstance, numerous paternal plastids were observed in the opposite female reproductive cell.

Although over 100 ovules were examined in detail during the course of this study, only 17 provided unambiguous data concerning the fate of the male cytoplasm. Of these, 16 demonstrated transmission of three or more paternal plastids into the cytoplasm of the egg or zygote. In one case of the 17 examined, the opposite pattern of transmission was observed. In all cases, fertilization appeared to be proceeding normally, with a male nucleus observed either approaching or in the process of fusing with the nucleus of the female reproductive cell which it entered. Given the normal abortion rate of approximately 5%, it is impossible to determine whether the ovule in which the pattern of fusion was reversed would produce viable seed or whether this might represent one possible source of abortion.

The preferential transmission of paternal plastids into the female gamete in 16 of 17 cases examined in detail implies that discrimination between the two male gametes occurs and represents a statistically compelling case. The probability of this result (16 of 17 cases) or better (17 of 17) arising by chance alone is less than one in 7,000 if one assumes an equal size sample and equal likelihood of selection (using the binomial distribution). One may therefore conclude that the two sperm cells differ very significantly in their ability to fertilize the egg under normal circumstances and that in regard to gametic fusion, the sperm cells display specificity. Presumably, this specificity is conferred by some distinguishing factor in or on the sperm cells.

The recognition of sperm cells by the female reproductive cells is presumably mediated by the same mechanisms influencing recognition events in other systems (Clarke and Knox, 1978); however, the identity and mode of action of any specific receptor is presently undetermined. The presence of unfused sperm cytoplasmic bodies (Russell, 1983) suggests that a threshold effect occurs, whereby only the main sperm cell body can effect gametic fusion. Differences in sperm cell volume, which may approach a ratio of 2:1 (Russell, 1984), may also be an important factor in discriminating between the delivered male gametes. Whether such recognition involves a specific receptor, the cooperation of multiple receptors, or whether differences in membrane charge and surface area alone might provide the mechanism for discrimination are central to further elucidation of the basis of sperm specificity during double fertilization.

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THE STRUCTURE OF SPERM CELLS IN BRASSICA

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Summary

In mature tricellular pollen of Brassica campestris L., the pair of sperm cells are held together within the common plasma membrane of the vegetative cell. The sperm cell most closely associated with the vegetative nucleus, besides containing most of the mitochondria in the sperm cells, has been shown to possess a long tail, > 10 um in length, that penetrates through a passage in the highly convoluted vegetative nucleus. In all grains examined, this long tail contained a forked array of microtubules linked to arrays aligned within the ridges of the sperm cell. Plastids were absent in both sperm cells.

Introduction

Sperm cell associations have been previously reported in three tricellular pollen systems. The first is Plumbago zeylanica, where an association between sperm cells and vegetative nucleus was reported in the mature pollen and pollen tube (Russell and Cass, 1981). Subsequently, similar associations were found in Brassica oleracea (Dumas et al. 1984), and spinach, Spinacia oleracea (Wilms and van Aelst, 1983). Here, we report the finding of a sperm cell association in Brassica campestris and have demonstrated its 3 dimensional structure.

Results and Discussion

Serial thin sections were cut of entire sperm cell associations of 3 pollen grains, and 3-dimensional coordinate information obtained by digitization using the Zeiss Videoplan Image Analysis system. Precise lengths and placement of the sperm cells and vegetative nucleus in three dimensions were computed and steresscopic images generated. These were confirmed by manually constructed 3-dimensional models.

The sperm cell association is suspended centrally in the cytoplasm of the vegetative cell. The sperm cells, like those of B. oleracea (Dumas et al., 1984) are surrounded both by their own plasma membranes, and the continuous inner plasma membrane of the vegetative cell. The two plasma membranes are separated by an electron lucent zone, which may contain various types of vesicles, or even what appear to be cytoplasmic connections.

Within the sperm cells, there is an uneven distribution of mitochondria and no plastids. The vegetative nucleus is remarkably convoluted. The cytoplasm in the embayments contains prominent circular profiles of ER which may or may not be coated with ribosomes. These appear to originate from the outer membrane of the nuclear envelope. Also present are portions of the tail of SC 1, the sperm cell most closely associated with the vegetative nucleus. This sperm contains more mitochondria than the other sperm cell (SC 2), and possesses a long tail that penetrates through a passage in the highly convoluted vegetative nucleus.

Several different arrays of microtubules have been detected in the cytoplasm adjacent to the plasma memebrane of the sperm cells. In SC1, an array is present in each of the two ridges of the tail. Towards the point of entry into the nucleus, the arrays fuse and continue as a single array into the tip, but end prior to the branches. Each array appears as a group of linearly arranged rows of microtubules. In SC2, several arrays extend along the major ridges to the end adjacent to SC1 where they terminate. The arrays of microtubules presumably provide a cytoskeleton for the sperm cell, giving the tail the strength to maintain the association with the vegetative nucleus.

Our quantitative data support the concept of the male germ unit recently developed for the sperm cell association in B. oleracea (Dumas et al., 1984). The importance of this concept lies in its relationship to double fertilization:

-- all the DNA of heredity, both cytoplasmic and nuclear, is held together in a single unit;

-- there is dimorphism in both the shape and mitochondrial content of the pair of sperm cells (see Russell and Cass, 1983) and some evidence of their non-random participation in the two fertilization events.

While it is accepted that these conclusions apply in only some tricellular pollen systems, taken together, these resuls suggest that fertilization may be targeted. The male germ unit therefore appears to provide a means by which the male gametes are transported to the female reproductive cells.

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SPERM CELLS IN THE FERTILIZATION PROCESS IN LYCOPERSICON ESCULENTUM

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Summary

In tomato, just before merging with the egg- and central cell, the sperm cells show conspicuous reduction in size and in amount of organelles.

Introduction

In few cases e.g. in barley (Cass, 1981) sperm nuclei and other organelles take part in the fertilization process. In Spinacia (Wilms, 1981) the sperm nuclei have no cytoplasm before they merge with the cytoplasm of the egg- and central cell.

Results and discussion

Electronograms present sections from tomato ovules fixed at 34 h after pollination with the method reported by Kadej & Kadej (1983). These sections come from the same egg apparatus from three levels of cutting. They have been intrepreted as follow: the sperm cells in the tomato before leaving the pollen tube continue their transformation. These are mainly visible in the rejection of the outer cytoplasmic layer with the majority of mitochondria (M), dictyosomes (D) and endoplasmic reticulum (ER) in them (Fig. 3). Plastids were not observed. The male gametes so formed cover themselves with a new cell membrane containing pollen tube material (Fig. 3, arrows). The preserved remnants of the sperm cell wall (Fig. 2, arrows) and a considerable cavity in the pollen tube as well as the nature of the cytoplasmic structures in it point to the fact that the whole reduced sperm cell gets into the egg cell.

The mechanism of the fusion of the male gametes with the egg- and central cell would be a simple fusion of two cells. This fusion is preceded by resorbing of the cell wall which would cause the opening of two areas.

The role of the cytoplasm and the organelles of the sperm cells could be however insignificant. The may soon undergo complete degeneration.

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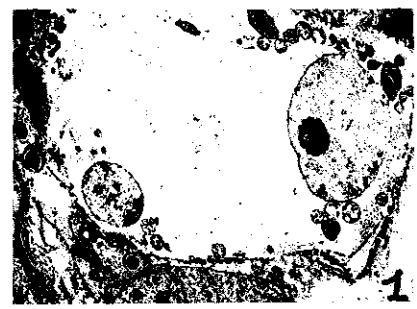

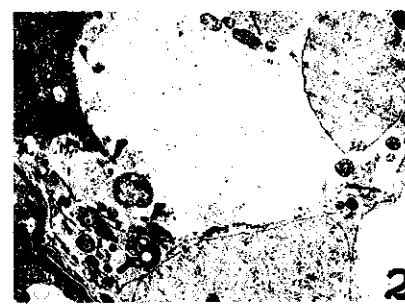

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SECONDARY NUCLEUS IN ROSOIDEAE

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Summary

In 16 examined species from 10 genera of the subfamily Rosoideae the three types of polar nuclei behaviour were found. Polar nuclei fused: 1. long or 2. shortly before fertilization, and 3. simultaneous triple fusion occurred(Table 1). In the last case no secondary nuclei were formed, or they were rare, in unfertilized embryo sacs and the degenerating polar nuclei lay attached one to another. The results confirmed the view that the behaviour of polar nuclei is one of the embryological characters of the species and it may vary within the genus.
Keywords: polar nuclei, secondary nucleus, Rosoideae.

Introduction

Schnarf (1931) and Davis (1966) emphasized the importance of the behaviour of polar nuclei in mature embryo sac as an embryological character which may be of taxonomic value. Davis, however, stressed that there was little exact information connected with this process.

The data for families of Angiospermae have been lately compiled by Poddubnaya-Arnoldi (1982). According to the cyto-embryological characteristics given in her monograph, in ca 80% of investigated families polar nuclei fuse forming a secondary nucleus which may be fertilized. Among the remaining familis the most interesting are those in which variation of the behaviour of polar nuclei was noted. One of them are Rosaceae. Poddubnaya-Arnoldi(1982) writes that in this family polar nuclei fuse either long before fertilization or at fertilization.

On the basis of this description of the character one can presume that in some taxa of Rosaceae, or under some special conditions, secondary nucleus is not formed. Such reports were published for the polyploid Rubus species investigated by Christen (1950), Berger (1953), Markarian et al. (1959) and Dowrick (1966), while Thomas (1940) and Virdi et al. (1969) observed early formation of the secondary nucleus in some diploids (ref. Czapik 1983 b).

The observations on polar nuclei in Rubus were comparatively more frequently performed than in other taxa of Rosaceae, where the published data for particular genera are fragmentary or completly lacking. Accidental observations are mostly insufficient and the studies on this character must be carefully planned to get the exact view when polar nuclei fuse and what kind of variation of their behaviour may be expected.

The problem was investigated by the authoress in Rubus (Czapik 1983 a, b). The four examined species of the subgenera Eubatus, Cyclactis and Chamaemorus differed in respect of this character and the following three types of behaviour of the polar nuclei were found.

1 Rubus chamaemorus type: polar nuclei fused long before fertilization, the secondary nucleus was always formed.

2 Rubus saxatilis type: polar nuclei fused shortly before fertilization, simultaneous triple fusion was noted in rare cases. In this type the secondary nucleus was present in old embryo sacs of unpollinated flowers.

3 Rubus caesius type: simultaneous triple fusion occurred. In old unfertilized embryo sacs no secondary nuclei were found, or they were very rare.

The influence of some external factors did not seem to contribute to the above types of behaviour of polar nuclei. Nor was it possible to correlate these differences, for certain, with any of the internal factors taken into consideration taxonomic relationship of the species, mode of reproduction or ploidy level of examined plants.

The aim of the present studies was to check whether the types of behaviour of polar nuclei described for Rubus occur in other genera of the subfamily Rosoideae.

Material and methods

16 species from 10 genera of the subfamily Rosoideae were examined. They were members of the four tribes:Filipenduleae, Rubeae, Potentilleae, Sanguisorbeae and Dryadeae (Table 1).

In each species at least two plants from two distant localities in Poland were examined, except Duchesnea indica and Waldsteinia geoides taken from the collection of the Kraków Botanic Garden.

The chromosome numbers of specimens were established (Table 1). The examined plants were potted and cultivated in the

experimental field in Modlnica where emasculation, isolation and controlled pollination were performed. The ovaries of various age as well as of pollinated and unpollinated flowers were fixed in acetic alcohol 1:3, 5 to 48 hrs or 4 to 6 days after anthesis. Microtome sections 8 - 12 µm thic were stained with Heidenhain's haemathoxylin, alone or combined with PAS or ruthenic red. The Feulgen method was used for some embryo sacs fixed at the time of fertilization.

Results

In normally developing flowers of the e examined plants embryo sacs were fully organized at the time of expansion of flower buds and just before anthesis. In gynoecea with many pistills some younger ovules might have occurred at that stage but the young embryo sacs were easy to recognize.

The behaviour of polar nuclei in examined taxa followed one of the three patterns established for Rubus (Table 1). In fact, the spead of the fusion was the point in which the main types of the polar nuclei behaviour differed. The nuclei approached each other and lay in close proximity during relatively long or short period of time.

In species assigned to type (1) Rubus chamaemorus, secondary nucleus was formed mostly within a closed flower bud before anthesis and pollination. In type (2) Rubus saxatilis fusion of polar nuclei took place at the time of pollination, sometimes just before fertilization. In the third type simultaneous triple fusion occurred and no secondary nucleus was formed.

The genera were represented by one species each except Rubus studied previously, and Potentilla. The species of the last two genera differed in respect of the examined character. The differences in Rubus led to the recognition of the three model groups of polar nuclei behaviour. In tetraploid Potentilla arenaria secondary nucleus was formed shortly before fertilization, while in hexaploid P. puberula secondary nuclei were rare in unfertilized embryo sacs. Such embryo sacs contained unfused polar nuclei laying closely attached to one another.

The fusion of polar nuclei before anthesis and pollination type(1) was noted in five species: three diploids from the tribe Dryadeae (Sibbaldia procumbens, Waldsteinia geoides, Dryas octopetala) and two polyploids from the tribes Rubeae and Potentilleae (Rubus chamaemorus and Duchesnea indica).

The polar nuclei fused shortly before fertilization in 7 species. In their unfertilized embryo sacs secondary nuclei were visible. However, in Rubus saxatilis, representing this type of polar nuclei behaviour, the simultaneous triple fusion was observed sporadically. It may be the result either of some delay of the polar nuclei fusion or of the quicker growth of the pollen tubes. It is highly possible that similar process may have occurred also in other plants with Rubus saxatilis type of polar nuclei behaviour.

Table 1. Behaviour of polar nuclei in examined species of Rosoideae.

Species	2n	Tribe
(1) Rubus chamaemorus type - polar nuclei fuse long before fertilization		
Rubus chamaemorus L.	56	Rubeae
Duchesnea indica Focke	42	Potentilleae
Sibbaldia procumbens L.	14	Dryadeae
Wald. geoides Willd.	14	Dryadeae
Dryas octopetala L.	18	Dryadeae
(2) Rubus saxatilis type - polar nuclei fuse shortly before fertilization		
Rubus saxatilis L.	28	Rubeae
Filip.hexasepala Gilib.	14	Filipenduleae
Pot. arenaria Borkh.	28	Potentilleae
Comarum palustre L.	42	Potentilleae
Fragaria vesca L.	14	Potentilleae
Geum urbanum L.	42	Dryadeae
Sang. officinalis L.	28	Sanguisorbeae
(3) Rubus caesius type - simultaneous triple fusion		
A. in unfertilized embryo sacs polar nuclei in pairs, secondary nuclei rare		
Rubus caesius L.	28	Rubeae
Pot. puberula Krašan	42	Potentilleae
B. in unfertilized embryo sacs all stages of fusion of polar nuclei		
Rubus Bellardii Weihe	28	Rubeae
C. in unfertilized embryo sacs pairs of unfused polar nuclei		
Agrimonia eupatoria L.	28	Sanguisorbeae

Wald. = Waldsteinia, Filip. = Filipendula
Pot. = Potentilla, Sang. = Sanguisorba

This type was represented by one species from each tribe, from Potentilleae - by three species. The plants were diploid (Filipendula hexasepala, Fragaria vesca) or polyploid (Rubus saxatilis, Potentilla arenaria, Comarum palustre, Sanguisorba officinalis, Geum urbanum/.

The third type of polar nuclei behaviour, i.e. simultaneous triple fusion, seemed to be a rule in the three tetraploids: Rubus caesius, R. Bellardii (Rubeae) and Agrimonia eupatoria (Sanguisorbeae)as

well as in one hexaploid - Potentilla puberula (Potentilleae). In Agrimonia eupatoria secondary nuclei were not formed in unfertilized embyro sacs till the sixth day after anthesis, when the degeneration started. Such state was observed in three specimens from two distant populations, in one of them during two following seasons. In Rubus caesius, R. Bellardii and Potentilla puberula secondary nuclei either were not formed or they were very rare in unfertilized embryo sacs.

There were some similarities between species of type (3) Rubus caesius and plants with multiple embryo sacs from the group (2) Rubus saxatilis. Whether they were essential or casual it was not obvious. Fragaria vesca, Waldsteinia geoides and Filipendula hexasepala developed 1 - 4 mature embryo sacs per ovule. The additional embryo sacs sometimes did not form secondary nuclei in unfertilized conditions. In some ovules the discrepancy could be explained by a somewhat younger age of the embryo sac with unfused polar nuclei. There were, however, embryo sacs for which such an interpretation seemed to be doubtful. In Waldsteinia geoides the longest, probably strongest, embryo sac contained always a secondary nucleus. The embryo sacs with unfused polar nuclei were shorter, often with converted polarity and situated in chalaza. Many of them seemed to have unreduced chromosome number (Czapik, in press). The hypothetical correlation between the apomictic nature of embryo sacs and the lack of secondary nucleus or the delay of its formation in unfertilized embryo sacs was considered previously (Czapik 1983 b). There is, however, no certain evidence which could help to verify that assumption.

Discussion

The observations showed that the behaviour of polar nuclei varies within the subfamily Rosoideae. It may be considered as one of the embryological characters of a species, at least in genera Rubus and Potentilla, where more than one species was examined and in which differences of the behaviour of polar nuclei were stated.

There are, however, several questions left unanswered. The first is-why in some species, such as Agrimonia eupatoria, or in some embryo sacs of Rubus caesius and R. Bellardii, the polar nuclei were unable to fuse without fertilization, or at least why their fusion was delayed.

The second question refers to the range of variability of the character both at the intra- and interspecific levels. The subtypes of type (3) Rubus caesius are an example of the interspecific variation. The intraspecific variation was noted in Rubus saxatilis. In a few embryo sacs of the the examined plants simultaneous triple fusion was observed, while the formation of secondary nuclei was a rule.

The influence of external factors on such a process as fusion of nuclei cannot be excluded. Some unknown factors caused that 1,7% of central cells of unfertilized embryo sacs contained unfused polar nuclei in Ranunculus investigated by Rutishauser (1954). Ormrod et al. (1967) found in Phaseolus vulgaris that the number of embryo sacs with fused polar nuclei was modified by temperature. However, nothing is known about the phenotypic control and very little about the degree of interspecific variation of the examined character in Rosoideae.

A separate problem for the variation studies is offered by plants with multiple embryo sacs where the main embryo sac contains a secondary nucleus and the additional ones have unfused polar nuclei. The hypothesis of the correlation between apomixis and the behaviour of polar nuclei requires examination of the developmental processes in ovules of the plants with multiple embryo sacs as well as of those with single sacs, in which secondary nuclei are not formed in unpollinated flowers.

The present report on the behaviour of polar nuclei in Rosoideae may be considered as the first step of further studies on one of the embryological characters of Angiospermae which has seldom been investigated in detail.

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EMBRYO SAC ULTRASTRUCTURE BEFORE AND AFTER FERTILIZATION IN CUCUMIS SATIVUS

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Summary

The composition and development of ovule and embryo sac of Cucumis sativus, from anthesis up to fertilization has been studied. The mature ovule of Cucumis is anatropous, bitegmic and crassinucellate, and the embryo sac is of the Polygonum type. Development, composition and ultrastructure of the embryo sac generally resemble the pattern as already described for many other angiosperm species. The pollen tube grows through the micropylar nucellar tissue towards the embryo sac and strongly proliferates. After discharge of part of its contents into one of the synergids, the pollen tube is disconnected from the degenerated synergid by the deposition of a plug.

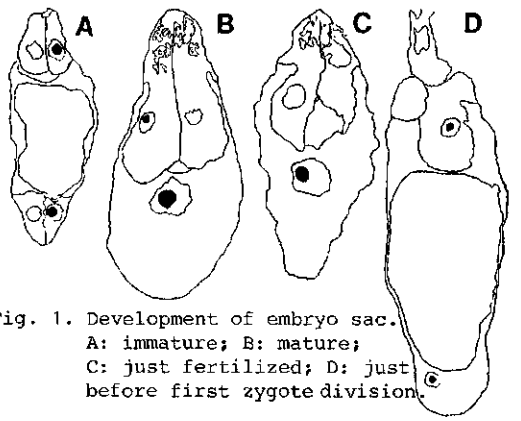

Fig. 1. Development of embryo sac. A: immature; B: mature; C: just fertilized; D: just before first zygote division.

Introduction

As basis for a breeding project on the partial transfer of male genetic material by pollination with irradiated pollen, an investigation on the normal fertilization in Cucumis has been started. This part describes and relates the ultrastructure of the different cell types of the embryo sac in some stages, from their formation until some time after fertilization. Special attention is paid to the cells which are directly related to the process of fertilization.

Material and method

Unpollinated (just before anthesis) and pollinated (respectively 25, 45 and 70 hrs after pollination) flowers of Cucumis sativus L. var. Hardwickii, were taken from a greenhouse culture. The ovaries were sectioned and fixed in 3.5% glutardialdehyde for 10 hrs and post fixed in 1% OsO_4 in 0.1 m sodium cacodylate buffer (pH 7.2) for 12 hrs at room temperature. Then they were dehydrated and embedded in epon.

Results and discussion

The mature ovule of Cucumis sativus is anatropous, bitegmic and crassinucellate, and the embryo sac is of the Polygonum type (Fig. 1). The ovule strongly enlarges during development and becomes flask shaped, with a long and narrow micropylar part of the nucellus (Fig. 3). The egg apparatus strongly enlarges within 30 hrs after anthesis, while the antipods disappear before maturity of the embryo sac. The cytoplasm of the various cells of the

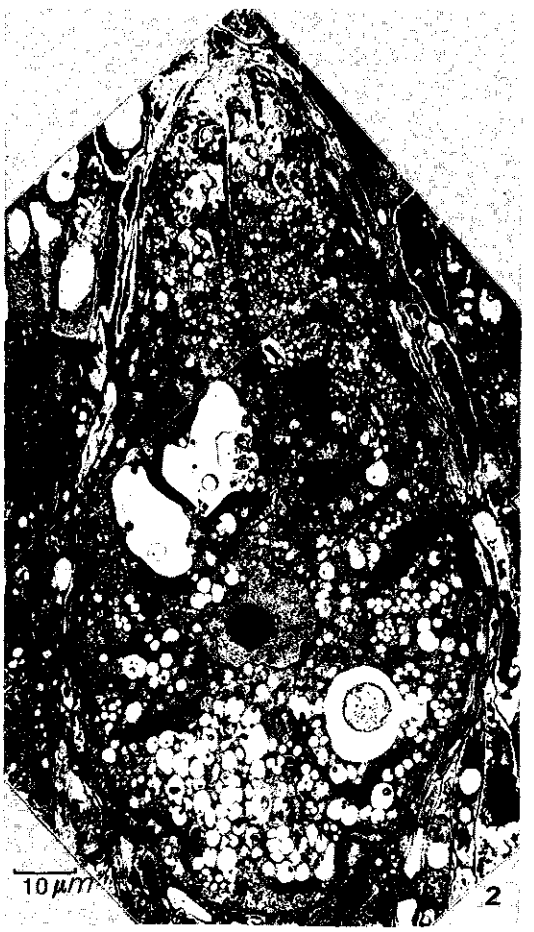

Fig. 2 Composition and ultrastructure of mature embryo sac

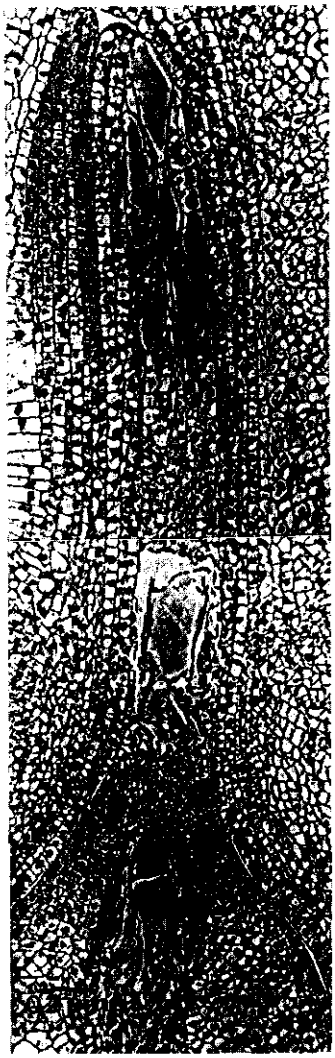

Fig. 3 Composition of nucellus after fertilization, showing proliferation of pollen tube.
Fig. 4 Ultrastructure of zygote and blocked pollen tube
Fig. 5 Ultrastructure of persistent and degenerated synergid

egg apparatus increases. The total volume of the embryo sac does not increase very much, nor does the volume of the central cell (Fig. 1). Simultaneously the cell types of the embryo sac each develop a specific morphology and ultrastructure. The mature egg cell is pear shaped and appear active with large RER accumulations, many mitochondria and dictyosomes (Fig. 2). Development, composition and ultrastructure of the embryo sac generally resembles the pattern as already described for many other angiosperm species. The filiform apparatus is well developed and highly convoluted (Fig. 2). Shortly before the pollen tube enters the embryo sac one of the synergids starts to degenerate. The pollen tube grows through the micropylar nucellar tissue towards the embryo sac and strongly proliferates in this nucellar tissue (Fig. 3). The pollen tube enters the embryo sac through the filiform apparatus and discharges part of its contents into the degenerated synergid (Fig. 5). After this discharge, the remaining pollen tube is disconnected from the embryo sac by the deposition of a plug (callose), just outside the embryo sac (Fig. 4). The remaining cytoplasm in the pollen tube and pollen tube proliferation shows very active mitochondria, many ER and dictyosomes, which indicates a high metabolic activity. Even after fertilization, the pollen tube continues to increase in volume, on account of the surrounding nucellar tissue (Fig. 3). This growth implies haustorial activity (Tilquin, 1983). This pollen tube proliferation may also form a blockage for other pollen tubes to penetrate the ovule.

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EMBRYO SAC ULTRASTRUCTURE IN PEPEROMIA BLANDA H.B.ET K. BEFORE AND AFTER FERTILIZATION.

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Summary.

In the paper the ultrastructure of all types of cells of the Peperomia blanda embryo sac is described.

Introduction.

Species of the genus Peperomia are characterized by the tetrasporic, sixteen-nucleate, polypolar embryo sac of the Peperomia type rather rare for angiosperms. The number of central cell nuclei and that of lateral cells, homologous to antipods, is varied. Problems concerning the number of synergids and functional specialization of egg apparatus cells are discussed in literature. The data on ultrastructure of the Peperomia type embryo sac are not reported.

Results.

All cells of the P.blanda embryo sac before and some time after fertilization are surrounded only by plasmalemma, whereas polysaccharide walls are absent. The ultrastructure of each type of cells is specific. A considerable quantity of plastids is typical of the egg apparatus cells, the number of which constantly equals 3. There is a small amount (relative to the cell volume) of plastids in the central cell. In the lateral cells plastids are rather rarely observed. Mitochondria are the most abundant in lateral cells and their quantity is also high in the central cell and moderate in the egg apparatus cells. Other characters of the ultrastructural organization of cells are similar. On the early stage, nuclei with even profile of the envelope and mainly diffuse chromatin are typical. The density of ribosome distribution is low. ER cisterns are not numerous. During the embryo sac differentiation the quantity of ribosomes rises to some extent, and almost complete reduction of ER in cells of all types occurs. In the egg cell, as well as in central and lateral cells, the nuclei become lobar. In synergids, the nuclei preserve the even envelope contour. In the both synergids, before the pollen tube enters the embryo sac, between membranes of organelles envelopes osmiophilic substances are accumulated. The pollen tube enters the embryo sac through a synergid. The fusion of gamete nuclei proceeds slowly. During this process the content of ribosomes and organelles in all types of cells increases. ER (cisternal and tubular) is developed. Cisterns of ER are most greatly developed in the central cell. The tubular reticulum is the most abundant in zygote. In all cells the nuclei have lobar shape, diffuse chromatin. The polysacharide cell walls are formed in the final period of nuclei fusion in zygote.

SEXUAL REPRODUCTION IN THE GENUS FUCHSIA. EMBRYOGENESIS IN F.FULGENS.

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Summary

Fertilization and embryo development in F.fulgens are investigated preliminarly in order to perform the fertilization in-vitro. The pollen tubes grow between the cells of the transmitting tissue and reach the loculus of the ovary three days after pollination. They enter the ovule through the micropyle and penetrate one of the synergids before to reach the egg-cell. The diploid zygote grows up in a coenocytic endosperm which becomes cellular after the 10th day. The mature seed is exalbuminate.

Introduction

The Fuchsia species belongs to the family of the Onagraceae. The research conducted on the fertilization and embryogenesis comes into the frame of the selection of new varieties in horticulture. Many Fuchsia lovers keep searching for new hybrids. The development of hybrids is in some cases hampered by barriers of incompatibility, be it before or after fertilization; these barriers might be removed through test-tube fertilization or through in-vitro cultivating aborting embryos. The study of embryogenesis in F.fulgens (section Ellobium) is a preliminary to this research.

Results and discussion

1. From pollination to fertilization.

The germination of pollen on the stigma, as well as the course of the pollen tubes in the style until entering in the ovary are followed thanks to smears made after 1, 2 3 days (stained with anilin-blue; fluorescence microscope). About 3 days are necessary for the pollen tubes to enter the ovary and fertilize the first ovules. It takes 24 hours more for all ovules of the ovary to be fertilized, i.e. a maximum of 85%. The entrance of the pollen tube into the ovule occurs through the micropyle; it crosses the nucellus and enters the embryo sac through a synergid where its content is discharged. The synergid becomes opaque. The second synergid degenerates at once.

2. Embryogenesis.

Research has been made on microtome slides cuts stained with haematoxyline-fast-green. The pollination day is represented by Do. The fertilization occurs on Do +4. The embryo is hidden by degenerated synergids. The primary endospermic nucleus undergoes the first division immediately. The inner and outer integuments have respectively 2 and 4 cell-layers. They will, very early, go through changes that will make the spermoderm out of them. Some mitoses can still be observed in the outer integument, whilst the cells of the inner integument are loaded with dark-brown coloured substances. Mitoses occur inside the nucellus. At Do +7, the albumen has about 10 nuclei. The nucellus cells become vacuolated which pushes the nucleus against the cell wall. No mitose can be found anymore. The pollen tube persists long after fertilization and probably assumes a haustorial function (Tilquin et al., 1983).
The pollen tube cytoplasm is highly concentrated. The outer integuments differentiate : the outer and inner layers get flat, whilst the middle layer cells get empty of their content. The cells are loaded with dark granules and the cell-walls get thicker.
At Do +10, the embryo has got rid of the micropylar dark stain and carries 1 to 6 cells. The albumen is coenocytic with scores of nuclei. At Do + 17, the embryo is globular with 45 to 140 cells. The albumen is cellularised around the embryo. Some nucellus cells still contain a nucleus, which does not divide anymore. At Do +20, the embryo is cotyledonary and counts over a hundred cells, inside of a celled albumen with several hundred cells. At the chalazael end of the ovule, the albumen remains coenocytic. From Do + 30, up to the maturity of the fruit (Do +50), the embryo develops, progressively fills in all the cavity of the seed, rebsorbs the albumen and scratches nucellus which disappears completely. At maturity, the seed is exalbuminate.

Reference

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A CYTOLOGICAL METHOD FOR DETECTING COEXISTENCE OF SEXUAL AND DIPLOSPORIC REPRODUCTIVE PROCESSES 1

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Summary

A study realized in hermaphroditic plants by linking up the concomitant stages of female sporogenesis and gametogenesis, on the one hand, and of male sporogenesis and gametogenesis, on the other hand, allowed to reveal differences in gametophytogenesises dynamics.

Each dynamics can be correlated with a definite reproductive process on basis of cytological observations. So, it was that the coexistence of sexual and apomictic reproductive processes could be brought to the fore in Brussel's chicory (Cichorium intybus L.), the term " apomictic " covering here diplospory sensu lato. There is some confirmation of the difference in dynamics between sexuality and diplospory within a same genus (Eragrostis).

In some cases, this method must allow to bring to the fore facultative apomixis in species thought to be entirely sexual or obligate apomicts. Moreover, it makes possible to estimate the degree of apomixis in plants where this reproductive process is appearing naturally or by hybridization.

Introduction

Each gametophytogenesis process (meiotic, apomeiotic, mitotic...) seems to have its own dynamics. This dynamics depends on the initiation and progress of the first division of the archespore. Indeed, any disturbance in the meiosis leading to unreduced gametophytes (mitotisation of the meiosis : Stebbins, 1941) gives rise to changes in gametophytogenesis progress. On another hand, a purely mitotic division generally gives rise to changes in gametophytogenesis initiation. Apparently, the more the mitotisation is important, the more there is a delay in the first division of the archesporial cell (Bergman, 1941).

Conceiving of the reproductive calendar

To evaluate the degree of such a delay, it is necessary to have a reference. With regard to the dynamics of the gametophytogenesis in the ovules, it was more often made reference in the litterature to the size and/or the differenciation of the ovule (for example : Esau, 1946), more rarely of the ovary or the flower (Pacini & Sarfatti, 1978). However, we found in a Compositae, Cichorium intybus L., little and underdeveloped ovules in old ovaries, preventing any reference to flower components. Sometimes too, it was used a strict time reference but, following Favre-Duchartre et al.(1979), estimates of angiosperm oogenesises durations, in absolute value, are extremely rare. It is indeed very insecure to evaluate the speed of a physiological process on a chronological basis because of the influence on the development of environmental factors (temperature, light, hygrometry...) and of genetic factors responsible for interplant variations.

So, we thought to use a more intrinsic factor of the flower development, i.e. the male gametophytogenesis. We established then a " reproductive calendar " where the stages of female sporogenesis and gametogenesis were correlated with the concomitant stages of male sporogenesis and gametogenesis in the same flower (Louant & Longly, 1981a; Longly & Louant, 1982).

In this view, the young stages of megagametophytogenesis, corresponding to the division of the archesporial cell, are the most important. In monosporic species, the " key stage" will be the tetrad one. However, older stages can be useful, for example when sexual and diplosporic embryo sacs are morphologically discernible.

Application of the reproductive calendar method

a) Within a species

So, in Cichorium intybus L., such a cytochronological detailed study has been undertaken. At the same time, it was seen there were several types of structures in the ovules of this species (Louant & Longly, 1981a and b; Longly, 1984). These structures produce generally the same type of gametophyte. Most of them can be correlated with a known mode of reproduction. Besides the normal progress of meiosis, we found several forms of apomeiosis and meiotic irregula-

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rities.

The forms of apomeiosis show a more or less important delay of the first division of the archespore compared with the meiosis. The more the deviation is starting soon, the more the delay is important. This is in accord with Bergman (l.c., cfr introduction). All these processes give rise to unreduced gametophytes.

The meiotic irregularities, on the other hand, give rise to tetrads or polyads with the same " timing " as in case of regular meiosis. The resulting gametophytes are here reduced and generally aneuploïd.

Here, only the reproductive calendar allowed to reveal cases of apomixis not structurally different of sexuality but showing a slight delay.

b) Within a genus

The method of reproductive calendar was also applied to compare two species of the genus Eragrostis Wolf (Graminae) : E. tef (Zucc.) Trotter and E. curvula (Schrad.) Nees.

E. tef follows sexual mode of reproduction (monosporic bipolar embryo sac of the " Polygonum " type : Mengesha, 1964).

Relating to E. curvula, cytomorphological studies of the megagametophytogenesis suggested (Brown & Emery, 1958) and then confirmed (Streetman, 1963) reproduction by diplospory.

Since the microgametogenesises seem to be identical in both of these species, we can get them to coincide in an only reference scale. The elaborated reproductive calendar enables to give prominence or to situate cytochronological and cytomorphological differences between apomixis and sexuality in both species. In this way, megaspores tetrads of E. tef are found at the same male stage as archespores in E. curvula. These archespores start to divide, at the earliest, at the same time as the mitotic division of the functional megaspore in E. tef. The resulting gametophytes (sexual and apomictic) are here different. Initially, the first ones are octonucleate while the second ones, tetranucleate.

At the time of reproductive calendar establishment, we discovered, in E. curvula, symptoms of sexuality besides apomictic structures. So, a tetrad of megaspores and an embryo sac with sexual appearence were observed at the same male stages as those corresponding to identical female development in E. tef. This facts confirm the facultative apomixis concept for the species (Voigt & Bashaw, 1973; Brix, 1974).

Limits of use

Nevertheless, there are limits to the use of the reproductive calendar method.

-The flowers have to be hermaphrodite. Moreover a too pronounced protandry or protogyny should make ineffectual the calendar.

-Detection of apomictic reproductive process by this way applies only in case of diplospory sensu lato. Somatic apospory is another problem. It concerns other cells than the archespore. Our calendar, only, allows to compare different dynamics of the same cell.

-When calendars have to be compared, in a same species or between different ones, the microgametophytogenesis processes must be identical or, in any case, superimposable. However , an abnormality doesn't change inevitably the progress of a microgametophytogenesis.

Fields of use

-The calendar is appropriate when it is difficult or not possible to discern sexual and apomictic ripe gametophytes.

-The microgametophytogenesis reference becomes indispensable when it is impossible to utilize quantitative characters such as the inversion of the ovule in case of anatropy, the ovules and ovaries size, the filling of the ovarian cavity... to estimate the progress of female gametophytogenesises. In the opposite situation, it can serve as a complement.

-The calendar prevents from looking for division figures in the archesporial cells. These are indeed seldom due to their quickness. It allows to take into account cytomorphological consequences of these divisions to define a reproduction mode.

-In natural or hybrid facultative apomicts, the use of a calendar allows to realise a screening apomixis versus sexuality within a same inflorescence, a same plant or a population, to specify the relative rate of apomixis. The samples can be analyzed either by means of paraffin sections or clearing squashes.

Conclusion and prospect

A progeny test combined with a cytological study of megasporogenesis and embryo sac development are generally required to confirm apomixis presence and to identify his mechanism (Bashaw, 1980). Nevertheless, according to Stebbins (1950), irrefutable evidence of either presence or absence of apomixis can only be provided by cytomorphological observations. The reproductive calendar replies to this requirement.

With the calendar method, it is above all possible to detect coexistence of sexuality and apomixis in a same species or in a same genus on basis of the non-equivalence of the dynamics of the reproductive modes.

In species interesting from the evolutive or agronomic point of view, the systematic application of our method would permit to bring up to date the unsuspected diversity of reproduction modes within so much a same spe-

cies as a same genus. This diversity is in connection with the thought of Asker (1979) according wich only facultative apomixis with various degrees occurs in species considered as obligatory apomicts.

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INITIATION OF ASEXUAL SEED PRODUCTION IN KENTUCKY BLUEGRASS, POA PRATENSIS L.

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Summary

The sexual embryo sac development of Poa pratensis follows the Polygonum type, while the apomictic embryo sac development follows the aposporous way as in Hieracium. Initiation of the asposporous cell occurs next to the chalazal part of the sexual cell. This initiation takes place at some stage of the development from megaspore to mature embryo sac. While developing, the aposporous cell enlarges in the direction of the micropyle. In the more sexual experimental variety of Poa pratensis L. in a short period of embryo sac development a huge complex of antipodals develops. It is possible that this complex restricts initiation and development of aposporous cells. The results of this study indicate that it is possible to predict the reproductive character of Poa pratensis plants by observing their very first flowers when the embryo sac development has reached the four-nucleate stage.
Keywords: Kentucky bluegrass, Poa pratensis L., apomixis, apospory, embryo sac development.

Introduction

Kentucky bluegrass, Poa pratensis L., is a grass species used for lawns and sportfields. It can produce seed in a sexual way (amphimictic), and/or in an asexual way (apomictic) (Winkler 1905). Plant breeders utilize this phenomenon: the sexual way is used when creating variability through intra-specific hybridization and then only those F_1 plants which deviate phenotypically from the female parent are selected for the assessment of turf performance and seed production; the asexual way is used when maintaining varieties in an uniform and stable way. Up till now in Poa pratensis the degree of apomixis is determined afterwards in field experiments by counting the deviating plants, which is a time consuming procedure. Therefore plant breeders have asked for a method to determine the reproductive character as soon as possible, preferably with the help of the very first flower. This reproductive character is determined by the way in which embryo sac development proceeds. So what one should look for are the crucial moments in this development.

According to the literature in ovules of apomictic Poa pratensis varieties one finds two developing embryo sac producing cells: A so called aposporous cell situated beside a haploïd sexual cell (Rütishauser, 1967). Both cells develop into embryo sacs. 'Sexual cell' indicates any stage of development from megaspore mother cell to mature embryo sac, while 'aposporous cell' indicates any stage of development from aposporous initial cell to mature aposporous embryo sac. The egg cell of the embryo sac derived from the aposporous cell (diploïd) can eventually undergo parthenogenesis, probably after fertilization of its central cell nucleus (Åkerberg, 1942). Therefore one can expect to originate from such Poa pratensis flowers seed with two diploïd embryos, one of sexual origin, the other of asexual origin (twins). Engelbert (1941) came across this phenomenon in up to 5% of studied Poa pratensis plants. In 0,5% of the germinated seeds he found twins, both of which showed matroclinic character. Hence these twins both have been of aposporous origin. The sexual embryo sac (cell) must have stopped developing at a certain time and it probably disappeared. In Poa pratensis varieties listed as obligate apomicts, none or very few plants deviate phenotypically from the female parent. In these varieties the sexual embryo sac development apparently stops very regularly. At what stage exactly the sexual cell aborts and is replaced by the developing aposporous sac is unknown (Bashaw, 1980). The same holds for the causes of this abortion and replacement (Grazi et al., 1961; Nygren, 1967; Van Dijk, 1971). While studying these problems it became apparent that it is difficult to distinguish the sexual cell from the aposporous one, especially in the more advanced stages of the gametogenesis (Bashaw, 1969, 1980).

Material and methods

Panicles were taken from plants of Poa pratensis L. varieties Parade and Enwarto, as representatives with a high degree of apomixis, and of a more sexual experimental variety. The plants had not yet flowered. The samples were preserved in formaldehyde acetic acid aethanol (FAA). 8 to 10 spikelets were taken from a panicle: the 4 or 5 uppermost spikelets of the panicle spindle and 4 or 5 spikelets of the lowest positioned side spindle (Fig. 1).

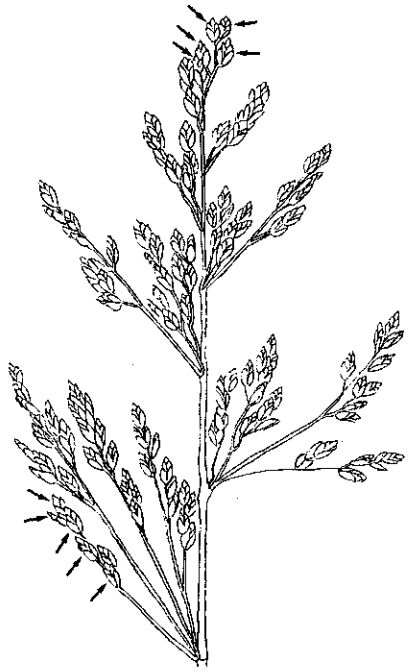

Fig. 1. Panicle of Poa pratensis-variety Parade. Arrows point at used spikelets.

A certain number of dissected ovaries was transferred to a clearing fluid (Herr, 1971). After clearing, the ovary tissue surrounding the ovule was removed and the ovules (5-15) were placed in clearing fluid on a slide (Fig. 2). They were examined with a Nomarski light microscope.

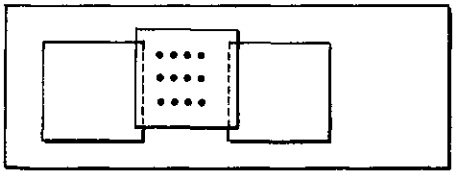

Fig. 2. Slide with one coverglass on each side of objects. Third coverglass on top of objects and other glasses.

Another number of ovaries was processed for embedding in plastic: the varieties Parade (cleared in Herr fluid) and Enwarto (fixed in FAA) were treated for 3x24 hrs in 100% aethanol and 3 hrs in 1% OsO_4 in aethanol. The variety Enwarto (FAA) 3.5 hrs in 1% OsO_4 in phosphate buffer. The fixed material was then washed in ethanol or buffer, dehydrated in graded 'ethanol' series and embedded in Epon. Sections (4 µm) were placed consecutively on a slide and examined with a phase contrast microscope.

Results

Sexual embryo sac development

Megaspore mother cells (mmc) were found in young hemitropous ovules of approximately 0,16 mm width (Fig. 3a). The megaspore mother cell stretches towards the chalaza. There is one layer of nucellus cells between the megaspore mother cell and the micropyle. The mmc nucleus is notably big, with a large nucleolus. The nucleus undergoes a meiotic division sagittaly (Fig. 3b). The division of the chalazal dyad cell is completed sooner than that of the micropylar one (Fig. 3c). The four developed megaspores are cylindrical to round (Fig. 3d). The size of each of them is roughly the same as that of the young megaspore mother cell. Their contours are clearly visible, presumably because of callose deposits. The chalazal megaspore

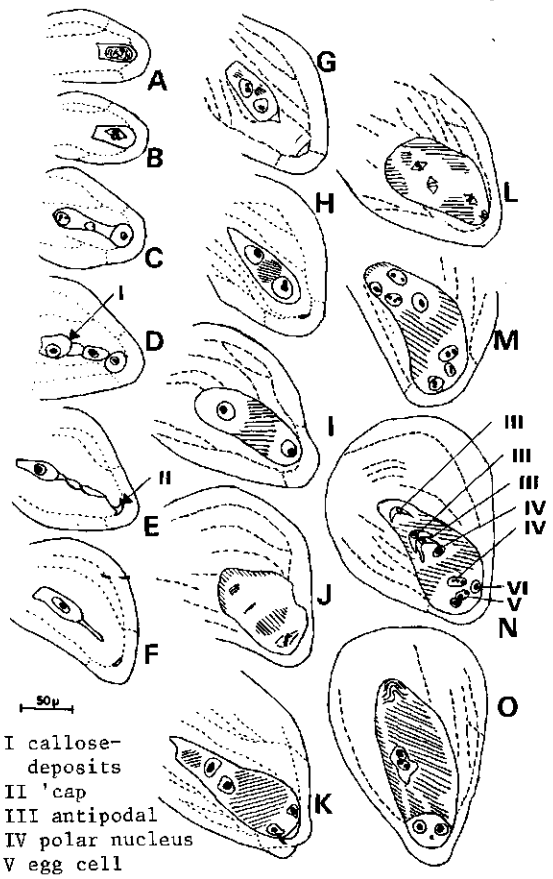

Fig. 3. Development of sexual embryo sac and surrounding nucellus tissue of Poa pratensis varieties Parade and Enwarto. Vacuole has been shaded, nucellus layers have been dotted. The ovaries surrounding these ovules have to be pictured with their styles upwards while their points of attachment are at the bottom.

grows, whereas the others degenerate (Figs. 3e, f). Conspicuous then becomes the 'cap' between the megaspores and the one layered nucellus near the micropyle (Figs. 3e, f, g, h). The functional megaspore can become twice as big while growing in the direction of the micropyle. The nucleus contains one or two nucleoli and is situated in the middle of the cell. A first cytoplasmic vacuole becomes visible. The nucleus undergoes a mitotic division sagittaly. The two nucleated cell is pear shaped and grows considerably (up to 6x its original size) (Figs. 3g, h, i). The vacuole enlarges as well. Its position is in between the two nuclei. A smaller vacuole is visible at the chalazal end. Through its enlargement, the cell gets closer to the micropyle (Fig. 3i). The two nuclei divide: the chalazal one sagitally, at right angles to the micropylar nucleus (Fig. 3j). The four nucleated cell is now separated from the micropyle by only one nucellar cell layer (Figs. 3k, 4).

Fig. 4. A four nucleated cell of Poa pratensis variety Parade (Herr clearing).

The nuclei are spherical with one, two or three nucleoli. The vacuoles have enlarged as well. When dividing for the third time mitotically, the cell grows broadwise. The direction of the division of the two outer nuclei is at right angles to the line micropyle chalaza. At the same time the direction of the division of one of the nuclei is at right angles to the direction of the division of the other. The two inner nuclei divide sagitally (Fig. 3l). The vacuoles in the eight nucleated cell resemble the vacuoles in preceding stages (Fig. 3m). Eventually the cell differentiates into an eight nucleated, seven celled embryo sac (Fig. 3n). There are three antipodals at the chalazal side. In a later stage their cytoplasm is darker. They are V-shaped, with their nuclei in the bend of the V. They do not have vacuoles. In a later stage the antipodals disappear or become extra large having nuclei with numerous very dark nucleoli. This antipodal complex was especially found in the more sexual experimental variety of Poa pratensis. It was remarkable that in this variety, in comparison with the other two varieties, more embryo sacs were found in a late stage. The central cell with two polar nuclei occupies two thirds of the embryo sac. Fused polar nuclei have not been found. The egg apparatus consists of an egg cell and two synergids. The egg cell is bell-shaped and contains a vacuole. The nucleus is about as large as a polar nucleus and at an early stage it is situated at the micropylar side, at maturity in the centre of the cell. The two synergids are oblong pyramidal. Their nuclei are in the centre. During the embryo sac development the ovule changes from a horizontal to a vertical position in the ovary (Fig. 3).

Aposporous embryo sac development

The aposporous initial cell is very characteristic (Fig. 5). Initially its shape is round, in a later stage oblong. Some small vacuoles are visible. The initiation of this aposporous cell takes place in the nucellus near the chalazal part of the developing sexual embryo sac cell. In the studied material ovules have been found with more than one aposporous initial cell. Sometimes the initial cell was found growing into the sexual cell. The aposporous embryo sac development is similar to the sexual embryo sac development described above. Only the eight nucleated aposporous cell has not been found. From the two nucleated stage on the cell grows towards the micropyle through nucellus tissue, passing the developing sexual cell. In one occassion the cell wall of the aposporous embryo sac was observed to have grown into that of the sexual embryo sac, in between the polar nuclei and the egg-apparatus. In a later stage the aposporous embryo sac reaches the micropylar opening. Moving towards the micropylar opening it sometimes grows through one or two layers of the inner integument.

The aposporous and the sexual cell have been found next to each other in the same and/or different stages of development. Data on these observations are given in Fig. 6.

Discussion

According to Rütishauser (1969) the sexual embryo sac development of Poa pratensis follows the Polygonum type, the aposporous embryo sac development follows the Hieracium type. If one has to decide whether one deals with a sexual or an aposporous

Fig. 5. Embryo sac of Poa pratensis-variety Enwarto with synergids (s), egg cell (e), polar nuclei (p), antipodal complex (ac) and aposporous initial cell (ai) (sectioned).

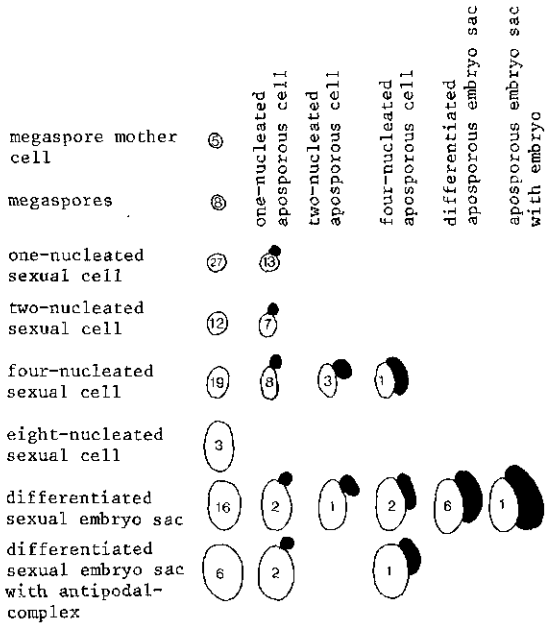

Fig. 6. Numbers of observed sexual and aposporous embryo sac development stages in Poa pratensis. The aposporous cells are shaded.

development, three characteristic stages one has to keep in mind:

In the one- and two-nucleated stage: the aposporous cell contains less cytoplasm and more vacuolar volume than the sexual cell; in the sexual development one can expect to find degeneration rests of the three micropylar megaspores.

In the two-, four- and eight-nucleated stage: while stretching towards the micropyle the aposporous cell bends round the sexual cell towards the micropyle and pushes nucellus and integument tissue aside.

In the mature embryo sac stage: in the aposporous embryo sac one can expect to find an embryo (without fertilization having taken place); in the sexual embryo sac one can expect to find an antipodal complex, which covers the largest part of the embryo sac and has pushed out nucellus tissue.

Observations of various authors confirm the characteristic distinctions given above: publications by Tinney (1940), Grazi et al. (1961) and Nygren (1967). Nygren shows drawings of the aposporous initial cell which correspond to the present observations, and drawings by Nielsen (1945) show degeneration rests of megaspores. From Fig. 6 it can be seen that initiation of the aposporous cell was found to occur next to a developing sexual one-nucleated or older cell, but it appears that up to and including the four nucleated stage most of the aposporous initial cells have come into existence. Next to an embryo

sac with an antipodal complex there were only few aposporous initial cells found. It is possible that the antipodal complex restricts initiation and development of the aposporous cells, since in the experimental variety relatively more sexuality is found in the F_1 plants.

From Fig. 6 it can also be seen that above all aposporous cells develop into embryo sacs when had been initiated in an early stage of development. When the sexual cell has reached the eight nucleated stage of development it fills up most of the space in the ovule. This leaves little room for aposporous embryo sac development. The latter only has a chance to develop next to the sexual cell when this sexual one is not yet fully grown. Occasionally the aposporous cell might even take the place of the sexual one.

The results of this study indicate that it is possible to predict the reproductive character of Poa pratensis plants by observing their very first flowers when the embryo sac development has reached the four nucleated stage or beforehand. If there is an aposporous cell at these stages, the plants will, in all probability, be aposporous. If there is not an aposporous cell, the plants will, in all probability, reproduce sexual.

Acknowledgements

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POLLINATION AND FERTILIZATION IN PICKLING AND SLICING CUCUMBERS

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Summary

Pollination of parthenocarpic Dutch slicing cucumbers often yields low number of seeds, because only the proximal segment of the fruit contains plump seeds. To clarify the reason for this inadequate seed set, in vivo pollen tube growth and ovule and seed development were studied in six cucumber cvs which differ for fruit length and parthenocarpy. Pollen parents had no significant effect on pollen tube growth or seed set. Pollen tubes never reached the basal fruit segments of long fruited types, because the fruit elongation proceeded more rapidly than pollen tube growth. Therefore, rather than parthenocarpy, the long ovary and rapid elongation of fruits is responsible for the low seed set in long fruited cucumber types.

Introduction

Pollination often induces a typical malformation of the fruit in Dutch slicing cucumbers: the blossom end of the fruit enlarges while the rest of the fruit remains slender. This thick fruit segment contains the seeds. It is called 'seed head'.

Pollinated fruits of American salad cucumbers and pickling cucumbers grow out evenly and contain seeds throughout the fruit. Seed yield per fruit of such types is much higher than can be obtained with Dutch slicers.

When the reason for the low seed yield of Dutch slicing types is known, it may become possible to improve the efficiency of seed production.

Dutch slicing type cvs are characterized by genetic parthenocarpy and large fruit length. Parthenocarpy generally reduces seed yields per pollination, but it is not well known how. In vivo studies of the growth of pollen tubes through the style and ovary and of the fertilization process may help to clarify the observed differences in seed set.

Experiments

Six different cucumber cvs were used: G6 (slicing type, parthenocarpic); Vestervang (slicing type, non parth.); Maram F1 (beth alpha type, parth.); Beth Alpha (non parth.); Nanet F1 (pickling type, parth.) and Unicurba F1 (pickling type, non parth.). All were self pollinated and cross pollinated with two tester cvs (Nanet and Vestervang). Glasshouse temperature ranged from 20°C (night) to 32°C (day). Four days after pollination six fruits per cross were removed, fixed, macerated and stained for UV-microscopic study of pollen tube growth and fertilization. The length of the 1-5 longest pollen tubes was measured as well as that of the main front of pollen tubes (at least 10-20 tubes).

Ovule and seed development of parthenocarpic and pollinated fruits of cvs G 6, Maram and Nanet were examined at anthesis and after 4,8 and 12 days.

Fruits were divided in five segments at maturity and ovules and seeds were counted per segment.

Results and discussion

At pollination time there were no size differences between ovules throughout the length of the ovary in all cvs. Enlargement of ovules became evident after 4 days. For G 6 and Vestervang this occurred in the blossom end of the fruit only.

In vivo pollen germination and tube growth was not affected by pollen parent, there were no differences between self and cross pollinations. At 4 days after pollination small, but significant differences in pollen tube growth existed between maternal parents, cv Maram ovaries had the longest pollen tubes.

Fruit length at 4 days after pollination differed greatly. The ovaries of G 6 were already long at pollination time, and tripled their length in 4 days. The short ovaries of the pickle and beth-alpha cvs elongated more slowly.

Fruits of the long fruited cvs contained very many ovules in their fruits, but only very few had grown out into plump seeds. Fruits of the short fruited cvs contained less ovules, but high percentages of seeds. In the long fruited cvs only the first two fruit segments contained seeds - the 'seed head'.

Seed set was not influenced by pollen parent, there were no differences between self and cross pollinations. Seed set was strongly influenced by maternal parent.

Conclusion

'Seed-heads' and low seed yield of Dutch slicing cvs are not caused by genetic parthenocarpy, but most likely by their large ovary size and very fast initial fruit elongation.

Pollen tubes of long fruited types have similar growth capacity as those of short fruited types. They are unable to reach the ovules past the proximal one third of the ovary. The ovules in the rest of the ovary -although they seem not to be different- remain unused. Selection for faster and longer pollen tube growth could improve the fertilization success and increase seed yields.

GAMETE COMPETITION IN OIL SEED RAPE: EFFECT OF POLLEN ß - GALACTOSIDASE DEFICIENCY ON FERTILIZATION

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Summary

We have recently reported the detection of a new haplophase mutation in crop plants that affects the expression of the enzyme β-galactosidase in pollen of oilseed rape, Brassica campestris. Plants heterozygous for the enzyme deficiency, Gal/gal shed pollen grains, 50% of which are enzyme deficient. We have compared male gamete transmission abilities of normal (Gal) and β - galactosidase deficient (gal) pollen and report that mutant pollen shows reduced competitive ability in fertilization.

Keywords: oil seed rape, Brassica campestris, β -galactosidase, fertilization.

Introduction

In higher plants, there are very few molecular genetic markers which can be used to study the physiological and biochemical basis of male gametophyte competition. Until now only three segregating loci were known, including waxy (wx), alcohol dehydrogenase (Adh) in maize pollen, and now β-galactosidase (gal) in oil seed rape pollen. Adh deficiency had no delaterious effect (Schwartz 1969), while wx pollen showed only a slight reduction in male gamete transmission (Sprague 1933). We report here the results of experiments on the effect of pollen β-galactosidase deficiency on fertilization.

Materials and Methods

Pollen quality was determined by the fluorochromatic reaction (FCR) test. The β-galactosidase activity in pollen was detected cytochemically by using 5-bromo-4-chloro-3-indoxyl- β -galactoside as substrate (Singh and Knox 1984).

Results and Discussion

When Gal/Gal is used as female and Gal/gal as male, the progeny should be 1 Gal/Gal : 1 Gal/gal. The latter shows pollen segregation for the enzyme deficiency and thus can easily be detected by the pollen staining test. Such a plant can only be obtained if gal pollen fertilized the egg cell. This criterion was used to determine percentage of progeny originating from enzyme deficient gal pollen (Table 1).

Table 1. Segregation of progeny of Gal/gal crossed reciprocally to wild type Gal/Gal. (expected ratio: 1 Gal/Gal : 1 Gal/gal)

CROSS	OFFSPRING Gal/Gal	Gal/gal	Chi Square df=1	
Gal/gal ♀x Gal/Gal ♂				
Observed	13	14	0.04	N.S.
Expected	13.5	13.5		
Gal/Gal ♀x Gal/gal ♂				
Observed	57	35	5.30	p 0.01
Expected	46	46		

The results show that there is normal transmission of gal through the egg cell. However, gal pollen grains show reduced competitive ability in fertilization. This is the first example in angiosperms of male gamete competition related to enzyme deficiency. Experiments are in progress to determine whether the selective advantage to Gal pollen is associated with the initial events of pollen adhesion, hydration and germination or later stages of pollen tube penetration and growth through the pistil.

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CYTOCHEMICAL INVESTIGATION OF LILIUM REGALE EMBRYOGENESIS AFTER POLLINATION WITH γ-IRRADIATED POLLEN

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Summary

Seven DH involved in different metabolic pathways were investigated cytochemically in the embryosac of L.regale after pollination with γ-irradiated pollen. Abnormalities in the embryogenesis and metabolism were observed. The activities of IDH,MDH,G6PDH,6PGDH and LDH in the embryosac were not changed as compared with controls, but ADH and GDH were completely inactivated.
Keywords: γ-irradiated pollen, dehydrogenases, cytochemistry, abnormal embryogenesis.

Introduction

Marked changes in the lily embryogeny and morphology of the seeds take place after pollination with γ- and X-irradiated pollen (Vassileva-Dryanovska,1966;Price , 1957; Poole et al.,1978). As the physiological peculiarities and the behaviour of the cells in the embryosac during early embryogenesis can be distinguished only cytochemically, we undertook the cytochemical investigation of 7 DH, involved in different pathways to reveal some metabolic disturbances following pollination with γ-irradiated pollen.

Material and methods

Flowers from Lilium regale W were pollinated with γ-irradiated pollen (10,25 and 50 krad). Fresh pistils 1-30d after pollination were used. The cytochemical localization of DH (IDH,MDH,G6PDH,6PGDH,LDH,ADH and GDH) was carried out by the method of tetrazoliumreductases (Lojda et al.,1979).

Results and discussion

It was established that the 3 doses under study stimulated fertilization and cell division in the embryosac and affected the appearance of similar cytoembryological abnormalities, which were better expressed at higher doses. The predominate ones was the formation of embryo as an irregular mass of cells and atypical free nuclear endosperm with small nuclei.
This kind of embryosac almost didn't grow and dye about 20 d after pollination.

In our previous work (Georgieva, 1983) was found that during the fertilization and early embryogenesis the enzyms of citrate cycle (IDH,MDH), of PPP(G6PDH,6PGDH), of alcoholic fermentation (ADH), of glycolysis (LDH) as well as GDH became activated in the embryosac of L.regale. In the present study it was shown that although the embryogenesis had been destroyed after pollination with γ-irradiated pollen the degradation of carbohydrates via the citrate cycle and PPP as well as the glycolysis were not affected. The rate of anaerobic breakdown of carbohydrates by ADH however drasticallydecreased with the increasing γ-ray dose. In the same time the connection between the metabolism of carbohydrates and amino acids mediated by the key enzyme GDH is disrupted because of the GDH inactivation. As the important metabolic pathways i.e. citrate cycle, PPP and glycolisis were not affected in the embryosac, the earliest stages of embryogenesis were characterized by enchanced growth. It can be admitted that the break-up of some metabolic chains (e.g. those in which ADH and GDH are involved) probably completely destroy the metabolism of the embryosac and provoke the seed failure

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Abbreviations: DH- dehydrogenases, IDH- isocitrate dehydrogenase, MDH- malate dehydrogenase, G6PDH- glucose-6-phosphate dehydrogenase, 6PGDH- 6-phosphogluconate dehydrogenase, LDH- lactate dehydrogenase, ADH- alcohol dehydrogenase, GDH- glutamate dehydrogenase, PPP- pentose phosphate pathway

SOME STRUCTURAL FEATURES OF THE EMBRYO/ENDOSPERM INTERACTION IN ANGIOSPERMS

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Summary

By the underlying examination of the early embryogenesis of selected species of angiosperms, three correlation types were marked off in embryo and endosperm development. They are bound to a definite type of endosperm, to a duration of endospermal function, to the dynamics of embryo growth and to the onset of the digestive or digestion stimulating function of the embryo.
Keywords: embryo, endosperm, correlation types.

Introduction

Correlation in the development of both embryo and endosperm is one of the basic supposition of normal seed development. The type of correlation is characteristic of the individual taxa of angiosperms. In various species of angiosperms both embryo and endosperm are developing with different absolute and relative speed. Our work aimed at ascertaining the differences in the relative speed of their development that characterize the type of correlation. The type of correlation appears to point at possible differences in the function of the endosperm as well as at differences in the growth and regulation activity of the embryo in the first phases of embryogenesis.

Material and methods

The early embryogenesis was studied of selected species of angiosperms /Papaver somniferum, Nicotiana tabacum, Jasione montana, Linum usitatissimum, Capsella bursa pastoris, Raphanus sativus, Pisum sativum, Phaseolus vulgaris/. Young seeds of these species were sampled from the field in 1 - 2 days intervals, starting with the day of pollination up to the 7th--12th day of seed development. They were embedded in paraffin, sectioned and stained with haematoxiline according to Heidenhain or by the PAS reaction. A part of the seeds was fixed according to Baker, embedded in paraffin and sections were analysed by histochemical methods to prove hydrolytic enzymes. To prove acid phosphatase the reaction with α - naphtylphosphate and FAST RED TR was used and to prove non-specific esterase the reaction with α - naphtylacetate and the FAST BLUE B was used.

On the individual sampling days the number of cells /nuclei/ of both endosperm and embryo was counted and the structural changes traced that reffered to the spatial and functional interaction of embryo and endosperm on the level of optical microscopy.

Results

The course of the early embryogenesis in selected species of angiosperms has allowed to characterize 3 types of correlation in embryo and endosperm development.

Type I is characterized by a marked advance in endosperm development in the first phases of embryogenesis /Fig. 1/.

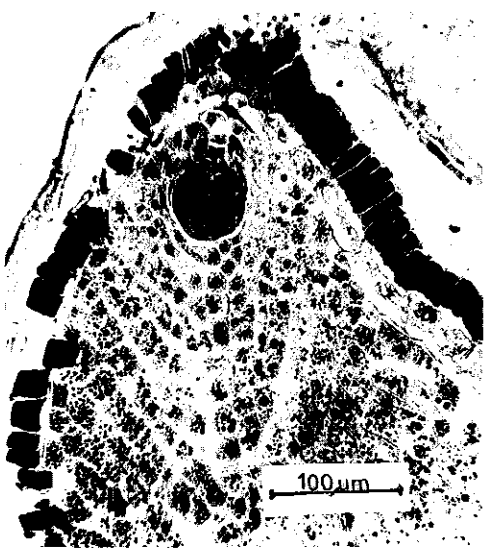


Fig.1 Globular embryo of Papaver somniferum in the advanced endosperm.

In this period there gradually fall to one embryo cell from more than ten cells /e.g. with Nicotiana tabacum, Jasione montana/ to several hundreds of cells / e.g. with Papaver somnife-

rum/ /Table 1/. The Ist type of correlation is usually bound to the cellular endosperm or to the nuclear endosperm that cellularizes relatively early, and to the albuminous type of seed. At the time when the endosperm reaches the final number of cells the embryo is in the globular phase or passes from the globular to transitional or to heartshaped phase of its development. At the same time it passes from the suspensoral to the surface nutrition in which it obtains supply material from the disintegrating cells of the endosperm. The embryo acquires, at a relatively early phase of its development, a digestive or digestion stimulating function.

Table 1

Embryo and endosperm cells number /in some selected days/

	embryo	endosperm
Papaver /I/ 7 days	16	4 034
Papaver 12 days	169	16 722[x]
Nicotiana /I/ 9 days	36	558
Nicotiana 11 days	304	2 522[x]
Linum /II/ 7 days	5 059	15 045
Pisum /III/ 7 days	24 533	19 357

x Data represent roughly the final or nearly final cell number.

The development of the embryo and endosperm in type II is the parallel one. It uses to be bound to a nuclear type of endosperm /Linum usitatissimum, Capsella bursa pastoris, Raphanus sativus/. In this type of correlation the number of endosperm cells falling to 1 embryo cell stabilizes soon after the start of embryogenesis and the embryo proliferates and differentiates parallel with the proliferation of the endosperm. /Fig.2/ Embryo and endosperm jointly fill the inner space of the embryo sac. Their spatial interaction, manifested also by the desintegration of endospermal cells round the embryo, sets in only at a more advanced stage of embryogenesis, at a time when the embryo is already in its late heartshaped or torpedo phase of development. In this type of correlation most of the supply material needed for germination is stored into the cotyledons. A small portion of the endosperm, however, may be preserved until seed maturity.

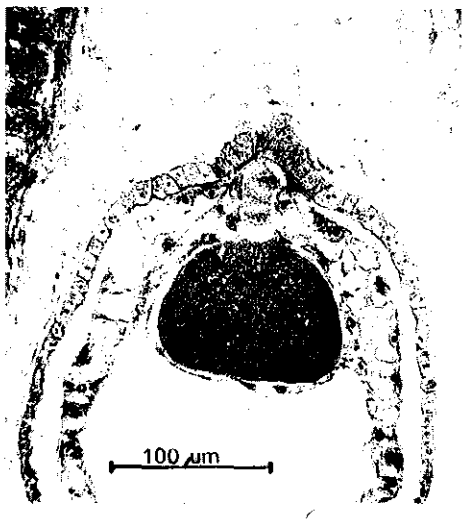


Fig.2 The embryo and endosperm of Linum usitatissimum in the parallel development.

Correlation type III occurs with species exhibiting markedly exalbuminous seed. After an initial short-term rise in the number of endospermal nuclei, it is the embryo that acquires dominance in the development /Fig.3/.

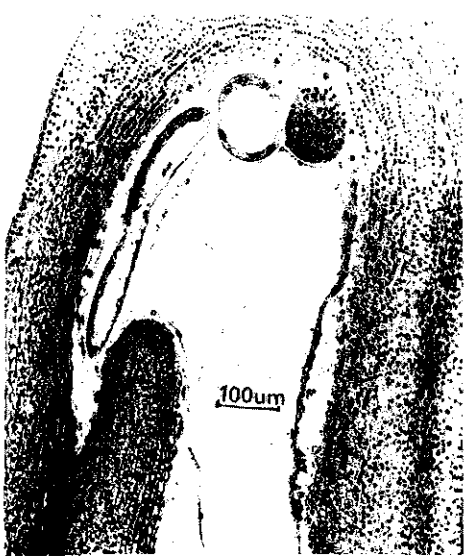
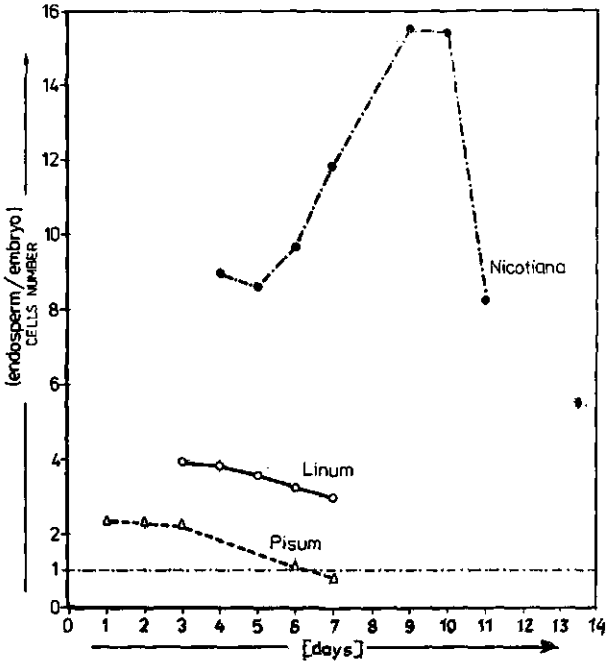


Fig.3 The advance of embryo development in the seed of Pisum sativum

The number of embryo cells soon exceeds the number of endosperm cells many times over. The endosperm development is very limited and all supply substances needed for germination are stored into the cotyledons. The weakly developed endosperm layer desintegrates as soon as it gets into spatial contact with the developing embryo, thus in the micropylar area of the embryo sac soonner and in the chalazal area later. This type of correlation is known, for example, in the family Viciaceae, and bound to the nuclear endosperm.

The portion of endosperm to embryo cells number during early embryogenesis in the species typical for all correlation types is indicated on the Fig.4.



In type II and III, embryos very often contain chlorophyll during embryogenesis.

Discussion

The knowledge and completion of results obtained by examining the early embryogenesis of selected species of angiospermous plants by literary data on the embryogenesis of other species /Davis 1966, Poddubnaja-Arnoldi 1982 and others/, has allowed to characterize three types of embryo and endosperm correlation. The facts referred to in the paper show that embryos of various species pass over from the suspensory to the endospermal nutrition in various phases of their development. Some already in the globular or early heartshaped phase /correlation type I/,other ones in the late heartshaped phase or later /correlation types II and III/. Not only the character and duration of the nutritive function of endosperm but also the character of the growth and digestive or digestion stimulating activity of the embryo are connected with this. The question of the character of embryo activity /digestive or digestion stimulating/ during embryogenesis remains open /Jones 1974, Olson 1981 and others/.

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ULTRASTRUCTURAL ANALYSIS OF EMBRYO-ENDOSPERM INTERACTIONS IN DEVELOPING MAIZE SEEDS (ZEA MAYS L.)

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Summary

The fine structure of developing caryopses from maize (Zea mays L.) was investigated. Samples were taken from 4 to 11 days after pollination. It is concluded that a strong interaction between embryo and endosperm exists and that nutrient supply of the embryo occurs by various pathways. The cytological evidence leading to this view is presented.

Introduction

There is a lack of studies dealing with embryo development in relation to the neighbouring tissues, as was pointed out recently by Smart & O'Brien (1983). Although embryo formation in maize has been studied extensively, an integrated study examining functional embryo-endosperm relationships at an ultrastructural level was not yet carried out. We therefore studied the early developmental stages of maize caryopses by electron microscopy, putting emphasis on cytological details indicating important functions of the endosperm surrounding the embryo in relation to nutrient synthesis and/or transport.

It was thought that - while in vitro culture methods using immature embryos of maize are becoming increasingly important (see Vasil et al., 1984) - more knowledge of the in vivo nutrient supply of these immature embryos is strongly required.

Results and conclusion

During the first week of seed development the young embryo is surrounded by a region of small, plasma-rich cells (Fig. 1, arrows). Electron microscopy of this "modified endosperm" bordering the embryo suspensor at the end of the first week showed a high level of Golgi and ER activity, while mitochondria were located near the plasma membrane adjacent to the embryo (Fig. 2). After the first week the large, highly vacuolated endosperm cells near embryo axis and scutellum degenerate (Fig. 3).

It is concluded that in the first week food supply of the immature embryo occurs mainly by the modified endosperm bordering the suspensor. Amino acids and proteins might be main constituents. After that period, also food uptake by the embryo axis and the scutellum takes place. Lipids and carbohydrates (cell wall remnants) seem to be the main nutrients present in that region.

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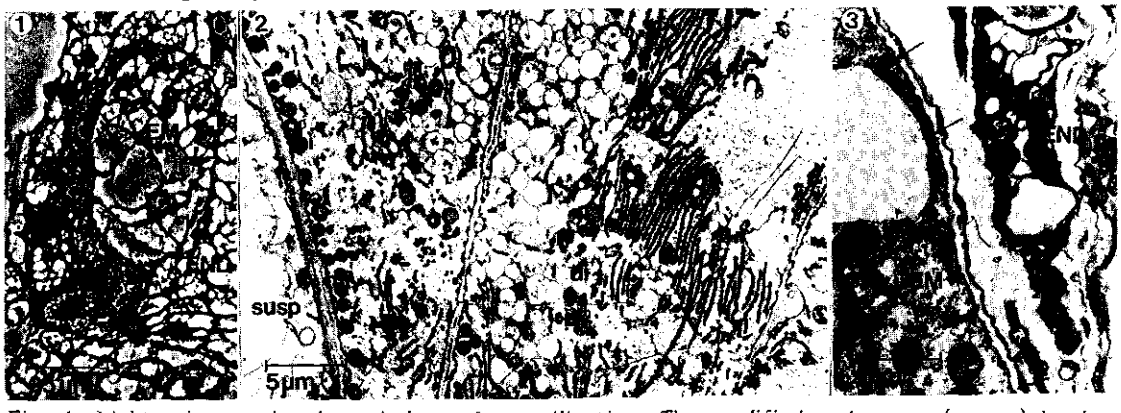

Fig. 1. Light micrograph; about 4 days after pollination. The modified endosperm (arrows) borders the embryo. Fig. 2. Electron micrograph of the modified endosperm at 7 d.a.p. Fig. 3. Degenerated endosperm near the scutellum (11 d.a.p.). Note the presence of lipid droplets (arrows).

REPRODUCTIVE PHYSIOLOGY OF LIMNOPHYTON OBTUSIFOLIUM (L) Miq.-A HISTOCHEMICAL ANALYSIS

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The embryo sac follows a bisporic Scilla pattern. All the eight female nuclei have different diameters but identical extinction values for DNA. The endosperm is helobial. Its polyploid nuclei have multiples of DNA C values. The DNA content per tapetl nucleus remains amplified because of fusions but after meiosis in microspore mother cells it decreases dramatically. -SH proteins showed peak values during periods of meiosis and mitosis indicating their functional role. The female gamete showed a distinct staining for Feulgen reaction confined only to the nucleus and even during maturation of the egg, DNA did not reveal qualitative or quantitative changes. Acidic proteins (NHCP) detection by toluidine blue O or by fast green FCF at acidic pH of 0.5 left firm unspecific binding with cell walls and cytoplasm of ovular tissues. The cell cb remains unicellular and ever enlarging while only ca gives rise to the organs of the embryo. The course of divisions thus depicts the Aponogetonad pattern. An intense reducing condition prevails in the hypertrophied cb. In fact ascorbic acid and -SH proteins ascend uniformly. The RNA and protein show vigorous fluctuations. cb multiplies its genome 16 times resulting in a large polyploid 32C nucleus. However, the acidic nuclear proteins (NHCP) content increases multifold followed by a sharp decline. The synthetic performance of the suspensor cell was at full swing in the quadrant and octant stages of embryo development but reached its peak value at the globular embryo where cells determine their developmental pathway The various tiers of proembryo do not show differential distribution of the metabolites indicating their even physiological state. At the end it is the globular embryo which starts revealing a dispersed reaction of metabolites followed by the differentiation of specific precursor cells. Three critical histogenic events appear in the interior of the globular embryo: (i) Two loci or initials of presumptive sites for the embryonal regions viz. hypocotyl and cotyledon. (ii) On such a spherical interior first axial and afterwards appendicular proeambial systems embark at the same time. (iii) Cotyledonary initial divides rapidly in a plane at right angles to the hypocotyledonary initial. One forms the cotyledonary mound while the other designs the embryo axis. Thus one half of the embryo consists of many large cells, less basiometric as compared to the hypocotylar half which has smaller cells but richly pyroninophilic. The first histochemical departure appears with the formation of the first indentation demarcating the germinal and abgerminal portions on the globular embryo. The intensity of ascorbic acid (AA), RNA and -SH proteins becomes more pronounced on hypocotylar face than the cotylar one. It initiates the change of symmetry. A SEM, ontogenic as well as histochemical profiles from the first dipression to mature embryo fabric manifest three different extensions of the single lateral cotyledon viz. sheath, middle piece and the haustorium. At the end the embryo has one lateral cotyledon and an axis bearing two growing poles-radicle and plumule. Surprisingly in the mature seed, various tissues of Limnophyton yield extremely variable quantitative levels of metabolites. Anther tapetal cells, meiocytes, suspensor, (cb), endosperm, vegetative cell and male gametes in the pollen grain, antipodals, seed coat cells and perianth tissues show varying ($2n = 22, 44, 52, 66$) chromosome set up in numbers on metaphase plates. Surprisingly in spite of the huge differences in the volume and chromatin make up in these nuclei, the hydrolysis curves ($60^{o}C$ 1 N HCl) for the above diverse cell nuclei showed a similar plateau value as with the vegetative shoot apical meristems. This is the virtual optimal period of hydrolysis. To arrest Feulgen hydrolysis at its peak, the preparations are dipped in cold 1N HCl for 1 minute and then rinsed with distilled water for 2 minutes. This step replaces 2-3 peaks and produces a flat curve. Meiocytes show two/three peak periods of Feulgen hydrolysis.

GRASS EMBRYO - A SEM AND HISTOCHEMICAL ANALYSIS

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Summary

Differentiation of the grass embryo proceeds by two primary indentations on the face of the globular embryo and several ones on the surface of the scutellum. All embryonal cells maintain equal but high metabolic potential till the embryo assumes a radially symmetrical form. Basically the embryonal form of the grasses is identical with the rest of the monocotyledons. Ours is an attempt to integrate the histochemical turnovers which seem to condition or control the unfolding of the axis (hypocotyl) as well as the appendage (cotyledon) in the grass embryo by localising different metabolites or substances during different stages of embryo development and correlate them with morphological changes.

Observations and conclusion

In an attempt to settle the wide gulf of the grass embryo, it heralds the basic plan that it has an axis known as the hypocotyl. On its one terminal lies the shoot apex on the other end the radicle. It bears a solitary senescing lateral cotyledon. Basically the embryonal topography of the grasses is identical with the rest of monocotyledons but its single cotyledon is functionally divergent like its leaf. The compact scutellum shields the embryonal axis leaving only the ends of the coleoptile and coleorhiza visible. The embryonal axis shows leaf primordia enclosed within the coleoptile and a primary root surrounded by the coleorhiza. In SEM, it has a boat or shoe-shaped scutellum wrapping the embryo axis leaving a broad mouth towards the radicle end only. This open plateau is guarded by an epiblast, a pair of auricles and ventral scales. These appendages even ensheath the inner coleoptile-coleorhiza cylinder. Thus embryo axis of the hypocotyl is protected by two concentric covers or veils-one of the coleoptile-coleorhiza and the other of the scutellum. Neverthless both are unit extensions and form a single lateral cotyledon. This results further in organised development of the embryo and the formation of tubelike coleoptiles-coleorhiza which emerge from the central region of the cup-shaped scutellum. The cotyledonary mound enlarges and ridges and furrows appear on the surface of the scutellum radiating from the surface of the scutellar node towards its periphery. In this way the peripheral part of the scutellum becomes lobed, deeply divided and nodular and forms 3 or 4 outgrowths. Coleoptile is scutellar in origin. The origin of leaf like structures is not at all associated with a shoot meristem. Future presumptive sites of these outgrowths are traced to subepidermal initials of the scutellum. Thus the grass embryo has two apertures one is guarded by auricles and scales and the other by the coleoptile. The ridges of tissues are cotyledonary or scutellar. Auricles and scales appear in ontogeny and develop after the covering of plumule by the coleoptile and radicle by the coleorhiza. The region of adnation between the scutellar node and the coleoptilar node is the mesocotyl. It is the fusion product of the hypocotyl and the scutellum. The attachment or the point of divergence between the two is an expanse running from 0.12 mm to 1 cm. Coleoptile develops from abgerminal sector and makes its appearencs as a circular ring before the shoot apex; it becomes a part of the cotyledon. Early appearance of coleoptile before shoot apex formation helps to protect embryonal shoot apex. Histochemical turn overs and statistical analyses for RNA, total proteins and total nucleic acids in maize reveal that the developing latency of all the constituent cells of octant (m and q) tiers is nearly the same and the fascimile of all the cell derivatives of ca remain exactly alike till the globular embryo. The origin of the organs cannot be assigned to specific tiers in the young embryo. The extinction values of five metabolites in four different cells of 1 and 1' tiers show the coefficient of

variation of 7.00 for polysaccharides, 10.08 for -SH proteins, 6.38 for RNA, 3.65 for histones and 5.39 for DNA. Thus the cells have not been summoned for different behaviours either at quadrant or octant stage. As the development proceeds the stain intensity (e. values) for DNA and histones in the derivatives of ca rises and reaches its peak values. At the elongated and indented embryo, the extinction values decline. The quantitative evidence from the extinction values, content per cell and concentration per unit area of DNA and histones, profile of the procambial strands, origin of the organelles of the cotyledon point to a positive correlation ($r = + 0.68$). DNA/histone ratio remains fairly constant. Its steep rise runs parallel and an intense reducing condition prevails in the suspensor cells till the embryo is globular. In fact ascorbic acid and -SH proteins are maximum at this stage. The globular stage does not inherit either any basic cytoplasmic variance or ultra-structural diversity to merit uneven potential of all the cells. In this light absence of differential detection of genetic as well as labile cellular molecules imprint equal but high physiological potency till the first depression appears. It separates the axial hypocotylar unit from the lateral cotyledonary unit. Two loci of the growing regions viz. hypocotyl and cotyledon lie in the interior of the radially symmetrical embryo and not at its distal end. Estimations of ascorbic acid, -SH proteins and total nucleic acids reach its peak values at the globular embryo in rice, ragi and oat and subsequently decline at first indentation or inlet. Globular embryo occupies a transitory but conclusive place between pro- and adult embryonic stages. It shows two faces -1 germinal or axial and 2. abgerminal or cotylar. The level of ascorbic acid and non-histone chromosomal proteins (NHCP) remain low on the abgerminal face than the germinal one. Differentiation in the globular embryo is expressed by two primary and several secondary successive indentations. Cytophotometric analysis of RNA, histones and DNA in 6 embryonal regions of mature embryos is Sorghum bicolor and Zea mays shows that scutellum, mesocotyl, coleoptile and coleorhiza possess higher cell area and nuclear volume as compared to that of plumule and radicle. In both the plants plumule and radicle have higher concentrations of RNA, DNA and histones as compared to that of the cotyledonary regions indicating a high metabolic turnover in these growing apiees. While scutellum, coleoptile, coleorhiza and mesocotyl possess higher content per cell/nucleus of DNA, histone and RNA. The mesocotyl occupies an intermediary position between the cotyledon and embryo axis. Extinction values for DNA are higher in the scutellum of Zea as well as Sorghum as compared to the rest of the regions probably indicating higher ploidy level in this storage sink of the cotyledon. E. values for histones are also higher in this region in both Zea and Sorghum suggesting the senescing fate of the scutellum. The concentrations of DNA in the plumule and radicle are 2-8 times higher than the cotyledonary region in Zea and 1 to 3 times higher in Sorghum. However content per cell shows a reverse trend the cotyledonary regions portraying 9-10 times higher values in Zea and 3-5 times more in Sorghum. There is a gradual diminution in the free and bound (ASG) ascorbic acid (AA) from 24 to 72 hr in all the regions of the embryo. There is a decrease in the AAU from 24 to 48 hr in the coleorhiza, radicle and plumule while it increases in the scutellum and coleoptile. RNA, proteis, ascorbic acid and -SH and -SS containing proteins exhibit wide fluctuations in embryonal tissues embarking on differentiation. Histone level remains higher in the cotyledonary regions than in the radicle and plumule. RNA, protein and total nucleic acids increase manifold in the plumule and radicle than the cotyledonary regions depicting high metabolic turn over. The estimation of coefficients of correlations elucidate that proteins have significant and positive correlations with significant and positive correlations with eight metabolites out of the nine studied such as DNA ($r = + 0.34$) RNA ($r = + 0.47$), total nucleic acids ($r = + 0.54$), basic ($r = + 0.29$), acidic ($r + 0.47$), -SH and -SS containing proteins ($= + 0.73$) and insoluble polysaccharides ($r = + 0.43$), indicating its intense role in growth and differentiation of the germinating embryo. Only ascorbic acid did not reveal any significant correlation with proteins ($r = + 0.05$). Proteins containing -SH and -SS groups are

highly significant and show positive correlations with DNA (r = + 0.59), RNA (r = + 0.52), total nucleic acids (r = + 0.63), acidic proteins (r = + 0.55) and total proteins (r = + 0.73). Ascorbic acid displays significant positive correlation with DNA (r = + 0.47) and insoluble polysaccharides (r = + 0.40) but a negative correlation (r = - 0.26) with acidic proteins. The colsest correlations of RNA are with total nucleic acids (r = + 0.92), total proteins (r = + 0.47), acidic proteins (r = + 0.72) and -SH and -SS groups (r = + 0.52). Thus it is the total proteins followed by total nucleic acids having maximum correlations with most of the macromolecules followed by -SH and -SS groups, acidic proteins, RNA and insoluble polysaccharides, which occupy the next positions. While DNA and ascorbic acid are highly selective in their correlations with the rest of the metabolites.

Changes in the levels of RNA and proteins indicate that cells of the apical meristems and leaf primordia have a high concentration of metabolites as compared to those of scutellar extensions. Moreover the mature embryo of monocotyledons the embryonal axis shows a profound staining intensity of RNA, DNA, proteins but they lack starch grains while scutellum and its annexures evince a feeble staining reaction but their cells are studded with starch grains. The scutellum and its organells however, contain the major quantum of these metabolites. Large starch grain in plenty a few fairly conspicuous fat droplets and many small peripherally positioned protenaceous granules constitute the major storage products. The amount of DNA and histones points to a positive correlation (r = + 0.86) in the expance of the cotyledon. Acidic proteins in Sorghum cotyledonary regions showed significant and positive correlations with total proteins (r = 0.77) total nucleic acids (r = 0.69), RNA (r = 0.72) and -SH and -SS proteins (r = 0.56) all significant above 99 %. However, there is no correlation found between DNA and acidic proteins indicating that the synthesis of acidic proteins is not consonant with that of DNA upto the end of embryogeny, the plumule radicle poles have a high level of ascorbic acid. But as soon as the axis is organised there is a surge of AA towards the scutellum. Probably this depletion of AA from the axis and its transfer to scutellum and pile of the basic proteins are responsible acts leading to dormancy while the reverse is the behavior of histones and ascorbic acid in Rhizophora - a viviparous plant. Differentiation of the hypocotyl is marked by the first and second indentations carring its distal and proximal terminals respectively along with a part of the cotyledon. The presumptive site of the cotyledon brings about a change in the symmetry of the embryo from radial to bilateral. As a result two organogenic centres are discerned. A longisection passing through these two sectors imprint certain histochemical differences too. One half of the globular embryo consists of many large cells, less basiochromatic as compared to the plumular half which are smaller in area but richly pyroninophylic. Further initially cells of the cotyladonar part are more active with respect to mitotic divisions, where as its complementary half shows little mitotic activity. The early divisions in the plumular half are anticlinal where as the cotyledonary cells divide in irregular planes to form a massive cotyledon. Thus the above data does not support either Hansteinian-Soueges nor Swamy-Haccius Schools on the organography of the embryo in monocotyledons. Histochemical staining reactions manifest the ontogenetic homogeneity among different extensions of the scutellum.

Extinction values of substances in four fertile cells of the grass octant embryo

	DNA	Histones	RNA	Total proteins	-SH and SS proteins
First cell of the tier 1'	0.066	0.266	0.097	0.0706	0.095
Second cell of the tier 1'	0.0643	0.28	0.098	0.0810	0.092
Third cell of 1	0.0703	0.28	0.097	0.076	0.080
Fourth cell of 1	0.073	0.29	0.084	0.084	0.074
Standard deviation	±0.0037	±0.0103	±0.006	±0.0049	±0.0086
Coefficient of variation	5.39	3.65	6.38	6.30	10.07

all the four cells have an identical developmental potential

DNA MEASUREMENTS IN THE CHALAZAL PROLIFERATING CELLS OF CAPSELLA BURSA-PASTORIS (L.) MEDIC(*)

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Summary

DNA content in chalazal proliferating cells of Capsella has been studied. Cytophotometric measurements showed some degree of endopolyploidy in all embryo developmental stages. A hypothetical role for this tissue is considered in relation to embryo nutrition. Its probable function as a substitute for the antipodals is discussed.

Introduction

Previous studies of chalazal proliferating tissue in Capsella (Pollock & Jensen, 1967; Schulz & Jensen, 1971) have clarified its origin and development. In order to find out more exaclty what role is played by this tissue, we have measured cytophotometrically the DNA content in the nuclei of the cells, already hypothetically attributed polyploid.

Results and discussion

Chalazal cellular populations examined in the globular (G), early heart-shaped (Ci) and heart-shaped (C) embryo stages are thus distributed in the following zones, with different DNA values (fig. 1):

A, distal (with respect to the embryo-sac): 2-12c in G and Ci; 2-8c in C

B, intermediate: 4-24c in G and Ci; 2-16c in C

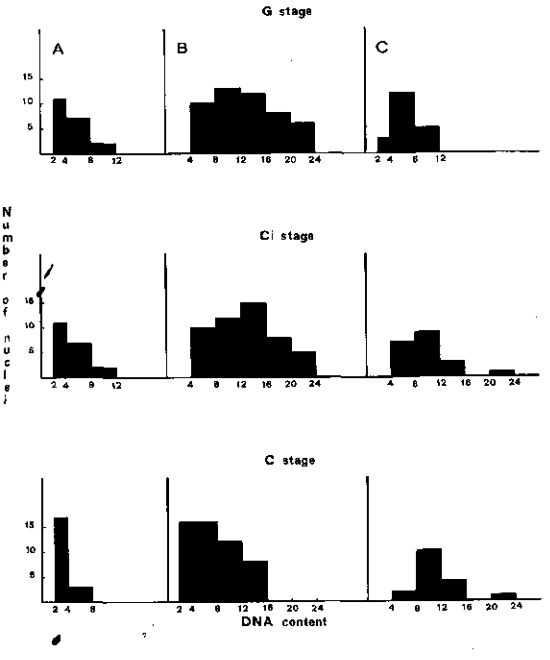
C, proximal: variable DNA values

The degree of ploidy in the cells has been calculated in relation to values 2c and 4c of the embryo cells.

Endopolyploidy, particularly evident in B, confirms the activity of the chalazal proliferating tissue in the earliest stages of embryo development and its rapid degeneration.

As discussed by Pollock & Jensen (1967), Schulz & Jensen (1971) this structure may hypothetically be attributed a role in the differentiation of the central cell and developing endosperm, with both of which it has a close plasmatic relationship.

Fig. 1. DNA contents (arbitrary units) in different zones of the chalazal proliferating tissue, at various embryo stages.



The very limited synthetic activity of the antipodals in this species and their brief duration strongly support the teory that chalazal proliferating cells may be a substitute structure.

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RESPIRATION RATE OF THE EMBRYO IN ITS NATURAL ENVIRONMENT IN VITRO

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Summary

Respiration rate (QO_2) of the embryo (Haemanthus Katharinae, Aesculus hybrida) inside the intact ovule, and in the central vacuole sap respectively is strongly restricted in vitro and in vivo. It has been suggested that main function in the QO_2 of the embryo and endosperm in intact ovule in vitro and in vivo is accomplished by pentose cycle. After preparation the coat, embryo and endosperm tissue from the ovule the glicolitic sequence pathway and tricarboxylic acid cycle is mainly used by these tissues in vitro.
Keywords: A. hybrida, H. Katharinae, embryo, ovule, respiration rate = QO_2 central vacuole sap = sap.

Introduction

There are only few papers (Cutter et al., 1952; Stanley, 1957; Forman, Jensen, 1965; Johri, Maheshwari, 1966; Ryczkowski, 1976; Ryczkowski, Szewczyk, 1978) containing the results on QO_2 of the developing embryo, endosperm, coat and ovule in higher plants in vitro.

These results and conclusions concerning the biochemical and energetic aspect of the embryo, endosperm and ovule in vitro QO_2 are not concordant.

The main aim of the performed measurments of the embryo QO_2 was to get some complementary data concerning its QO_2 inside the ovule in vitro and indirectly in vivo. This paper presents the results concerning: 1.QO_2 of an ovule containing the embryo, and an ovule without it of the same age as the previous one, 2.QO_2 of an ovule containing the embryo, and the embryo of the same age as the ovule with the embryo, 3.QO_2 of the embryo placed in the central vacuole sap i.e. in its natural environment in vitro.

Material and methods

Ovules of Haemanthus Katharinae Bak. (of the age 132-150 days after the perianth wilted, stationary phase of growth), and embryos of Aesculus hybrida D.C. (A.octandra x A.pavia, exponential phase of growth) were used as experimental material collected between 8 and 9 a.m.. The number of days counted from the day the perianth wilted to the day of sampling and the dimensions of embryos (H.Katharinae) or else the dimensions of embryos (A.hybrida) only were the adopted criteria of their development. It should be noticed that plants of H.Katharinae produced a small number of ovules of normal dimensions but without embryos.

The procedure of measuring the dimensions of embryos was described in paper (Ryczkowski, 1962b), and the technique of the sap collection in paper (Ryczkowski, 1962a). Before use the collected sap was filtered through a Schott 11G3 filter.

QO_2 of the ovules and embryos was determined by means of Warburg apparatus at 25° ± 0,2° C in darkness. QO_2 measurements of H.Katharinae ovules or embryos were performed in moist atmosphere (Ryczkowski, 1976).

Measurements of QO_2 of A.hybrida embryos were carried out in two different environments: a.moist atmosphere-main compartment of the manometric vessel type - WA 0110 contained 10 ml of distilled water, and its side arm 0,5 ml of 20% KOH, embryos were placed on a plastic perforated plate above the water, b.sap - main compartment of the manometric vessel contained 10 ml of the sap and embryos, and its side arm 0,5 ml of 20% KOH.

The vessels were equilibrated for 30 min., and pressure changes recorded every 5 or 10 min. dependent on embryo dimensions for one hour.

After QO_2 measurements the ovules and embryos were weighted to determine their fresh weight. Before that A.hibrida embryos were washed in distilled water and dryed with a pice of wood-wool.

The results presented in Tables 1,2 and 3 are mean values of 2-3 measurements of QO_2, and are expressed in mg CO_2/h/ovule respect. embryo, and in mg CO_2/h/g fresh wt.

Results

H.Katharinae. QO_2 (mg CO_2/h/ovule) of the ovule with and without embryo at age 135 - 150 day-old lay within the limits 0,0179 - 0,0329 mg CO_2 respect. (Table 1). The QO_2 difference between these ovules (of the same age) were 0,0206 - 0,0366 and

Table 1. H.Katharinae. QO_2 of the ovule with embryo, and the ovule without it (the same age) in moist atmosphere; + higher QO_2 the ovule with embryo; - higher QO_2 the ovule without embryo.

Age days	Fresh wt. g 1 ovule with embryo	without embryo	QO_2 in mg CO_2/h per ovule with embryo	without embryo	difference	per g fresh wt. with embryo	without embryo	difference
135	0,4372 <	0,4583	0,0206 >	0,0179	0,0027+	0,0462 >	0,0391	0,0071+
135	0,6440 >	0,6432	0,0366 >	0,0329	0,0037+	0,0569 >	0,0511	0,0058+
150	0,4875 >	0,4007	0,0297 >	0,0270	0,0027+	0,0610 <	0,0674	0,0064-

Table 2. H.Katharinae. QO_2 of the ovule with embryo, and embryo (the same age as the ovule with embryo) in moist atmosphere.

Age days	Dimensions of embryos in mm	Fresh wt. g ovule	embryo	QO_2 in mg CO_2/h per ovule	per embryo	per ovule	per g fresh wt. embryo
132	9,9x2,8	0,4962	0,0343	0,0215	0,0054	0,0432	0,1587
135	9,8x2,6	0,5166	0,0321	0,0211	0,0058	0,0408	0,1832
142	9,6x2,6	0,4761	0,0308	0,0186	0,0046	0,0390	0,1465

within the range 0,0027-0,0037 mg CO_2.

QO_2 (mg CO_2/h/g fresh wt.) of the ovule with embryo at the age 135-150 day-old lay within the limits 0,0462-0,0610 mg CO_2, and this value of the ovule without embryo was within the range 0,0391-0,0674 mg CO_2 (Table 1). Differences in the QO_2 between both types of ovules lay within the limits 0,0058-0,0071 mg CO_2/h/g fresh wt.

The QO_2 (mg CO_2/h/embryo) of 132-142 day-old embryos was within the limits 0,0046-0,0058 mg CO_2, and constituted one fourth of the QO_2 of the ovules with embryos (Table 2).

QO_2 of the embryo (mg CO_2/h/g fresh wt.) was within the range 0,1465-0,1832 mg CO_2 and was 3,7-4,5 times higher than this value established for the ovule with embryo (Table 2).

A.hybrida. QO_2 (mg CO_2/h/embryo) of the developing embryo placed in the sap or moist atmosphere increased irreglarly from 0,0285 to 0,1310 and from 0,0514 to 0,4680 mg CO_2 respectively (Table 3).

QO_2 of the embryo (mg CO_2/h/g fresh wt.) placed in the sap or in the moist atmosphere decreased from 0,4814 to 0,1160, and from 0,8684 to 0,4146 mg CO_2 respectively (results not placed in Table 3).

Table 3. A. hybrida. QO_2 of the developing embryo in the central vacuole sap and in moist atmosphere.

Dimensions of embryos in mm	Fresh wt.g 1 embryo	QO_2 in mg CO_2/h per embryo sap	moist atm.
12,7x5,5	0,059	0,0285 <	0,0514
17,9x5,6	0,060	0,0389 <	0,0476
20,9x5,8	0,159	0,0492 <	0,0957
20,1x8,0	0,201	0,0687 <	0,1210
25,1x8,6	0,272	0,0454 <	0,1631
28,3x10,7	0,566	0,0744 <	0,2477
30,1x10,7	0,509	0,0732 <	0,1161
34,8x14,6	1,129	0,1310 <	0,4680

Discussion

Basing upon the obtained results, concerning the QO_2 of the embryo of different age in its natural environment i.e. inside the ovule or in the sap, and in moist atmosphere, the following facts wer established :

1. Difference between the QO_2 of the ovule with embryo and QO_2 of the ovule without it (H.Katharinae, independent on their age, and small difference in their weight) lies within the range of biological variability and methodical errors. 2. The QO_2 of the embryo (H. Katharinae) constitutes about one fourth of the QO_2 of the ovule with embryo in vitro, in the moist atmosphere. 3. The QO_2 of the embryos (both species, moist atmosphere) in vitro is much higher than that of these embryos in their natural environments in vitro (Table 1,2,3) i.e. in the ovule or in the sap. 4. The QO_2 of the A. hybrida embryos are 1,8-3,6 times lower in the sap than in moist atmosphere (Table 3). Hence, it results that QO_2 of the embryos (both species) is strongly restricted in the ovule in vitro and in vivo. This conclusion

is in agreement with the very low oxgen tension inside the ovule (Ryczkowski, 1973), and unpublished data and its anatomy (Netolitzky, 1926).

On the basis of literature data (Forman, Jensen, 1965; Johri, Maheshwari, 1966; Kolloffel, 1970) and the author's previous results (Ryczkowski, 1976; Ryczkowski, Szewczyk, 1978), and present investigations on the QO_2 of the ovule, coat, endosperm tissue and embryo it should be taken into account that these tissues are potentially capable of respiring by: a. glicolitic sequence pathway and tricarboxylic acid cycle, b. direct oxydation of sugars - pentose cycle, and c. glicolitic sequence and anaerobic respiration.

It seems that the main function in the respiration rate of the endosperm tissue and embryo in intact ovule, in vivo and in vitro is accomplished by the pentose cycle (Barnet et al., 1953; Gibbs, Beevers, 1955; Wager, 1983). Most probably it occurs in big ovules (H.Katharinae, A.hibrida) in which oxygen penetration inside the ovule is strongly restricted (Ryczkowski, 1973; Ryczkowski, Szewczyk, 1978).

On the other hand after isolaiting the coat, endosperm tissue, and embryo from the ovule, and in dependence on its dimensions and anathomy, these tissues, organs respire in an aerobic way - glicolitic sequence pathway and tricarboxylic acid cycle (Stanley, 1957; Forman, Jensen, 1965; Johri, Maheshwari, 1966). This suggestion is also confirmed by a distinct increse of QO_2 of these tissues after their preparation in comparison with the QO_2 of an intact ovule (Ryczkowski, 1976; Ryczkowski, Szewczyk, 1978).

In the case of the ovule with green embryos capable of photosynthesis in vitro (Ryczkowski, Szewczyk, 1975) and probably in vivo too, the oxygen from this procces could be used in situ in the procces of embryo and endosperm tissue respiration. This fact makes the problem of respiration of these tissues in intact ovule still more obscure.

Anaerobic respiration of the embryo and endosperm tissue in intact ovule in vivo and in vitro seems improbable but not exclude at a determined developmental stage.

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ELEMENT CONCENTRATIONS IN THE SAP SURROUNDING THE DEVELOPING EMBRYO

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Introduction

In recent literature there is a lack of quantitative investigations on elements in the ovule during embryogenesis (Maheshwari, 1963; Wardlaw, 1965).

The present abstract contains the results of element determinations in the central vacuole sap (= sap) carried out with the aim to establish: a. the number of elements occurring in the sap, b. their concentrations and changes during embryogenesis.

Material and method

Ovules of Aesculus hybrida D.C. (A. octandra x A. pavia) were used as experimental material. The dimensions of embryos were the adopted criteria of their age. The procedure of measuring embryos was described in previous paper (Ryczkowski, 1962b) and the technique of the sap collection in paper (Ryczkowski, 1962a).

Spectrophotometer Perkin-Elmer Model 503 (AAS - method) was used for determination of K, Ca, Mg, Mn, Fe, Zn in air-acetylene flame (standard condition), and HGA74 Graphite Furnance for Cu.

Results and discussion

It was established: a. Concentrations of K, Ca and Mg were distincly higher in the sap than concentrations of Fe, Mn, Zn and Cu (Table). b. The lowest concentration was characteristic of Cu (Table). c. Total concentration of K+Ca and Mg+Fe+Mn+Zn+Cu respectively, changed during the embryo development. At the begining of embryo differentiation there was a small increase in these concentration, and during further development these concentrations decreased. These changes are in agreement with changes of osmotic value of the sap (Ryczkowski, 1962a). d. The elements occurring in the sap were used up by the developing embryo. This conclusion is concordant with two facts: decrease of these elements in the sap, and occurrence of the same elements in the embryo (preliminary investigations).

The occurrence of elements in the sap is in aggreement with literature data (Tammes, 1959).

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Table. A. hybrida. Element concentrations in the sap surrounding the embryo.

Embryo mm Dimensions	K	Ca	Σ K Ca	Mg	Fe	Mn	Cu	Zn	Σ Mg Fe Mn Cu Zn
	mg/cm³			μg/cm³					
0,87x0,54	3,20	1,27	4,47	113,00	8,17	3,40	0,080	3,35	128,00
1,23x0,53	3,20	1,35	4,55	125,00	7,40	3,15	0,150	3,36	139,06
3,80x2,00	3,50	1,43	4,93	125,00	6,00	2,40	0,050	2,90	136,35
4,70x2,10	3,20	1,33	4,53	108,00	7,75	2,90	0,160	3,43	122,24
16,10x5,40	3,00	1,15	4,15	88,00	7,65	2,53	0,150	2,53	100,86
25,30x9,70	3,20	1,10	4,30	115,00	4,40	1,90	0,125	2,30	123,73
28,10x14,0	2,45	1,00	3,45	80,00	6,45	2,63	0,095	1,55	90,73
39,90x19,2	1,50	1,10	2,60	100,00	3,20	1,60	0,080	0,90	105,78
S.D.	0,30	0,28		11,48	0,15	0,75	0,008	0,55	

HYBRIDIZATION BETWEEN PINUS NIGRA AND PINUS SYLVESTRIS AND MORPHOLOGY OF CHROMOSOMES

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Summary

The karyotypes of parent trees Pinus nigra Arn. (tree ni 221) and P. sylvestris L. (tree sy 77) have been compared with the karyotype of hybrid tree P. x nigrosylvis Vid. (tree nisy 410). The differences of chromosome total and arm lengths, as well as the centromeric position between parent trees are less expressed than the differences of the same paterns between each of the parent trees when compared to the hybrid.

Introduction

One of the six verified hybrid trees P. x nigrosylvis (the tree nisy 410), grown since 1968, was used to get the karyotype for the comparison with the karyotypes of parent trees P. nigra (tree ni 221) and P. sylvestris (tree sy 77). Borzan presented karyotypes of these parent trees in 1981.

Work method, results and discussion

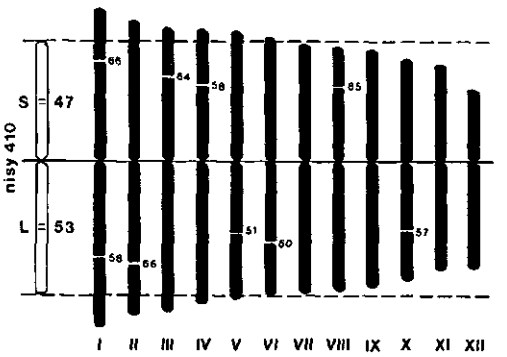
18 suitable cells of the endosperm in development were used for working out the karyotype of the hybrid tree nisy 410. All of the cells have originated from one ovule only, i.e. the same slide made by Feulgen squash method (Darlington & La Cour, 1962). In 1981, Borzan described karyotype analysis and data processing. The comparison of numerical karyotype values of trees ni 221, sy 77 and nisy 410 was done using t-test on a computer.

Table 1. Hybrid tree nisy 410 numerical karyotype values.

Chromos. number	Relative chromosome lengths: Total S + L	Short arm S	Long arm L	Arm ratio S/L	Centromeric index 100S/S+L
I	125.26 (7.97)	59.87 (4.24)	65.39 (4.73)	.917 (.056)	47.80 (1.58)
II	116.32 (5.16)	55.30 (2.26)	61.02 (4.57)	.911 (.070)	47.59 (1.99)
III	111.94 (3.62)	52.51 (2.60)	59.43 (4.20)	.889 (.087)	46.95 (2.57)
IV	108.40 (3.05)	51.60 (2.94)	56.80 (2.70)	.911 (.078)	47.59 (2.22)
V	105.47 (2.84)	50.93 (1.56)	54.54 (1.97)	.935 (.037)	48.30 (1.01)
VI	102.26 (2.51)	49.19 (1.87)	53.07 (2.39)	.929 (.061)	48.12 (1.70)
VII	99.03 (2.00)	46.67 (3.21)	52.36 (3.29)	.897 (.104)	47.13 (3.10)
VIII	96.71 (3.06)	45.27 (2.90)	51.45 (3.28)	.885 (.100)	46.81 (2.83)
IX	93.50 (3.19)	43.92 (2.16)	49.59 (2.44)	.888 (.062)	46.97 (1.80)
X	87.83 (4.98)	40.79 (4.14)	47.04 (1.75)	.867 (.087)	46.33 (2.67)
XI	81.47 (5.39)	38.05 (2.93)	43.42 (3.88)	.882 (.087)	46.74 (2.55)
XII	71.80 (6.01)	28.44 (4.64)	43.36 (4.38)	.663 (.134)	39.51 (4.83)

From the standpoint of incompatibility, it is interesting that P. nigra x P. sylvestris hybrids have been produced, but not vice versa. With 20 year experience in crossing these two species, it seems that different methods in overcomming the incompatibility can only increase the number of seeds, that can, in small numbers be produced without irradiation, mentor or other pollen treatments, for the P. nigra x P. sylvestris combination.

Figure 1. The idiogram of the hybrid tree nisy 410.



On the contrary the P. sylvestris x P. nigra crossing seems to be completely incompatible. Back crosses of the P. x nigrosylvis hybrids, with both parent species, are easy to do, and the number of fertile seeds per cone is the same as after intraspecific hybridization. From this point of view, the results of karyotype analysis of individual trees ni 221, sy 77 and their progeny nisy 410 are interesting. In conclusion, the differences of chromosome morphology patterns between parent trees are less expressed than the differences of the same patterns between each of the parent trees when compared to the hybrid. It is interesting that the chromosome XI of the progeny nisy 410 is not submetacentric, according to Saylor's (1961) definition. This is why the differences between that chromosome compared to the chromosome XI of the parent trees are significant. To understand these results it is worth stressing the heterozygosity existing in both parental pine trees, large possible variability of the gamets forming the nisy 410 zygote, as well as the fact that the endospermal nisy 410 tissue, used to work out the karyotype, has been developed after the meiosis took place.

Table 2. Results of tested differences between investigated trees.

Tested differences between trees	Total chromosome lengths	Short arm lengths	Long arm lengths	Arm ratios S/L	Centromeric indexes 100S/S+L
ni 221 / sy 77	No signif. differences	No sign. differ.	X*	No sign. differ.	No signif. differences
nisy 410 / ni 221	No signif. differences	V*; XI**	X*; XI**	XI**	V*; XI***
nisy 410 / sy 77	VII*	XI***	V*; XI*	V*; XI***	V**; XI***

* Significant differences at 5% level; ** 1% level; *** 0.1% level.

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IN VITRO EMBRYOGENESIS OF FLAX (LINUM USITATISSIMUM)

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Summary

Embryos of flax can be cultivated in vitro begining with their globular stage (the first critical stage). After initial enlargement of both length and width, cotyledons started to differentiate. Width is more increasing than the length. The globular embryo reaches the second critical stage on the 8th - 10th day of cultivation. The widened globular stage is reaching the second critical point on 6th - 8th day and the heart stage embryo attains the second critical stage already on 2nd - 6th day, or the embryo immediately passes into the exponential growth. Kinetin added into the cultivation medium promotes the transition of the embryos into the second critical stage.

Introduction

The method of embryo cultivation in vitro became a very effective method for plant breeding. This paper studies completing of embryogenesis in vitro, mainly under the effect of kinetin.

Material and Methods

For our experiments embryos of flax (Linum usitatissimum L. cv. Viera) were used. The method was described in details in our previous paper (Preťová, 1983). To the basic Monnier's medium (Monnier, 1978) we added kinetin in concentrations of 5.10^{-4} mol, 5.10^{-6} mol and 5.10^{-8} mol.

Results and Discussion

In the process of flax embryogenesis in vitro, some differences were observed in embryo development in dependence on the stage in which the embryos were excised (Preťová 1983). When kinetin was added to the cultivation medium in concentrations described above, a promotion of the embryo development, of growth of embryos and of the finishing of their embryogenesis was observed. Postembryonal development - the germination of embryos and the growth of seedlings were stimulated, too. The greatest stimulation effect was observed at the concentration of 5.10^{-8} mol of kinetin. Kinetin in concentration of 5.10^{-4} mol caused unorganised growth and callus formation. Kinetin , mainly in the concentration of 5.10^{-8} mol, speeded up reaching of the second critical stage (in an average 2 - 3 days earlier than the control embryos) in vitro.

Kinetin was also increasing the percentage of embryos completing their embryonal development. At the globular stage it was increased from 20 % to 35 - 40 %. At the excised more proceeded embryos this percentage increased from 60 % to 85 %.

The stimulative effect of kinetin on the development of excised embryos is perhaps due to the ability of kinetin to stimulate cytokinesis (Köller et al. 1962, Ioffe & Zhukova, 1965). This can be also in a connection with the confirmed stimulation of proteosynthesis (Klämbt, 1976).

It seems that kinetin, in a suitable concentration, can be used for speeding up the in vitro embryogenesis of flax embryos.

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DIRECT SOMATIC EMBRYOGENESIS FOR RAPID GENOTYPE PROPAGATION

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Summary

By direct somatic embryogenesis in vitro a clone of aseptic plantlets can be raised from a single immature zygotic embryo of Trifolium repens (white clover) within about six weeks from pollination. Somatic embryoids arise directly from superficial cells of the hypocotyl with no visible callus phase. Similar systems for direct primary somatic embryogenesis have also been developed for T. pratense (red clover), T. resupinatum (Persian or annual strawberry clover), Medicago sativa (alfalfa), Lotus corniculatus (birdsfoot trefoil) and Brassica campestris (oilseed rape). In addition, secondary cycles of direct somatic embryogenesis on subcultured embryoids have also been achieved for T. repens and B. campestris.

Introduction

We are developing methods for propagation by direct somatic embryogenesis or direct multiple shoot formation on immature zygotic embryos, with the aims:

1. to minimise culture-induced variation by avoiding cellular destabilization;
2. to develop simple, rapid, space-efficient propagation techniques which can be applied as early in the life cycle as possible;
3. to develop continuous embryogenic cultures with potential for mechanized propagation and harvesting.

In outbreeding self-incompatible species such as Trifolium repens and Brassica campestris, natural populations are normally highly heterozygous and variable. Consequently, individual seeds, even from the same plant, tend to vary widely in genotype. Although many perennial species such as T. repens can be propagated by cuttings, this procedure is slow and cannot provide aseptic material equivalent to seedlings. For annuals such as a number of B. campestris varieties, propagation by cuttings may be extremely difficult. Furthermore, when self-incompatible annual plants are artificially self-pollinated to generate homozygous inbred stocks, these lines must normally be maintained by artificial manipulations such as bud pollination.

Rapid propagation in tissue culture offers a potentially convenient technique for multiplying selected genotypes of outbreeding species and bypassing sexual reproduction with its attendant genetic recombination. Since cellular destabilization (Meins 1983) in callus and suspension cultures is now well known to result in genetic and cytological alterations ("somaclonal" variation, Larkin et al. 1984), pathways of morphogenesis such as direct somatic embryogenesis and multiple shoot induction which show no gross de-differentiation, are more likely to give true clonal regenerants. Here we report on systems for somatic embryogenesis from zygotic embryos.

Results and Discussion

Immature zygotic embryos were dissected from surface-sterilized pods at the "torpedo" stage (cotyledons initiated, embryo filling 1/2 - 2/3 embryo sac). This stage showed a marked proliferative response to appropriate hormone treatments. Embryos were placed on nutrient medium in sealed Petri dishes and incubated at 22°C in continuous light.

Primary somatic embryogenesis was achieved on the media:

T. repens, T. pratense, T. resupinatum, M. sativa	EC6 (Maheswaran & Williams 1984) with 1 gl^{-1} yeast extract plus 0.05 mgl^{-1} BAP
L. corniculatus	EC6 (without YE) plus 1-2 mgl^{-1} BAP
B. campestris	(B5 (Gamborg et al. 1968) (plus 0.05 mgl^{-1} BAP; or (modified SH ((Bhattacharya & Sen 1980) (plus 1 gl^{-1} YE, 0.022 (mgl^{-1} 2,4-D and 0.00216 (mgl^{-1} kinetin

Secondary direct embryogenesis from subcultured primary embryoids was achieved on the media:

T. repens	EC6 with 0.25-1.0 gl^{-1} YE plus 2 mgl^{-1} BAP
B. campestris	B5 with 2% sucrose, no hormones.

Primary and secondary embryoids of all species were readily separated and rooted on the corresponding media without hormones, before transfer to soil.

In T. repens the origin of primary embryoids was examined by scanning electron microscopy and light microscopy of sectioned

material. Embryoids arose superficially by proliferation of one or a few hypocotyl epidermal cells, without attachment to the vascular system of the parent embryo.

The uniformity of regenerants has not yet been confirmed. However, if this proves to be satisfactory, direct somatic embryogenesis from zygotic embryos may provide not only a means for propagation, but also "seedling" clones for multiple screening in host/pathogen or host/Rhizobium studies, or experiments where breeding is combined with destructive sampling. Also, since embryoids appear to arise from one or a few cells, direct somatic embrogenesis after mutagen treatment of immature embryos in vitro may improve recovery rates of whole-plant mutations and reduce chimerism in mutation breeding.

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APPLIED ASPECTS OF EMBRYOLOGY

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Introduction

Two main trends can be distinguished in modern embryology.The first is the establishing of regularities of the evolutionary process and solution of questions of phylogeny and systematics.The basic method of these investigations still remains the comparative-descriptive method.The second line of investigation is the clearing out the laws of individual development concerning plant reproduction (sexual, asexual, agamic)which has wide application in practice.The method of investigation is both comparative-descriptive and experimental.Both these trends are closely connected with each other. Still more significance has acquired at present the experimental line.One of the tasks of experimental embryology is to attain some possibilities of controlling separate stages of ontogenesis.Plant embryology is a necessary basis for practical investigations concerning the questions of plant reproduction.At present we can concretely determine some trends in works on selection and plant genetics which urgently demand knowledge of embryology and application of some cytoembryological methods.Some directions and methods will be considered in detail.

Distant hybridization

The development of investigations in the field of comparative and experimental embryology of wild and cultured plants made it possible to consider in a new light the possibilities of control and regulation of processes of distant hybridization and establish some causes of non-crossability.There is one example.Selectionists attempt to obtain hybrids with the participation of diploid T.monococcum L.,because this species of wheat is distinguished by its high resistability to many diseases.However, any attempts to obtain a cross of one-grain wheat with other species of wheat have been seldom succesful.Investigation of normal embryology of wheat in typical representatives of the polyploid series(T.monococcum,T.dicoccum Scrank,T.aestivum L.)(Batygina 1959-1974) was carried out. This enabled us to study all embryological processes in distant hybridization in the genus Triticum.

The analysis of literature and the authors data made possible to establish a series of common regularities of development common for the processes taking place in distant hybridization.Incompatibility in distant crosses can be expressed in three stages:1)Interrelationship of the pollen and pollen tube with the tissues of the pistil.2)The process of fertilization (syngamy and triple fusion). 3)The development of the embryo and endosperm.

The manifestation of incompatibility at the following stage is not always connected with the picture of the course of processes in the previous stage.For example, a good growth of the pollen tube does not always secure the success of fertilization.

Comparison of the character of disturbances of embryological processes in distant hybridization with the normal course of development enables us to distinguish two main types of disturbances: converted and non-converted.To converted disturbances belong such deviations which do not disturb homeostasis in the system pollen-pollen tube-egg cell-zygote-endosperm-surrounding maternal tissues of the ovule-the ovary.They are mainly connected with slowing down the tempo of development and seldom - with the acceleration of the whole correlative intergrated system.

To the non-converted belong the disturbances which change correlations in the development of some or other structures.These deviations pass the limits of self-regulation. Non-converted disturbances, in their turn, can be divided into several groups.1.Disturbances connected with the germination of the pollen and the growth of pollen tubes.2.Disturbances connected with an anomalous effusion of the pollen tube contents into the embryo sac cavity and anomalies connected with the disturbances of the mechanism of divergence of sperms: for example, one or several sperms are retarded in the synergid, where they gradually undergo lysis and the endosperm is not formed.All the sperms are in the central cell.The egg cell is not fertilized - the embryo is not formed.3.Disturbances of correlations at syngamy and triple fusion, the development of the embryo and endosperm. The character and the degree of expre-

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ssion of anomalies are different at different stages of embryo and endosperm. That is why while working out the methods of getting viable seeds in incompatible crosses it is important to take in mind not only the character of disturbances, but also the time of their expression. In the reciprocal crossing of one-grain(T.monococcum) with hexaploid species (T.aestivum var.Diamant) the stage of blastomerization in the embryo takes place without any special disturbances. However, in direct crossing the embryo stops its development at the moment of turning from blastomerization to organogenesis. In reversed(back) crossing the formation of all organs is usually observed in singularly formed embryos, and their development passes without anomalies which are met with at different stages of development.

In distant hybridization reciprocal crossings are almost always "effective" in one direction. In the combinations of crossing discussed, most successful seems to be the back crossing, that is, when hexaploid wheat T.aestivum is used as the maternal organism, as relatively full grains were obtained.

Thus, embryological investigation has shown that incompatibility in reciprocal crossing manifests itself in all the three stages discussed above, but the character of disturbances and the time of their manifestation in direct and back crossings are different We have discussed only the morphological manifestation of various disturbances. One of the most possible reasons for the disturbances in these processes, as can be seen from vast literature (Linskens 1959-1983) are the peculiarities of the immunological relationships between the tissues of the maternal plant, pollen and the pollen tubes, the hybrid embryo and the endosperm, which leads to the disturbances of regulation of development both of a separate organ and the whole system of structures as a whole. This gives possibility to geneticists and selectionists to work out certain approaches and use different methods.

The culture of embryos. Autonomy

One of the methods for overcoming non-crossability in vitro is the culturing of the embryo in vitro. In the majority of works devoted to embryo culture, are used mainly the embryos isolated at relatively late stages of them development which have passed in the maternal organism most part of their development.

The culture of embryos at early stages of embryogenesis is one of the most complicated, as well as most important problem of embryology.

Since 1970 the Laboratory of embryology of the Botanical Institute of the Academy of Sciences has carried on work on studying the behaviour of isolated embryos in different species of flowering plants. We have worked out a system approach, permitting already today, to manage some morphogenetic processes.

The problem of independence of the embryo was discussed in literature. However, the authors' solution has been one-sided. The dependence of the embryo from the maternal organism, in particular, from the endosperm, was considered in connection with some aspects of phylogeny and systematics, and only the correlation of sizes of the embryo and the endosperm was considered (Tachtajan 1966). It was also discussed from the viewpoint of physiological independence (metabolic independence) and ability to germination (Raghavan 1976). The progress achieved in the field of experimental science enable us to solve this problem in a new way.

We have established a new feature- the e m b r y o a u t o n o m y. Autonomy is a special structural-functional state of the embryo, which reflects its independence from surrounding tissues and is manifested in its ability to complete normal embryogenesis outside the maternal organism, and develop into a normal plant (Batygina, Vasilieva 1977-1984). This is the most important phase of embryogenesis, because just with it begins a relatively independent (autotrophic) development of the embryo. The stage of autonomy can be established by the method of culture in vitro. It should be specially noted that the development of the plant in embryoculture in the species studied begins with the stage when the embryo acquires independence from exogenic hormones. It means that by this time it contains a certain amount of endogenous stimulants, physiologically active substances which supply the normal further differentiation of the embryo and its germination. The stage of autonomy is species-specific for each taxon. Probably we can speak of progressive autonomization of ontogenesis and consider it in certain taxons as a directed process of evolution of living systems.

The wide-spread opinion about the fact that the stage from which the embryo becomes independent of the mater-

nal organism is similar for all angiosperms and is characterized by differentiation of cotyledons and the point of growth, has not been confirmed by us.

The studies of all stages of embryogenesis, and, first of all, the stage of autonomy, is a necessary theoretical precondition for applied investigations such as overcoming of incompatibility in distant hybridization, obtaining of haploproduction, shortening of the period of rest of seeds, choice of optimal time of crops, and some others.

Our data on the autonomy of the embryo are also used as a basis for other works, and find their confirmation in them (Davoyan,Batygina 1982,Lukyaniuk 1982,1983 and others) and can be considered as one of new approaches of untraditional technology of selection

A perspective line of investigation in selection is the use of dihaploid lines obtained by means of a haploproducer - Hordeum bulbosum(Ho,Jones 1980, Simpson,Snape 1981,Lukjanuk, Ignatova 1980,1982). But Kasha (1975) has shown cytologically that in crossing H.vulgare with H.bulbosum there takes place elimination of chromosomes in the embryo sac, which results in haploid embryos of H.vulgare.After this they are diploidized.The difficulty of this work is the perishing of embryos at early stages of development because their endosperm degenerates in the first days of development(Jensen 1977,Lukyanuk 1982). Working out of the time of their isolation is necessary.S.F.Lukyanuk has carried out a a detailed research of studying autonomy in haploid and diploid embryos of barley which were obtained with the help of haploproducer (Hordeum bulbosum) and has got a number of precious forms.It was established that the diploid embryo becomes autonomic by the 13-14th day after pollination when its size is more than 2,0 mm. At this, as we have shown, the formation of its autonomy takes place during two days (that is 11-12th days after pollination in culture).It was necessary for the haploids of such size to germinate, to choose the media with addition of different physiologically active substances.It means that they were only relatively autonomic.

It is necessary to mention here that the selection process includes several stages, and one of the most important is the creation of new initial material.The callus obtained from embryos and its organs and other organs of plants, especially after variuos exposures (for example, irradiation), can be used as one of the possible methods of obtaining new forms of plants, in particular, rice (Davoyan 1979, Davoyan,Batygina 1982,1984,Davoyan 1983).

C o m p l e t e a u t o n o m y is a special structural-functional state of the embryo reflecting its independence from surrounding tissues and shown by its ability to complete normal embryogenesis outside the maternal organism and develop into a normal plant.Autonomy of the embryo is determined mainly not by conditions of growing in culture in vitro. Speaking about the autonomy of the embryo and some other structures it is necessary, for convinience, to introduce the term "the degree of autonomy". Naturally, in nature there are no structures which would develop in complete autonomy from a complex of different factors (physiological, gravitative , electric, etc.)By relative autonomy of the embryo must be understood the ability of the embryos to develop into a whole plant in culture in vitro on different media.

Naturally, the stage of isolation will be absolutely different and will be determined mainly by the conditions of culturing specific for each taxon.

Culturing of anther in vitro. Haploids

Together with the culture of isolated embryos, still more serious attention for practical aims should be given to the working out of the theoretical foundations of culture in vitro of both vegetative and generative structures such as the anther, the ovary, the ovule, the embryo sac, the endosperm and the embryo.The antner being a complex intergrated system, from the methodical viewpoint, doubtlessly is a simpler model for investigation in situ, in vivo and in vitro, because on it it is easier to observe and establish separate morphogenetic processes and their correlations.For a more succesful study of the anther as a model system it is necessary, first of all, to work out a methodical basis of studies: unification of terminology of stages of development, working out of the main problems involved in the investigation, unified studies of the ways of morphogenesis of the anther in culture in vitro in different species of angiosperms, elaboration of questions of joint studies of all the structures of the stamen, the anther and the fe-

male reproductive sphere of plants.

Because of the fact that for the purposes of selection it is necessary to obtain a mass quantity of haploids, in literature is widely discussed, for example, the use for culturing in vitro of stages of development of sporogenic tissue, of microspores, of microspores, pollen, the state of donor plants. There is given evidence of the influence of various physiological factors on morphogenesis.The different ways of forming embryoids are described.However,in the majority of cases, it can not be said for certain - what will be the result of regenerants, in which way of morphogenesis will go their development, why the ways of morphogenesis are different in different species. These questions, first of all, need a detailed knowledge of the object investigated which we should advise to study from the viewpoint of system approach elaborated by us and secondly the study of morphogenesis ways on the system of reproduction in natural conditions and in culture in vitro,that is, the method of differentiation of various morphological structures.

Nowadays, in spite of the effectiveness of such approaches as haploidy, parasexual hybridization and induction of regenerants from somatic cells, no physiologist or biochemist dealing with these processes knows just how the regenerants are formed from the pollen.It has been known theoretically that generative and vegetative cells may participate in this, either one of them, or the microspore (Sunderland and oth.). However, nobody has studied step by step with the help of a ceitrafer the successive stages of differentiation of regenerants from the pollen.Deep and wide analysis of literature on morphogenesis in culture in vitro and our personal data show that it is not clear: from what pollen haploids are formed: normal or abnormal, a certain per cent of which is always present in the anther.

Brilliant works of Indian scientists and others who have discovered this phenomenon have established that a certain percent of haploids in Datura is connected with a certain percent of anomalies. Stow (1936) have discovered the changes of sexualization and forming of the embryo sacs in the anther, though it is known that with the help of different factors (for example, temperature) the microspore may be changed, that is, to obtain only equal division (Norrell 1973 and oth) and a 100 % regenerants.

Probably, the choice of the stage of the anther and pollen development for putting them into culture in vitro can be exactly determined only by successive detailed studying of the stamen of the investigated object only from the viewpoint of system approach.This stage in different species will be always determined by different complex of factors and in certain sense is species-specific.For the managing of the process of getting of haploids are necessary additional theoretical findings.

Generative structures and factors of enviroment (microelements).

A rich experimental material has been collected on the reaction to the factors of environment at different periods of ontogenesis. Many data witness to the important role of microelements in the grain crop,in particular, boron.Its shortage during the growth of wheat results in the disturbance of the processes of ear formation, their outlet from the tubes, and also leaf formation (Troitskaya, Batygina 1970).The detailed embryological investigation of their development in wheat in the case of lack of boron, are practically absent.

Our experiments (Batygina,Troitskaya Alimova 1966) have shown that the sterility of the spike in the lack of boron is the result of disturbances occuring in the anther. Anomalies were found in every anther.They affected both sporogenic tissue and pollen (mitosis and meiosis), as well as the wall of the microsporangia. In all ovaries there were developed normal embryo sacs which, because of the lack of pollen, degenerated. In artificial pollination of these ovaries with the pollen from plants grown with boron, a 100 % formation of grains was observed.In connection with the introduction of Triticale into crop industry were carried out all-biological investigation of the perspective example AD 537. Manufacturing tests have shown that it was a remarkable silos culture. However, its drawback is weak production of grains and alternation of grains.It was found that a large quantity of sterile pollen is developed in the anthers - 51,1 % (2,8 - in rye, 7,7 - in wheat).All this leads to the lack of sufficient amount of normal pollen necessary for fertilization. In connection with the above-mentioned, experiments of addition of boron to AD 537 were conducted for many years, at the stage of

forming leaves, that is when the formation and development of female and male generative structures took place, as a result we managed to increase the productivity of the spike. The preliminary results show that the amount of the fertile pollen has increased at the expense of decrease of asynchronity of the process of its development. On the same lines were carried out works on the study of apomixis in different species of Poa (Batygina 1977, Batygina, Freiberg 1977, Batygina,Mametieva 1979). In Poa pratensis all types of apomixis have been discovered for the first time and two methods of formation of nucellar embryos (embryoids from one nucellar cell, similar to the zygotic, sexual and throug the embryonic cell complex (ECC).The analysis of literature on the problem of morphogenesis in the reproduction system of flowering plants in natural conditions and in culture in vitro (Batygina 1976-1984) made it possible to put forward some theoretical and practical ideas concerning reproduction and closely connected with the most complicated problem of differentiation of various embryonic structures (fig. 1).

Besides the aspects discussed, where knowledge of embryology is absolutely necessary, we can note the following:

1.The determination of viability of the ovule, stigmas, egg cells, pollen for establishing the time of artificial pollination and possible borders of works on artificial pollination.

2.Elaboration of methods of preservation of viability of pollen.

3.Cytoplasmic male sterility (CMS).

4.Elaboration of biological methods of struggle with parasitic plants.

5.Obtaining of parthenocarpic fruits and suppression of this process.

6.Application of the method of artificial pollination and fertilization injection of suspension into ovary, combined cultivation of pollen and the ovules.

7.Determination of the type of apomixis for possible conservation of necessary valuable features.

8.Determination of the type of sterility and elaboration of the methods of its overcoming (becrosses, obtaining of amphiploids, microclonal reproduction, conditions of growing etc.

9.Use of nucellar polyembryony, for example, for obtaining wildings and stocks resistant to draught, diseases etc.

10.Elaboration of methods of accelarated reproduction: microclonal reproduction of separate generative and vegetative structures in culture in vitro (anthers, ovules, etc.).

11.Creation of new forms of plants from callus (including the influence of different factors) obtained from different reproductive structures (inflorescence, flower).

12.Shortening and elimination of the resting period in seeds.

13.Creation of the bank of plant tissues of valuable species of plants (from different generative and vegetative structures).

14.Finding out of the ways of morphogenesis in the system of reproduction.It is especially important for the managing of morphogenetic processes occuring in cultivation of generative and vegetative structures and organs.

15.Establishing of the stages of anther development in different species of flowering plants, of pollen for the effective obtaining of regenerants.

All enumerated problems, approaches and methods in some degree are connected with genetics-selection works. At present it is difficult to imagine that the problem of reproduction could be solved without embryologists. However, all these questions are all-biological. This is why for the aims of conscious managing of separate stages of ontogenesis wide theoretical generalizations are necessary which can be done only in complex with other sciences (selection, genetics, morphology, cytology, physiology, physics, chemistry,etc.).

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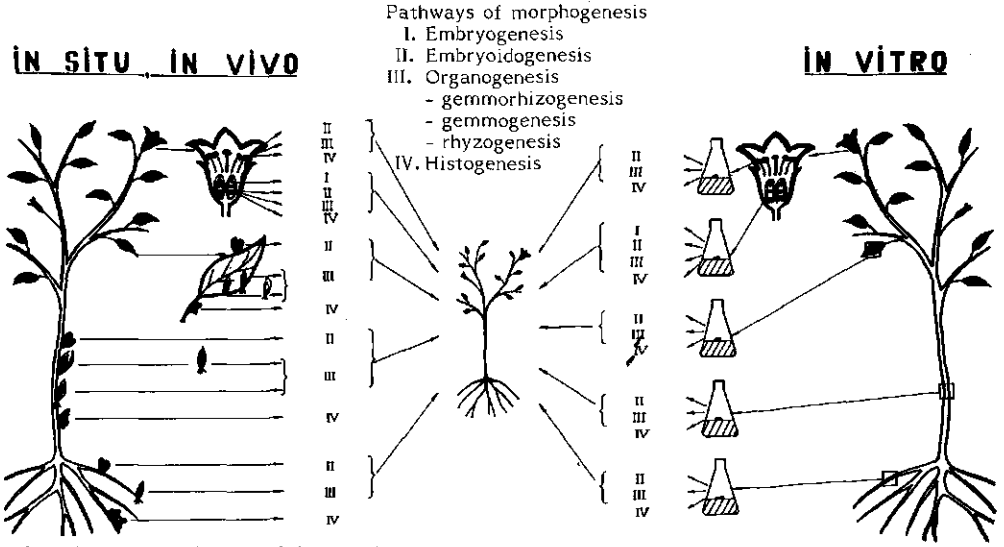
THE UNIVERSALITY OF THE PATHWAYS OF MORPHOCENESIS IN FLOWERINC PLANTS IN NATURAL CONDITIONS AND IN CULTURE IN



II. - from the flower: Allium odorum (Hegelmaier, 1897; Modilevski, 1925, 1930), Erythronium americanum (Jeffrey, 1895), Eulophea epidendraea (Swamy, 1943) and other.
- from the stem: Linum usitatissimum (Poddurnaya-Arnoldi, 1976).
- from the leaves: ?
- from the roots: ?

III. - from the flower: Poligonum viviparum, Poa, Festuca, Eringium viviparum (Braun, 1956) and other.
- from the stem and the roots: Bryophyllum (Ossenbeck, 1927), Pogonia ophioglossoides (Carlson, 1938, 1950), Lilium tigrinum, Malus domestica, vitis vinipera, Prunus, Aristolochia (Sinnot, 1960) and other.
- from the leaves: Linaria (Van Tieghem, 1887), Cardamine pratensis (Goebel, 1908), Begonia rex (Prevot, 1938, 1939), Bryophyllum calycinum (Berge, 1877; Yarbrough, 1932), Kalanchoe rotundifolia (Sinnot, 1960) and other.

IV. - from the flower: ?
- from the stem: in different woody plants.
- from the leaves: ?
- from the roots: Salix (Sinnot, 1960).

I. - from the flower: Hordeum (Norstog, 1961), Paeonia (Batygina, Butenko, 1981), Nelumbo nucifera, Dactylorhiza maculata (Batygina, Vasilyeva, 1981) and other.

II. - from the flower: Nicotiana tabacum (Tanaka, 1968), Ranunculus sceleratum (Norrell, 1973), Paeonia (Batygina, Butenko, 1981) and other.
- from the stem: Ranunculus sceleratum, Foeniculum vulgare (Konar, Nataraja, 1965), Petunia hybrida (Rao et al., 1973) and other.
- from the leaves: Daucus carota, Coffea robusta (Norrell, 1973), Petunia hybrida (Rao et al., 1973) and other.
- from the roots: Daucus carota (Reinert et al., 1970, 1973), Petroselinum (Vasil, Hildebrandt, 1966), Panax ginseng (Butenko et al., 1968) and other.

III. - from the flower: Cuscuta reflexa (Maheswari, 1961), Paeonia, Dactylorhiza maculata (Batygina, Vasilyeva, 1979, 1982) and other.
- from the stem: Asparagus (Steward, Maple, 1971), Petunia hybrida (Rao et al., 1973) and other.
- from the leaves: Macleaya cordata (Kohlenbach, 1965).
- from the roots: Panax ginseng (Butenko et al., 1968).

IV. - from the flower: Daucus carota (Hildebrandt, 1970), Paeonia (Batygina, Butenko, 1981) and other.
- from the stem: ?
- from the leaves: ?
- from the roots: Daucus carota and other (Hildebrandt, 1970).

TO THE MORPHO-FUNCTIONAL GAMETOGENESIS STATUS OF HIGHER PLANTS

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Summary

In this communication an attempt was made to summarize the accumulated data about gametogenesis and to indicate some pecularities of gamete formation principles. Being in a full agreement with the opinion about the role of the whole in the formation of a particular part (sexual cells) we want also to throw some light on the embryonal differentiation problem.

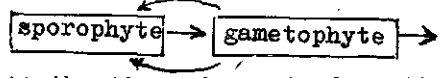
Introduction

The gametogenesis occupies the central place in the sexual reproduction of higher plants. It's a prerequisite to the fertilization and to some degree it canalises the subsequent events of the embryonal determination. Gametogenesis hides in itself important ontogenesis initiations, individual development, its stability and adaptability. These questions are concerned directly the problem of adaptive reaction norme (Shmalgausen, I940). Gametogenesis is the echo of darwinian selection.

The retrospective analysis of available information concerning the gametogenesis biology of different taxa is far from fullness. V.A.Poddubnaja-Arnoldi (I982) has shown that of 438 families of angiosperms I40 ones are far from the complete embryological investigation or aren't investigated at all.

Results and discussion

In the previous studies with the light and electron microscopes (Chebotaru, I960, I972) the structural and functional pecularities of male and female differentiation of gametophyte sporogenous cells are analysed. The attention was drawn to the interrelation within the system: maternal organism → the sporogenous tissue → the sexual cells → the zygote → the embryo → the seed. It was shown that the formation and the differentiation of sporogenous and particularly the haploid structures occurs to a great extent due to the specific back links of the sexual cells with the maternal organism (see the arrows at the scheme).



At the time of gamete formation these relations are manifested in the building of growthactivated substances like auxins, hormones etc., stimulating the growth of pericarp, the formation of parthenocarpic or apomictic fruits (seeds), the strengthening of trophical and physiological reconstruction of maternal organism in the whole.

The other widespread events, concomitant the sexual cells formation - this is the degeneration of the surrounding sporophyte cells (that's of the maternal organism). While studuing gametogenesis one is surprised by the constancy of the meiotic division number with strictly repeating chromosomal and biochemical rearrangements observed at all higher plants rangs. And though gametogenesis is for a long time considered as a main source of initial material for the darwinian natural selection its understanding is rather contradictory. At the sporo-gametogenesis (beginning from the archesporial cell an ending with the building of sexual cells) there is seen a constantly repeated separating of the common into uneven or potentially uneven formations that strengths the spectre of shedding-elimination of a great part of apparently little adapted haploid structures. The geterogenous structure

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formation at the micro-, macrosporo- and gametogenesis in different systematic phyla is regularly accompanied by the dropping out (elimination) of the genetically defective haploid structures called by us a) the principle of elimination of genetically defective structures which exhibits at the phylo-ontogenesis as a peculiar evolutionary sieve of the natural selection on the cell level. In this process a genetically fixed way of elimination of the "exhausted slags" (in Goldshmidt's understanding) and the providing of the geterogenous potential of sexual cells from one generation to another is also seen. From this point of view the tendency of gamete variability within the ecotype, population, phytocenosis having specific amplitudes of the reaction normes is quite clear. It arises as a result of the realisation of darwinian selection pressure on the past of the previous parental basis and this in its turn causes an additional elimination of the gametes themselves, the embryo abortion or appearance of a sterile progeny.

It is necessary to point out the fact that gametogenesis that is the haploid structure building occurs only in the strictly polarised coenocyte formations. Outside the coenocyte the sexual cells are not differentiating. Consequently micro- and macrospores before taking the road of sexual cell formation are subjected to complex structural and physiological rearrangements of the coenocyte pattern. Ultrastructural analysis of the male and female gametes of the different species of flowering plants (Chebotaru, I972, I976; Willemse, I972) has revealed a striking similarity of male gametes. Thus it suggests the second important conclusion that the sexual cells are arising as elements of integrated evolutionary-fixed phylogenetical systems. The small differences between the male and female gametes (Paolilo, I969; Chebotaru, I976 et al.) within the species, population are rather elements which supplement each other. All this permitts to formulate the following rule -
b) the principle of equipotentiality of haploid formations (♀ and ♂). This principle is manifested in the building of full genotypical specificity in the cource of gametogenesis. The equipotential features of the gametes are realised in the cource of fertilization.

This way of sex formation appearing to a great extent "unstable" provides nevertheless a steady genotypical renewal which is canalised by panmixis.

S.G.Navashin (I950) has revealed the enantiomorphical nature of the sister spermcells. This permitted to understand their biological activity. Apparently the same is occuring in the female gametophyte, though there the differentiation of egg and central cell is an individual and very specific event. Amphimixis of enantiomorphical structures has left a general biological imprint on all the flowering plants and it is formulated by us as c) the priciple of synergism of enantiomorphical formations. It is the synergism of enantiomorphical of male and female formations that results in arising of racemical labile genome systems (ontogenesis) which are based on the morphogenetical homeostasis. The latter in addition canalises the adaptive variability of sporo-gametophytes at the onto-phylogenesis.

Gametogenesis is an instance where it is possible to follow the sources of organic (bioecological) advisability, the beginning of adaptive reaction norme (after Shmalgausen) and other very important regularities. In gametogenesis the action of darwinian selection is followed and the manifestation of the Vavilov's law of the homological rows. In gametogenesis it occurs a hard selection on the level of meiosis, fertilization and zygotogenesis. There the compatibility of paternal(previous) genomes is fitted (in the manner of a zipper.

In the gametogenesis Baer's law of integrated determination finds its confirmation. Through gametogenesis there is the way of building of species diversity, without which the evolution is unthinkable. Therefore it is impossible to agree with the opinion that the sexual reproduction as a such bears only the potential possibilities of the species renewal (Levina, I982). The higher plants gametogenesis has to be considered as an original yet unknown to the end alphabet by which the evolutionary way of biogeochemical past development (the plant world) is written. And if the mountain formations are reminding the past of our planet by their hardened into stone silence, then the flowering plants make this by their constant

ontogenetical renewal. The genomes once arised in the geological past are repeated in the numerous generations through gametogenesis.

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SEXUAL REPRODUCTION OF SEEDPLANTS, FERNS AND MOSSES. A BACKGROUND

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The flower

The flower, the organ of reproduction, shows during its development many functions, ultimately intended to produce a renewed individual with good changes of survival. The flower is a continuously developing organ which, during a long period of time and at the cost of much energy, will produce viable seeds. Each plant species has its own characteristic in sexual reproduction, being marked by variations within a series of different steps. These steps comprise: induction of meiosis, meiosis itself, cell isolation by a callosic wall, attraction and recognition, cell fusion and a resting period. These are the standard events of sexual reproduction in green plants.

From water to land

The biotope for unicellular algae is water. In water the gametes are attracted by very specific substances produced by one partner. Such substances are also operative during the recognition processes. After attraction and recognition gamete cell fusion will follow.

In the course of life, algae as well as fungi show the reproductive characteristics of induction of meiosis, meiosis itself, cell isolation, attraction and recognition, cell fusion, and a resting period. Also alternation in ploidy of individuals within a generation exists. From unicellular algae to multicellular algae few new elements are added to sexual reproduction. Only the organisation of gamete producing organs became more specialized.

The role of the sexual game in thallic and complex algae becomes more specialized within the basic characteristics of sexual reproduction. Multicellular or thallic algae have extend their strategies by a higher level of differentiation, partly due to their diploid level and the possibility to use each unicellular phase within their course of life as a starting point for a new organism With these characteristics the land biotope can be conquered. The possibility to change from a haploid to a diploid level enlarges the chance of occupying more areas with different environmental conditions. The first land plants inherited the possibility to alternate from ploidy level, oogamy, attraction and recognition mechanisms, monoecy and dioecy and the spore as the unit for their dispersal. The conquest begins with the use of the ability of sexual reproduction and during the colonization the diversity in land plants increases.

The sporophytic nature of the plant, diploidy, prevails and also gene exchange by sexual reproduction. The preference for cross fertilization develops further. Vegetative propagation decreases strongly.

Mosses, ferns and seedferns

The earliest small archegoniates on land, ferns, but also the mosses need, besides light, very humid conditions to survive. Both for sexual reproduction and the excretion of attractive substances, dispersed in all directions to promote fusion of dissimilar gametes, water is necessary. Plant dispersal proceeds with spores relying on abiotic factors such as wind and high or low humidity. The germination of the spore remains dependent on water. That characteristic, being adapted partly to a wet and partly to a dry biotoop during the course of their life, is demonstrated by mosses. Spore germination, protonema, mossplant and especially sexual reproduction, are dependent on water. Dispersal is a function of the sporophyte and occurs in dry conditions. Ferns also show this ability to survive in wet and dry biotoops, with spore germination and sexual reproduction depending on water and spore dispersal being adapted to land life.

Within the mosses and ferns there is a great variety in constitution and form. Their sporangium starts to develop in two different ways, preparing the heterospory. In some mosses and even more ferns a difference in sexuality in the spores is expressed, promoting the possibility of interbreeding. The number of spores produced in ferns change. The number of microspores, with their dependence of dispersal by wind, remains numerous. The shape of the megaspore enlarges and their number is greatly reduced, in special cases to one. The dimension limits their dispersal in the vicinity of the plant. This means that the task of sexual crossing is mainly restricted to the microspore, the megaspore determines the rate of dispersal. The problem for the gametes to meet each other is beautifully demonstrated by the appearence of appendages on the spores to promote a collective spore dispersal. Horsetails and waterferns show haptera and anchor mechanisms. The interbreeding, however, is not obvious from the point of view of one individual. The heterospory culminates in the seedferns were the megaspore is reduced to one, receives its nutrient supply from the sporophyt and remains there till after 'pollination'. In this respect also some of the waterferns show seedfern characteristics. In seedferns diploid tissues

appears around the megaspore and the separation of the megaspore is dependent upon abscission of sporophytic tissues. Within this tissue the megaspore and megagametophyte develops in isolation of the surrounding tissues. In the transition from diploid to haploid during the meiosis, this isolation is preceeded by the formation of a callose wall.

Within the mosses and ferns, self-fertilization as a survival strategy remains clearly possible, along with cross-fertilization. Incompatibility mechanisms should not be excluded although incompatibility is scarcely reported in mosses and ferns.

From seedfern to seedplant

The longer retention and feeding of the megaspore on the sporophyte permits a further development and employment of the gametophyte and sporophyte for sexual reproduction. This longer stay on the sporophyte and the presence of specialized surrounding tissue permits the micro- and megaspore to develop into a highly specialized gametophyte in which cells get a high level of differentiation.

This differentiation means for the microgametophyte the preparation for an abiotic or, in the case of angiosperms also a biotic transport, the possibility to rest, thereafter to germinate and to produce sperm cells. It becomes manifest that mature pollen grains of different plants strongly differ in their level of development when anthesis occurs especially in relation to transport, recognition and germination.

From the megagametophyte in gymnosperms a relative large prothallium develops with one to several very specialized archegonia surrounded by follicle cells, or there is a simple egg cell. An egg cell receives one or two sperm cells, depending on the level of differentiation of this egg cell and on the way of transport of the sperm cells.

In angiosperms the size of the megagametophyte is reduced, but its consituting cells are highly specialized. In fact, to speak of 'the reduction of the gametophyte' draws attention to only a minor aspect in comparative gametophytogenesis. An egg cell contains but little storage substances and will receive one sperm cell. The egg cell is surrounded by two synergids and by the central cell, commonly soon diploid. A degenerating synergid will receive the two sometimes connected sperm cells. Then the other synergid and usually the antipodals degenerate. After fertilization all haploid cells dissappear. Only the ploidy level of the embryo and that of developing endosperm differs. This difference in ploidy level after fertilization is common in seed plants. Probably this difference between embryo and the nutritive surroundings, haploid in gymnosperms, tri- or polyploid in angiosperms, is partly a result of a difference in the start of the development of the new plant. The sporophyte provides a great deal of the food supply in a relative short period of time to form a seed within one growing season as occurs in most angiosperms. This initiation of embryogenesis is triggered by pollination and by double fertilization, which starts the embryo and also the endosperm. In gymnosperms this endosperm development is started after pollination during the genesis of the megagametophyte.

The reason for the high differentiation grade of the megagametophyte is its function in the formation of the seed. In relation to dispersal of the plant, seed development shows a lot of differences partly related to the biotic or abiotic factors, partly to the expected different germinating conditions. In that respect the seedferns develop a strategy to colonize the land by the formation of a presemen. In seed ferns also sporophytic elements become involved in the seed dispersal.

The sporophytic development for the use of sexual reproduction of seedplants

The abiotic and biotic way of transport of the microgametophyte becomes an important fact of wich the sporophyte plays a role and will adapt.

The pollen of gymnosperms is prepared for wind transport over, in general, a long distance and during a long period. Ultimately sucked in by a pollination droplet, the pollen will reach the upper part of nucellar tissue. Recognition reactions occurs between sporophytic nucellar tissue with its exudate and the microgametophyte, surrounded in general by an uncovered pollen wall. This mechanism of recognition is unclear.

In angiosperms the pollen lands on the stigma. The pollen wall is covered with sporophytic remnants containing recognition substances. The first pollen-stigma contact occurs in fact between sporophyte and sporophyte. The pathway of the pollen tube permits to add recognition between the sporophytic stylar tissue and substances originating from the pollen tube, a recognition between sporophyte and gametophyte. A recognition between pollentube and nucellar tissue as in gymnosperms, occurs also in some angiosperms. In seedplants the classic attraction between sperm cell and egg cell seems mainly to be shifted to an attraction after recognition between pollen tube and egg cell in gymnosperms and probably the synergids in angiosperms. In angiosperms not only the recognition but also aspects of the attraction are mainly taken over by the sporophyte. This coincides with the development of the pistil in relation to promotion of cross pollination. The mixture of different relationships between sporophytic-gametophytic recognition to sporophytic recognition systems and the effort to avoid self-pollination mainly by appealing on biotic pollination, makes this system very complicated.

The primitive flower construction devel-

ops as a nutrition source for different kinds of animals. Pollen, nectar, and the complex flower shape are involved in the life cycle of different animals, mostly insects. Colour, odour, shape and position of the flower may attract animals in different ways. Sepals, petals, stamens and pistil all can be involved in this function. In this way the whole flower is pre-emintly an organ of communication functional in cross pollination. Within these plant- animal relationships the diversity in flowers increases strongly. The shape of the flower, its expression in signalling, its nutritive value, the way of recognition, all vary to characterize their individuality.

The gametophytic development for the use of sexual reproduction of seed plants

Also within the structures and mechanisms of haploid tissues directly involved in sexual reproduction of gametophytic origin a range of changements occur.

Pollen development, as a process from mother cell to sperm cell, is a programmed process and prepares the future functions of the pollen. However, the mature pollen grain shows several variable characteristics when its transport starts, depending on the species involved. This is not only expressed in the number of cells present, but principally in the possibility to germinate directly after maturity or at a later point of time, its dryness and the amount of Pollenkitt present. The sculpture and composition of the pollen wall are highly specific and sustain the palynology. Although the relation is not yet known between the shape of the pollen and the sculpture of the pollen wall on the one side and the receiving stigma surface on the other. The more or less physically determined sticking forces may not be the only true factor. Also in the nature and place of recognition many possibilities are well known and yet the knowledge about the basal mechanism in recognition is not unambiguous , partly because of the diversity in interaction patterns.

Embryo sac development too is a process of several steps, which can be reduced to the number of mitotic divisions versus the formation of cell walls, the position of the spindle and nuclei and the nuclear migration or its degeneration. In fact a mixture of various conditions maybe present in the embryo sacs of ovules within the same ovary. Thereby possibilities to escape from sexual reproduction within the embryo sac or the ovular tissue are well known as apomixis. In the submorphology of the embryo sac many diversifications can be noted at the moment of its maturation.

Of the original functions connected with the egg cell, such as attraction, recognition and prevention of polyspermy, several alternations can be observed. Recognition occurs on, or by means, of sporophytic tissues: attraction can be a function of the egg cell in a number of gymnosperms; whereas in angiosperms probably some attraction features are taken oven by the synergids and extend to the central cell as well. The pathways of the pollen tubes are partly fixed by the sporophytic tissues in stigma and style. The arrival of two sperm cells is actual in some gymnosperms but is resolved by a selective degeneration of one of the sperm cells in the egg cell. Otherwise the ability to make a barrier around the egg cell after penetration of one sperm cell seems to be lost in seed-plants.

A rare feature of the embryo sac is the post-zygotic abortion which occurs as a result of ovular incompatibility In ferns and mosses a kind of zygotic abortion seems to be unknown.

The origin of the ovule and anthers from sporangia is basically comparable and similar, especially in their functions. Heat shock and breeding experiments, however, show that the ovule can produce pollen and the anther embryo sacs, the opposite type of gametophyte.

Both mega- and microgametophytes determine the very specific individuality of the sexual reproduction of each distinct species.

The flower, organ of communication

In the foregoing paragraphs the sexual reproduction is put forward as a developmental process during the course of time, strongly evolved in relation to the biotope, abiotic and biotic, in order to develop diversity and to renew the individual species. On land the flower develops, above all to avoid self-pollination. The gametophytes developing within the flower progressively attain a higher level of differentiation. In conquering landlife, the flower, in its overall functioning, such as pollination and seed dispersal, became involved in both abiotic and biotic surroundings, which evolved too.

During this conquest the plant uses the inherited steps in sexual reproduction, acquired in the biotope, water. These possibilities are amongst otherones the use of each unicelluar step during the life cycle to form an organism, the alternation within a generation, from a diploid to a haploid level and the other way around, which enables the plant to live in different circumstances and which leads to a highly differentiated sporophyte and gametophyte. The recognition and attraction mechanism shifts for the greater part from the gametes to the sporophyte and to the gametophyte. The prologation of the retention of the female gametophytes on the sporophyte ultimately results in a more simply constructed egg cell and the formation of a seed. New and manifest is the construction of the flower, a product of the sporophyte which reflects the relation mainly with the biotic environment. In this respect the flower is the organ of communication, representative of a high level of specialization.

Communication is an very important basic characteristic of life. In this context the gamete fusion is the first step, the function of the flower a manifest accentuation. The communication leads to a further diversification and to a higher level of differentiation.

Reproduction research

Reproduction research studies the flower in its development, complexity and diversity. Some of the experimental results can already be incorporated in our cognizance of reproduction, but owing to our insufficient in sight into the normal complicated development of sexual processes some cannot be fitted in as yet. The realization of the existence of a reproductive calendar, the characteristics of development, the external circumstances, the course of the normal process and the multiple function of the flower, are some of the points to keep in mind during experimental research.

Budding, flowering and fruiting all are closely related to sexual reproduction and seed dispersal. Research on sexual reproduction, a fascinating complex study, is flowering, and will be fruiting at both the fundamental and applied levels.

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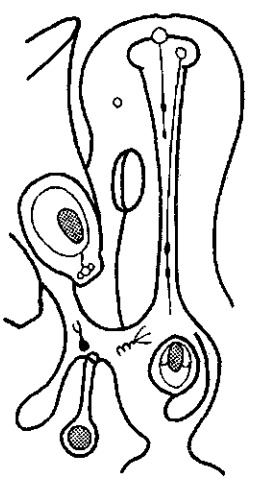

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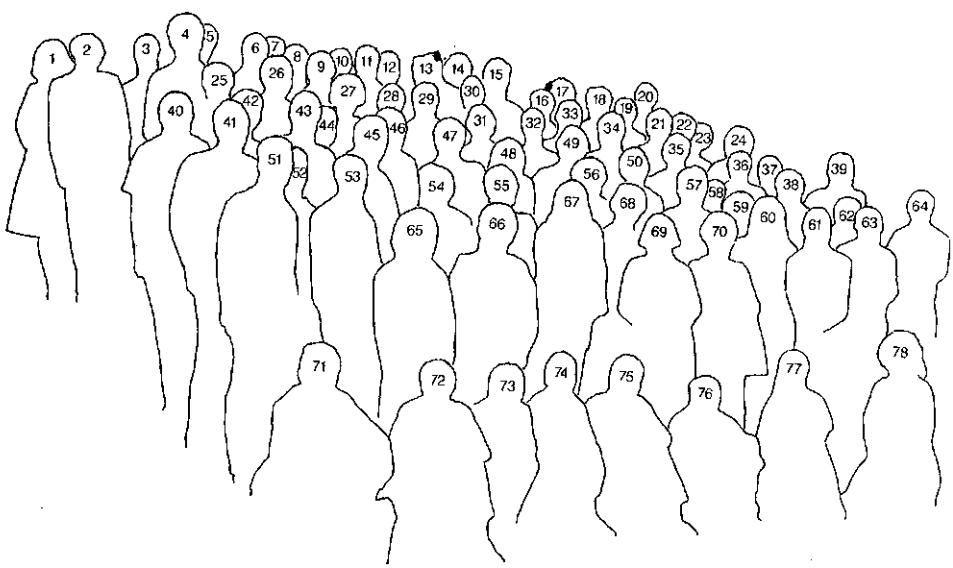

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