FLOWER DEVELOPMENT OF *Begonia franconis* LIEBM.

Promotor: Dr. J. Bruinsma, hoogleraar in de fysiologie der planten.

J. Berghoef

FLOWER DEVELOPMENT OF *BEGONIA FRANCONIS* LIEBM.

PROEFSCHRIFT
TER VERKRIJGING VAN DE GRAAD VAN
DOCTOR IN DE LANDBOUWWETENSCHAPPEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS,
DR. H.C. VAN DER PLAS
HOOGLERAAR IN DE ORGANISCHE SCHEIKUNDE,
IN HET OPENBAAR TE VERDEDIGEN
OP VRIJDAG 7 SEPTEMBER 1979
DES NAMIDDAGS TE VIER UUR IN DE AULA
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN.

ISN 104858_03

STELLINGEN

I

Regulatoren hebben geen direkte invloed op de geslachtsdifferentiatie van de bloemen van *Begonia franconis* Liebm.

Dit proefschrift.

II

Voor de differentiatie van vrouwelijke bloemen van *Begonia franconis* Liebm. is het gehalte aan assimilaten een beperkende factor.

Dit proefschrift.

III

Als bewijs voor de direkte beïnvloeding van de geslachtsbepaling bij *Cucurbitaceae* door regulatoren is het werk van Galun e.a. als enig concreet voorbeeld onvoldoende.

E. Galun, Y. Jung en A. Lang. Develop. Biol. 6, 370-387 (1963).

IV

De conclusie van Zieslin en Halevy dat de bloemknopatrofie bij de roos een gevolg is van een daling van de gibberelline activiteit wordt door hen niet bewezen.

N. Zieslin en A.H. Halevy. Physiol. Plant. 37, 317-335 (1976).

V

Waargenomen hormoonconcentraties in plantenweefsel zijn vaker een gevolg van een bepaalde orgaanontwikkeling dan de oorzaak hiervan.

VI

Bij de vegetatieve vermeerdering *in vitro* wordt ten onrechte meestal onvoldoende rekening gehouden met de groeiomstandigheden van het uitgangsmateriaal.

VII

Het vermelden van concentraties in mg/l bij de *in vitro* cultuur maakt een onderlinge vergelijking van voedingsmedia tot een rekenkundig in plaats van een fysiologisch probleem.

VIII

De groeibevorderende werking *in vitro* door toevoeging van ammonium aan nitraatbevattende media moet in de eerste plaats worden toegeschreven aan de snelle opname van ammonium.

IX

De verkoop van snijbloemen wordt geremd door de hardnekkige handel in onrijpe snijbloemachtige produkten.

X

Bolbloemen zijn energievriendelijk.

XI

Hogere beloning voor onaantrekkelijk werk geeft een grotere bijdrage tot vermindering van de werkeloosheid dan aftopping van de hogere inkomens.

J. Berghoef
Wageningen, 7 september 1979

INHOUD

VOORWOORD

Gaarne maak ik van de gelegenheid gebruik om allen te bedanken die aan dit proefschrift hebben meegewerkt.
In de eerste plaats gaat mijn dank uit naar mijn promotor, Prof.dr. J. Bruinsma, die door zijn enthousiasme en goede raadgevingen een stimulans voor mij betekende om het onderzoek te volbrengen.

Alle medewerkers van de vakgroep Plantenfysiologie LH dank ik voor de waardevolle suggesties en de prettige werksfeer.
In het bijzonder wil ik Mw. Y.E. v. Oosten-Legro bedanken.
Yvonne, zonder jouw uitstekende assistentie zou dit proefschrift nooit compleet zijn geweest. Ook de heer H. van Oeveren en de studenten Ben, Ben en Kees ben ik voor hun bijdrage dankbaar.

De vakgroep Plantencytologie en -morfologie LH ben ik erkentelijk voor de verleende faciliteiten voor het anatomische gedeelte van het onderzoek. De heer A.C. van Aelst dank ik voor het maken van de foto's van de microscopische preparaten.

Door de goede verzorging van de planten door de heren J. Verburg en G. Geerenstein was er altijd voldoende materiaal voor het onderzoek beschikbaar. Het fotowerk werd verricht door de heren T. Zaal en R. Jansen, het tekenwerk door Mw. S. Mastenbroek-Kuyper en de heer H. van Lent, waarvoor mijn dank. De verzorging van het type-werk was in goede handen van Joke en Corry terwijl de afdeling Tekstverwerking LH de definitieve versie verzorgde. De omslag werd verzorgd door Mariet de Geus.

De Landbouwhogeschool ben ik erkentelijk dat zij mij dit werk als promotie-assistent heeft laten doen.

Tenslotte bedank ik mijn ouders die dit alles mogelijk hebben gemaakt door hun vertrouwen en geduld tijdens mijn studie.

INLEIDING

Bloemen zijn voor de plant noodzakelijk voor het in stand houden van de soort. De essentiële organen hierbij zijn de meeldraden en de stampers als respectievelijk de mannelijke en vrouwelijke geslachtsorganen. In een bloem kunnen beide geslachtsorganen aanwezig zijn (hermafrodiete bloemen) of slechts een van de twee (eenslachtige bloemen). De eenslachtige, mannelijke of vrouwelijke, bloemen kunnen op één plant voorkomen (eenhuizig) of op verschillende planten (tweehuizig). HESLOP-HARRISON (1972) geeft een overzicht van de verschillende geslachtstypen bij Angiospermen. Volgens dit overzicht is het geslacht *Begonia* overwegend eenhuizig, dat wil zeggen dat de mannelijke en vrouwelijke bloemen voorkomen op dezelfde plant. Bij het merendeel van de *Begonia*-soorten staan de mannelijke en vrouwelijke bloemen in één bloeiwijze, waarbij de mannelijke bloemen worden aangelegd voor de eindstandige vrouwelijke bloemen.

Mogelijkheden tot regulering van de geslachtsexpressie kunnen belangrijk zijn bij de produktie van bloemen en vruchten en bij de plantenveredeling. Bij *Begonia* zou een vergroting van het aantal vrouwelijke bloemen de sierwaarde kunnen verhogen, omdat mannelijke bloemen een sterkere neiging tot abcissie vertonen dan de vrouwelijke bloemen. (HANISCH TEN CATE e.a., 1975).

Bij veel planten is de geslachtsexpressie afhankelijk van uitwendige factoren zoals temperatuur, lichtintensiteit en minerale voeding. Een uitgebreid overzicht hiervan geven NAPP-ZINN (1967) en HESLOP-HARRISON (1972). Bij *Begonia* werd een toename van de vrouwelijke bloei gevonden bij een verbetering van de minerale voeding (MATZKE, 1938) en bij een stijging van de temperatuur (HEIDE, 1969). Verder zijn er aanwijzingen dat een hoge lichtintensiteit de vorming van vrouwelijke bloemen bevordert (NOACK, 1962; LICHTENBERG, 1971; PREIL, 1974).

Algemeen wordt aanvaard dat de geslachtsexpressie gereguleerd wordt door de hormonale samenstelling van het weefsel tijdens de bloemknopdifferentiatie. In de meeste gevallen bevorderen gibberellinen de mannelijke bloei, terwijl ethyleen en auxinen de vrouwelijke bloei stimuleren (bijv. GALUN, 1959; PETERSON en ANHDER, 1960; BHANDARI en SEN, 1973; CORLEY, 1976). Cytokininen kunnen vervrouwelijkend werken door een bevordering van de uitgroei van de stamper (DE JONG en

BRUINSMA, 1974; POOL, 1975). Het meeste onderzoek naar de regulatie van de geslachtsexpressie is gedaan bij de *Cucurbitaceae* maar ook andere genera zijn gebruikt, bijv. *Cannabis* (KOHLER, 1964; MOHAN RAM en JAISWAL, 1970), *Carica* (JINDAL en SINGH, 1976) en *Begonia* (HEIDE, 1969). In een aantal gevallen worden resultaten vermeld die afwijken van het eerder genoemde algemene patroon. Bij mais (KRISHNAMOORTHY en TALUKDUR, 1976) en *Begonia* (HEIDE, 1969) geven gibberellinen vervrouwelijking, terwijl auxinen bij *Mercurialis* vermannelijkend werken (CHAMPAULT, 1969).

In het algemeen worden de effecten van regulatoren bestudeerd door behandeling van de gehele plant. Een bezwaar is dat hierdoor vele processen in de plant kunnen worden beïnvloed, waardoor de groei en ontwikkeling van de plant kunnen veranderen. We kunnen dan niet bepalen of een verschuiving in de geslachtsexpressie een direkt gevolg is van de toegepaste regulator of slechts een indirekt gevolg hiervan is door een veranderde groei en ontwikkeling van de plant. Door bepaling van de endogene hormoongehalten zou men hierin meer inzicht kunnen verkrijgen. Echter, een waargenomen hormoonpatroon behoeft niet de oorzaak te zijn van de gevonden geslachtsexpressie maar kan hiervan juist een gevolg zijn. CONRAD en MOTHES (1961) vonden dat vrouwelijke planten van *Cannabis* een hoger auxinegehalte bezitten dan mannelijke planten. Wanneer echter de auxinebepalingen werden gedaan voor de aanleg van de bloemen, was er geen verschil tussen de planten (CONRAD, 1962).

De enige methode waarmee de direkte invloed van regulatoren op de geslachtsexpressie kan worden bepaald is de kweek van bloemknopprimordia *in vitro*. Hierbij is interferentie van andere delen van de plant uitgesloten. GALUN e.a. (1962) gebruikten deze methode als eersten bij bloemen van de komkommer, waarna de *in vitro* methode ook bij andere planten werd toegepast (bijv. BLAKE, 1969; BILDERBACK, 1972; DE JONG en BRUINSMA, 1974). Voor een juiste interpretatie van de resultaten is het noodzakelijk dat de bloemen *in vitro* een normale ontwikkeling vertonen. Bij bloemknopprimordia van *Aquilegia* vonden TEPFER e.a. (1966) een positief effect van auxinen op de aanleg van vruchtbladen. De verdere ontwikkeling van de bloemknoppen was echter gering. BILDERBACK (1972) gebruikte een medium waarop de bloemknoppen zich beter ontwikkelden en vond geen effect meer van auxinen op de vorming van de vruchtbladen. Omdat de samenstelling van het voedingsmedium voor elke plant verschillend kan zijn, is het belangrijk dat hieraan veel aandacht wordt besteed. Slechts dan is het mogelijk de invloed van regulatoren op de bloemknopdifferentiatie te bestuderen.

Het onderzoek naar de regulatie van de geslachtsexpressie bij *Begonia* is ge-

daan omdat het een belangrijk probleem is, in verband met de knopval van mannelijke bloemen, terwijl de resultaten in de literatuur bij verschillende *Begonia*-soorten uiteenlopend zijn. *Begonia franconis* Liebm. is gekozen omdat de mannelijke en vrouwelijke bloemen bij deze plant een vaste positie hebben in de bloeiwijze en zeer vele bloeiwijzen per plant worden gevormd. Daarnaast was de geringe grootte van de bloemen en de bloeiwijzen belangrijk, vooral ook bij de kweek *in vitro*.

In het eerste gedeelte wordt de bouw van de bloeiwijzen beschreven. Verder wordt de invloed van uitwendige factoren en van behandelingen met regulatoren op de samenstelling van de bloeiwijzen gegeven. In het tweede en derde gedeelte wordt de cultuur van bloeiwijzen *in vitro* beschreven. Het tweede gedeelte behandelt de invloed van voedingsstoffen en regulatoren op de groei van de bloemknoppen *in vitro*. In het derde gedeelte wordt de invloed van regulatoren en van saccharose op de differentiatie van organen in bloemknopprimordia besproken. Het is echter mogelijk dat bij bloemknopprimordia het geslacht reeds in een zeer vroeg stadium is vastgelegd en een verandering *in vitro* niet meer mogelijk is. Daarom wordt in het vierde gedeelte de vorming van adventieve bloemknoppen beschreven en de invloed van regulatoren en saccharose op het geslacht van deze adventieve bloemknoppen.

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FLOWER DEVELOPMENT OF *BEGONIA FRANCONIS* LIEBM. I EFFECTS OF GROWTH-REGULATING SUBSTANCES AND ENVIRONMENTAL CONDITIONS ON THE COMPOSITION OF THE INFLORESCENCE.

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Summary

The effects of growth-regulating substances and environmental conditions on the composition of *Begonia franconis* Liebm. inflorescences were analysed. The inflorescences are generally composed of two male flowers and one terminal female flower.

Auxins, gibberellins, and cytokinins, added to the branch apices, as well as low light intensity, promoted male flowering by increasing the number of male flowers. Removal of branches as well as application of cytokinins induced ramification of the inflorescences by outgrowth of the normally dormant axillary bud in the bract of male flowers. A transition of male flowers into female flowers was not observed.

A model of the sex-regulating mechanism in relation to hormones and environmental conditions is put forward. On the principle that female development requires a high nutritional level it is suggested that the level of assimilates is important in regulating sex expression, hormones influencing the sex of flower buds through their regulation of the flow of assimilates.

Key words: Begonia, sex expression, environment, growth-regulating substances.

Abbreviations: ABA, abscisic acid; BA, N^6-benzyladenine; chlormequat: (2-chloroethyl)trimethylammoniumchloride; Ethephon, (2-chloroethyl) phosphonic acid; GA_3, gibberellin A_3; GA_{4+7}, gibberellins A_4 and A_7; IAA, indolyl-3-acetic acid; NAA, 1- naphtaleneacetic acid; 2iP, N^6-isopentenyladenine.

Introduction

Regulation of flower development has been extensively investigated, in particular the factors regulating sex expression (NAPP-ZINN, 1967; HESLOP-HARRISON, 1972). The sex of flowers in many monoecious and dioecious plants was found to be influenced by growth-regulating substances and environmental conditions.

Most reports deal with monoecious cucurbit plants in which auxins, ethylene, and ABA generally promote female flowering and gibberellins enhance male flowering (*e.g.* GALUN, 1959; PETERSON and ANHDER, 1960; RUDICH and HALEVY, 1974). This was also found with *Cannabis sativa* (KOHLER, 1964; MOHAN RAM and JAISWAL, 1970), *Carica papaya* (GHOSH and SEN, 1975; JINDAL and SINGH, 1976) and other plants (*e.g.* BHANDARI and SEN, 1973; CORLEY, 1976). However, auxins have occasionally been found to promote male flowering (CHAMPAULT, 1969) and gibberellins to enhance female development (KRISHNAMOORTHY and TALUKDUR, 1976; HEIDE, 1969). ABA showed different effects when applied on monoecious or gynoecious cucumber plants (FRIEDLANDER *et al.*, 1977). Moreover, cytokinins enhance the formation of female organs in a number of plants (*e.g.* DURAND, 1969; HASHIZUME and IIZUKA, 1971; DE JONG and BRUINSMA, 1974b).

With monoecious *Begonia* species hormonal factors and environmental conditions also cause variations in the ratio between male and female flowers. MATZKE (1938) found a decrease of the male:female ratio by a higher nutrition level with *Begonia semperflorens*. NOACK (1962) noted a positive correlation between the duration of sunshine and the transformation of male into hermaphrodite flowers with *Begonia cathayana*. LICHTENBERG (1971) and PREIL (1974) found indications that a high light intensity promotes the formation of female flowers of *Begonia semperflorens*. HEIDE (1969) investigated the sex expression of *Begonia x cheimantha*. High temperature and long days promoted the formation of female flowers on this short-day plant. Both IAA and GA_3 decreased flowering and enhanced female sex expression, while these regulators had no effect on *Begonia semperflorens* (LICHTENBERG, 1971).

Because, on the one hand, the data obtained with different *Begonia* species are conflicting and, on the other hand, sex expression is an important feature, especially with cultured varieties liable to drop of male flower buds, a detailed study of the effects of growth-regulating substances and environmental conditions was initiated. For this purpose *Begonia franconis* Liebm. was selected because this species forms a great many small inflorescences of male and female flowers in predetermined positions. The limited size of flowers and inflorescences makes them also suitable for studies *in vitro* as will be described in subsequent papers.

Material and Methods

A clone of *Begonia franconis* Liebm. was cultivated in the glasshouse, minimum temperatures 21° C (day) and 18° C (night). The photoperiod was maintained at 16 hrs by additional illumination from high-pressure mercury lamps (Philips HLRG 400W) at 18 W m^{-2}.

Solutions of BA, zeatin, 2iP, kinetin, GA_3, GA_{4+7}, and ABA were made by dissolving the regulators in a few drops of 0.1 N NaOH and dilution with distilled water. NAA and IAA were used as the potassium salts. The pH of all solutions was adjusted to 6.0 with HCl. 50 ppm Triton X-100 was added as a wetting agent. The regulators were applied daily (5x/week) by putting a droplet of 10 µl on the apex of the branches. To indicate the start of the treatments, the smallest visible inflorescence was removed, preliminary experiments showing no effect of this removal. The first two inflorescences formed were not taken into consideration to ascertain that all recorded inflorescences were initiated during the treatments. The composition of the inflorescences was recorded every 10 days for a period of 6 - 8 weeks.

Each treatment consisted of 4 - 7 plants of which 4 branches were treated. The experiments were repeated at least once. Unbranched plants were obtained by taking a top-cutting of a flowering branch of which all lateral buds became inflorescences, resulting in a plant without side branches. Per treatment 10 - 20 unbranched plants were used. A low light intensity was achieved by using tents of cheese cloth.

Results

Composition of the inflorescences. In *Begonia franconis* Liebm., the axillary bud of every leaf develops into an inflorescence. The inflorescences are generally composed of two male flowers and one female flower. The male flowers always have two bracts and are never terminal, whereas the female flower has no bracts and is invariably the terminal one. Frequently, deviating inflorescences occur, carrying a different number of male and female flowers. The inflorescences can be divided into three types, depending on the numbers of male flower primordia initiated before formation of the female flower(s) (Fig. 1).

Type I: One male and one female flower. The first inflorescence on a branch is always of this type, which seldomly occurs at other positions on the branch. As type I has never been found in relation to a treatment and its occurrence is always less than 1%, it is omitted from the Figures and Tables.

Type II: Two male flower primordia are initiated before formation of the female flower primordium. Type IIa is the commonly encountered inflorescence. With types

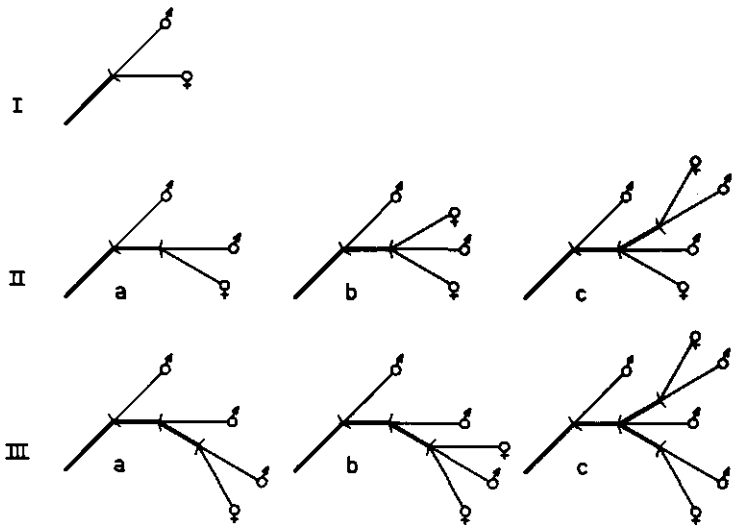

Fig. 1: Classification of inflorescences. For explanation see text.

IIb and IIc the lateral bud in the axil of the bract of flower primordium 2 (the second male flower) is activated. This activated bud can be female (IIb) or male (IIc), in the latter case one or two terminal female flowers are formed in the axils of the bracts of this male flower.

Type III: Three male flower primordia are present before the female flower is in-

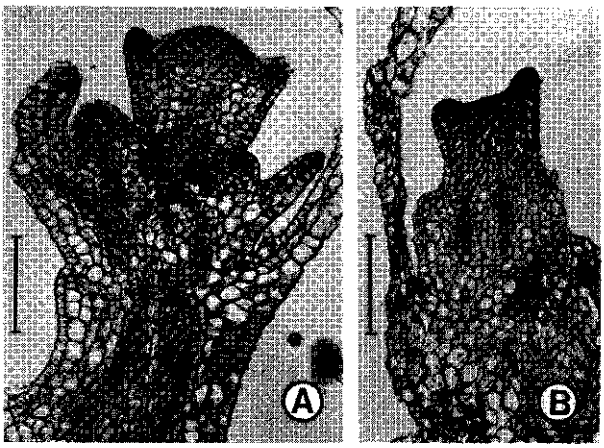

Fig. 2: Male (A) and female (B) bud with perianth primordia initiated. Bars represent 100 µm.

itiated. In some cases more flowers are formed (IIIb and IIIc, or even more complex forms) by bud activation as described for type II.

Anatomical studies indicated a difference between male and female buds from the start of differentiation of the perianth, male buds being round-shaped and female buds flattened (Fig. 2).

Because there was no sign of pistil primordia in male buds or of stamen primordia in female buds, the sex of these flowers is not a result of selective growth of stamens or pistils, as in Cucurbit flowers, but is determined at a very early stage of development already. Male flowers have four perianth leaves and female flowers five, so the sex determination presumably takes place before or during the initiation of these perianth primordia. Very rarely, however, intermediate flowers were found, female flowers with only four perianth leaves or hermaphrodite flowers with naked superior ovaries, 1 - 3 pistils and some stamens, indicating that a shift of sex is possible at later stages of development.

The number of branches on a plant has an effect on the composition of the inflorescences (Table 1). In this experiment the unbranched plants were obtained as described in Material and Methods, the plants with four branches by removing all 20 - 30 branches except four. Removal of branches activated the bud in the bract of the second male flower, so that the percentage of higher developed inflorescences (type IIb and c) increased. The number of primary male flowers was not increased, the percentage of type III remaining at 1%.

Table 1. Effect of branching of plants on inflorescence composition.

	Type of inflorescences (%)				Total number of
	IIa	IIb	IIc	III	inflorescences
unbranched	71	22	6	1	204
4 branches	86	10	3	1	258
branched	94	3	2	1	189

Growth-regulating substances. Preliminary experiments showed that addition of 50 ppm Triton X-100 to the treatment solutions increased the effect of the growth-regulating substances and did not affect the composition of the inflorescences. The addition of growth regulators to the apices of particular branches never affected the inflorescence composition of nearby untreated branches.

The effects of IAA, GA_3, zeatin, and BA on inflorescence composition are

presented in Fig. 3. The auxin, IAA, had little effect, at high concentrations only it led to the production of a third primary male flower, giving at most 20% type III. It decreased the size of the male and female flowers but never completely inhibited their formation. 10^{-3} M IAA caused some apical necrosis. NAA gave similar results as IAA at one tenth of the IAA concentrations, i.e. necrosis already at 10^{-4} M.

GA_3 similarly promoted male development, at 10^{-4}M over 40% of the inflorescences being of type III. Likewise flower size diminished but the pedicel elongated considerably. At 10^{-5}M GA_3 5%, and at 10^{-4}M 8% of the inflorescences had

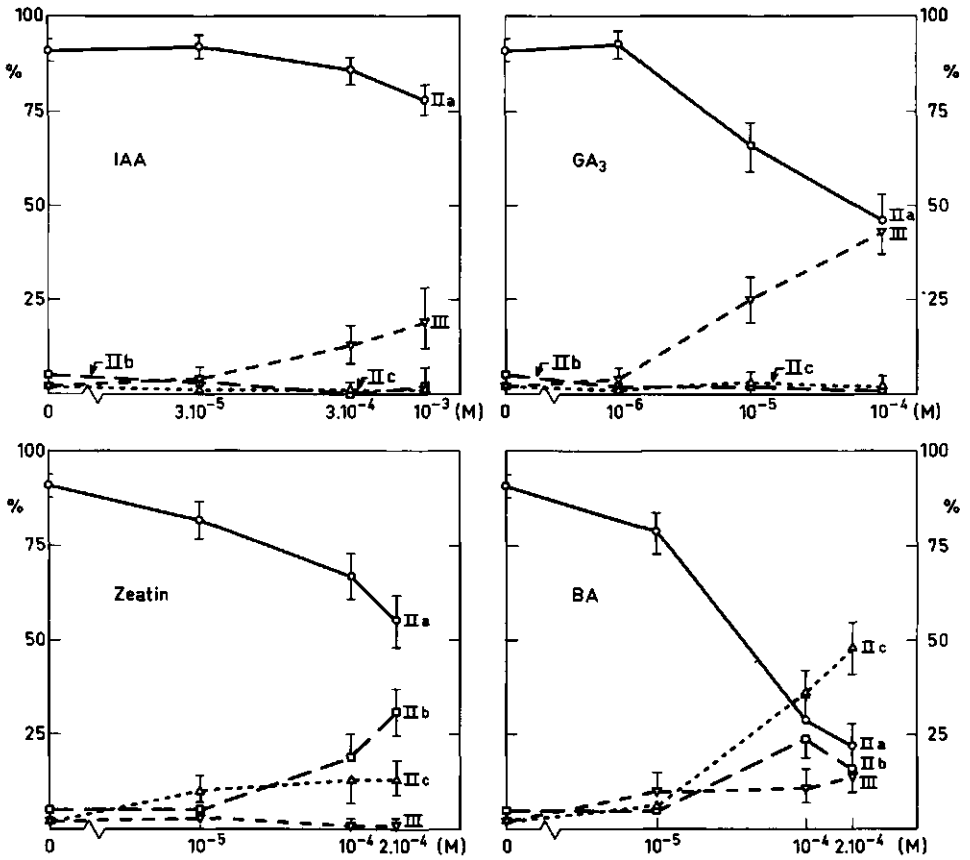

Fig. 3: Effect of growth-regulating substances on inflorescence composition. Bars indicate 95% confidence limits.

no female flower at all, and of these male inflorescences 95% was reduced to a single male flower. The pedicel of the male flower still had two bracts, but the subsequent primordia did not develop further. GA_{4+7} gave the same results as GA_3.

The cytokinin, zeatin, had a contrary effect. A third male flower never developed, instead the axillary bud of the bract of the second male flower was activated so that at 2.10^{-4}M nearly half of the inflorescences developed into the types IIb and c. Zeatin also increased the size of male and female flowers, the latter often contained more than the usual three carpels and pistils.

Whereas kinetin and 2iP were completely inactive, BA had even stronger effects than zeatin. At 2.10^{-4}M the normal type IIa was reduced to below 25%. Even type III was significantly enhanced, 65% of it being IIIc, inflorescences with 7 - 12 flowers frequently occurred.

ABA, ethephon, and chlormequat were tested over a wide concentration range, up to levels that completely inhibited growth, but none of the treatments significantly affected inflorescence composition. ABA and ethephon did not reduce the effect of gibberellins.

Auxins, gibberellins and cytokinins also affected the internode length, as well as the number of inflorescences per branch, indicative of the rate of development, as the lateral bud of every leaf became an inflorescence (Table 2). Both cytokinins and gibberellins accelerated the development, whereas auxins were slightly inhibitory. Similarly GA_3, and to a lesser extent BA, promoted, and IAA decreased internode length.

Table 2. Effect of growth-regulating substances on number of inflorescences per branch and mean internode length in a typical experiment

	mean number of inflorescences/branch	mean internode length (cm)
control	8.3a	2.3a
10^{-4}M BA	11.0b	2.9b
10^{-4}M GA_3	10.2b	4.1c
3.10^{-4}M IAA	7.2c	1.8d

Numbers followed by different letters differ significantly (P = 0.05).

Environmental factors. The effect of *light intensity* on inflorescence composition of branched and unbranched plants is given in Table 3. Shading with cheese cloth reduced ramification of the inflorescences (types IIb and c) and promoted

its extension (type III), with both branched and unbranched plants. A difference between branched and unbranched plants was not apparent in the shaded condition. Accordingly in summer more type IIb and c inflorescences were recorded than in winter but the effect of shading was similar in both seasons.

The effect of *temperature* was analysed in a phytotron at 17, 21 and 25° C,

Table 3. Effect of shading on inflorescence composition of branched and unbranched plants

Plants	light intensity	Type of inflorescence (%) IIa	IIb	IIc	III	Total number of inflorescences
branched	100%	92	7	1	0	278
	30%	91	0	0	9	149
unbranched	100%	65	26	9	0	140
	30%	89	0	1	9	106

Experiment from november 1975 until january 1976

however, no significant effect on the composition of the inflorescences was established. To see whether *mineral nutrition* affected inflorescence compositions, an experiment was done at 3 nutritional levels by the addition of 0, 2 and 6g/l N:P:K fertilizer every ten days. Although fertilizer treatment stimulated growth considerably, there was no difference in the composition of the inflorescences, neither in branched nor in unbranched plants.

Because roots are a primary source of endogenous cytokinins, plants were cultivated in different *pot volumes* (0.25, 0.50 and 1.1 l) to obtain differently developed root systems. Again, although vegetative growth was greatly affected, the composition of the inflorescences was the same in all treatments.

Interaction of environmental factors and growth-regulating substances.

Since removal of branches and treatments with cytokinins increased ramification, whereas gibberellins, auxins, and low light intensity promoted inflorescence extension, the possible relationship between these factors was explored by applying growth-regulating substances alone or in combinations to branched and unbranched plants under normal and shaded conditions.

Table 4 shows the effects of growth-regulating substances on branched and unbranched plants. The auxin, IAA, prevented ramification of the inflorescences of

the branched plants but not of the unbranched plants. It also did not break through the inhibition of inflorescence extension of the unbranched plants. GA_3 gave similar results as IAA. The ramification of the inflorescences of unbranched plants was increased by the cytokinin, BA. Combined application of BA and GA_3 induced ramification of the inflorescences (type IIc] and even diminished the inhibition in the unbranched plants of the formation of a third primary male bud (type III). Combined application of BA and IAA gave similar results. The effects of growth-regulating substances on branched plants in the normal and shaded conditions are shown in Table 5. The effects of shading were enhanced by IAA and GA_3 giving inhibition of ramification of the inflorescences and promotion of the formation of a third primary male bud. BA removed the inhibition of ramification in low light intensity and promoted the extension of the inflorescences under both

Table 4. Effect of growth-regulating substances on inflorescence composition of branched and unbranched plants

		Type of inflorescence (%)				Total number of inflorescences
		IIa	IIb	IIc	III	
control	br.	73	10	10	7	191
	unbr.	68	18	13	1	88
3.10^{-4}M IAA	br.	79	0	2	19	126
	unbr.	71	20	6	3	65
10^{-4}M GA_3	br.	60	1	5	29	176
	unbr.	54	22	20	4	81
10^{-4}M BA	br.	31	16	47	6	165
	unbr.	17	27	53	3	70
BA + GA_3	br.	48	6	24	22	188
	unbr.	22	14	55	9	78
BA + IAA	br.	51	11	25	13	153
	unbr.	26	17	51	6	74

br. = branched; unbr. = unbranched.
Experiment from july until september 1976.

light conditions. This was also brought about by the combined applications of BA and GA_3 and of BA and IAA, by which the extension of inflorescences in particular was promoted.

Table 5. Effect of growth-regulating substances on inflorescence composition in normal and shaded conditions.

	light-condition	Type of inflorescence (%) IIa	IIb	IIc	III	Total number of inflorescences
control	100%	85	8	5	2	352
	30%	94	0	0	6	212
3.10^{-4}M IAA	100%	77	6	3	14	125
	30%	75	0	0	25	77
10^{-4}M GA_3	100%	65	0	1	31	194
	30%	64	0	2	31	128
10^{-4}M BA	100%	46	15	25	14	195
	30%	71	7	8	14	141
BA + GA_3	100%	46	6	12	36	218
	30%	30	9	11	50	116
BA + IAA	100%	58	13	10	19	174
	30%	57	7	8	28	129

Experiment from february until april 1976.

Discussion

The general feature emerging is that sex expression in *Begonia franconis* Liebm. differs from that in other *Begonia* species and cultivars in that it is quite stable. The sex of the flowers is determined at a very early stage of primordium differentiation already (Fig. 2) and growth-regulating substances and environmental conditions mainly affect sex expression indirectly, by influencing the number of flowers, that is, the position of the flowers in the inflorescence.

The inflorescence is a sympodial cyme (Fig. 1) so that every flower develops from an apical bud and the inflorescence continues by development of an axillary bud in the bract of a flower. This only occurs with male flowers, the inflorescence is always terminated by a female flower without bracts. The first inflorescence at a branch may develop only one male flower (type I), normally two male

flowers develop (type II), sometimes three (type III) before formation of a female flower. Moreover, the inflorescence may branch by the development of the axillary bud of the second (or third) male flower (types IIb and c, IIIb and c).

The sex of the first male flower is never changed and the second bud is female only in the rather underdeveloped first inflorescence on a branch (Type I). The third bud is very interesting from the point of sex expression. Normally this is a terminal female bud (Type II) but gibberellin, and to a lesser extend auxin, cytokinin and shading, can induce a third male bud (Type III), combinations of these factors having enhanced effects.

Removal of branches inhibited the formation of this third male bud (Table 4) except at low light intensity. Therefore, unbranched plants must have a factor inhibiting inflorescence extension that can be neutralized by low light intensity. SAITO and ITO (1963) reported about specific substances produced by the leaves of cucumber and regulating sex expression, low levels promoting male flowering and high levels stimulating female development. TSE *et al.* (1974) found an increase in the level of assimilates in the shoot tips and a better development of the inflorescences of *Bougainvillea* by removal of the young leaves, probably by removing the sink activity of these leaves. The same was found with tomato (KINET, 1977), while DE JONG and BRUINSMA (1974a) noted that removal of mature, assimilate-exporting leaves of *Cleome* inhibited pistil development. NOACK (1962), LICHTENBERG (1971) and PREIL (1972) all found promotion of female flowering by improved illumination conditions of various *Begonia* species.

Thus, assimilate level may be one of the factors involved, high levels being required for female development. As unbranched plants have larger leaves and branches do not compete mutually, a higher level of assimilates would explain why unbranched plants only gave a third male bud at low light intensity.

On this principle of the requirement of high assimilate levels for female differentiation the following model for inflorescence differentiation can be postulated. At the start of differentiation of the inflorescence its sink capacity will be relatively small, the vascular system being not yet well developed (Fig. 2), whereas the vegetative apex close above will compete considerably. The resulting low assimilate input induces the buds to differentiate as male. During this differentiation the inflorescence will increase its sink activity by the hormone production of the developing buds (*e.g.* JEFFCOAT and HARRIS, 1972; PATRICK and WAREING, 1972; GOLDSCHMIDT and HUBERMAN, 1974). The vascular system becomes more developed, so that a higher level of assimilates enables female development, normally when the third bud starts to differentiate.

The effects of growth-regulating substances on sexual differentiation can be indirect through a redirection of the flow of assimilates. The addition of GA_3 and BA to the vegetative apex considerably enhances its vigor and, as with apical dominance, can divert the sap flow from the developing inflorescence. This leads to reduced assimilate levels during flower differentiation, resulting in a third male flower. This may also be the case in such other species as cucurbits, where ethylene decreases growth and promotes female development, whereas gibberellins increase growth and promote male development (*e.g.* IWAHORI *et al.*, 1970; SPLITT-STOESSER, 1970; RUDICH and HALEVY, 1974].

Moreover, the findings of FUCHS *et al.* (1977] that gibberellin treatment of cucumber did not give a reversal of future pistillate flowers but rather an abortion of these and growth of primordial staminate buds, that normally never reach an appreciable development, also indicate that the effect of gibberellins on the sex-regulating system is indirect.

The effects of cytokinins are more complex. Firstly, they activate the axillary bud of the second male flower bract, resulting in branched inflorescences. Secondly, they promote male development by increasing the number of primary male buds (type III). Thirdly, they also promote female development in that they increase the numbers of carpels and pistils. However, this may not be a direct promotion of female development, but merely a distortion of the generative apex, comparable with cytokinin-induced fasciations.

However, the present results do not exclude a direct effect of growth-regulating substances on inflorescence composition. Such an effect can only be verified by the culture of young flower buds *in vitro*. Results of these studies will be described in subsequent papers.

Acknowledgements. The skilful technical assistance of Mrs. Y.E. v. Oosten-Legro is gratefully acknowledged.

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FLOWER DEVELOPMENT OF *BEGONIA FRANCONIS* LIEBM. II EFFECTS OF NUTRITION AND GROWTH-REGULATING SUBSTANCES ON THE GROWTH OF FLOWER BUDS *IN VITRO*.

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Summary

The development of flower buds of *Begonia franconis* Liebm. was studied *in vitro*. Inflorescences with two male and one female bud primordium were inoculated on chemically defined media to analyse the requirements for optimum growth.

Omission of agar increased growth of the buds, on a liquid medium the buds reached a normal and complete development. Growth required both nitrate and ammonium. A cytokinin was also necessary for bud growth, the optimum cytokinin concentration for the female bud being 10 to 30 times higher than that for the male buds.

IAA and ethephon had no effect on bud size, but ABA decreased growth if applied together with cytokinin. Although GA_3 had no effect, GA_{4+7} promoted the length of the male perianth.

Key words: Begonia, flower bud, in vitro culture

Abbreviations: ABA, abscisic acid; BA, N^6-benzyladenine; GA_3, gibberellin A_3; GA_{4+7}, gibberellins A_4 and A_7; IAA, indolyl-3-acetic acid; 2iP, N^6-isopentenyladenine.

Introduction

Growth regulators can affect the composition of inflorescences of *Begonia franconis* Liebm. (BERGHOEF and BRUINSMA, 1979a). Using entire plants, it is impossible to distinguish whether the effects of the regulators are direct or indirect. Therefore, inflorescence primordia were isolated and studied deprived of influen-

ces from other plant parts *in vitro* culture.

Since GALUN *et al* (1962) reported about the culture of cucumber flower buds *in vitro*, several studies on this subject have been published (*e.g.* BLAKE, 1969; HICKS and SUSSEX, 1970; BILDERBACK, 1972; DE JONG and BRUINSMA, 1974). However, only in a few cases normal development of the flower buds was achieved, propably due to a sub-optimum composition of the medium. A study to render the basal medium optimum should precede any investigation using the *in vitro* technique (DE JONG *et al.*, 1974).

The optimum composition of the medium for the growth of flower buds *in vitro* is developed in the present study. Also the effects of growth-regulating substances on bud growth are analysed.

Material and Methods

The culture of the clone of *Begonia franconis* Liebm. plants was described earlier (BERGHOEF and BRUINSMA, 1979a). Young inflorescences were collected when the largest male bud had reached a length of 2.0 - 2.5 mm, the second male bud of about 1.0 mm and the female bud of 0.4 - 0.7 mm (Fig. 1). The inflorescences were sterilized in 0.6% NaClO + 50 ppm Triton X-100 during 4 min and rinsed extensively with sterile tap water.

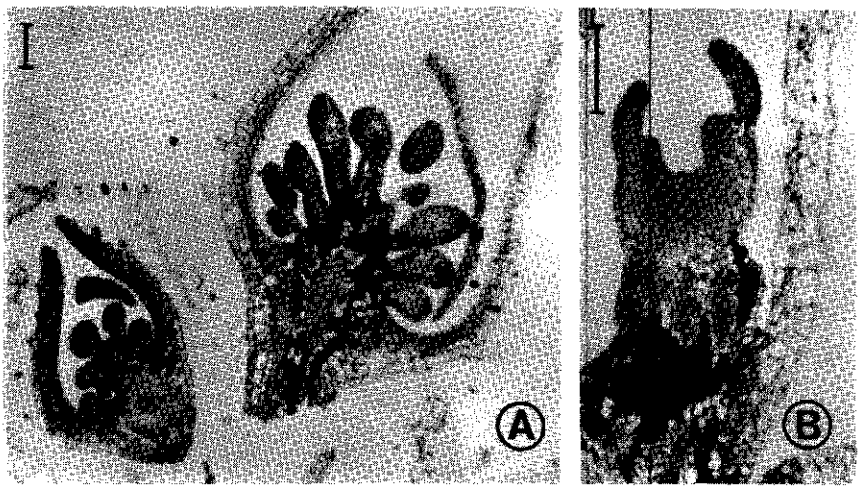

Fig. 1 Stage of flower bud differentiation at the onset of the experiments. A: first and second male bud. B: female bud. Bars represent 100 µm.

The composition of the original basal medium, BM, is given in Table 1. Erlenmeyer flasks of 100 ml containing 25 ml BM, closed with a Steristop, were

autoclaved for 20 min at 110° C. When amino acids were added, these were filter-sterilized (0.45 µm) and added to the autoclaved medium at about 50° C. The later developed liquid, new basal medium, NBM, had the same composition except that the agar was omitted and the calcium content reduced to 1.0 mM. It was completely filter-sterilized.

Experimental samples always consisted of 4 Erlenmeyer flasks, each with 5 inflorescences. The experiments were repeated at least once. The Erlenmeyer flasks were incubated in climate rooms, 16 hrs per day illuminated by fluorescent

Table 1: Composition of the basal medium (BM)

MACRO-ELEMENTS (mM):		FeEDTA:	
KCl	13.0	5 ml/l of a solution containing:	
KNO_3	5.0	Na_2EDTA	7.45 g/l
NH_4NO_3	5.0	$FeSO_4.7H_2O$	5.57 g/l
$CaCl_2.6H_2O$	2.8		
$MgSO_4.7H_2O$	1.6	VITAMINS (mg/l):	
KH_2PO_4	1.5	nicotinic acid	5
		pyridoxin-HCl	0.5
MICRO-ELEMENTS (mg/l):		thiamine-HCl	0.5
$MnSO_4.4H_2O$	25	biotin	0.05
H_3BO_3	10	folic acid	0.5
$ZnSO_4.7H_2O$	10		
$Na_2MoO_4.2H_2O$	0.25	meso-inositol	100 mg/l
$CuSO_4.5H_2O$	0.025	sucrose	30 g/l
		agar	8 g/l

and incandescent lamps (blue, red, and far red 4.8, 4.9, and 1.6 $W.m^{-2}$, respectively) at 25° C, and 8 hrs dark at 20° C. After 21 days the length of the perianth and of the inferior ovary of the female bud, as well as the length of the second male bud, were measured to 0.1 mm using a dissecting microscope. Stamen

and pistil development were regularly evaluated and found to be normal if not mentioned otherwise.

In the Figures average values with their standard errors are presented. In the Tables significant differences are given as determined by analysis of variance coupled with the Student-Newman-Keuls mean comparison test (SOKAL and ROHLF, 1969).

Results

In most of the experiments only the growth of perianth and ovary of the female bud is presented, perianth growth of the male buds responding similar unless mentioned otherwise.

Table 2: Effect of acidity on the length of female bud parts. Inflorescences grown on BM + 10^{-6}M BA for 21 days.

	length of perianth and ovary in mm					
initial pH	4.0	4.5	5.0	5.5	6.0	6.5
perianth	3.16a	2.87ab	2.99a	2.89ab	2.53bc	2.42c
ovary	3.76a	3.34b	3.27b	3.23b	2.84c	2.84c

Numbers following by different letters differ significantly (P = 0.05)

In an initial comparison of several culture media using different levels of nitrogen, the medium of DE JONG *et al.* (1974) modified as to nitrogen content proved to be most satisfactory. This medium (BM, Table 1) was varied to determine optimum concentrations of its components. Preliminary experiments showed the presence of a cytokinin in the medium to be essential for growth of the buds. All cytokinins tested: BA, zeatin, 2iP, and kinetin were active. 10^{-6}M BA gave optimum growth and was used in all experiments, except if mentioned otherwise.

DE JONG *et al.* (1974) showed that a low *pH-value* can favor the growth of floral organs. The growth of *Begonia* buds also was improved at lower pH values (Table 2). At pH 4.0, however, the buds became glassy. Independent of the acidity at the start of the experiments, the final pH after 3 weeks of culture was 4.8 -

5.0, the media being not especially buffered. In subsequent experiments a pH of 5.0 was used, obtained by titration of the media with NaOH or HCl.

Sucrose was used as a carbohydrate source, 30 g/l gave optimum growth, higher concentrations were inhibitory.

Nitrate and *ammonium* are important components of the medium, different species or organs requiring specific nitrate and ammonium levels for optimum growth (*e.g.* PETERSON, 1973; DE JONG *et al.*, 1974). Fig. 2 shows the effect of nitrate and ammonium on the growth of perianth and ovary. In this experiment nitrate was added as $NaNO_3$, ammonium as NH_4Cl, potassium was replenished as KCl. Growth was poor with either nitrate or ammonium as the sole nitrogen source. Optimum growth was attained at 5 to 10 mM NO_3^- and 5 mM NH_4^+. Further addition of nitrogen decreased growth, the perianth being somewhat more sensitive than the ovary. Increasing ammonium concentrations tended to decrease the pH-value at the end of the culture. Without ammonium the final pH was 5.0, with 10 mM 4.6. Nitrate had no effect on the final acidity.

BILDERBACK (1971) found an improvement of the development of *Aquilegia* flower buds by adding *amino acids* to the medium. The growth of callus cell suspension cultures is also sometimes improved by amino acids (*e.g.* GAMBORG, 1970; MURTHY REDDY and NARAYANA, 1974). We have tested alanine, γ-amino butyric acid, arginine, asparagine, and glutamine at 1.7, 5.0, and 15.0 mM. None improved growth, and the higher concentrations invariably were inhibitory. The amino acids were also added to media from which nitrate, ammonium, or both were omitted. In all cases growth was less than on the basal medium. *Casein hydrolysate* did not significantly influence growth whereas *urea* decreased growth. Urea could not replace ammonium in the combination with nitrate.

Potassium was applied as KNO_3, KH_2PO_4 and KCl. Without potassium hardly any growth occurred, half the normal amount was almost optimum already, higher concentrations had no inhibitory effect. Without *phosphate* growth was absent, the buds turned black and died within a few days. A quarter of the normal amount turned out to be sufficient already. Between phosphate and FeEDTA an interaction occurred (Fig. 3). At a low phosphate and a high FeEDTA concentration the buds died within a few days, propably due to precipitation of the phosphate by Fe^{3+} released from the EDTA.

Calcium decreased growth at increasing concentrations, the best growth on an agar medium occurring without calcium (Fig. 4). On the later adopted liquid medium NBM (see below) the buds became large without calcium but also glassy, about 1.0 mM calcium being optimum.

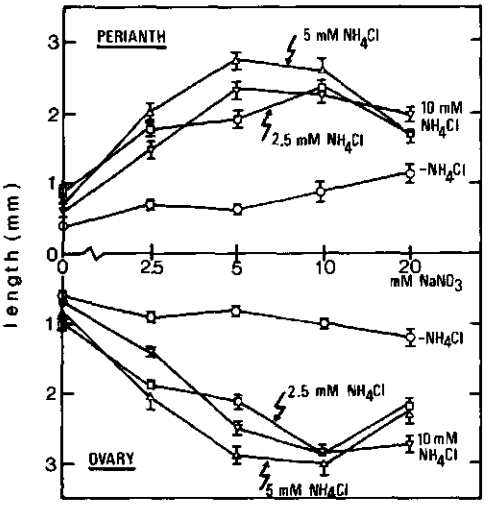

Fig. 2 Effect of nitrate and ammonium on the length of female bud parts. Inflorescences grown on BM + 10^{-6}M BA for 21 days.

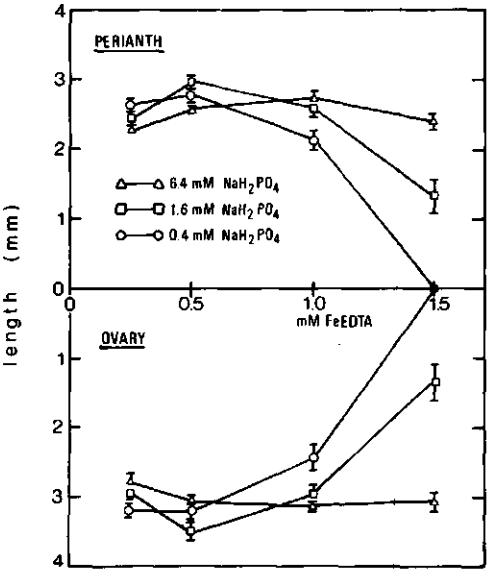

Fig. 3 Effect of FeEDTA at different phosphate levels on the length of female bud parts. Inflorescences grown on BM + 10^{-6}M BA for 21 days.

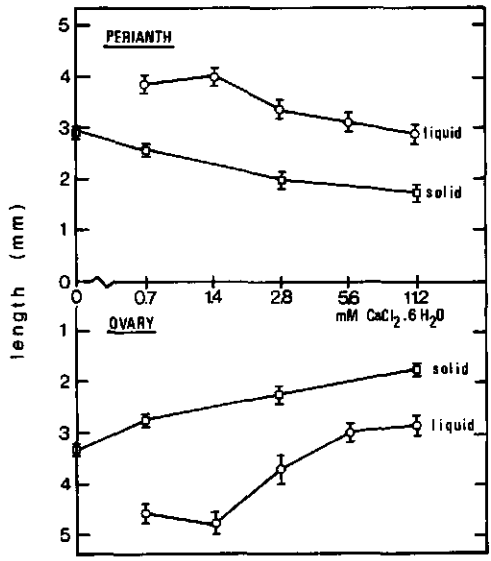

Fig. 4 Effect of calcium in a liquid and a solid medium on the length of female bud parts. Inflorescences grown on BM + 10^{-6}M BA for 21 days.

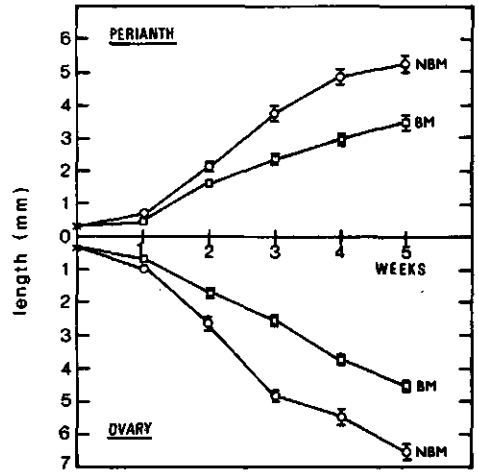

Fig. 5 Growth of inflorescences on original, agar-containing, and new, liquid, basal media (BM and NBM), with 10^{-6}M BA.

Table 3: Effect of agar on the length of female bud parts. Inflorescences grown on BM + 10^{-6}M BA for 21 days.

	length of perianth and ovary in mm					
concentration (%)	0.5	0.6	0.7	0.8	0.9	1.2
perianth	3.04a	3.01a	2.98a	2.68b	2.51bc	2.31c
ovary	3.67a	3.56a	3.67a	3.21b	2.97b	2.59c

Numbers followed by different letters differ significantly (P = 0.05)

Variation of the standard amount of *vitamins* and *micro-nutrients* over a wide range of concentrations did not significantly affected bud growth.

The presence of *agar* decreased growth of the buds (Table 3), the use of highly purified agar did not significantly improve growth. On a liquid medium, NBM, in which the agar was omitted and the calcium content reduced to 1.0 mM, growth was substantially improved (Fig. 5). The inflorescences were placed on a filter paper, touching the solvent surface and supported by glass beads

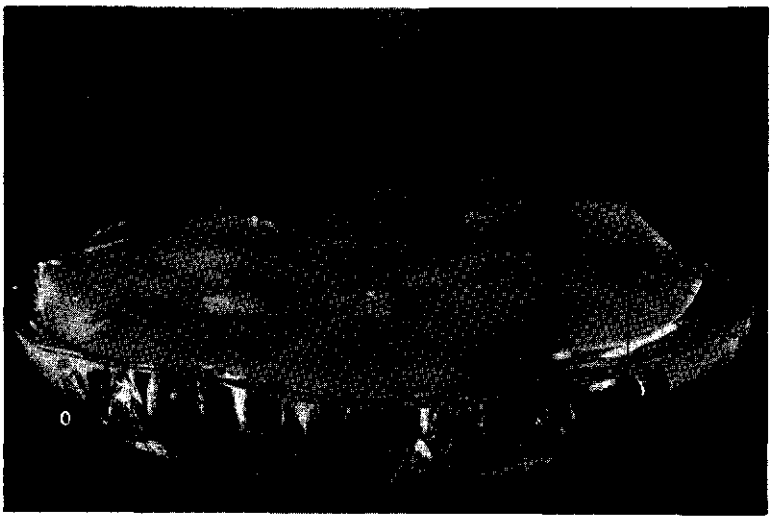

Fig. 6 Erlenmeyer flask with liquid medium. Inflorescences placed on a filter paper supported by glass beads.

(Fig. 6). Growth on the liquid medium was the same whether the medium was autoclaved or filter-sterilized. After 5 weeks on the liquid medium, the female buds reached a length comparable to that of the buds *in vivo* just before anthesis.

With the aid of the new basal medium, NBM, the effects of *growth-regulating substances* on bud development were analysed. The cytokinins tested were BA, zeatin, 2iP, and kinetin (Fig. 7). All cytokinins promoted bud growth, but markedly differed in activity. BA was the most active, kinetin needed a 10 to 30 fold higher concentration to give the same effect, while zeatin and 2iP were intermediate. Adenine, not presented in Fig. 7, did not support growth at all. The ovary was more sensitive to cytokinin than the perianth, showing optimum growth at 3.10^{-7} to 10^{-6}M BA, higher concentrations decreasing the final size of the ovary. The optimum for perianth growth was 3.10^{-6}M BA, higher concentrations being hardly inhibitory. Similar effects occurred with the other cytokinins according to their different relative activities.

Table 4: Effect of cytokinins on the percentage of first male buds reaching anthesis and on the length of second male buds. Inflorescences grown on NBM for 21 days

		percentage of first male buds reaching anthesis				
concentration (M)	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	3.10^{-5}
BA	71	80	85	25	0	0
zeatin	79	63	50	47	10	0
2iP	62	63	100	89	89	29
kinetin	42	67	100	100	100	12
		length of second male buds (mm)				
BA	3.49a	4.09a	4.79b	6.46c	7.22d	8.76e
zeatin	4.23a	4.46ab	4.47ab	4.97b	6.13c	7.21d
2iP	3.73a	3.78a	4.55a	5.47b	6.95c	7.19c
kinetin	3.58a	3.59a	3.56a	3.32a	5.31b	5.59b

Numbers followed by different letters are significantly different (P = 0.05)

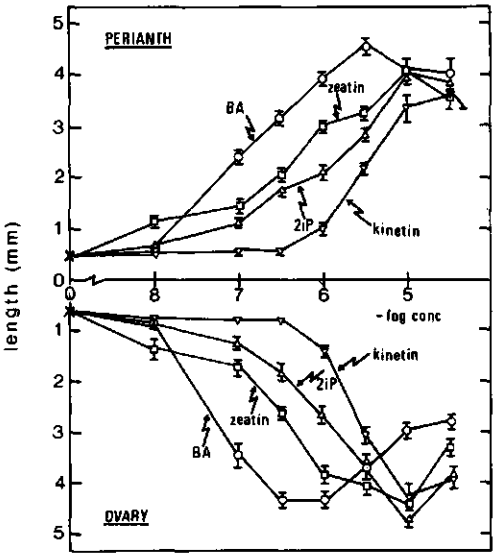

Fig. 7 Effect of cytokinins on the length of female bud parts. Inflorescences grown on NBM for 21 days.

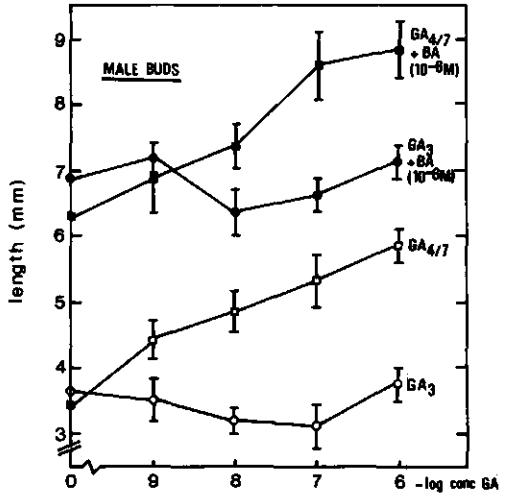

Fig. 8 Effect of GA_3 and GA_{4+7} on the length of 2nd male bud. Inflorescences grown on NBM for 21 days.

The length of the male buds, too, was promoted by the cytokinins (Table 4), without inhibitory effects at the higher cytokinin concentrations. High cytokinin concentrations, however, inhibited stamen development. This is expressed in Table 4 as the percentage of first male buds reaching anthesis, which only occurred if the stamens were fully developed. *In vivo* the male buds reached a length of about 5 mm just before anthesis, the perianth turning white and the anthers yellow at anthesis. *In vitro* this occurred at 10^{-8} to 10^{-7}M BA. At higher concentrations the perianth became larger and remained green, whereas the anthers remained small and green or colourless.

IAA, GA_3, ABA, and ethephon, in concentration ranges of 10^{-9} to 10^{-5}M, had no effect on the growth or development of the male or female buds in the absence of BA. Together with 10^{-6}M BA, these regulators did not affect the growth either, only ABA decreasing growth at the higher concentrations. Unlike GA_3, GA_{4+7} increased growth of the male buds, both in the absence and in the presence of BA (Fig. 8). However, only the perianth was enlarged, stamen development being unaffected.

Discussion

The present paper demonstrates that flower buds of *Begonia franconis* Liebm. can be succesfully grown *in vitro* from young inflorescences composed of two male and one female bud. As with *Cleome* flower buds (DE JONG *et al.*, 1974), the growth of *Begonia* flower buds was promoted at a pH value of 4 to 5. Most of the experiments *in vitro* with flower buds have been performed at higher pH values (*e.g.* BLAKE, 1969; HICKS and SUSSEX, 1970; BILDERBACK, 1972).

Acidity and nitrogen uptake are closely correlated. The former is influenced by the nitrate:ammonium ratio (VELIKY and ROSE, 1973; SCHLETZ, 1974) and, moreover, the pH can influence nitrogen uptake. MARTIN and ROSE (1976) found a decrease of nitrate uptake and an increase of ammonium uptake when the pH was raised from 4.8 to 6.4. In a non-buffered system this will result in a final pH where nitrate and ammonium uptake are balanced. For *Begonia* inflorescences this might be the case at the final pH 4.8 - 5.0, although experiments with especially buffered media are required to verify this.

Both nitrate and ammonium must be present. Interaction between nitrate and ammonium has frequently been found in cell suspension cultures (*e.g.* GAMBORG, 1970; WETHERELL and DOUGALL, 1976). DE JONG *et al.* (1974) also demonstrated an interaction of nitrate and ammonium with *Cleome* flower buds, which might be ascribed to a direct use of ammonium for nucleic acid synthesis (JONES *et al.*

1973) or to an increase of nitrate reductase synthesis (MOHANTY and FLETCHER, 1976).

Growth of the flower buds was progressively inhibited at increasing concentrations of agar. WERNICKE and KOHLENBACH (1976) showed an inhibitory effect of agar on adventitious sprouting of anthers of *Nicotiana* which could be partially overcome by adding active charcoal to the medium or by dialyzing the medium. This points to a chemical rather than a physical effect, contrary to the suggestion by ROMBERGER and TABOR (1971). We found no improvement of growth with highly purified agar, the calcium concentration of which, however, is twice that of the normally used bacto-agar. This might have counteracted a possible positive effect of the higher degree of purity. With a liquid medium growth was optimum and the buds reached the same size as *in vivo*.

A cytokinin was necessary for growth of the flower buds. This was also found by HICKS and SUSSEX (1970) with *Nicotiana* and by BILDERBACK (1972) with *Aquilegia* flower buds. BLAKE (1969), on the other hand, found no effect of cytokinins on flower bud growth with *Viscaria*, whereas DE JONG and BRUINSMA (1974) demonstrated that cytokinin was required in *Cleome* flower buds for the development of the pistil. Different results might be explained by the size of the buds used in the different experiments. If the buds are rather large already, most of the cell divisions will be completed or, alternatively, the endogenous cytokinin content may be sufficient for sustaining the remaining development.

The female buds needed a 10 to 30-fold higher cytokinin concentration than the male buds for a normal development. High cytokinin concentration inhibited stamen development, as was also found by BLAKE (1969) and HICKS and SUSSEX (1970). Although the male and female buds are positioned closely together in the inflorescence primordium, the female bud may obtain more cytokinin by its higher sink activity due to its higher auxin content (HANISCH TEN CATE *et al.*, 1975).

Auxins had no effect on the growth of the flower buds. Similar observations were made by BLAKE (1969), HICKS and SUSSEX (1970) and BILDERBACK (1972).

Gibberellins, too, failed to affect growth, only GA_{4+7} promoted the length of the male buds. The same was found by DE JONG and BRUINSMA (1974), who observed a promotive effect of specifically GA_{4+7} on the petal growth of *Cleome* flowers.

The buds invariably remained male or female as according to their positions in the inflorescence, a change of sex or intermediate forms were never observed. Apparently at the time of isolation the buds had differentiated already into a stage at which the initiation of the floral organs was terminated (Fig. 1).

After having determined the optimum culture conditions, therefore, in a subsequent study (BERGHOEF and BRUINSMA, 1979b) the effects of growth-regulating substances on the initiation of the floral organs will be investigated, using very small inflorescence primordia in which the flower buds have not yet differentiated.

Acknowledgements

The authors gratefully acknowledge the skilful technical assistance of Mrs. Y.E. van Oosten-Legro and Mr. H. van Oeveren and the co-operation of Mr. L.E. Groen and Mr. B.J. v.d. Knaap.

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FLOWER DEVELOPMENT OF *BEGONIA FRANCONIS* LIEBM. III EFFECTS OF GROWTH-REGULATING SUBSTANCES ON ORGAN INITIATION IN FLOWER BUDS *IN VITRO*.

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Summary

The regulation of sex expression in *Begonia franconis* Liebm. was studied by analyzing the effects of growth-regulating substances on the initiation of floral organs in inflorescence primordia *in vitro*.

In the absence of growth-regulating substances or with IAA, ABA, and Ethephon, no differentiation of flower buds occurred. With 10^{-6}M BA all floral organs were initiated and the flower buds reached anthesis. Gibberellins promoted organ initiation in the buds. However, removal of the first bud of the inflorescence primordium strongly reduced organ initiation in the remaining buds by gibberellins. The growth-regulating substances did not change the sex of the buds as determined by their position in the inflorescence. However, female differentiation was inhibited at low sucrose levels that increased the number of male flowers.

It is suggested that sexual differentiation is regulated endogenously by the central region of the inflorescence primordium, the carbohydrate level being a limiting factor for female differentiation.

Key words: Begonia, flower bud, in vitro culture, differentiation, sex expression

Abbreviations: ABA, abscisic acid; BA, N^6-benzyladenine; Ethephon, (2-chloroethyl)phosphonic acid; GA_3, gibberellin A_3; GA_{4+7}, gibberellins A_4 and A_7; IAA, indolyl-3-acetic acid.

Introduction

In a previous study (BERGHOEF and BRUINSMA, 1979b) the effects of growth-

regulating substances on the growth of flower buds of *Begonia franconis* Liebm. inflorescence primordia were analysed *in vitro*. Cytokinins proved to be essential for bud growth, female buds having a higher requirement than male ones. Growth-regulating substances did not change the sexual development of floral organs, which turned out to be already determined at the start of the experiments. To examine the effects of growth-regulating substances on the initiation of floral organs, very small inflorescences with still undifferentiated flower bud primordia were used in the present study.

Material and Methods

The culture of the clone of *Begonia franconis* Liebm. plants and culture conditions *in vitro* were described earlier (BERGHOEF and BRUINSMA, 1979a and b). Young inflorescence primordia were collected when the first bud had reached a length of 0.20 - 0.25 mm, using a dissecting microscope. The developmental stage of the flower buds in these primordia is given in Fig. 1. The primordia were sterilized in 0.5% NaClO + 5 ppm Triton X-100 for 2 min and rinsed extensively with sterile tap water. The primordia were transferred to the liquid, filtersterilized medium using a small brush in order to minimize damage of the tiny inflorescences. All experiments were repeated at least once.

After 28 days of culture the inflorescences were either transferred to FAA (formalin:acetic acid:ethanol = 5:5:90) or the developmental stage of the buds was determined using a dissecting microscope. The inflorescences stored in FAA were dehydrated through a tertiary butanol concentration series and embedded in paraplast. Sections were cut 8 µm thick and stained with safranin fast green by the method of JENSEN (1962).

Results

Fig. 1 shows the developmental stages of the flower buds in the inflorescence primordia at the start of the experiments. Bud 1 had initiated perianth primordia, buds 2 and 3 were still undifferentiated. In all experiments the sex of the flower buds was found to be according to their position in the inflorescence, buds 1 and 2 initiated stamen primordia and never pistils and bud 3 pistil primordia without stamen. The primordia of perianth, stamens or pistils were recorded as present when they were distinctly recognizable. As bud 1 gave initiation of perianth and stamen primordia in all treatments, these were omitted from the results.

In the absence of growth-regulating substances in the medium there was only a slight differentiation of bud 2 and hardly any differentiation of bud 3. Table 1 shows the promotion of development by BA, 10^{-7}M being minimally required to

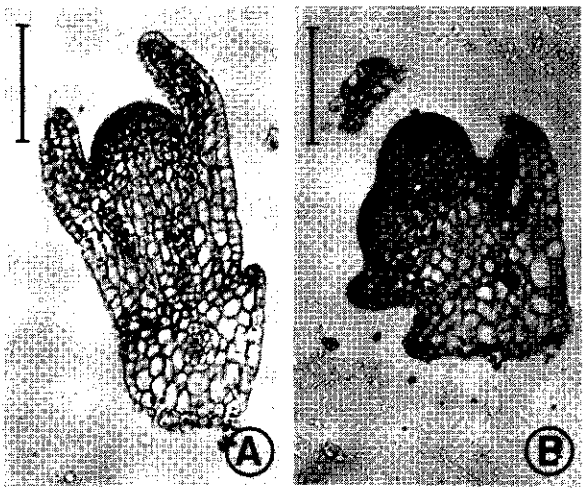

Fig. 1 Stage of flower bud differentiation at the onset of the experiments. A: Bud 1. B: Bud 2 and 3. Bars represent 100 µm.

always give development of the 2d male bud, 10^{-6}M for the 3d, female bud. However, 10^{-7}M BA was insufficient to give complete male development. The developmental stages of the buds on varying BA concentrations are shown in Fig. 2. The Figure is representative for those inflorescences of which all three buds had differentiated, the larger part of the inflorescences on the low BA concentrations showing less differentiation. Both with the male (bud 2) and the female

Table 1: Effect of benzyladenine on organ initiation in second and third bud. Inflorescence primordia grown on liquid medium for 28 days.

	number of inflorescences	second bud perianth	second bud stamens	third bud perianth	third bud pistils
control	19	15	8	2	1
BA 10^{-8} M	18	18	13	8	2
BA 10^{-7} M	18	18	18	18	14
BA 10^{-6} M	21	21	21	21	21

(bud 3) differentiation was enhanced by increasing concentrations of BA. There was a close dependance of bud 3 on development of bud 2. A well developed bud

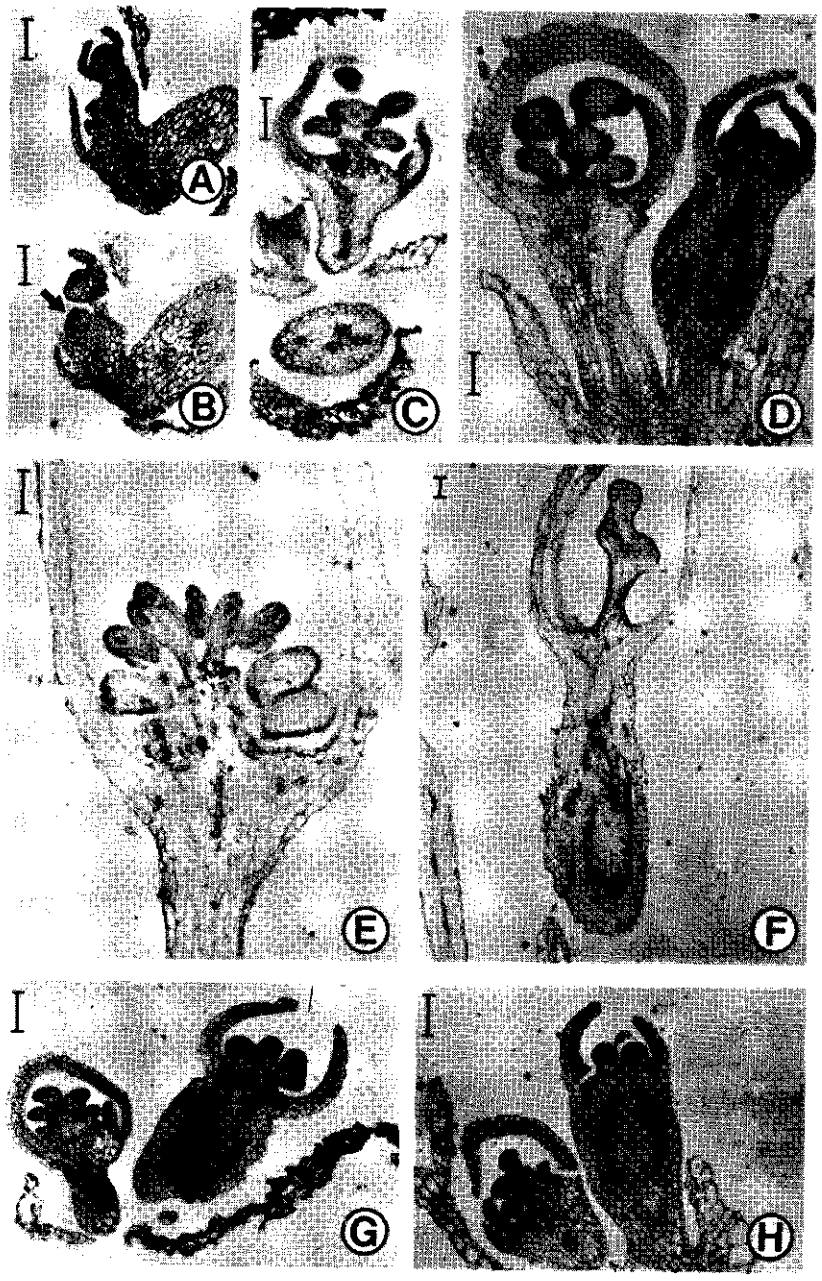

Fig. 2 Stage of flower bud differentiation after 28 days in vitro. A: Control, bud 2. B: Control, bud 3 (Arrow). C: 10^{-8}M BA, Bud 2 and 3. D: 10^{-7}M BA, Bud 2 and 3. E: 10^{-6}M BA, Bud 2. F: 10^{-6}M BA, Bud 3. G: 10^{-7}M GA_3, Bud 2 and 3. H: 10^{-6}M GA_3, Bud 2 and 3. Bars represent 100 µm.

Table 2: Effect of regulators on organ initiation in second and third bud. Inflorescence primordia grown on liquid medium without BA for 28 days

	number of inflorescences	second bud perianth	second bud stamens	third bud perianth	third bud pistils
control	37	34	11	4	0
IAA 10^{-7} M	14	14	4	2	0
IAA 10^{-6} M	22	16	3	1	0
GA_3 10^{-7} M	18	15	12	10	7
GA_3 10^{-6} M	22	22	22	19	19
ABA 10^{-7} M	17	12	5	2	0
ABA 10^{-6} M	15	9	2	2	0
ethephon 10^{-7} M	18	18	3	1	0
ethephon 10^{-6} M	14	13	4	0	0

2 coincided always with a strong differentiation of bud 3, while differentiation of bud 3 was never observed without bud 2 having initiated stamen primordia.

IAA, ABA, and Ethephon had no effect on differentiation of the flower buds, while GA_3 was clearly promotive (Table 2). Both male and female development were enhanced by GA_3 (Fig. 2), although the differentiation was less pronounced than with BA. GA_{4+7} gave similar results as GA_3.

Table 3: Effect of regulators on organ initiation in second and third bud. First bud removed. Inflorescence primordia grown on liquid medium for 28 days.

	number of inflorescences	second bud perianth	second bud stamens	third bud perianth	third bud pistils
control	8	2	0	0	0
BA 10^{-6} M	11	11	11	10	10
GA_3 10^{-6} M	9	6	4	3	2
IAA 10^{-6} M	10	6	0	0	0

Time course studies with BA or GA_3 revealed that bud 3 started to differentiate when stamen primordia of bud 2 had just become visible. Thus, we might compare the differentiation of the flower buds in these inflorescence primordia with the sequential initiation of floral organs in a hermaphrodite flower bud. HESLOP-HARRISON (1963) postulated in his hypothesis that initiation of floral organs is regulated by specific inducers produced by the preceding floral organ. When differentiation in an inflorescence is comparable with that in a hermaphrodite flower, this would implicate that there could be no differentiation of bud 3 after removal of bud 1 and 2. Table 3 shows the effects of growth-regulating substances on differentiation after removal of the first bud. Without growth--regulating substances (control) or with 10^{-6}M IAA there was hardly any differentiation of the remaining buds. With 10^{-6}M BA both buds showed a normal development, while 10^{-6}M GA_3 gave differentiation of both buds in a few inflorescences only. GA_{4+7} gave similar results in all experiments, and Table 4 shows the combined results of all experiments with gibberellins on intact inflorescences and those of which bud 1 was removed. Removal of bud 1 decreased differentiation of both buds 2 and 3, again the third bud only developed when the second had initiated its stamen primordia, but no shift to male differentiation occurred in the 3d bud.

Table 4: Effect of removal of the first bud on organ initiation in the subsequent buds in the presence of gibberellin (10^{-6}M GA_3 or GA_{4+7})

	number of	second bud		third bud	
	inflorescences	perianth	stamen	perianth	pistils
intact	76	76	76	60	60
bud 1 removed	33	26	18	16	10

The second bud could not be removed before inoculation without damaging bud 3. Therefore, inflorescences of which bud 1 was removed were inoculated on the basal medium + 10^{-6}M BA for 4 days, whereafter the second bud, which had just initiated its perianth primordia, was cut away with a small piece of razor-blade under a dissecting microscope. The remaining, undifferentiated, third bud was transferred to the basal medium supplemented with 10^{-6}M BA, 10^{-6}M GA_3, 10^{-6}M IAA, or without growth-regulating substances. Bud 3 still showed a normal, female development in all treatments, although about 25% of the buds did not develop,

propably owing to damage at removal of the second bud.

Cytokinins, gibberellins, and auxins affected the composition of the inflorescences *in vivo* by increasing the number of male and/or female flowers (BERGHOEF and BRUINSMA, 1979a). Also *in vitro*, BA increased the number of flowers per inflorescence (Fig. 3). As *in vivo*, this was caused by activation of the dormant bud in the axil of the bract of the second flower, giving branched inflorescences (types IIb and c, BERGHOEF and BRUINSMA, 1979a). *In vivo* GA_3 promoted the formation of a third primary male bud (type III), whereas *in vitro* this was never found.

Combinations of 10^{-7}M or 10^{-6}M BA with 10^{-6}M IAA or GA_3 gave identical results as when BA was used alone, neither the number of buds per inflorescence

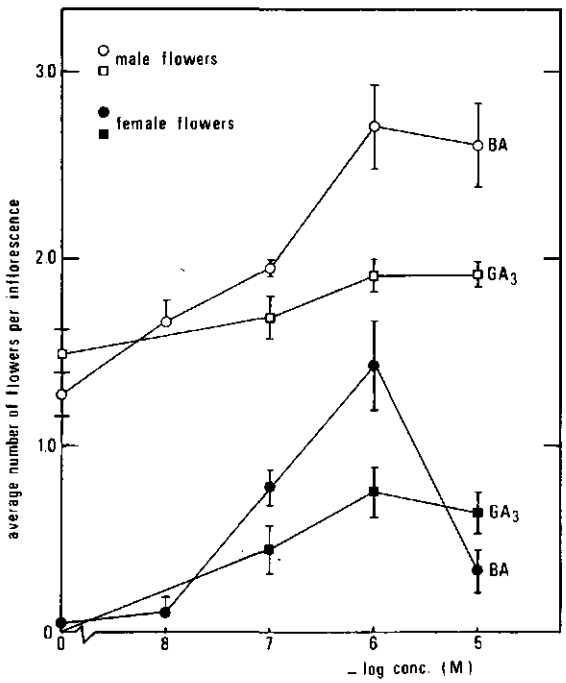

Fig. 3 Effect of BA and GA_3 on number of male and female flowers. Inflorescences grown on NBM for 28 days.

nor their developmental stage being affected by IAA or GA_3. This might be explained by a dominating effect of BA on development, obscuring any effects of IAA or GA_3. For this reason, experiments were done in which the inflorescence primordia, inoculated on liquid medium + 10^{-6}M BA, were transferred after a short

period to the liquid medium with only IAA or GA_3. The inflorescences were transferred after 3 days, at that time bud 1 had initiated stamen primordia and bud 2 perianth primordia. After transfer on a medium without growth-regulating substances the buds showed the normal, complete differentiation. Neither IAA nor GA_3, at 10^{-7}M or 10^{-6}M, caused any changes in the development.

The experiments *in vivo* demonstrated that assimilates may be important in sex expression (BERGHOEF and BRUINSMA, 1979a). The sucrose level in the liquid medium was 30 g/l, providing optimum growth of the buds (BERGHOEF and BRUINSMA, 1979b). To test the effect of sucrose on differentiation, a concentration series was made of 0, 1, 3, 10, 30, and 60 g/l, supplemented with 10^{-6}M BA. Without sucrose the buds died, with 60 g/l 40% of the inflorescences died, propably due to the high osmotic value. With 1 g/l, buds 1 and 2 differentiated the perianth, but no stamen primordia were visible. With 3 g/l buds 1 and 2 had well developed stamen primordia. Bud 3 was undifferentiated in 50% of the inflorescences, the other half had 3 or more well developed male buds, whereas no undifferentiated bud could be found. Pistil development was not observed with 3 g/l. With 10, 30, and 60 g/l the buds showed the normal development, 2 male and 1 female buds per inflorescence primordium. This shows that with low sucrose concentrations only male development was initiated.

Discussion

The present data show that initiation of organs in undifferentiated flower primordia of *Begonia franconis* Liebm. can be achieved in culture *in vitro*. Occasionally the experiments were continued to demonstrate the eventual anthesis of the flower buds. GALUN *et al.* (1963) with *Cucumis*, HICKS and SUSSEX (1970) with *Nicotiana*, and BILDERBACK (1972) with *Aquilegia*, used flower buds in which some of the floral organs still had to be initiated, however, their flower buds failed to develop to maturity. BLAKE (1966) with *Viscaria*, and BRULFERT and FONTAINE (1967) with *Anagallis* observed development to maturity, but only when leaves or roots were present, respectively. In other studies, *e.g.* BLAKE (1969), DE JONG and BRUINSMA (1974) and POOL (1975), flower buds were used with all floral organs already initiated. When the effects of growth-regulating substances on the initiation and differentiation of floral organs are studied, a medium is required giving fully normal development. For *Aquilegia* flower buds, for instance, TEPFER *et al.* (1966) reported the beneficial effect of IAA on the initiation of floral organs, using coconut milk in the medium, while BILDERBACK (1972) found no effect of auxins on a chemically defined medium.

In the present study, growth-regulating substances in the medium were not

required for the initiation of the stamen primordia in bud 1 and the perianth primordia in bud 2. However, a cytokinin or gibberellin was essential for further organ initiation of the buds in the inflorescence. With *Aquilegia*, gibberellins (TEPFER *et al.*, 1966) or kinetin (BILDERBACK, 1972), increased the number of floral organs initiated, although some were formed in the absence of growth-regulating substances. Flower primordia of *Nicotiana* initiated all floral organs without growth-regulating substances being required (HICKS and SUSSEX, 1970). Thus, a number of floral organs can be initiated in the absence of growth-regulating substances, propably due to the endogenous hormone level at the time of excision. The number of primordia to be initiated in *Aquilegia* flower buds or *Begonia* inflorescences is larger than in *Nicotiana* flower buds, which may account for the apparent differences in requirement of growth-regulating substances for the initiation of floral organs.

We found that auxins had no effect on the differentiation of the buds. GALUN *et al.* (1963) with cucumber, and TEPFER *et al.* (1966) with *Aquilegia*, noted a promotive effect of IAA on the initiation of female floral organs. In both studies the media were supplemented with coconut milk. Possibly the positive effect of IAA was a result of an interaction with some substance in the coconut milk, the more so because BILDERBACK (1972) found no effect of IAA with *Aquilegia* on a chemically defined medium.

In *Begonia franconis* Liebm., a cytokinin proved to be necessary for the initiation and development of the floral organs. As with *Nicotiana* (HICKS and SUSSEX, 1970), the development of all organ primordia was enhanced by cytokinin. This supports the results *in vivo* where cytokinins increased the formation of both male and female flower buds (BERGHOEF and BRUINSMA, 1979a). Thus, cytokinins do not necessarily have a direct effect on sex differentiation, they function primarily as a promotor of cell divisions. Gibberellins, too, promoted differentiation of the buds, however, removal of the first bud strongly reduced this effect. This indicates that a substance is present or synthesized in bud 1, required for the promotive effect of gibberellins on the initiation of primordia. HESLOP-HARRISON (1963) suggested that initiation of floral organs is regulated by specific inducers, produced by the preceding primordia. According to this hypothesis, in *Begonia* differentiation of a bud would be regulated by the preceding one. However, BA gave differentiation of bud 3 in the absence of bud 1 and bud 2. This shows that no specific inducers are required. NITSCH (1967) reported about a synergistic effect of gibberellic acid with kinetin in tobacco tissue culture. Possibly the promotive effect of gibberellins on differentiation was caused by

the synergistic effect with the endogenous cytokinins present at excision. By removal of bud 1, involving the larger part of tissue of the inflorescence primordia, the level of cytokinins might have become below a required threshold value. Like cytokinins, gibberellins equally promoted the initiation of male and female organs. However, for the further development of these organs, the presence of cytokinin was essential (BERGHOEF and BRUINSMA, 1979b).

The present experiments demonstrate that none of the growth-regulating substances, applied singly or in combinations, directly affects the sex-regulating mechanism. This indicates that the sex of the flower buds is endogenously regulated, either by the already established primordia (HESLOP-HARRISON, 1963) or by the central region of the inflorescence primordium. JENSEN (1971), in his experiments with bisection of flower promordia of *Aquilegia*, concluded that the nature of primordia to be initiated was under the influence of the already established primordia. HICKS and SUSSEX (1971) suggested that the initiation was regulated by the central region. They found that after median bisection of flower primordia of *Nicotiana* organs were initiated, although the preceding floral organs were not in the immediate proximity. With *Begonia* the presence of preceding buds was not required for differentiation. Irrespective of whether buds 1 and 2 were present or not, bud 3 differentiated into a female bud. We must conclude, therefore, that the regulation of the nature of the primordia to be initiated takes place in the central region.

Particularly the nutritive state of this region might be decisive. At a low level of sucrose bud 3 often initiated stamen primordia and no pistils, higher levels being required for female differentiation. However, at high levels of sucrose buds 1 and 2 never initiated pistil primordia. This indicates that the level of sucrose is a limiting rather than a regulating factor for female differentiation. In the experiments *in vivo* (BERGHOEF and BRUINSMA, 1979a) a low light intensity as well as gibberellins applied to the apex of the branch were found to cause elongation of the inflorescence, the extra flower(s) invariably being male, the terminal flower always remaining the female one. This is most likely a result of a decreased level of assimilates in the central region of the inflorescences. For gibberellins this can be caused by the accelerated development of the vegetative apex, increasing the competition for assimilates at the cost of the inflorescences. The resulting low nutritive state prevents the third bud to develop female. Thus, *in vitro* the male developing bud may or may not be followed by a terminal female one. It is concluded that the nutritive state of the inflorescence primordium determines the sex of the developing flower buds, in that a

limited food supply prevents female development.

The regulation of sex expression was finally analysed by studying the formation of adventitious flower buds on explants *in vitro*. The results of this study will be presented in the next paper.

Acknowledgements

The authors gratefully acknowledge the skilful technical assistance of Mrs. Y.E. v. Oosten-Legro, the co-operation of Mr. C.J.J. Neefjes, and the help of the Department of Plant Cytology and Anatomy in the microscopic studies including the microphotography.

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FLOWER DEVELOPMENT OF *BEGONIA FRANCONIS* LIEBM. IV ADVENTITIOUS FLOWER BUD FORMATION ON EXCISED INFLORESCENCE PEDICELS *IN VITRO*.

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Summary

On stem segments of *Begonia franconis* Liebm. *in vitro*, only vegetative buds could be induced. On inflorescence pedicels, on the contrary, the formation of adventitious flower buds was abundant. Both cytokinin and auxin were required, the latter during the first 10 days. After the promotion of callus formation, the auxin became inhibitory to flower bud formation. Gibberellin strongly repressed flower bud development.

None of the growth regulators tested affected the ratio of male to female flower buds. Pistillate buds never differentiated directly on the callus, but originated at the bracts on the pedicels of the staminate ones. A high sucrose level was required for female differentiation, particularly.

It is concluded that the sex of *Begonia franconis* Liebm. flower buds is determined by the nutritional state of the tissue as a limiting factor for female differentiation.

Key words: Begonia, adventitious flower buds, in vitro culture.

Abbreviations: BA, N^6-benzyladenine; chlormequat, (2-chloroethyl) trimethylammoniumchloride; ethephon, (2-chloroethyl)phosphonic acid; GA_3, gibberellin A_3; IAA, indolyl-3-acetic acid; NAA, 1-naphtalene-acetic acid.

Introduction

In a previous study (BERGHOEF and BRUINSMA, 1979c) we have shown that growth-regulating substances have no direct effect on the sex expression of flower buds

of *Begonia franconis* Liebm. cultured *in vitro*. Undifferentiated flower buds in inflorescence primordia developed into male or female buds as determined by their position in the inflorescence. However, although there was no visible sign of differentiation at the time of excision, we could not entirely exclude that their differentiation was determined already. This would require formation of new flower buds after excision.

RINGE and NITSCH (1968) demonstrated adventitious flower bud formation on explants of various *Begonia* species. We investigated the possibility of adventitious flower bud formation with explants of *Begonia franconis* Liebm. and the effects of growth-regulating substances on the sex expression of these buds.

Material and methods

The culture of the clone of *Begonia franconis* Liebm. plants was described earlier (BERGHOEF and BRUINSMA, 1979a). Explants were taken from the inflorescences of which two flowers had just reached anthesis. They were cut as shown in Fig. 1, sterilized in 0.5% NaClO + 50 ppm Triton X-100 during 2 min, and rinsed with sterile tap water.

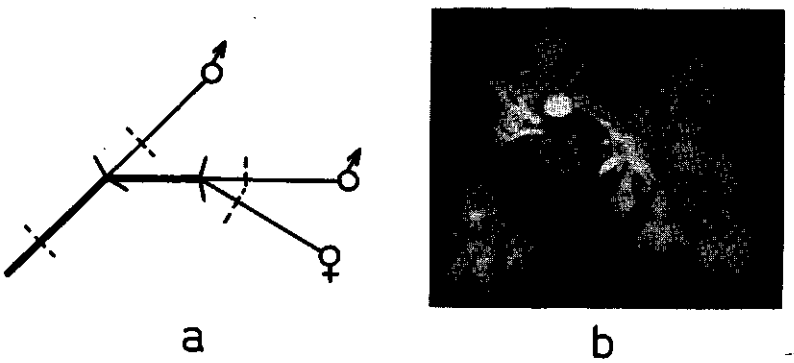

Fig. 1 Pedicel explant. A: schematic, dotted lines indicate excision. B: after 8 weeks on medium with 10^{-6}M BA + 10^{-7}M NAA.

The final composition of the medium is given in table 1. The pH was adjusted to 5.0 with NaOH or HCl. Tubes with 15 ml medium were autoclaved for 20 min at 110°C. When GA_3 or Ethephon were added, these were filter-sterilized and added to the autoclaved medium at about 50°C.

Experimental samples consisted of 8-12 tubes, one explant per tube. The experiments were repeated at least once. Culture conditions were the same as in the culture of inflorescences (BERGHOEF and BRUINSMA, 1979b). After 7-8 weeks

Table 1: Composition of the basal medium.

Macro-elements (mM):		FeEDTA:	
KNO_3	5.0	0.5 ml/l of a solution containing:	
NH_4Cl	1.5	Na_2EDTA	7.45 g/l
$CaCl_2.6H_2O$	1.0	$FeSO_4.7H_2O$	5.57 g/l
$MgSO_4.7H_2O$	0.5		
$NaH_2PO_4.H_2O$	0.2	Vitamins (mg/l):	
		nicotinic acid	0.5
Micro-elements (mg/l):		pyridoxine-HCl	0.05
$MnSO_4.4H_2O$	2.5	thiamine-HCl	0.05
H_3BO_3	1.0	folic acid	0.05
$ZnSO_4.7H_2O$	1.0	biotin	0.005
$Na_2MoO_4.2H_2O$	0.025	meso-inositol	10 mg/l
$CuSO_4.5H_2O$	0.0025	sucrose	30 g/l
		agar	8 g/l

the numbers of male and female buds were counted using a dissecting microscope.

In the Figures average values with their standard errors are presented. In the Tables significant differences are given as determined by analysis of variance coupled with the Student-Newman-Keuls mean comparison test (SOKAL and ROHLF, 1969).

Results

First a medium had to be developed on which adventitious flower bud formation was achieved. Adventitious roots and sprouts were to be avoided because they might interfere with exogenous growth-regulating substances in their effect on sex expression.

Three media were tested with a low, medium and high level of the constituents. The amounts of macro-nutrients were according to the low, medium and high levels used by FOSSARD *et al.* (1974). The levels of micro-nutrients, FeEDTA, and vitamins were 1/10, 1/2, and 2 times the concentrations of the basal medium used for flower bud growth (BERGHOEF and BRUINSMA, 1979b). Sucrose was added at 1, 3, and 5%. To each of these three media two auxins, NAA plus IAA, and two cytokinins, BA plus kinetin, were added at 0, 10^{-7}, 10^{-6}, and 10^{-5}M each, in all possible combinations, so that 48 different media were tested. Two types of explants were used, stem segments of 1.5 cm and inflorescence stalks as shown in Fig. 1. Only four replicates were used.

Table 2: Effects of auxins and cytokinins on regeneration of adventitious organs on excised inflorescence pedicels. Low level of nutrients.

BA plus kinetin (M)	NAA plus IAA (M)			
	0	10^{-7}	10^{-6}	10^{-5}
0	-	r	r	r
10^{-7}	-	r	r	r
10^{-6}	v(f)	vf	vr	r
10^{-5}	v	vf	v	-

r = roots; v = vegetative buds; f = flower buds; - = no regeneration.

The results with the inflorescence stalks at the low level of nutrients are shown in Table 2. On the low nutrient level, flower bud formation was found with the cytokinins at 10^{-6} or 10^{-5}M, and the auxins at 0 or 10^{-7}M. Without the auxins only explant developed flower buds, in later experiments we never observed flower bud formation without auxins being present.

At the medium nutrient level only one combination of cytokinins (10^{-6}M) and auxins (10^{-7}M) gave flower bud formation. Whereas on the low nutrient level, only few vegetative sprouts developed, on the medium nutrient level vegetative sprouting was abundant. The number of adventitious roots increased at higher auxin concentrations. The latter was also found at the high nutrient level, but neither vegetative nor generative sprouts were observed at this level. On stem segments the formation of roots and vegetative sprouts was similar as on inflorescence

Table 3: The effect of sucrose on the number of adventitious male and female flower buds per explant. Explants on medium + 10^{-6}M BA + 10^{-7}M NAA for 7 weeks.

sucrose (%)	mean number of male buds	mean number of female buds
1	4.5 a	0 a
2	12.9 b	1.9 a
3	15.7 b	9.3 b
5	3.1 a	0 a

Numbers followed by different letters differ significantly (P = 0.05).

stalks, but flower buds were never found.

The low nutrient medium, with the cytokinins at 10^{-6}M and the auxins at 10^{-7}M, was varied to determine the optimum concentrations of its components. The effect of sucrose is presented in Table 3. It shows that at 1% sucrose few and only male buds were formed, whereas with 3% sucrose many more staminate and also pistillate buds were found. All explants showing regeneration started with the formation of callus on which the flower buds were formed. However, female flower buds never originated directly from the callus but were formed in the axils of the bracts of male bud pedicels. As *in vivo* the pedicels of the male buds had two bracts, the axillary buds which may develop into female flowers. In all subsequent experiments 3% sucrose was used.

Nitrate and ammonium were tested in a wide concentration range. Nitrate proved to be essential for adventitious organ formation. Concentrations above 5 mM hardly influenced flower bud formation but increased the formation of vegetative sprouts. Ammonium was not required, but 1.5 mM considerably enhanced flower bud formation, higher concentrations promoted vegetative sprout formation. Phosphate, potassium, calcium, magnesium, and FeEDTA, too, promoted vegetative sprout formation at higher concentrations, whereas they did not affect the number of flower buds.

With the resulting low nutrient medium as given in Table 1, hardly any vegetative sprouts were formed. None of the mineral nutrients tested significantly affected the ratio of male:female flowers.

Table 4: The effect of BA on the number of adventitious male and female flower buds per explant. Explants on medium + 10^{-7}M NAA for 7 weeks.

BA (M)	mean number of male buds	mean number of female buds
0	0 a	0 a
10^{-7}	0 a	0 a
10^{-6}	18.7 b	13.0 b
10^{-5}	27.5 c	23.1 c

Numbers followed by different letters differ significantly (P = 0.05).

In the experiments described above two cytokinins, BA and kinetin, were present at 10^{-6}M each. The effect of BA alone is given in Table 4. Without or at 10^{-7}M BA no flower buds were formed. At 10^{-6} and 10^{-5}M BA many flower buds were formed,

but at 10^{-5}M they were difficult to distinguish from the vegetative buds. Kinetin was less effective and gave only a few flower buds at 10^{-5}M. Addition of 10^{-6}M kinetin to 10^{-6}M BA had no supplementary effect and was omitted in the latter experiments.

Auxins were present as NAA and IAA at 10^{-7}M each. The effects of NAA are given in Table 5. Without NAA the explants became brown and showed no regeneration at all. With 10^{-8}M NAA some callus was formed but no buds. Flower buds were formed at 10^{-7} and 10^{-6}M NAA, the latter concentration also producing adventitious roots. IAA gave about 30-50% more flower buds than NAA, but also considerably enhanced vegetative sprouting. Therefore, in the latter experiments only 10^{-7}M NAA was given.

Table 5: The effect of NAA on the number of adventitious male and female flower buds per explant. Explants on medium + 10^{-6}M BA for 7 weeks.

NAA (M)	mean number of male buds	mean number of female buds
0	0 a	0 a
10^{-8}	0 a	0 a
10^{-7}	15.3 b	9.8 b
10^{-6}	9.1 c	3.7 c

Numbers followed by different letters differ significantly (P = 0.05).

Neither the cytokinins nor the auxins had any significant effect on the ratio male:female flower buds.

The effects of GA_3, chlormequat, and ethephon, were analysed in the presence of 10^{-6}M BA and 10^{-7}M NAA. The effect of GA_3 is given in Fig. 2. Higher concentrations progressively decreased the formation of both male and female buds, until at 10^{-4}M there was no differentiation at all. Chlormequat had little effect on the numbers of male and female flowers but it was inhibitory at 3.10^{-4}M (Fig. 3). Ethephon had no effect on the number of staminate buds, but the formation of pistillate buds decreased at increasing concentrations of ethephon (Fig. 4). At 10^{-6}M and 10^{-5}M most of the male buds were brown at the end of culture. At 10^{-4}M the explants died within a few weeks.

Because BA and NAA might interfere with each other in their effects on sex expression, explants were transferred after 10 days from the medium with BA and

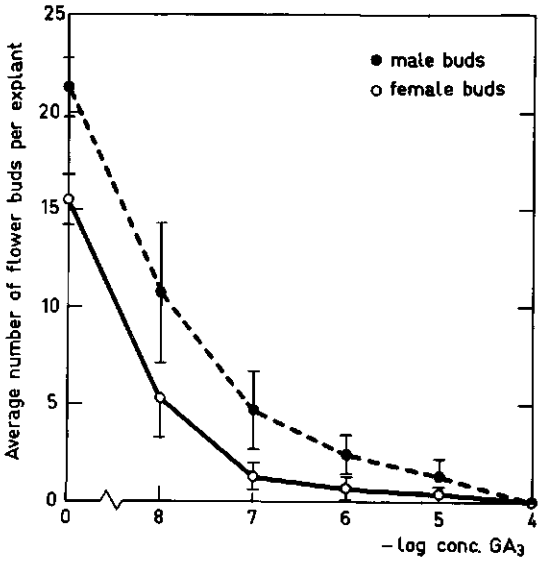

Fig. 2 Effect of GA_3 on the numbers of male and female buds. Explants on medium + 10^{-6}M BA + 10^{-7}M NAA for 7 weeks.

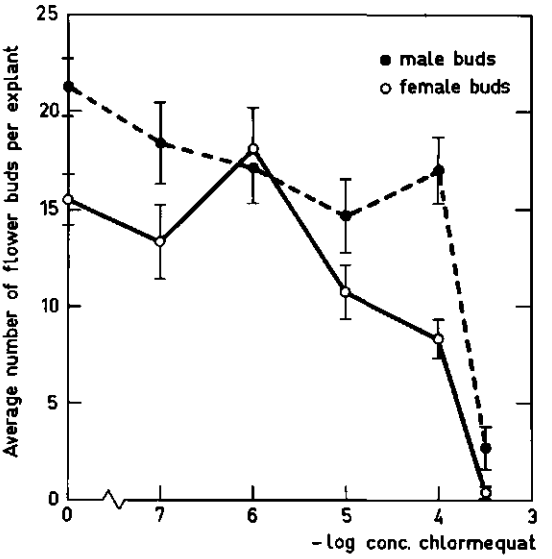

Fig. 3 Effect of chlormequat on the numbers of male and female buds. Explants on medium + 10^{-6}M BA + 10^{-7}M NAA for 7 weeks.

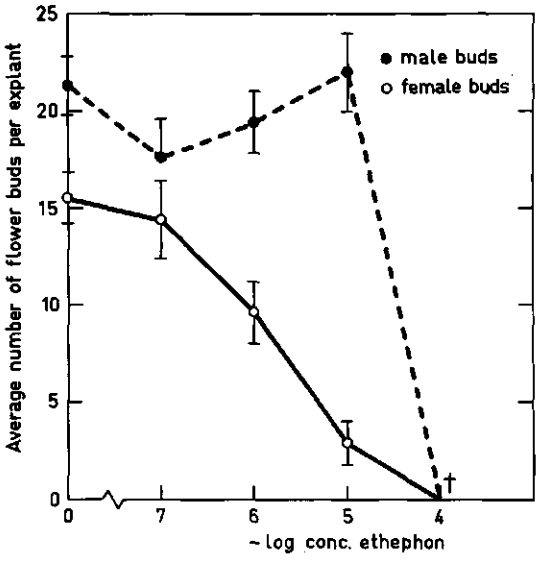

Fig. 4 Effect of ethephon on the numbers of male and female buds. Explants on medium + 10^{-6}M BA + 10^{-7}M NAA for 7 weeks.

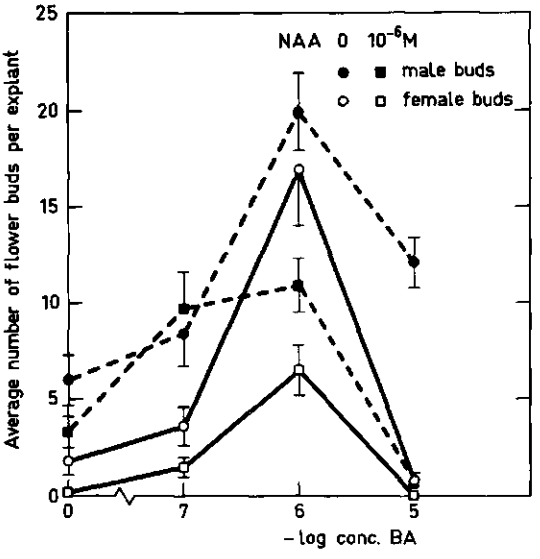

Fig. 5 Effect of BA and NAA on the numbers of male and female buds. Explants first grown on medium + 10^{-6}M BA + 10^{-7}M NAA for 10 days. Flower buds recorded after 8 weeks.

NAA to media in which their concentrations were varied. Time course studies revealed that after 10 days only some callus was formed. The first male buds became visible after about 3 weeks, while female buds were formed after approximately 5 weeks.

Fig. 5 shows that after transfer to media without BA and NAA the explants formed few male and female buds. BA strongly promoted flower bud formation, again particularly at 10^{-6}M. At 10^{-5}M BA female buds were hardly formed and the male buds had poorly developed stamen and were difficult to distinguish from the vegetative buds. NAA generally decreased the number of flower buds. The perianth of the buds was small on media with NAA and without or with 10^{-7}M BA, whereas stamen and pistil development were normal under these conditions. 10^{-8} or 10^{-7}M NAA also decreased the formation of flower buds, though less pronounced than 10^{-6}M.

We also studied the effects of GA_3, chlormequat, and ethephon after an initial period of 10 days on the basal medium with BA and NAA. The explants were transferred to media with GA_3, chlormequat or ethephon, supplemented with 10^{-6}M BA or 10^{-7}M NAA, or without these substances. The effects of GA_3, chlormequat, and ethephon were similar with and without BA or NAA, and comparable with the effects as shown in Fig. 2, 3, and 4, respectively. Thus, 10^{-8} or 10^{-7}M GA_3 decreased both male and female flower bud formation. Chlormequat had no effect at 10^{-6} or 10^{-5}M, and 10^{-6} or 10^{-5}M ethephon decreased the formation of female buds without affecting the number of male buds.

Discussion

The present paper demontrates that adventitious male and female flower buds can be induced abundantly *in vitro* on explants of *Begonia franconis* Liebm. The flower buds always developed on parts of the inflorescence, on stem segments only vegetative buds were found. This was also reported by RINGE and NITSCH (1968), and with other plant species *e.g. Nicotiana* (AGHION-PRAT, 1965), *Lunaria* (PIERIK, 1965), and *Cichorium* (MARGARA, 1974), although vegetative parts of *Plumbago* may produce generative buds (NITSCH and NITSCH, 1967).

Both cytokinins and auxins were required at specific concentrations. Without auxin the explants showed no regeneration at all and died off within a few weeks. After callus induction, however, auxin generally decreased the number of flower buds as was shown by the experiments (Fig. 5). RINGE and NITSCH (1968) suggested the function of auxin to be to maintain the vitality of the explants until meristems are organised. However, since the flower buds invariably originate from callus, a main function of auxin may be the promotion of callusing at

the explants.

In all experiments the first flower buds formed were always of the male type. As *in vivo*, the male buds had bracts on their pedicels. The axillary buds at these bracts may develop so that inflorescences are formed. Pistillate buds were never found to differentiate directly on the callus, they invariably originated from the axillary buds in the bracts of the pedicels of staminate buds. This indicates that either a generative meristem must be developing before female organs can differentiate, or that the male buds produce a specific factor required for female differentiation.

This factor might be the presence of functional phloemvessels. In accordance with previous observations, both *in vivo* and *in vitro* (BERGHOEF and BRUINSMA, 1979a and c), the differentiation of pistillate buds requires a high level of carbohydrates (Table 3). A high sucrose level in the medium, however, never induced female buds directly on the callus. Since preceding male buds do not specifically regulate female differentiation (BERGHOEF and BRUINSMA, 1979c), their effect by way of enhancing the nutritional state of the tissue is very likely.

Neither cytokinins nor auxins affected the ratio of male to female flowers, while gibberellin only generally decreased reproductive regeneration. Ethephon decreased the number of female flowers without affecting the number of male flowers. However, this is probably not a direct effect of ethephon on the sex-regulating system. The higher concentrations of ethephon caused early wilting of the male buds and pedicels. Because also the bracts and the axillary buds wilted, lack of development of female buds is an obvious consequence.

The results in the present paper confirm that the sex of the flower buds of *Begonia franconis* Liebm. is determined by the nutritional state of the tissue as a limiting factor for female differentiation.

Acknowledgements

The skilful technical assistance of Mrs. Y.E. v. Oosten-Legro is gratefully acknowledged.

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SAMENVATTING

In de inleiding is erop gewezen dat uitwendige factoren en regulatoren invloed kunnen hebben op de geslachtsexpressie. In deel I is beschreven hoe deze factoren de geslachtsexpressie beïnvloeden bij *Begonia franconis* Liebm. De bloeiwijzen van deze *Begonia* hebben in het algemeen twee mannelijke bloemen en een eindstandige, vrouwelijke bloem in de bractee van de bloemstengel van de tweede mannelijke bloem. Bloeiwijzen met een afwijkende samenstelling komen echter herhaaldelijk voor. Op grond van het aantal mannelijke bloemen dat wordt aangelegd voor de vrouwelijke bloem(en) is een indeling van de bloeiwijzen gemaakt in drie types. De eerst gevormde bloeiwijze op een tak heeft slechts een mannelijke en een vrouwelijke bloem. Geen van de toegepaste behandelingen gaf dit type bloeiwijze op andere posities aan de tak. Auxinen, gibberellinen en cytokininen, toegediend aan de vegetatieve toppen, alsmede een lage lichtintensiteit gaven een verlenging van de bloeiwijzen door de aanleg van meer mannelijke bloemen voor de vrouwelijke bloem. Behalve verlengen kan de bloeiwijze zich ook vertakken door het uitlopen van een okselknop in een bractee op de mannelijke bloemstengel. Dit kwam voor bij planten waarvan de zijtakken waren verwijderd en na behandeling met cytokinine. Een verandering van mannelijke bloemen in vrouwelijke bloemen werd in geen enkel geval waargenomen.

De resultaten tonen aan dat de toegepaste regulatoren het geslacht van de bloemen niet direct beïnvloeden. Alleen de verlenging van de bloeiwijze zou een direct vermannelijkend effect kunnen zijn. Belangrijk voor het geslacht van de bloemen lijkt de voedingstoestand te zijn, waarbij voor de differentiatie van de vrouwelijke bloem een hoger gehalte aan assimilaten nodig is dan voor de differentiatie van de mannelijke bloemen.

In deel II wordt beschreven hoe voedingsstoffen en regulatoren de groei van de bloemknoppen *in vitro* beïnvloeden. Hierbij bleken de zuurgraad, de samenstelling van de minerale voeding en de saccharoseconcentratie van groot belang te zijn voor een normale ontwikkeling van de bloemknoppen. Agar remde de groei, op een vloeibaar medium bereikten de bloemknoppen een grootte als *in vivo*. De aanwezigheid van een cytokinine in het medium was onmisbaar voor de groei. De opti-

male concentratie voor de vrouwelijke bloemen was ongeveer 10 tot 30 maal hoger dan voor de mannelijke bloemen. Het auxine IAA en ethephon beïnvloedden de groei van de knoppen niet, terwijl het abscisine ABA de groei remde in aanwezigheid van een cytokinine. Van de gibberellinen had GA_3 geen effect, terwijl GA_{4+7} de groei van de mannelijke bloemen bevorderde. Er was geen effect van de gebruikte regulatoren op het geslacht van de bloemen.

De invloed van regulatoren op de differentiatie van organen in bloemknopprimordia is behandeld in deel III. Hiervoor werden bloeiwijzen gebruikt waarvan de bloemknoppen nog ongedifferentieerd waren. Voor de differentiatie van de bloemknoppen was de aanwezigheid van een cytokinine of een gibberelline noodzakelijk. IAA, ABA en ethephon hadden geen effect. Het geslacht van de bloemknoppen werd door geen van de gebruikte regulatoren beïnvloed. De aan- of afwezigheid van de eerste of tweede knop had geen invloed op het geslacht van de derde (vrouwelijke) knop. Verwijdering van de eerste knop verminderde wel het positieve effect van gibberelline op de aanleg van bloemorganen in de twee overblijvende bloemknoppen. Een vermindering van het saccharosegehalte van 30 g/l tot 3 g/l verhinderde de differentiatie van vrouwelijke bloemknoppen volledig. De vorming van de mannelijke bloemknoppen werd hierdoor niet beïnvloed, terwijl in de helft van de gevallen een of meer mannelijke bloemknoppen extra werden gevormd.

Hoewel de bloemknoppen in de bloeiwijzeprimordia bij het begin van de cultures *in vitro* nog geen zichtbare differentiatie vertoonden is het mogelijk dat het geslacht van de bloemknoppen toch al bepaald was bij het begin van de experimenten. Daarom is ook de vorming van adventieve bloemknoppen *in vitro* bestudeerd en de invloed van regulatoren en saccharose op het geslacht van deze bloemknoppen. De resultaten hiervan worden gegeven in deel IV. Op de bloeiwijzestengels konden zich talrijke adventieve bloemknoppen vormen. Hiervoor waren zowel een cytokinine als een auxine in het medium noodzakelijk. Het auxine was alleen nodig gedurende de eerste tijd, na 10 dagen remde het de vorming van adventieve bloemknoppen. De eerst gevormde bloemknoppen waren altijd mannelijk, de vrouwelijke bloemknoppen ontstonden uit okselknoppen van bracteeën op de mannelijke bloemstengel. De vorming van vrouwelijke bloemknoppen direct op het callusweefsel is nooit waargenomen. Ook hier vonden we dat bij een verlaging van het saccharosegehalte van drie naar een procent de vorming van mannelijke bloemen nog wel mogelijk was, maar dat er geen vrouwelijke bloemknoppen werden gevormd. De gebruikte regulatoren, auxinen, cytokininen, gibberellinen, chlormequat en ethephon hadden geen invloed op de verhouding van de gevormde mannelijke en vrouwelijke bloemknoppen.

Uit de vier delen kan de conclusie worden getrokken dat regulatoren geen directe invloed hebben op het geslacht van bloemknoppen bij *Begonia franconis* Liebm. Veeleer blijkt de voedingstoestand van het weefsel tijdens de differentiatie bepalend te zijn. Voor de aanleg van de vrouwelijke bloemknoppen is een hoog gehalte aan assimilaten nodig. Dit blijkt zowel uit de verlenging van de bloeiwijzen bij lage lichtintensiteit (deel I) als uit de differentiatie van uitsluitend mannelijke bloemknoppen bij lage saccharosegehalten *in vitro* (delen III en IV). Regulatoren kunnen het geslacht slechts indirect beïnvloeden door hun invloed op de assimilatenstroom in de plant, bijv. via regulering van de sinkactiviteit. Dit verklaart de in deel I besproken verlengende effecten van regulatorentoedieningen aan de vegetatieve toppen.
In vivo zowel als *in vitro* worden altijd eerst mannelijke bloemknoppen gevormd. De oorzaak hiervan zou de aanleg van vaatbundels tijdens de ontwikkeling van de mannelijke knoppen kunnen zijn. Eerst wanneer deze vaatbundels voldoende assimilaten kunnen toevoeren kan differentiatie van de vrouwelijke bloemknoppen plaats vinden.

CURRICULUM VITAE

J. Berghoef werd geboren op 28 maart 1945 te Aalsmeer. In 1961 behaalde hij het einddiploma MULO en in 1964 het diploma van de Rijks Middelbare Tuinbouwschool te Aalsmeer. Na het behalen van het einddiploma HBS-B aan het Casimir Lyceum te Amstelveen in 1966, begon hij in september van dat jaar met zijn studie aan de Landbouwhogeschool te Wageningen. In januari 1974 slaagde hij met lof voor het doctoraal examen in de richting Tuinbouwplantenteelt met als hoofdvakken Tuinbouwplantenteelt en Plantenfysiologie en als bijvak Bodemvruchtbaarheid.
Van juli 1974 tot en met augustus 1977 was hij werkzaam bij de vakgroep Plantenfysiologie LH als promotieassistent. Van september 1977 tot en met februari 1978 was hij leraar Biologie aan de Rijks Middelbare Tuinbouwschool te Aalsmeer.
Vanaf maart 1978 is hij als wetenschappelijk ambtenaar verbonden aan de vakgroep Tuinbouwplantenteelt LH, sinds mei 1979 als wetenschappelijk medewerker eerste klas.