Flowering and vegetative propagation of pyrethrum (Chrysanthemum cinerariaefolium Vis.) in vivo and in vitro

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Abstract

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The influence of climatic conditions was investigated on flowering behaviour of pyrethrum (*Chrysanthemum cinerariaefolium* Vis.). At low temperatures high numbers of plants initiated high numbers of flower heads. Both the development of the initiated flower heads and the vegetative development of the plants were stimulated by higher temperatures.

The second aspect was the development of methods of vegetative propagation in vivo and in vitro. Through a culture of peduncle explants in vitro detailed information was obtained about the initiation and development of adventitious roots. With these data the process of adventitious root formation of shoot cuttings in vivo was optimalized. In practice this method of vegetative propagation in vivo may be useful for a fast multiplication of selected healthy plants with high yields of pyrethrins. Vegetative propagation was also achieved by initiation and development of adventitious shoots on capitulum explants cultivated in vitro and subsequent adventitious root formation of detached shoots. This procedure was applicable for vegetative propagation of several other *Compositae*.

Keywords: regeneration, organogenesis, adventitious, root formation, rhizogenesis, shoot formation, shoot cutting, explant, peduncle, capitulum, flower head, pyrethrins, Kenya, *Compositae*.

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The author graduated as Doctor in de Landbouwwetenschappen on 17 December 1976 at the Agricultural University in Wageningen on a thesis with the same title and contents.

Contents

1 General introduction

Pyrethrum (*Chrysanthemum cinerariaefolium* Vis.) is a plant species with daisy-like capitula (flower heads), belonging to the *Compositae*. This perennial rosette plant with deeply lobed leaves of variable shape and length is cultivated mainly in Kenya and other East African countries at an altitude of at least 1900 m above sea level. At this elevation and its low temperature, the capitulum is borne on a branched leafy stem, rising from a compact crown of foliage, whereas at a higher temperature in lower regions, the plants almost exclusively develop vegetatively (Gnadinger, 1945; Glover, 1955).

Pyrethrum is one of a few commercially grown plant species which produce compounds used as insecticides. The six constituents with insecticidal properties, collectively called pyrethrins, are located in all plant parts, but the ovaries and achenes of the disc and ray florets of the capitulum contain by far the highest concentration and largest amount (Head, 1966; Brewer, 1973). The capitula are picked by hand, dried and ground. The powder and the extract prepared therefrom contain these pyrethrins, which have unique properties, such as:

- high degree of suitability for combination with synergists (Chadwick, 1963),
- repellent, knockdown and toxic effects for a great variety of insects (van Rijn, 1974),
- almost complete harmlessness to men and warm-blooded animals (Griffin, 1973),
- rapid breakdown and no persistence of residues,
- hardly any buildup of resistance in insect populations (Busvine, 1960; Fine, 1963).

These properties permit the use of pyrethrins against insect pests in the house, on crops (even when a treatment is required just prior to harvest), stored food and livestock. Because of an increasing consciousness of the risks associated with the widespread use of many synthetic insecticides, like toxicity to mammals, persistence of residues and insect resistance, the demand for pyrethrins is growing.

Kenya provides about 60% of the world production of pyrethrins and this natural insecticide is among the principal export products of this country. In 1975 the production of about 100 000 farmers, 90% being small-scale producers, amounted to approximately 15 000 tons of dried flower heads, with a value of 15 million U.S. dollars (Glynne Jones, 1973; van Rijn, 1974).

Synthetic compounds resembling some of the six constituents of the natural pyrethrins have been manufactured (Elliot, 1967). Even though these synthetic pyrethroids are more toxic to insects, it is not likely that they will replace natural pyrethrins in the near future, because the latter are effective against a wider range of insects, more suitable for combination with synergists (Chadwick, 1963) and less expensive (Winney, 1973).

The application of natural pyrethrins is still restricted, because they are more expensive than many other insecticides. To strengthen the position of the natural

pyrethrins on the market it is of utmost importance to decrease their costs by increasing the annual production of pyrethrins per acreage. The yield is determined by several components, such as number, size, percentage dry matter and content of pyrethrins in the flower heads.

In pyrethrum breeding programmes the main objectives are the increase of the fresh flower yield (the product of number and size of fresh flower heads) and the pyrethrins content.

The fresh flower yield is determined by many factors, such as:
- the genotype (Parlevliet, 1969),
- the climate (rainfall, temperature, etc.; Glover, 1955; Parlevliet et al., 1969; Muturi et al., 1969; Parlevliet, 1970a),
- the soil (fertility, irrigation, ridging, spacing, weeding, etc.; Kroll, 1962, 1963; Weiss, 1966; Parlevliet et al., 1968; Mwakha, 1974),
- diseases and pests (like the root-knot nematode *Meloidogyne hapla*; Bullock, 1961; Robinson, 1963; Parlevliet & Brewer, 1970, 1971; Parlevliet, 1971).

The pyrethrins content is chiefly influenced by:
- the genotype (Head, 1967; Glynne Jones, 1968),
- picking interval and flowering stage (Head, 1966; Parlevliet, 1970b),
- the climate (Kroll, 1964; Parlevliet et al., 1969; Muturi et al., 1969),
- drying methods (Githinji, 1973; Head, 1973).

From 1940-1965 the pyrethrum breeding programme in Kenya was principally directed towards the production of good hybrids. Hybridization could easily be achieved, because most plants are self-incompatible and cross-compatible (Kroll, 1958; Brewer, 1968; Brewer & Parlevliet, 1969; Brewer & Henstra, 1974). As the genetic properties of the parent plants are widely divergent, seedling populations exhibit a considerable genotypic and phenotypic variability. Breeding and selection may further be complicated by a negative correlation between flower yield and pyrethrins content so that individual plants with both a high flower yield and a high pyrethrins content are very rare. In a population, however, both genetic characteristics can be improved in a fairly short time (Contant, 1963; Parlevliet, 1974).

The fixation of desired genetic properties is one of the greatest problems in the breeding of a cross-pollinated, highly heterozygous crop. Genetic characteristics of selected plants can be preserved by vegetative propagation. Selection and asexual multiplication of superior clones may be an even better method of improvement than just the production of good hybrids (Brown, 1965; Glynne Jones, 1968; Brewer & Parlevliet, 1969; Parlevliet, 1969; Parlevliet & Contant, 1970).

The actual breeding, selection and vegetative propagation of outstanding clones was started by Contant in Kenya in 1962 (Contant, 1963). Through his initiative and thoroughness and with financial support of the Ministry of Foreign Affairs of the Netherlands, a developmental project was set up in Kenya. Two Dutch plant breeders and several groups of Netherlands volunteers were posted in Kenya and charged with the breeding and selection of high yielding plants, and the vegetative propagation of selected plants on large-scale nurseries, respectively.

Because research on pyrethrum in Kenya lacked continuity and because this species

was of interest to several research institutes, fundamental research on pyrethrum in Wageningen was started in 1964. In 1966 the work was formally organized in a research project by Contant at the Association Euratom-Ital. At first this research was concentrated on plant breeding, but at the end of 1969 the investigations, which are described in this report, were shifted towards more practical aspects like flowering and vegetative propagation. The project was terminated at the end of 1972.

A vegetative propagation of pyrethrum can be achieved in several ways, like: multiplication from splits (the normal procedure in Kenya) and multiplication from shoot cuttings. The practical importance of propagation by splits has been indicated by several authors (Drain & Shuey, 1934; Martin & Tattersfield, 1934; Cormack, 1935; Chamberlain & Procter, 1947; Osbourn, 1961; Contant, 1963; Brown, 1965). The use of cuttings has also been reported by many authors (Drain & Shuey, 1934; Cormack, 1935; Chamberlain & Procter, 1947; Collings-Wells & Contant, 1963; Canham, 1968).

A substantial percentage of the root systems of pyrethrum plants in Kenya is severely attacked by the root-knot nematode *Meloidogyne hapla* so that flower yield decreases. If a selected stock-plant is infected, splitting results in a contamined clone, whereas with shoot cuttings a healthy clone is built up. Shoot cuttings may also have the advantage that a much higher multiplication rate may be achieved than with splits. Hence, a healthy clone of selected high yielding plants is probably obtained more rapidly from shoot cuttings than from splits.

This study deals with flowering and vegetative propagation of pyrethrum in vivo and in vitro. The influence of temperature and photoperiod on flowering behaviour of intact plants is discussed in Chapter 2. Chapters 3, 4 and 5 describe methods of vegetative propagation in vivo and in vitro. In Chapter 3 root formation is examined of peduncle explants cultivated in vitro. This method may provide detailed information on root organogenesis as affected by various factors. To what extent this knowledge can be applied to ensure a reliable root formation of shoot cuttings in vivo, is investigated in Chapter 4. In Chapter 5 is discussed whether a vegetative propagation can be achieved by the cultivation of capitulum explants in vitro.

2 Flowering behaviour of intact plants

2.1 INTRODUCTION

Pyrethrum plants almost completely fail to produce flower heads at high temperatures which occur in the lowlands (below 1900 m above sea level) of tropical countries. For an adequate production of large numbers of flower heads pyrethrum is grown in the highlands at altitudes between 1900 and 2800 m, where lower temperatures prevail (Martin & Tattersfield, 1934; Gnadinger, 1945; Parlevliet, 1969).

The temperature requirements for flowering under natural conditions in East Africa were investigated by Glover (1955). The flowering behaviour of intact plants under controlled conditions in growth chambers, however, has never been studied. Such studies may show how factors, like temperature and photoperiod, affect the vegetative and generative development of the plants. A vegetative development is desirable for the maintenance and multiplication of clonal plant material and a generative development is essential for the commercial production of pyrethrins from the flower heads.

2.2 MATERIAL AND METHODS

Stock plants of Clones 1087, 4331 and Ma 63/1889 were grown in a moderately heated greenhouse. Depending on the weather conditions outside, the temperature ranged from 20-35° in summer and from 10-20°C in winter. In the winter natural light was supplemented with high vapour mercury lamps. At the higher temperatures during summer the plants almost exclusively developed vegetatively and shoot cuttings were taken from stock-plants of 1-2 years old. Shoot cuttings of the three clones were rooted according to the procedure described in Section 4.2. Subsequently, the young plants were exposed for a few weeks to a constant temperature of 20°C under long-day conditions (Philips fluorescent tubes MF 140 W/33RS supplemented with Philinea incandescent lamps at a day-length of 14 h) in a growth chamber, before transfer to various temperatures and photoperiods in the phytotron[1].

Data were collected about the rate of floral initiation, the number of flowering plants and flower heads and the developmental stage of the flower heads. With respect to the vegetative development attention was paid to the shape and length of the leaves.

1. The phytotron of the Department of Horticulture (Agricultural University, Wageningen) has been described by Doorenbos (1964).

2.3 FACTORS INFLUENCING FLOWERING

2.3.1 Temperature

2.3.1.1 Constant temperature

Experiment 1. Young plants of clone Ma 63/1889 were exposed to constant temperatures of 9, 13, 17, 21 or 25°C for a daylength of 16 h and with 20 plants per temperature.

About 10 weeks after transfer the first flower heads developed simultaneously at 9 and 13°C. Table 1 illustrates the situation at the end of the experiment, 24 weeks after exposure. Apparently, low temperatures of 9 and 13°C are essential for floral initiation. The highest number of flower heads was initiated at 9°C. The subsequent development of

Fig. 1. The influence of constant temperatures (9-25°C) on the generative and vegetative development of plants of Clone Ma 63/1889, 19 weeks after exposure.

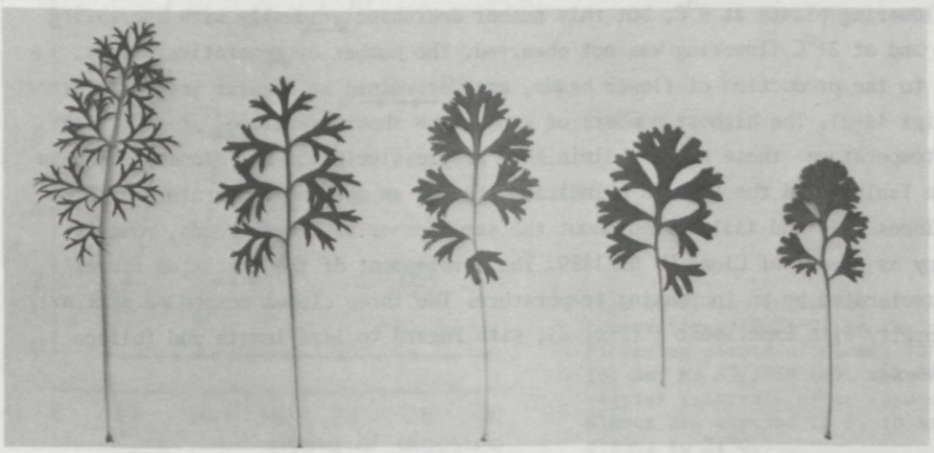


Fig. 2. The influence of constant temperatures (9-25°C) on the shape and length of leaves of plants of Clone Ma 63/1889, 19 weeks after exposure.

Table 1. The influence of constant temperatures (9-25°C) on the generative and vegetative development of plants of Clone Ma 63/1889, 24 weeks after exposure.

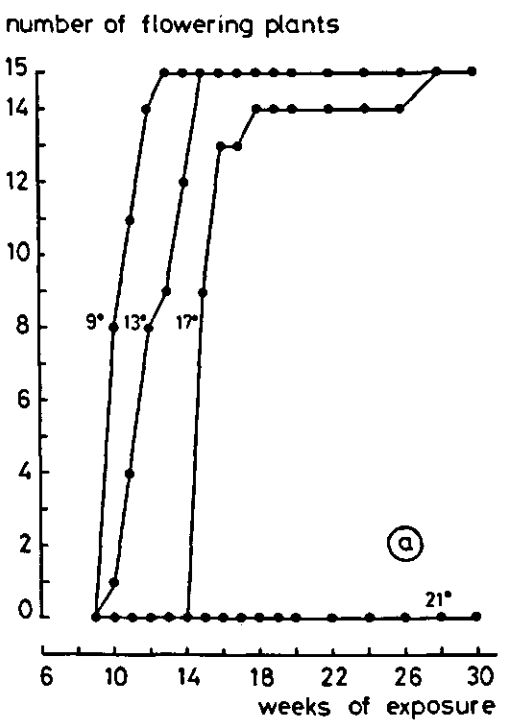
Temperature (°C)	Number of flowering plants (out of 20)	Number of flower heads	Number of flower heads per flowering plant	Average leaf length in cm
9	18	55	3.1	14.1
13[1]	13	24	1.8	14.3
17	0	0	0	12.8
21	0	0	0	11.0
25	0	0	0	11.0

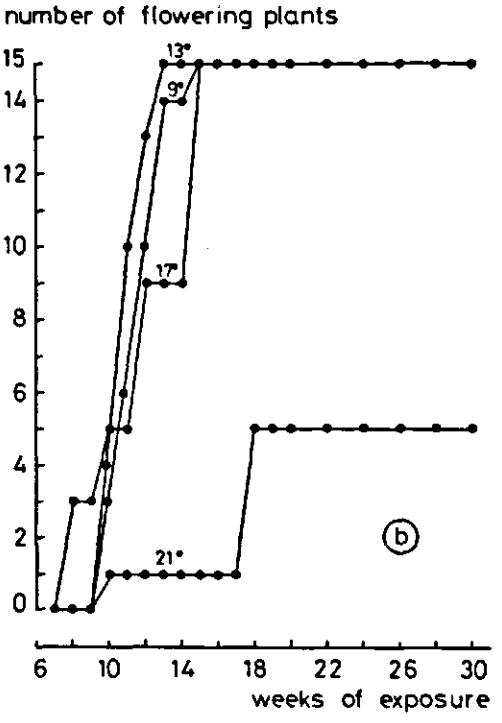
1. At 13°C one plant did not survive.

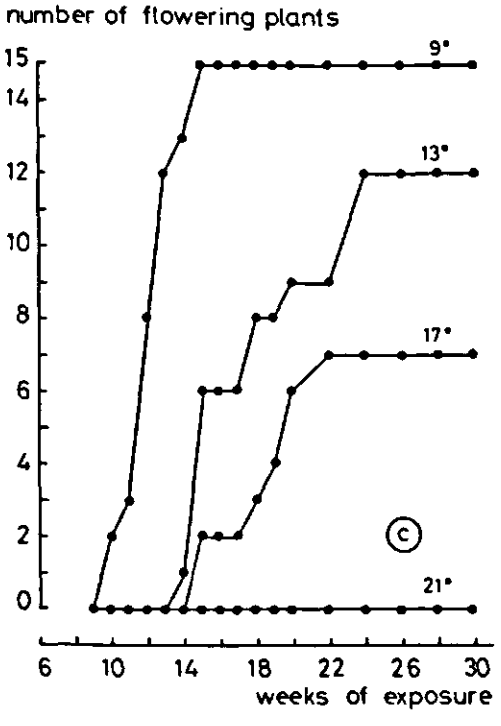
the initiated flower heads, however, was stimulated at 13°C, which can be observed in Fig. 1, where at 9°C flower heads are in the bud-stage and at 13°C are already in full bloom. At 17, 21 and 25°C the plants remained in a vegetative state. The appearance of the plants varied strongly with temperature, which was not only due to flowering but also to the length of the leaves (Table 1) and to pronounced differences in foliage type (Fig. 2).

Experiment 2. Young plants of Clones 1087, 4331 and Ma 63/1889 were exposed to constant temperatures of 9, 13, 17 or 21°C for a daylength of 16 h. Per clone 15 plants were exposed to 9, 13 or 17°C and 5 plants to 21°C.

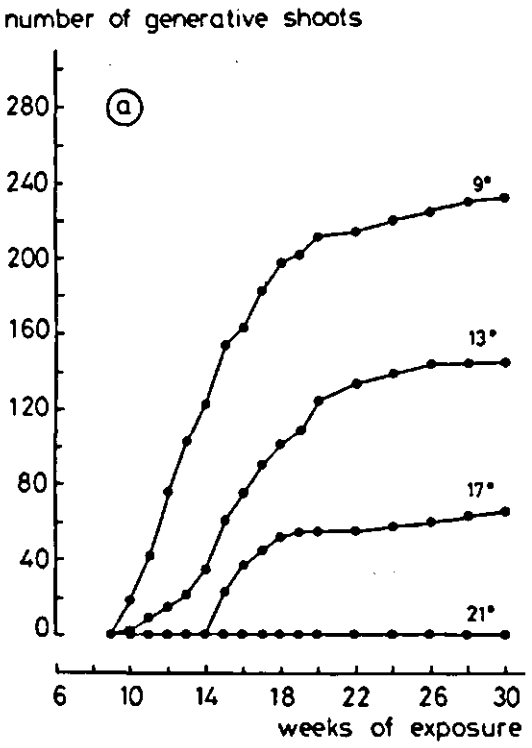
The three clones developed the first flower heads almost simultaneously 9 weeks after transfer; Clone 1087 first at 9 and 13°C and afterwards at 17°C (Fig. 3a), Clone 4331 at the same time at the four temperatures (Fig. 3b) and Clone Ma 63/1889 in first instance at 9°C and subsequently at 13 and 17°C (Fig. 3c). Clone 4331 yielded the maximum number of flowering plants at all temperatures, as did Clone 1087, except at 21°C, at which temperature flowering did not occur. Clone Ma 63/1889 had the maximum number of flowering plants at 9°C, but this number decreased gradually with increasing temperature and at 21°C flowering was not observed. The number of generative shoots, giving rise to the production of flower heads, was determined at regular intervals after exposure (Figs 4a-c). The highest numbers of generative shoots developed at 9°C; with increasing temperature these numbers diminished progressively for all clones. The data presented in Table 2 and the Figs 5a-c indicate that at an optimum temperature of 9°C plants of Clones 1087 and 4331 formed about the same number of flower heads, roughly twice as many as plants of Clone Ma 63/1889. The development of the initiated flower heads was accelerated by an increasing temperature. The three clones responded similarly and in conformity with Experiment 1 (Fig. 2), with regard to leaf length and foliage type (Figs 6a-c).

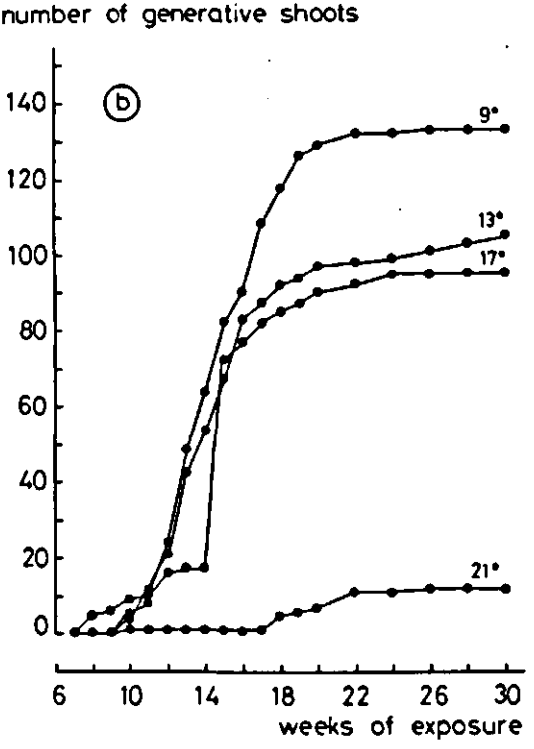


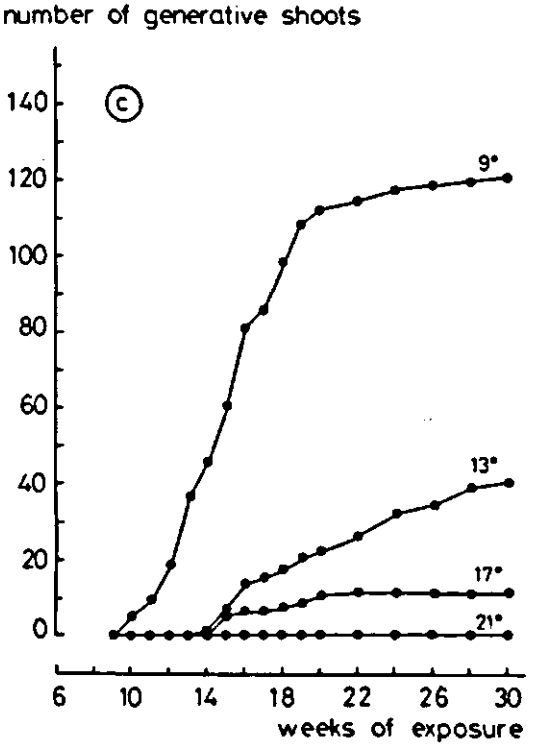




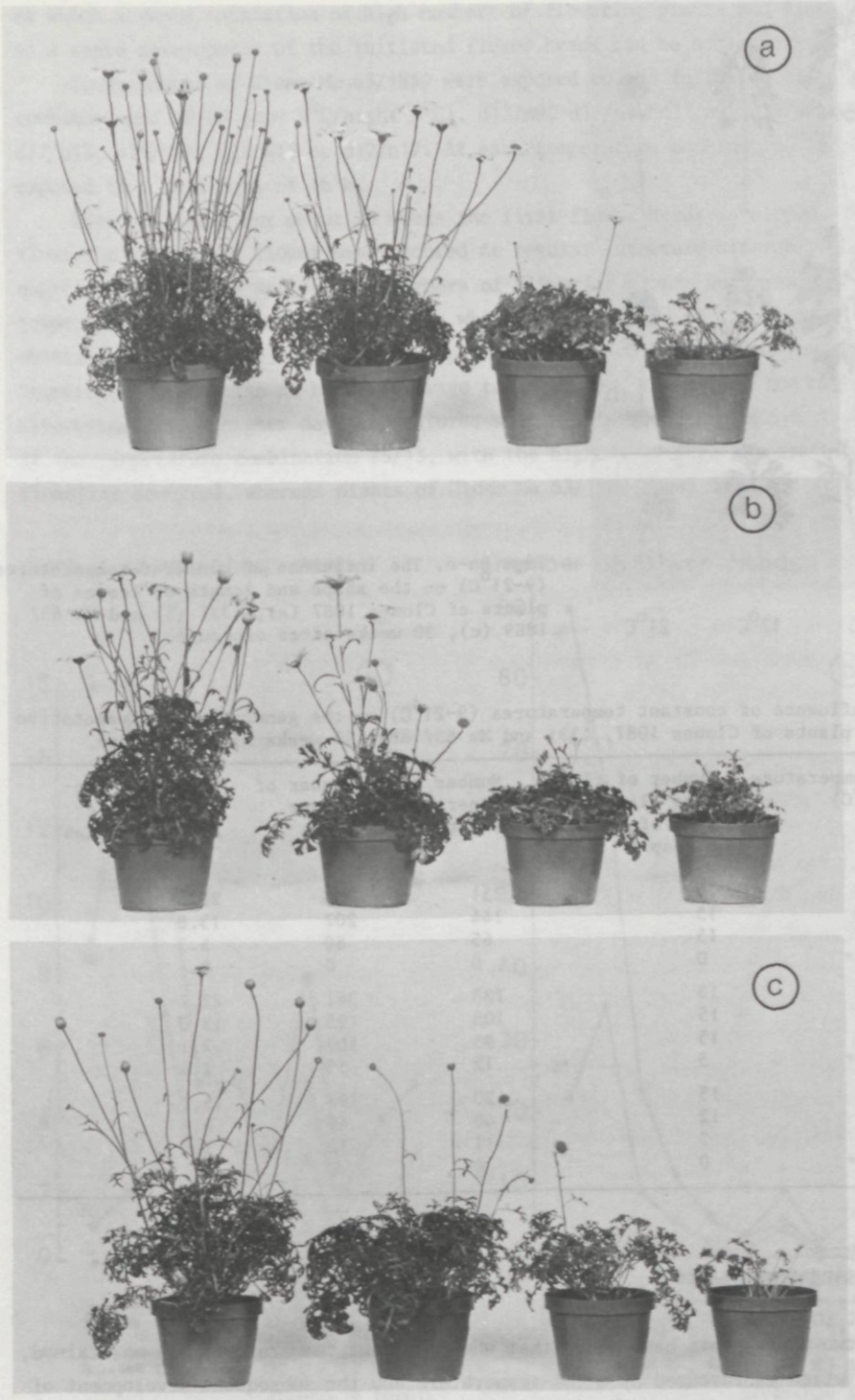
Figs 3a-c. The influence of constant temperatures (9-21°C) on the number of flowering plants of Clones 1087 (a), 4331 (b) and Ma 63/1889 (c), measured at regular intervals after exposure. (15 plants are exposed to 9, 13 and 17° and 5 plants to 21°C.)







Figs 4a-c. The influence of constant temperatures (9-21°C) on the number of generative shoots of plants of Clones 1087 (a), 4331 (b) and Ma 63/1889 (c), measured at regular intervals after exposure. (15 plants are exposed to 9, 13 and 17° and 5 plants to 21°C.)



Figs 5a-c. The influence of constant temperatures (9-21°C) on the generative and vegetative development of plants of Clones 1087 (a), 4331 (b) and Ma 63/1889 (c), 30 weeks after exposure.

Figs 6a-c. The influence of constant temperatures (9-21°C) on the shape and length of leaves of plants of Clones 1087 (a), 4331 (b) and Ma 63/1889 (c), 30 weeks after exposure.

Table 2. The influence of constant temperatures (9-21°C) on the generative and vegetative development of plants of Clones 1087, 4331 and Ma 63/1889, 30 weeks after exposure.

Clone	Temperature (°C)	Number of flowering plants (out of 15 and 5*resp.)	Number of generative shoots	Number of flower heads	Number of flower heads per flowering plant
1087	9	15	231	383	25.5
	13	15	144	207	13.8
	17	15	65	80	5.3
	21*	0	0	0	0
4331	9	15	133	341	22.7
	13	15	105	195	13.0
	17	15	95	107	7.1
	21*	5	12	12	2.4
Ma 63/1889	9	15	120	196	13.1
	13	12	40	49	4.1
	17	7	11	12	1.7
	21*	0	0	0	0

2.3.1.2 Fluctuating temperature

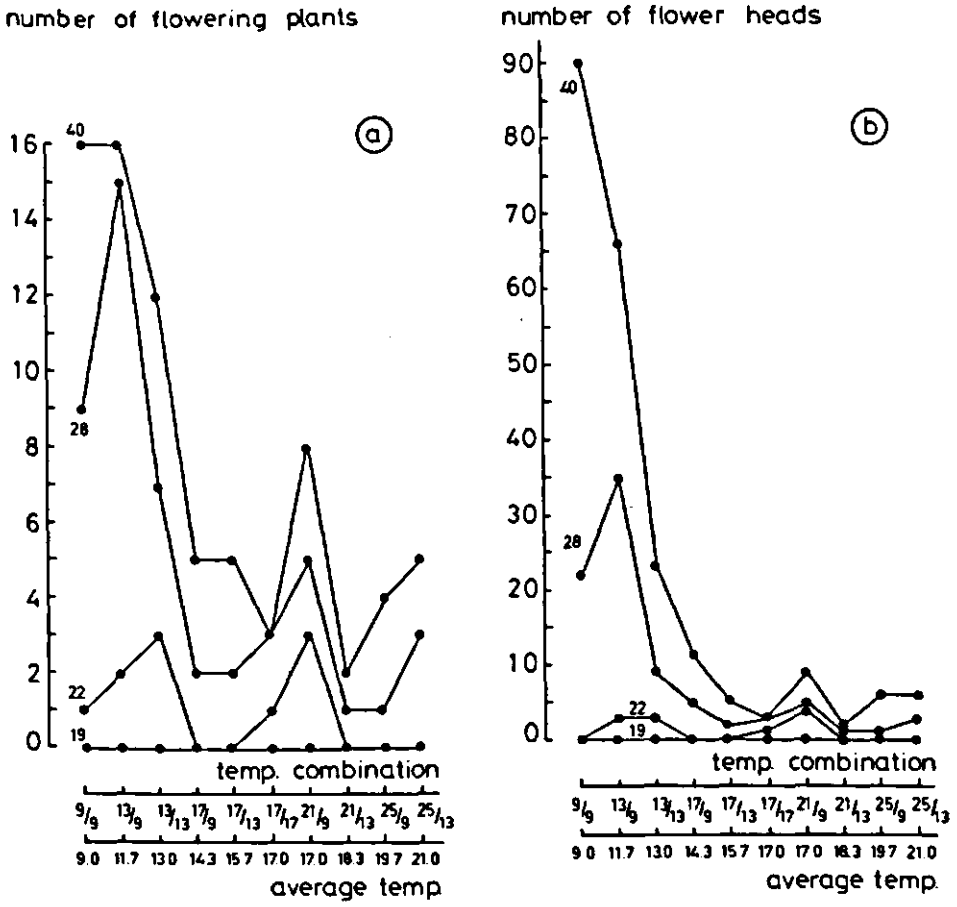
The first two experiments have shown that when constant temperatures are maintained, the floral initiation is favoured by a low temperature and the subsequent development of the initiated flower heads is stimulated by a higher temperature.

Experiment 3. In this experiment is examined whether there is a temperature combination

at which a rapid initiation of high numbers of flowering plants and flower heads as well as a rapid development of the initiated flower heads can be achieved.

Young plants of Clone Ma 63/1889 were exposed to the following temperature combinations: d9/n9 (day 9°C/night 9°C), d13/n9, d17/n9, d21/n9, d25/n9, d13/n13, d17/n13, d21/n13, d25/n13 or d17/n17. At each temperature combination 16 plants were exposed to a daylength of 16 h.

After exposure for about 20 weeks the first flower heads developed. The numbers of flowering plants and flower heads scored at regular intervals after exposure are expressed in Figs 7a and 7b. The numbers of flowering plants were maximum at the temperature combinations 9/9 and 13/9, while a high number of flowering plants was observed at 13/13 (Table 3). However, plants did not only flower at these lower average temperatures, but also at higher average temperatures, in which a low night-temperature alternated with a higher day-temperature, some plants becoming reproductive. For instance, at the temperature combination 25/13, with the highest average temperature of 21°C, flowering occurred, whereas plants of Clone Ma 63/1889 never flowered at a constant



Figs 7a-b. The influence of fluctuating temperatures on the number of flowering plants (a) and of flower heads (b) of plants of Clone Ma 63/1889, measured 19, 22, 28 and 40 weeks after exposure.

Table 3. The influence of fluctuating temperatures on the generative and vegetative development of plants of Clone Ma 63/1889, 40 weeks after exposure.

Temperature combination day/night (°C)	Average temperature (°C)	Number of flowering plants (out of 16)	Number of flower heads	Number of flower heads per flowering plant
9/9	9.0	16	90	5.6
13/9	11.7	16	66	4.1
13/13	13.0	12	23	1.9
17/9	14.3	5	11	2.2
17/13	15.7	5	5	1.0
17/17	17.0	3	3	1.0
21/9	17.0	8	9	1.1
21/13	18.3	2	2	1.0
25/9	19.7	4	6	1.5
25/13	21.0	5	6	1.2

temperature of 21°C (Experiments 1 and 2). At night temperatures of 9 and 13°C, maintained for 8 h, flower heads were initiated, even though day temperatures of 21 and 25°C were maintained for 16 h. At night temperatures of 9 and 13°C the flowering response decreased as the day temperature increased from 9 or 13 to 25°C. The flowering response also decreased when at day temperatures of 13, 17, 21 and 25°C the night temperature increased from 9 to 13°C or 17°C. Considerable numbers of flower heads were produced when day temperatures of 9 or 13°C were combined with night temperatures of 9 and, to a lesser extent, 13°C. The development of the initiated flower heads was accelerated by high day-temperatures of 17, 21 and 25°C. Vegetative characteristics, like the shape and length of the leaves, were determined by the day temperature to which the plants were exposed for 16 h. Consequently, leaves from plants of the temperature combinations 17/9, 17/13 and 17/17 showed a similar appearance. The foliage type and the length of leaves from plants cultivated at day temperatures of 9, 13, 17, 21 or 25°C corresponded with those of leaves from plants grown at the same constant temperatures (Fig. 2).

Experiment 4. Experiment 3 showed that a period of a continuous low temperature is essential for the production of high numbers of flowering plants and flower heads. But how long should this cold period last, before plants of Clone Ma 63/1889 can be exposed to a higher temperature, at which the initiated flower heads develop rapidly?

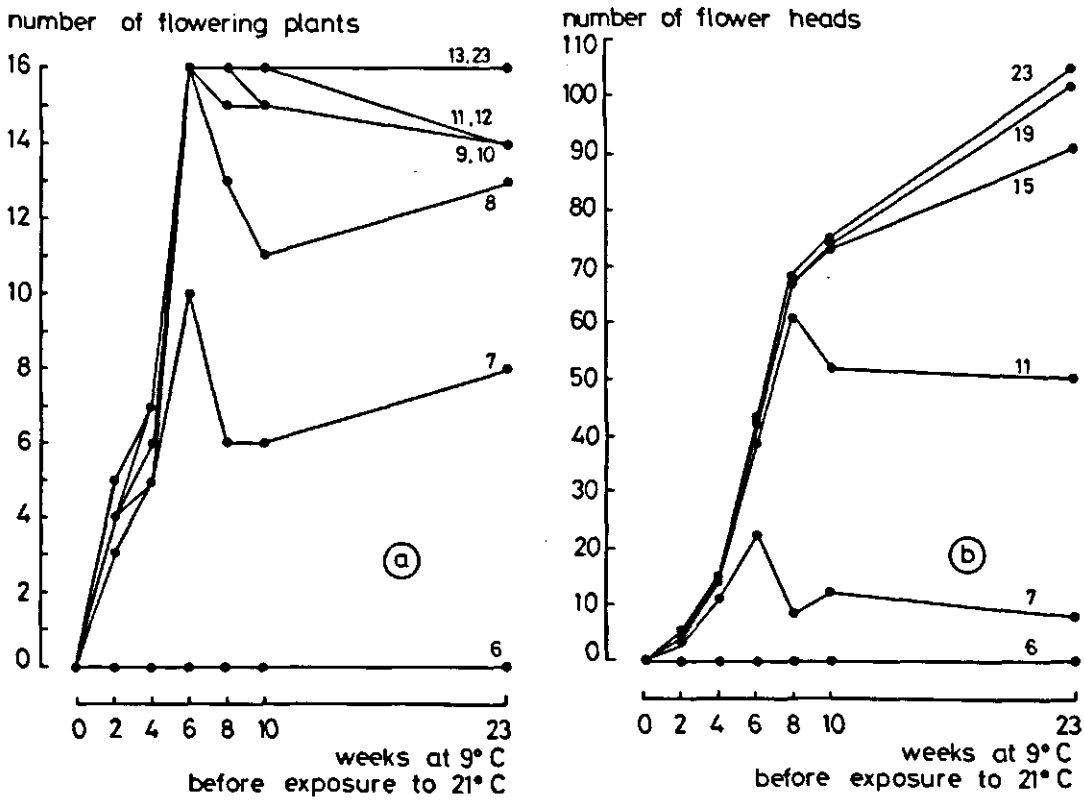
This question was examined in an experiment with young plants of Clone Ma 63/1889, which were subjected for 0, 2, 4, 6, 8, 10 or 23 weeks to 9°C before exposure to 21°C. Per treatment 16 plants were exposed to a photoperiod of 16 h.

In conformity with previous experiments flowering did not occur after exposure to a constant temperature of 21°C (Table 4). Fig. 8a shows that after a cold period the first plants started to flower 7 weeks after transfer, irrespective of whether 9°C was maintained for 2, 4, 6 or 7 weeks. Cold periods for 2 and 4 weeks were inadequate for an optimum generative development, because few plants became reproductive at 21°C. Seven weeks after transfer, the number of flowering plants was highest for those plants sub-

Table 4. The influence of exposure for 0, 2, 4, 6, 8, 10 or 23 weeks to a constant temperature of 9°C, before exposure to a constant temperature of 21°C, on the generative and vegetative development of plants of Clone Ma 63/1889, 23 weeks after exposure.

Weeks 9°C ⟶ 21°C		Number of flowering plants (out of 16)	Number of flower heads	Number of flower heads per flowering plant
0	23	0	0	0
2	21	5	5	1.0
4	19	7	15	2.1
6	17	16	43	2.7
8	15	16	68	4.3
10	13	16	75	4.7
23	0	16	105	6.6

jected to 9°C for 6 weeks, probably because they had been exposed for 1 week to 21°C, while plants of the groups to be exposed for 8, 10 or 23 weeks to 9°C were still at 9°C, at which temperature flower heads developed more slowly than at 21°C. Seven weeks after transfer, plants that had to be subjected to cold periods of 8, 10 and 23 weeks were still exposed to 9°C. Theoretically these treatments should not differ, but in reality after 7 weeks in the group to be exposed for 23 weeks to 9°C two more plants were



Figs 8a-b. The influence of exposure for 0, 2, 4, 6, 8, 10 or 23 weeks to a constant temperature of 9°C, before exposure to a constant temperature of 21°C, on the number of flowering plants (a) and of flower heads (b) of plants of Clone Ma 63/1889, measured 6-23 weeks after exposure.

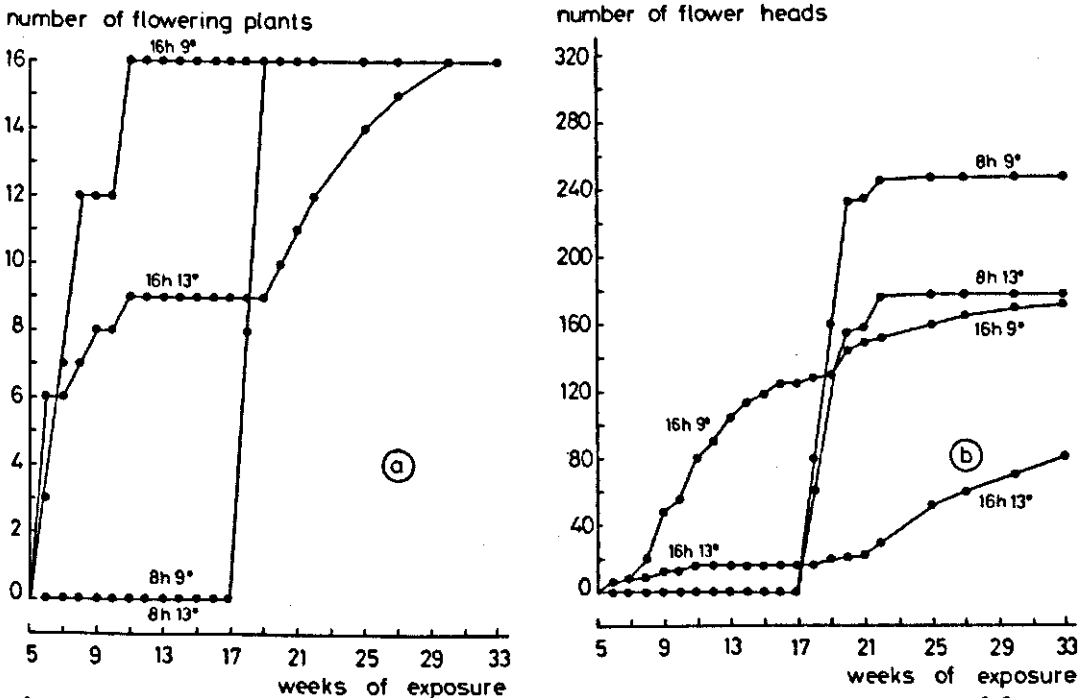
flowering than in the groups to be exposed for 8 or 10 weeks to 9°C (Fig. 8a). Such slight divergences from horizontal lines, which also occurred 8, 9 and 10 weeks after transfer, can also be observed in Fig. 8b. Maximum numbers of flowering plants in the groups to be exposed for 6, 8, 10 and 23 weeks to 9°C can be observed 8, 10, 11 and 13 weeks after transfer, respectively (Fig. 8a). Thus chilling for 6 weeks was sufficient for floral initiation of all plants. Initiated flower heads developed most rapidly when the plants were exposed to 21°C after a cold period of 6 weeks. The development of the flower heads was progressively retarded with an increasing duration of the cold period. Thus 7 and 11 weeks after transfer the numbers of flower heads were highest in those groups, where chilling persisted for 6 and 8 weeks, respectively (Fig. 8b). Despite the delayed development of the flower heads, however, 15 weeks after transfer the number of flower heads was highest in the group where chilling still persisted (group 23 weeks of 9°C). Finally, 23 weeks after transfer, the number of initiated flower heads increased with an increasing duration of the cold period; the highest number of flower heads was produced at a constant temperature of 9°C. Plants subjected first to 9°C and subsequently to 21°C, developed two foliage types, characteristic for both temperatures (Fig. 2).

2.3.2 Photoperiod

As many species of the genus *Chrysanthemum* are sensitive to daylength, it is of interest to check whether *Chrysanthemum cinerariaefolium* also responds to the photoperiod.

Young plants of Clone Ma 63/1889 were exposed to photoperiods of 8 (short day) and 16 h (long day), at 9, 13 or 17°C. Unfortunately, plants exposed to 17°C, both under short-day and long-day conditions, had to be discarded prematurely because of an attack by red spiders.

As can be observed in Fig. 9a the first plants became reproductive 5-6 weeks after transfer to long-day conditions, both at 9 and 13°C. At a daylength of 8 h the generative development of the plants was considerably delayed and the first flower heads developed 17-18 weeks after transfer, at 9 and 13°C. Flowering under long-day conditions occurred more gradually than under short-day conditions, where within 2 weeks all plants became reproductive. Ultimately, maximum numbers of flowering plants were observed in all treatments. Initially, flower heads were initiated under long-day conditions only, but afterwards a very sudden appearance of flower heads under short-day conditions was noticed (Fig. 9b). Finally, substantially higher numbers of flower heads were initiated under short-day conditions than at the corresponding temperatures under long-day conditions. At both photoperiods more flower heads were produced at 9 than at 13°C (Table 5). The flower heads developed faster at 13°C than at 9°C and faster under long-day conditions than under short-day conditions. At a daylength of 8 h the flower heads remained smaller than under long-day conditions, where flower heads of a normal size were produced. With respect to the shape and length of the leaves, it was observed that under long-day conditions at 9, 13 and 17°C normal leaves developed, whereas under short-day conditions at 9, 13 and 17°C the leaves were smaller, but with the same leaf-shape as for long days (Fig. 10).



Figs 9a-b. The influence of photoperiods of 8 and 16 h, at constant temperatures of 9 and 13°C, on the number of flowering plants (a) and of flower heads (b) of plants of Clone Ma 63/1889, measured at regular intervals after exposure.

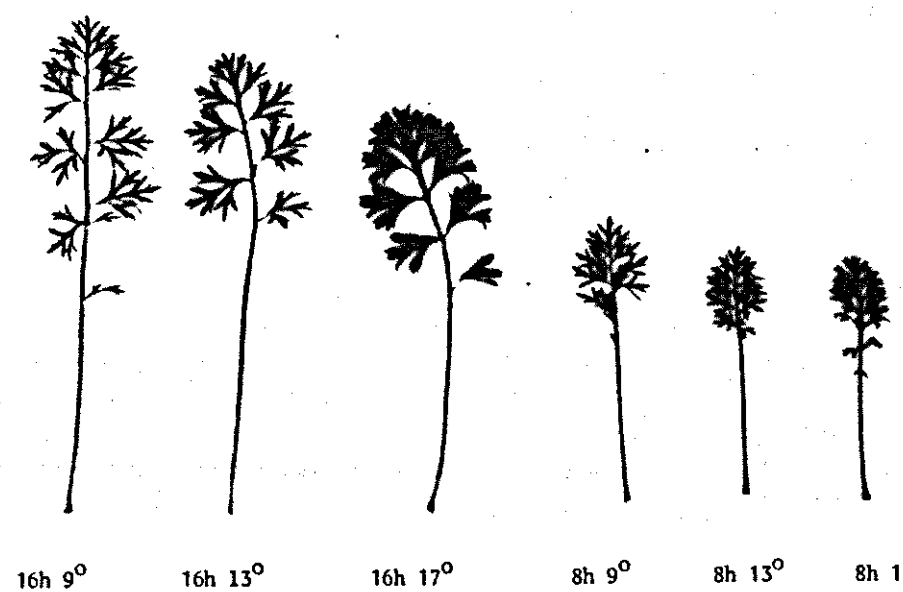


Fig. 10. The influence of photoperiods of 8 and 16 h, at constant temperatures of 9, 13 and 17°C, on the shape and length of leaves of plants of Clone Ma 63/1889, 17 weeks after exposure.

Table 5. The influence of photoperiods of 8 and 16 h, at constant temperatures of 9 and 13°C, on the generative and vegetative development of plants of Clone Ma 63/1889, 33 weeks after exposure.

Photoperiod (h)	Temperature (°C)	Number of flowering plants (out of 16)	Number of flower heads	Number of flower heads per flowering plant
8	9	16	248	15.5
	13	16	178	11.1
16	9	16	173	10.8
	13	16	82	5.1

2.4 DISCUSSION AND CONCLUSIONS

The plant has to pass through different stages before it flowers, namely ripeness-to-flower, floral induction and initiation and development of the flower heads. The young pyrethrum plants, which originated as cuttings from adult plants, reached the stage of ripeness-to-flower.

The vegetative and the generative development of young plants of Clones 1087, 4331 and Ma 63/1889 were markedly affected by temperature. All clones showed essentially the same reaction to constant temperatures. As the temperature decreased, more flower heads were initiated, the best floral initiation was obtained at 9°C. It was not investigated whether still lower temperatures would stimulate the initiation of flower heads further. The subsequent development of the initiated flower heads was stimulated by temperatures of 17, 21 and 25°C.

The temperature requirements of Clones 1087 and Ma 63/1889 were *qualitative*, so that at a constant high temperature (21°C) the plants did not flower at all, whereas the reaction of Clone 4331 proved to be *quantitative*, i.e. the plants also flowered at a constant high temperature (21°C). Thus plants of Clone 4331 may already have been induced at the constant temperature of 20°C, to which the plants were exposed in the growth chamber before transfer to the phytotron, while plants of Clones 1087 and Ma 63/1889 cannot have been induced at this temperature.

In conformity with observations in practice, Clone Ma 63/1889 showed the most pronounced cold requirement for flowering. This clone was selected in the Marindas district in Kenya at an altitude of about 3000 m, where the prevailing temperatures are constantly low. At lower altitudes, with higher temperatures, the flowering response of this clone was inadequate (Hoekstra, pers. commun.).

Since some variation in the flowering responses of the clones was observed, it is recommended to investigate the flowering behaviour of each selected new clone, to find out the most suitable region in which the clone should be cultivated.

A fluctuation of the temperature with day (16 h) and night (8 h) demonstrated that plants of Clone Ma 63/1889 flowered at an average temperature of 21°C (16 h day 25° and 8 h night 13°C), whereas flowering was never observed at a constant temperature of 21°C. For an optimum initiation of flower heads on plants of Clone Ma 63/1889, however, a

period with a continuous low temperature of 9° and, to a lesser extent, 13°C, was required. All plants of Clone Ma 63/1889 flowered when they were subjected to 9°C for at least 6 weeks, but chilling for a more prolonged period improved the initiation of flower heads. The number of initiated flower heads was about proportional to the duration of the cold period.

These findings correspond well with the data of Glover (1955), who investigated the temperature requirements for flowering under natural conditions in East Africa. He observed in field trials that the yield of pyrethrum flowers was directly related to the number of days spent by the plant at or below 15°C, some three months earlier. In vegetative plants flower heads were already initiated after exposure to 15°C for 10 days. Persistent chilling was more effective in stimulating bud production than more severe chilling maintained at night and alternated with periods of higher day-temperatures. If a mean temperature of 24°C was prolonged for one week or more, the production of flower heads was inhibited.

Under field conditions in Kenya flower heads are produced approximately 10 weeks after the start of a rain period with low temperatures. Depending on the district there are 2 or 3 rain periods during each year, alternating with dry periods of higher temperatures. In an ideal situation the plants initiate flower heads during the rainy season and these flower heads develop rapidly in the dry period.

In addition, to ensure a large-scale vegetative propagation in nurseries, it is recommended to cultivate pyrethrum at high temperatures in the lowlands to stimulate the vegetative development and to avoid or greatly reduce the generative development of the plants.

Vegetative characteristics, like the shape and length of the leaves, depend in the first place upon the genotype, but the different clones showed an analogous reaction to temperature. Both shape and length of leaves of Clone Ma 63/1889 were strongly affected by temperature, while the photoperiod only influenced the length of the leaves.

Apart from the temperature, the flowering behaviour of plants of Clone Ma 63/1889 also responded to the photoperiod. The first plants became reproductive under long-day conditions and it seemed that pyrethrum could be regarded as a long-day plant. Under long-day conditions the first flower heads were initiated rapidly and afterwards reasonable numbers of flower heads of a normal size developed gradually. Under short-day conditions, however, the initiation was strongly retarded, while suddenly considerably higher numbers of smaller flower heads developed. These results may be attributed to a lower rate of photosynthesis in 8 h than in 16 h of light. In conclusion, pyrethrum has to be regarded as a *quantitative* short-day plant, so that both under long and short days flowering is achieved, but the highest number of flower heads is produced under short-day conditions. These photoperiodic effects are of no direct practical importance for the cultivation of pyrethrum in tropical countries, where throughout the year the daylength is approximately 12 h. Thus the reaction of pyrethrum to the photoperiod was not further studied. From a fundamental point of view, however, it may be very interesting to analyse this very strange photoperiodic response.

3 Root formation of peduncle explants in vitro

3.1 INTRODUCTION

Vegetative propagation of many crops can be achieved through the formation of roots on basal stem portions of shoot cuttings. Unless roots originate from primordia that already existed at the time of making the explant, as in certain cases, roots result from a genuine initiation process. This means a dedifferentiation of cells which, after having reverted to the primary meristematic state, are organized to give a root meristem (Gautheret, 1966a). When cells of the root meristem divide, differentiate and elongate a root primordium, an adventitious root, and finally a root system develop.

As indicated in Chapter 1 vegetative propagation of pyrethrum from shoot cuttings would be desirable. Before developing a practical method (Chapter 4), it would be useful to have detailed information about the influence of various factors on this process of adventitious root formation. This can be achieved by culture in vitro, the technique of growing plants or plant parts aseptically on artificial nutrient media in test tubes.

The following advantages of culture in vitro, in comparison with the cultivation in vivo of plants or plant parts in pots or cutting beds (Pierik, 1969), can be mentioned:

- By culture in vitro, plant growth and development can be studied under satisfactorily controlled aseptic conditions, both above and within the medium in the test tube. A variation of a single growth factor in vitro hardly influences other factors involved in root formation and consequently the effect of separate factors can be examined. Because conditions in vivo are more variable, variation of one factor brings about a variation in other factors so that an evaluation of a single growth factor is complicated.
- Substances like growth regulators, which are applied in very small quantities to the medium, can easily be taken up by the explant in vitro, especially through a wound cut, but not so easily under conditions in vivo.
- Explants in vitro can be more easily manipulated than whole plants in vivo.
- Using small plant parts the quantity of plant material required for experiments in vitro is relatively small. The culture of explants requires less space than that of whole plants in vivo.
- One of the difficulties of investigations in vivo is due to correlations, which are certain relations between different parts of the plant. These correlations may be involved in regeneration phenomena. The method of culture in vitro, with small plant parts, provides a means of circumventing these correlations to some extent and is therefore more precise than the use of whole plants (Gautheret, 1966a).
- In general young more or less undifferentiated tissues show the most rapid and best regeneration. Plant material in a young developmental stage can be cultivated in vitro under aseptic conditions, but dies off in vivo when attacked by micro-organisms.

- Root formation can be observed in vitro at any desired moment without disturbing the experiment. An examination of root formation at regular intervals in vivo is impracticable. As a rule, only one comprehensive observation can be made, after which the plant material has to be discarded and the experiment is at an end.

The following disadvantages of culture in vitro can be indicated:
- More specific knowledge, skill and technical equipment are needed for culture in vitro than for the cultivation of plants under conditions in vivo.
- Often detailed data from culture in vitro will not completely correspond with those found under conditions in vivo. Consequently, one has to be very careful with the interpretation of results in vitro in relation to their validity in vivo.

Because of its many favourable aspects, the method of culture in vitro was used to examine the effect of separate factors on root organogenesis (Section 3.4). Studies on the anatomical aspects of this process of adventitious root formation in pyrethrum are described in Section 3.3. They are made to supply additional information about the location and kind of tissues involved in the initiation and development of adventitious roots and how root formation progresses in time.

It was found that different plant parts of pyrethrum are able to initiate adventitious roots in vitro, namely explants of the hypocotyl, the leaf blade, the petiole and the peduncle. Root organogenesis, however, was too sporadic to afford any hope of a rapid determination of the underlying conditions, except for explants of the peduncle. So peduncle explants, which produced numerous adventitious roots at high percentages, were chosen as test material.

3.2 MATERIAL AND METHODS

3.2.1 *Material*

As explants from plants growing outside could not be sterilized satisfactorily, stock plants were grown in a moderately heated greenhouse (Section 2.2). At low temperatures in winter flower heads were initiated, while with a rising temperature in spring, the initiated flower heads developed fast and plants started blooming. Flowering continued for about half a year and during this period the upper halves of peduncles of stock-plants of 1-2 years old of the three Kenya Clones 1087, 4331 and Ma 63/1889 were excised (Fig. 11, left). From the upper portions of 20 peduncles, derived from 20 different plants, the bracts and flower heads were excised (Fig. 11, middle). The peduncles were sterilized in a 5% (50 g/l) calcium hypochlorite filtered solution for 20 min and subsequently rinsed several times with sterilized tap water for 30 min. To prevent leaching, the water was poured off immediately after washing.

In an inoculation room, previously sterilized by ultraviolet irradiation, the sterile-culture technique was applied (Gautheret, 1959). The top and base portions of the peduncle were cut off, after which it was divided into segments of about 1.5 cm, in such a way that the nodal sites, where the bracts were emplanted, were excluded. The explants were further wounded at one side, by excising a strip of the cortex over the whole length of the explant, and cut slantingly at the morphological base to

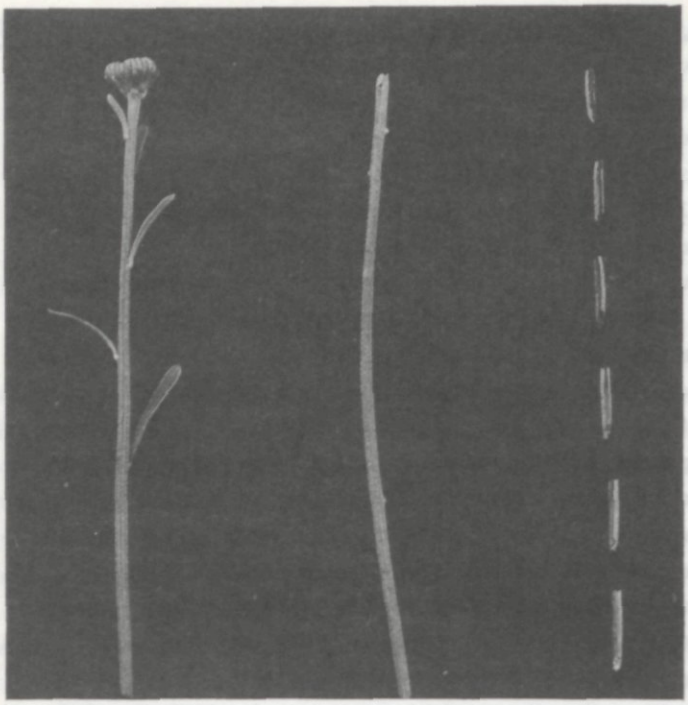


Fig. 11. Upper portion of a peduncle in a young flowering stage; from left to right:
- flower head and bracts attached,
- flower head and bracts excised,
- divided into 6 wounded explants of about 1.5 cm.

distinguish it from the horizontally cut morphological top (Fig. 11, right). Finally the explants were placed horizontally with the wounded side on the culture medium (Fig. 13).

3.2.2 Methods

The basic culture medium was composed of pyrex-distilled water, 'Difco' Bacto-agar 0.6% (6 g/1), the half strength of both Knop's major and Heller's minor salts (cf. Gautheret, 1959), sucrose 2% (20 g/1) and β-indolebutyric acid (IBA) at 10^{-5} g/ml. The pH of the medium was adjusted to 5.8 before autoclaving. About 25 ml of this medium was poured into pyrex test tubes with a length of 16 cm and a diameter of 2.7 cm. The tubes were plugged with cotton and covered by aluminium caps. The medium was autoclaved at 115°C for 20 min; tap water and filter paper at 120°C for 30 min.

The experiments, which are described in Section 3.4, were carried out by varying one growth factor. Generally each experiment consisted of 6-9 treatments and consequently the upper portions of 20 peduncles were each divided into 6-9 explants (Fig. 11, right), which were distributed systematically and in a balanced way over the various treatments. Per treatment 20 explants were exposed for 4 weeks to 20°C in darkness. To estimate the rate of adventitious root formation, about 1 h of illumination per week was necessary for observations. The explants in culture were examined with a hand

lens and in doubtful cases with a stereomicroscope (Wild type M5). At the end of the experiments, 4 weeks after incubation, the following variables were determined:
- rooting percentage (over 20 explants per treatment),
- the average number of roots (per rooted explant),
- the average length per root in mm,
- the average dry root weight (per rooted explant) in mg (measured after drying for 4 h at 80°C).

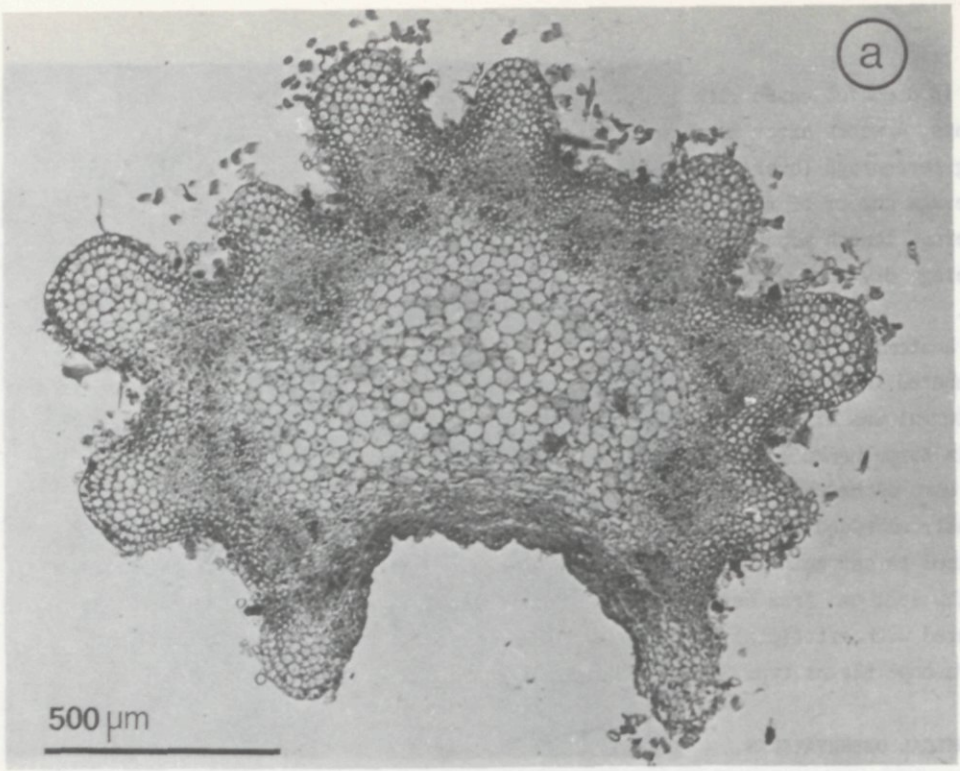
For anatomical observations (Section 3.3) explants of the peduncle of Clone 1087 were incubated in vitro under conditions suitable for adventitious root formation and the plant material was fixated 0, 1, 2, ... or 10 d after incubation in Carnoy's fluid for 5 min and in Karpechenko's modified Navashin fluid (Johansen, 1940) for at least 2 d. The segments were washed with tap water and dehydrated with ethyl and butyl alcohol and subsequently embedded in paraffin for sectioning with a microtome. Sections of 12 µm were stained in safranin with fast green as a counter stain. Mayer's adhesive was used to prevent the sections from being washed off during staining and finally the sections were mounted with artificial Canada balsam (Rhenohistol). Pictures were made with a photomicroscope (Zeiss type Standard universal).

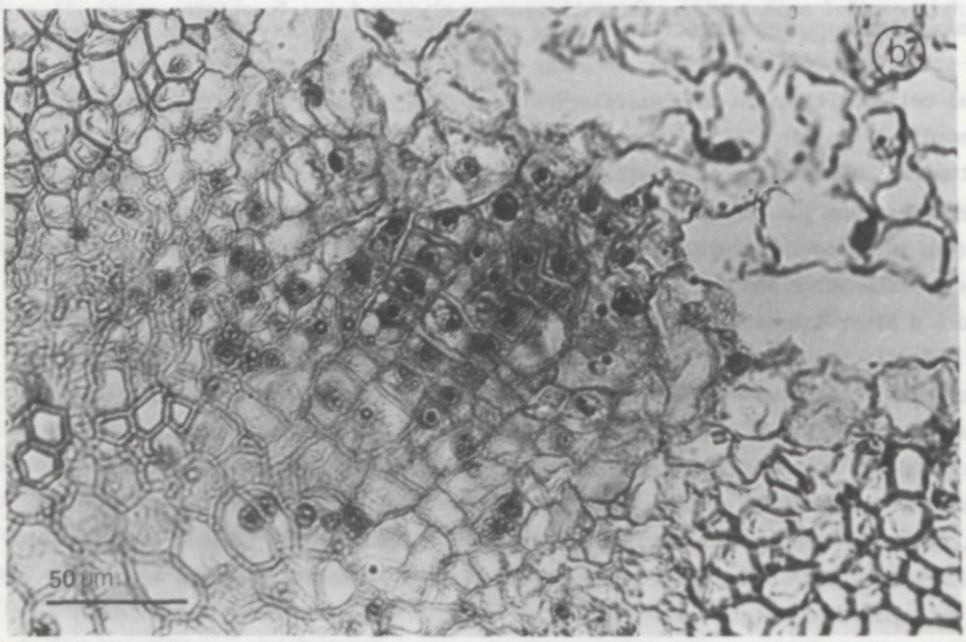
3.3 ANATOMICAL OBSERVATIONS

In many plant species adventitious roots originate endogenously from the pericycle of young stems (Libbert, 1956/1957). In stems of *Chrysanthemum morifolium* Ram. periclinal cell divisions in the interfascicular pericycle give rise to the formation of root primordia (Stangler, 1956).

Anatomical observations of pyrethrum were quite similar. Transverse sections showed the peduncle explant just before incubation in vitro (Fig. 12a) and periclinal cell divisions in the interfascicular pericycle 3-4 d after incubation. Subsequent cell divisions in transverse and anticlinal direction occurred and a root primordium was initiated 6 d after incubation (Fig. 12b). The cells of the root primordium divided, enlarged and differentiated rapidly during further development and, growing from the interfascicular pericycle in anticlinal direction to the epidermis, the root primordia brought about a collapse of cells of the endodermis (identifiable by Casparian strips), the cortex and the epidermis 8 d after incubation (Fig. 12c). The adventitious root penetrated the epidermis 10 d after incubation (Fig. 12d), just between the ribs of the peduncle, where the cortex consisted of a very loose chlorenchymous tissue, easy to penetrate. Penetration of the tight collenchymous cortex tissue of the ribs would probably be impossible and moreover the distance to be bridged would be considerably longer. Presumably, after initiation several pericyclic cells adjacent to the cell(s) of the root initial divided, became meristematic and were incorporated into the root primordium. Undoubtedly in a later stage the cambium also contributed cells to the growing root primordium, because a connection developed between the xylem of the original explant and the xylem of the initiated root.

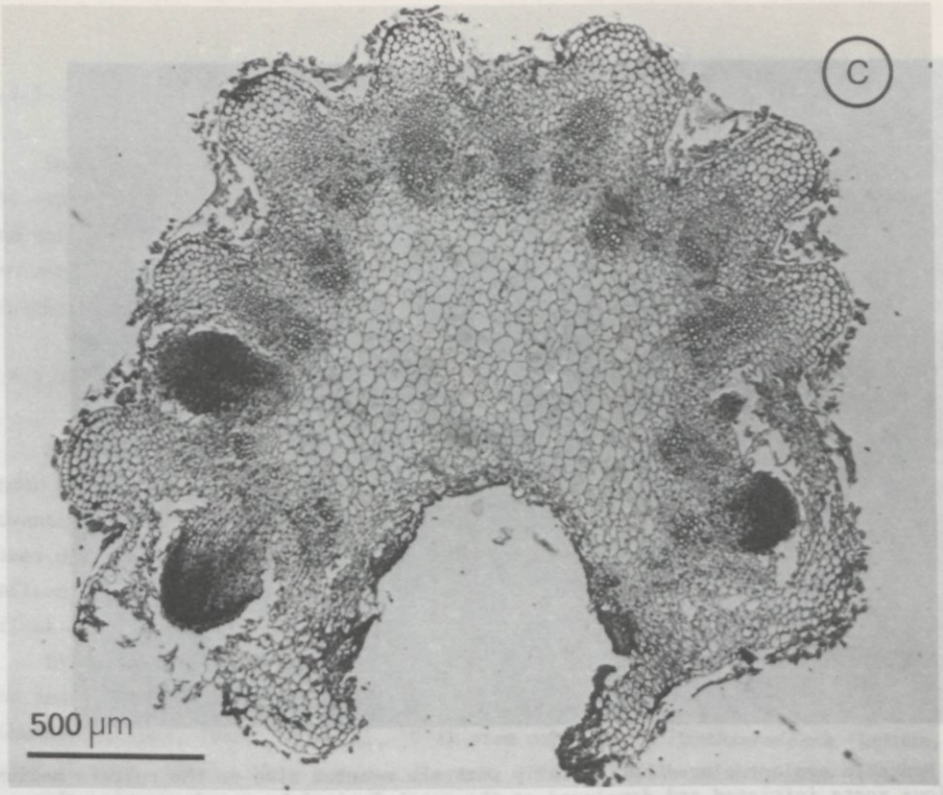
Adventitious roots were usually initiated during the first 2 weeks of incubation, while during the subsequent 2 weeks of incubation the root initials developed. The roots

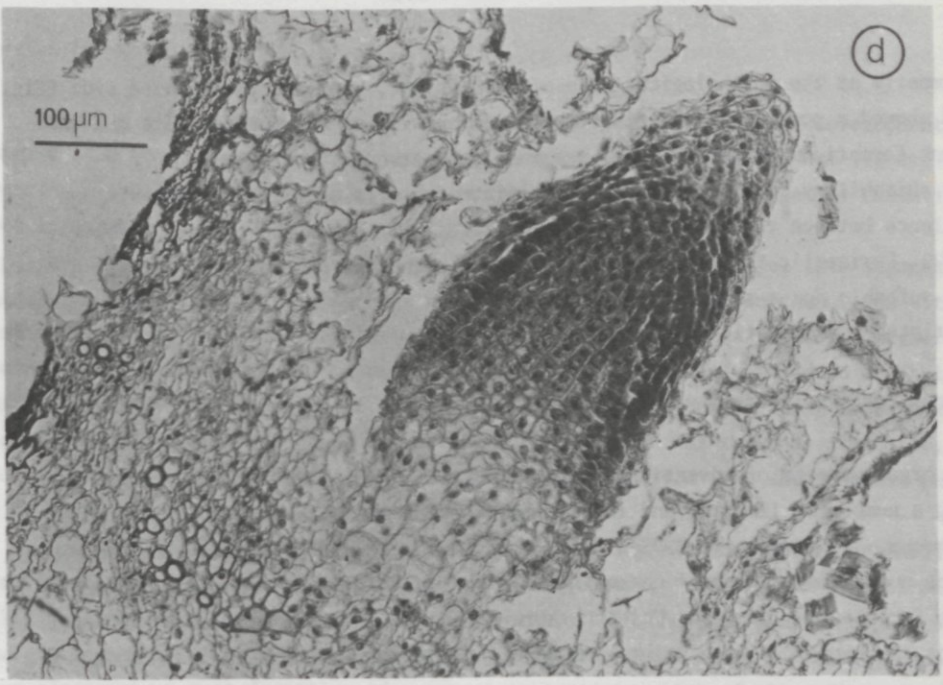




Figs 12a-d. Transverse sections of peduncle explants:

a. Note the ribs which enclose the vascular bundles and the wounded side (below), just before incubation.
b. A root primordium initiated in the interfascicular pericycle, 6 d after incubation.
c. Three root primordia arise between the vascular bundles and bring about a collapse of cells of the endodermis, the cortex and the epidermis, 8 d after incubation.
d. An adventitious root penetrates the epidermis between the ribs of the explant, 10 d after incubation.


c
500 µm


d
100 µm

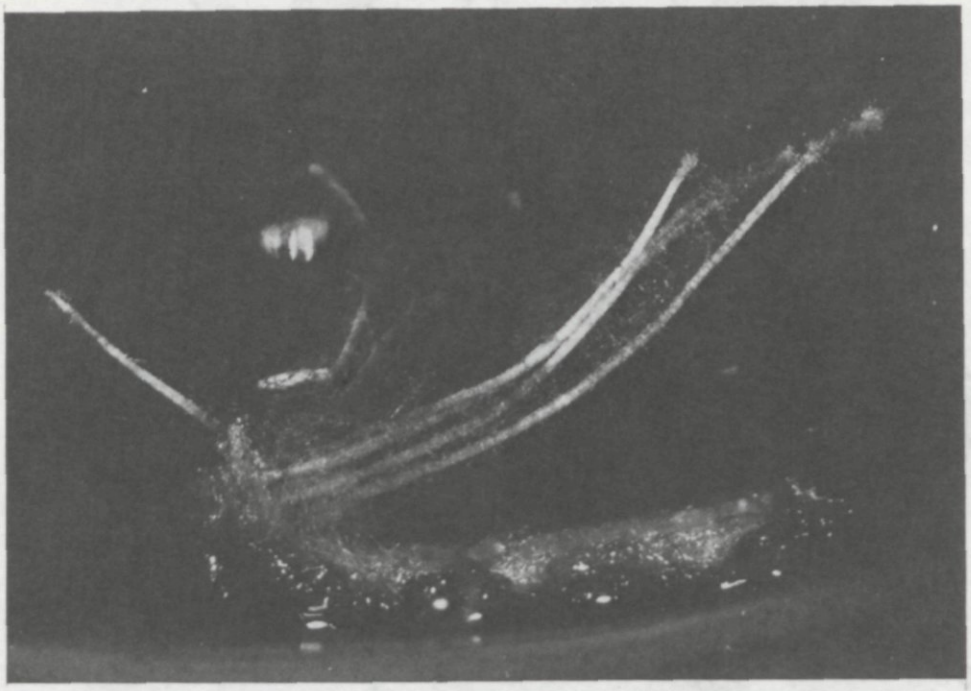


Fig. 13. Peduncle explant placed horizontally with the wounded side on the culture medium. Adventitious roots initiated and developed at the morphological base and opposite the wounded side, 4 weeks after incubation.

emerged mostly at the morphological base of the explant, opposite the wounded side (Fig. 13), and showed a positive geotropic response. Branching of the (main) roots and the consequent formation of lateral roots was rarely observed.

The suitability of the pericycle to initiate roots is explained by an existing inequivalence between the ontogenetic and physiological age of the tissues (Libbert, 1956/1957). Pericyclic tissue is ageing more slowly and therefore is less differentiated and more suitable for a subsequent dedifferentiation and regeneration of roots, than more differentiated adjacent tissues.

3.4 FACTORS INFLUENCING ROOT FORMATION

The organogenesis of adventitious roots in vitro depends upon a complex system involving a number of limiting and interacting factors (Libbert, 1956/1957; Gautheret, 1969c; Leroux, 1973; Reinert, 1973). Such factors are related to properties of the (ex) plant, the nutritional/hormonal composition of the medium and the climatic conditions to which the (ex)plant is exposed. Unless otherwise stated, the chosen standard growth conditions (Section 3.2) were maintained in all experiments described in this section.

3.4.1 Plant factors

Some of the plant factors which were evaluated concern the genotype and the age of the explant, which last characteristic finds expression in the flowering stage of the peduncle and the position of the explant in the peduncle. The other plant factors, like explant length, wounding and polarity, that were investigated, were related to how the explant was manipulated.

3.4.1.1 Genotype

In most experiments peduncle explants of Clones 1087, 4331 and Ma 63/1889 were used. Under various suitable conditions, explants of these clones regenerated high numbers of adventitious roots at high percentages. However, rooting parameters of the clones sometimes differed. These different rooting responses can be ascribed to the genotype influencing anatomical and physiological properties of the (ex)plant, which in their turn affect adventitious root formation.

Differences in root formation between various plants, within one species or within one genus, were also found in vitro with rhizome fragments of *Helianthus tuberosus* (Gautheret, 1961, 1969c; Tripathi, 1974) stem explants of *Parthenocissus* (Leroux, 1965), *Salix* (Leroux, 1966), *Pisum sativum* (Leroux, 1968ab, 1973). *Rhododendron* (Pierik, 1969; Pierik & Steegmans, 1975c) and *Prunus* (Feucht & Dausend, 1976).

3.4.1.2 Flowering stage

From the stock plants peduncles were excised in various stages of development, ranging from young (upper portion just long enough to yield 6 segments of 1.5 cm) to old (bearing overblown flower heads), to determine the most suitable stage for adventitious root formation.

The young, soft peduncles could easily be divided with a scalpel into segments, but this procedure proved to be difficult with old, woody peduncles. In comparison with explants from young peduncles, explants detached from old peduncles yielded a higher contamination percentage and a retarded root formation (Fig. 14). The data of Table 6 further indicate that segments of young peduncles elongated considerably more than segments of old peduncles. Whatever rooting characteristic was taken as a measure, the best regeneration of roots was obtained with explants excised from the youngest peduncles, which material was chosen for standard experiments.

A young developmental stage was found to be also advantageous for rhizogenesis of stem segments cultivated in vitro of *Rhododendron* (Pierik, 1969; Pierik & Steegmans, 1975c), petiole explants of *Lunaria annua* (Pierik, 1972) and leaf midrib fragments of *Gerbera jamesonii* (Pierik & Segers, 1973). Olieman-van der Meer et al. (1971) reported that adventitious root formation of epicotyl explants in vitro of *Phaseolus vulgaris* was not markedly influenced by the developmental stage.

Libbert (1956/1957) gave an explanation for the positive influence of a young developmental stage on adventitious root formation. He suggested that adventitious root

Table 6. The influence of various flowering stages of the peduncle (1 is young to 6 is old) on elongation and root formation of peduncle explants.

Clone	1	2	3	4	5	6
average explant length in mm						
1087	23.3	21.1	17.4	-[1]	16.2	16.3
Ma 63/1889	26.1	25.0	16.3	16.5	16.1	15.5
rooting percentage						
1087	65	50	50	-[1]	35	25
4331	80	95	85	90	90	45
Ma 63/1889	100	95	40	65	75	25
number of roots per rooted explant						
1087	23.8	25.6	25.1	-[1]	4.6	3.3
4331	45.6	25.1	15.5	18.6	9.9	5.8
Ma 63/1889	38.9	26.5	8.8	9.5	6.6	2.8
average root length in mm						
1087	5.6	5.3	5.0	-[1]	2.8	1.9
Ma 63/1889	6.1	6.0	5.0	2.3	2.4	1.7
dry root weight per rooted explant in mg						
1087	2.4	1.9	1.7	-[1]	0.1	0.1
4331	3.7	3.5	1.3	1.2	0.7	0.2
Ma 63/1889	4.7	4.1	0.7	0.4	0.3	0.0

1. Treatment discarded.

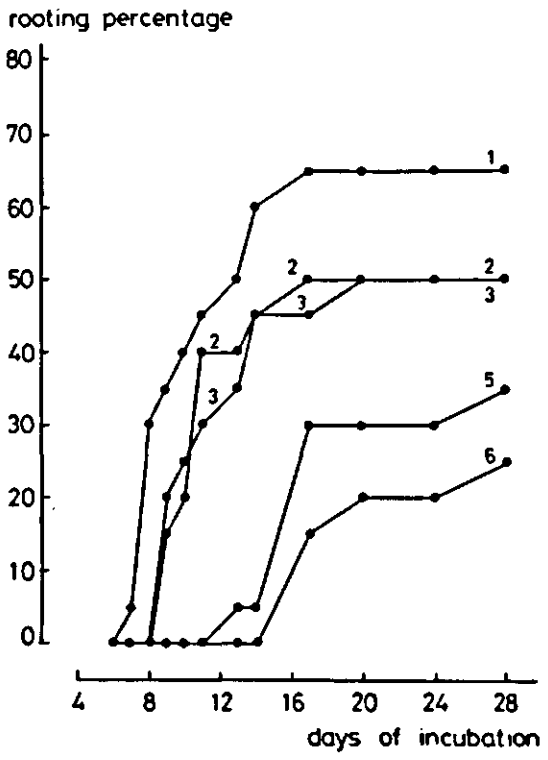


Fig. 14. The influence of various flowering stages of the peduncle (1 is young to 6 is old) on root formation of peduncle explants of Clone 1087. (Stage 4 was discarded.)

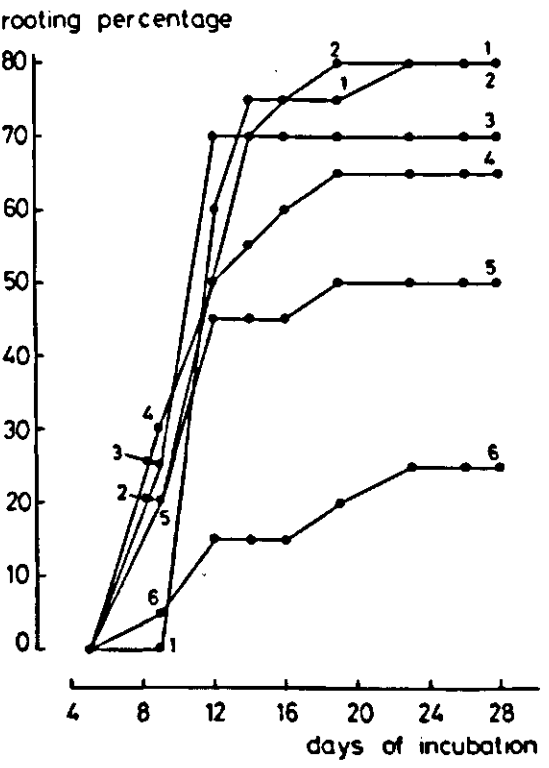


Fig. 15. The influence of various positions of explants from the upper half of the peduncle (1 is apex to 6 is base) on root formation of peduncle explants of Clone 1087.

formation becomes more difficult as the cells of the explant, which have to give rise to root initials, become progressively more differentiated. Apart from this anatomical aspect, which is also involved in pyrethrum, differences in root regeneration between various developmental stages of the peduncle may also be attributed to differences in physiological properties, like the nutritional/hormonal conditions.

3.4.1.3 Explant position

Explants can be isolated from different positions of the young peduncle. Explants excised from the lower half of the peduncle proved to be unsuitable for tissue culture, because of high contamination percentages. Explants detached from the upper half of the peduncle could be cultivated aseptically in a satisfactory way and these segments, located at 6 different positions, were examined for their rooting ability.

From the distal to the proximal part of this upper half of the peduncle, the segments became progressively more woody. The young, soft distal parts of the peduncle could be divided into explants more easily than the proximal older and more woody parts. Apical explants of Clones 1087 (Fig. 15) and Ma 63/1889 initiated adventitious roots more rapidly than more basal explants, while explants derived from 6 different positions of the upper part of the peduncle of Clone 4331 all showed about the same rooting rate. Explants excised from the apex of peduncles of Clone Ma 63/1889 elongated more than explants from the basal part of the upper half of the peduncle (Table 7). Evidently, the rooting response of the three clones decreased from the apical to the basal part. Although top explants yielded the best root formation, the whole upper halves of young peduncles were used, because otherwise too many peduncles would have been required for each experiment.

These observations agree with those reported by other scientists who found that

Table 7. The influence of various positions of explants from the upper part of the peduncle (1 is apex to 6 is base) on elongation and root formation of peduncle explants.

Clone	1	2	3	4	5	6
average explant length in mm						
Ma 63/1889	19.5	17.6	16.8	16.5	16.4	16.4
rooting percentage						
1087	80	80	70	65	50	25
4331	75	95	100	95	100	95
Ma 63/1889	100	90	80	95	85	80
number of roots per rooted explant						
1087	14.9	13.1	13.0	12.0	8.7	4.8
4331	32.1	32.4	23.3	20.6	20.6	16.1
Ma 63/1889	27.4	13.1	8.2	4.9	5.8	5.0
average root length in mm						
Ma 63/1889	5.1	5.0	3.6	3.5	3.2	3.5
dry root weight per rooted explant in mg						
1087	1.8	1.6	1.5	1.3	0.8	0.7
4331	4.9	5.3	3.5	3.3	3.2	2.6
Ma 63/1889	4.2	2.3	1.6	0.8	0.9	0.8

regeneration at different regions of a stem was influenced by so-called gradients of organogenesis. Gorter (1965) observed that when a young shoot of *Asparagus officinalis* was cut into segments of 0.5 cm and cultured in vitro, there was a gradient of rooting capacity which declined from the tip towards the base of the shoot. Pierik (1967) noticed that root formation on explants of the elongated axis of *Lunaria annua* cultivated in vitro started from the terminal part and later from the medial and basal part of the axis. The percentage of rooted explants at the end of the experiment decreased from the terminal to the basal part of the plant. These results pointed to the existence of a rooting gradient along the main axis of a flowering *Lunaria* plant. The position of *Rhododendron* stem explants had no effect on rooting (Pierik & Steegmans, 1975c).

Rooting gradients are caused by differences in the developmental stage which exist between explants isolated from various positions; top explants are in a younger stage of development than more basal explants. The effect of the developmental stage on root formation was already discussed in Section 3.4.1.2.

3.4.1.4 Explant length

The effect of the initial length of the explant on rhizogenesis was studied on segments of the upper half of young peduncles with a length of 0.5, 1, 1.5, 2, 2.5 or 3 cm. Explants of Clone 4331 were turned upside down, whereas explants of Clone Ma 63/1889 were placed horizontally.

The rate of root formation in Clone 4331 decreased with an increasing explant length (Fig. 16), while in Clone Ma 63/1889 all explant lengths yielded about the same rooting rate. For all characteristics the rooting response of the vertically placed explants decreased with an increasing explant length, whereas the horizontally placed explants showed a good rooting for all explant lengths (Table 8).

Differences in rooting response between both clones may be ascribed to the polarity (orientation) of the explants (Section 3.4.1.6). Explants of Clone 4331 were turned upside down to about half their lengths in the culture medium. Hence, with an increasing explant length substances in the medium, which proved to be of essential significance for rooting, like sugar (Section 3.4.2.3) and auxin (Section 3.4.2.4), have to be transported over an increasing distance before adventitious roots are initiated at the morphological base of the explant. Probably this transport becomes progressively limiting with an increasing explant length. Explants of Clone Ma 63/1889 were placed horizontally, with their wounded sides on the culture medium. Under these circumstances the uptake of essential substances is independent of the explant length. Hence, explants of all lengths yielded a good rooting response, while adventitious roots were formed over the whole length of the explants.

The effect of the initial explant size on root formation varies greatly with the plant species. Gorter (1965) observed that root initiation on excised shoot tips of *Asparagus officinalis*, placed upright in the medium, decreased with an increasing explant length. However, Lindner (cf. Dore, 1965) found the number of new organs initiated on root pieces of horseradish to be proportional to the length of the pieces used and also Nanda et al. (1968) reported the best rooting response for relatively long stem

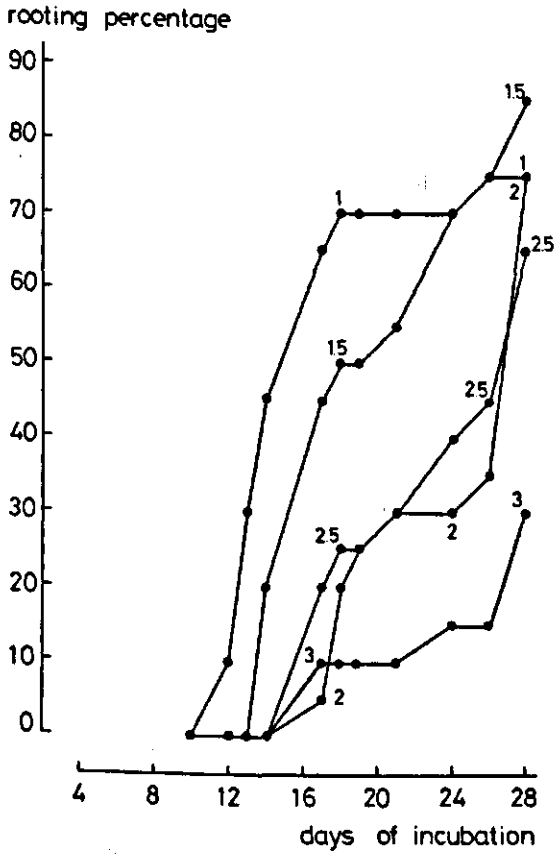


Fig. 16. The influence of various explant lengths (1-3 cm) on root formation of peduncle explants of Clone 4331, which were turned upside down.

Table 8. The influence of various explant lengths (0.5-3 cm) on root formation of peduncle explants. Explants of Clones 4331 and Ma 63/1889 were turned upside down and horizontally, respectively.

Clone	0.5	1	1.5	2	2.5	3
rooting percentage						
4331	-[1]	75	85	75	65	30
Ma 63/1889	70	60	50	65	60	55
number of roots per rooted explant						
4331	-[1]	17.9	11.6	5.3	7.4	6.0
Ma 63/1889	6.1	10.6	14.4	12.2	14.4	25.8
average root length in mm						
4331	-[1]	4.6	3.0	2.9	2.4	1.8
Ma 63/1889	9.7	9.6	8.1	7.1	7.5	5.7
dry root weight per rooted explant in mg						
4331	-[1]	0.5	0.2	0.1	0.1	0.1
Ma 63/1889	1.5	3.1	3.4	2.2	3.0	2.5

1. Treatment not included.

segments of *Populus nigra*, placed vertically. Finally, Pierik & Steegmans (1975c) noticed the same amount of root regeneration in stem segments of various lengths of *Rhododendron*, turned upside down.

It remains unexplained why horizontally placed long explants of pyrethrum do not yield the same rooting response per unit of length as horizontally placed short explants, when nutrients and growth regulators are available in sufficient quantities in the culture medium. Evidently, an internal limiting factor exists within long explants, of which the nature is as yet unknown.

Explants of pyrethrum yielded the best root formation per unit of length with short explants of 1 and 1.5 cm. A length of 1.5 cm was chosen as initial explant length in standard experiments. It has been shown that the initial length of the explant does not remain the same during the subsequent incubation and that explants become longer, dependent on the developmental stage of the peduncle (Section 3.4.1.2) and the position of the explant in the peduncle (Section 3.4.1.3). Finally, if relatively short explants are used, there is less chance of contamination of the explant and the quantity of experimental plant material can be reduced.

3.4.1.5 Wounding

To determine the effect of wounding, rooting was compared of explants which, apart from the basal and apical wounds, were not further wounded, with rooting of explants which were additionally wounded, by excision of the cortex at one side over the whole explant length. The latter were placed with the wounded side on the medium.

Such an additional wound cut slightly stimulated rooting rate, but considerably enhanced the other rooting parameters, as expressed in Table 9. As standard treatment the peduncle explants were wounded by excision of the cortex at one side over the whole explant length.

The favourable effect of wounding on rhizogenesis is a recognized fact and was also affirmed by Pierik & Steegmans (1975c) with stem explants of *Rhododendron* cultivated in vitro. Libbert (1956/1957) stated that the most reasonable explanation seemed to be the

Table 9. The influence of wounding on root formation of peduncle explants of Clones 1087, 4331 and Ma 63/1889.

	1087		4331		Ma 63/1889	
	unwounded	wounded	unwounded	wounded	unwounded	wounded
rooting percentage	65	70	75	90	40	55
number of roots per rooted explant	12.1	24.7	15.6	26.4	7.6	20.1
average root length in mm	4.2	4.5	5.5	5.3	3.8	7.4
dry root weight per rooted explant in mg	1.4	2.1	2.1	2.6	2.0	4.7

breaking of a mechanical barrier. Such a barrier may limit the penetration and transport of water, minerals, sugar and auxin within the (ex)plant. Moreover the oxygen supply may be restricted; the importance of a good aeration for root formation was pointed out by Dore (1965).

3.4.1.6 Polarity

In the previous sections it was determined which developmental stage and which part of the peduncle had to be chosen as test material and how this plant material had to be prepared for a good rooting response. The next question concerns the polarity (orientation) of the explants. Adventitious root formation was investigated of explants inserted vertically in the medium to about half their lengths, in upright position or upside down, and of explants placed horizontally with the wounded side on the medium.

Adventitious roots were always initiated opposite the wounded side. Roots sometimes developed in the medium, which were short and thick, whereas more elongated thin roots were regenerated above the medium. Adventitious root formation of explants inserted upright was strongly retarded in comparison with root formation of upside down or horizontally placed explants (Fig. 17). Explants inserted upright developed roots at the

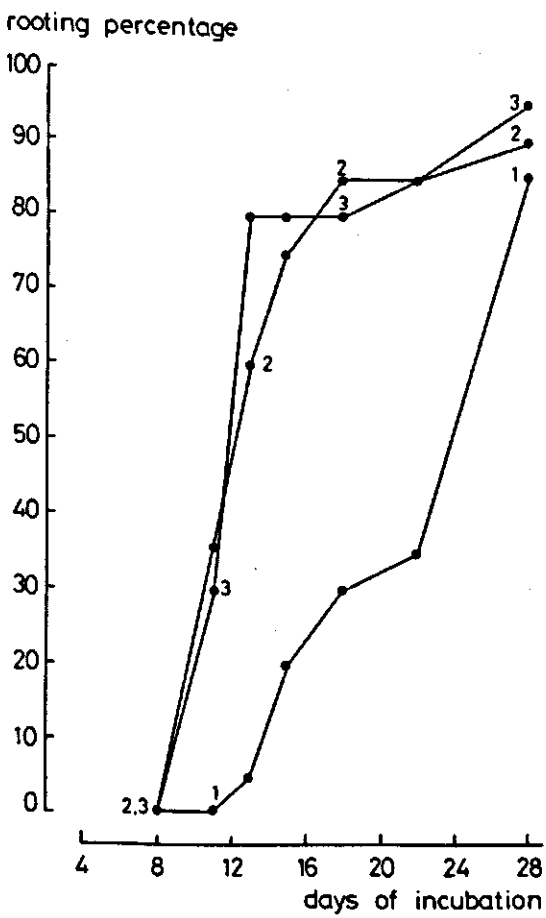


Fig. 17. The influence of various polarities on root formation of peduncle explants of Clone 4331. 1 is explant upright, 2 is explant upside down and 3 is explant horizontal.

Table 10. The influence of various polarities on root formation of peduncle explants of Clones 4331 and Ma 63/1889.

	4331			Ma 63/1889		
	upright	upside down	horizontal	upright	upside down	horizontal
rooting percentage	85	90	75	50	100	70
number of roots per rooted explant	20.6	21.5	15.5	12.5	20.1	21.3
dry root weight per rooted explant in mg	1.4	1.5	3.3	2.2	2.3	5.4

morphological base in the medium and just above the medium at the middle region of the explant. On explants turned upside down adventitious roots were regenerated exclusively at the morphological base above the medium. Explants placed horizontally initiated roots above the medium over the whole length, but especially at the morphological base (Fig. 13). No one treatment was preferable to the others with respect to rooting percentage and average number of roots (Table 10). Dry root weights, however, were much higher for horizontally than for vertically placed explants. In standard experiments explants were placed horizontally with the wounded side on the culture medium.

The unfavourable effect of an upright explant position on rooting in vitro has been described in the literature. Root formation of isolated stem segments of *Rhododendron* was strongly inhibited by placing the explants with their basal ends down; inverted explants, however, rooted easily above the medium and yielded more and heavier roots (Pierik, 1969; Pierik & Steegmans, 1975c). Similar results were obtained with petiole segments of *Lunaria annua* (Pierik, 1972), leaf midrib explants of *Gerbera jamesonii* (Pierik & Segers, 1973) and stem segments of *Helianthus tuberosus* (Tripathi, 1974). In this last case rooting was even better of inverted than of horizontally placed explants.

Substances which are essential for rooting, like sugar (Section 3.4.2.3) and auxin (Section 3.4.2.4), are transported in a polar (basipetal) direction within the (ex)plant. When segments are turned upside down these nutrients have to bridge the distance from the medium to the morphological base of the segment, where rooting occurs. When explants are inserted in an upright position the morphological base is directly exposed to the nutrients in the medium. Nevertheless, the root formation in the medium was poor, probably because of the bad oxygen supply in the medium. The importance of a good aeration was emphasized by Dore (1965). Horizontally placed explants are assured of a good aeration and a good uptake of essential substances. Consequently, in pyrethrum adventitious roots were initiated over the whole length of the explant. However, as a result of basipetal transport most roots were still initiated at the morphological base of the explant, which was also found with stem segments of *Helianthus tuberosus* (Tripathi, 1974).

Table 11. The influence of various agar concentrations (0-1%) on root formation of peduncle explants of Clone 4331.

	0	0.2	0.4	0.6	0.8	1
rooting percentage	90	100	75	85	95	100
number of roots per rooted explant	13.1	16.7	22.9	19.7	30.1	22.2
dry root weight per rooted explant in mg	0.9	1.6	2.8	3.9	4.6	3.9

3.4.2 Nutritional/hormonal factors

3.4.2.1 Agar

Agar is a product manufactured from seaweed and used as a solidifying agent. The organic and inorganic composition of agar is rather complex and varies with the time of the year the product is manufactured. The most critical nutritional work can be done when tissue cultures are grown in chemically defined nutrient media in which a complex component like agar is absent, i.e. in a liquid medium.

To determine whether a liquid medium is adequate for pyrethrum, the rooting response of explants of Clone 4331 was investigated at different concentrations of 'Difco' Bacto-agar (0, 0.2, 0.4, 0.6, 0.8 and 1%). In a liquid medium and at agar 0.2% filter paper was used to support the explants (Heller, 1953).

The observed rooting rate was about the same for all treatments. In a liquid medium rooting response was poor, while at agar 0.8% root formation was optimum (Table 11).

A promoting effect of liquid media on root organogenesis was reported by Gorter (1965) for shoot tips of *Asparagus officinalis* and by Pierik & Steegmans (1975c) with *Rhododendron* stem segments. This stimulation may be attributed to a better aeration in a liquid than in an agar medium. According to Romberger & Tabor (1971) it may also be partly due to increased diffusion rates of large molecules in liquid media, or possibly to unknown agar-borne inhibitors.

It remains unexplained why a detrimental effect of a liquid medium on root formation has been found with rhizome fragments of *Helianthus tuberosus* (Tripathi, 1974) and in the present experiments with peduncle explants of pyrethrum. In standard experiments with the latter an agar concentration of 0.6% was chosen, as at that moment it was not yet known that optimum root formation would occur at 0.8% agar.

3.4.2.2 Minerals

For the examination of the influence of the mineral nutrition on rhizogenesis, Knop's major and Heller's minor salts (cf. Gautheret, 1959) were added to the culture medium in different concentrations and combinations. The concentrations 0, ½, 1 and 2 indicate the absence and the presence of the minor and major salts in half, normal and double strength, respectively. The following treatments (with corresponding treatment numbers) were applied:

Table 12. The influence of various concentrations and combinations of Heller's minor salts and Knop's major salts on root formation of peduncle explants. (Treatments 1-6 are explained in the text.)

Clone	1	2	3	4	5	6
rooting percentage						
1087	20	10	80	70	55	15
4331	20	5	45	65	55	60
Ma 63/1889	10	10	50	65	45	25
number of roots per rooted explant						
1087	1.3	5.5	13.2	10.4	13.6	7.7
4331	3.3	4.0	13.3	11.3	16.1	14.4
Ma 63/1889	5.0	5.0	17.8	7.7	14.3	10.6
average root length in mm						
1087	1.4	4.9	2.8	4.9	3.0	2.0
4331	1.0	5.0	7.9	7.9	7.0	5.2
Ma 63/1889	8.6	10.4	6.2	7.6	6.2	6.1
dry root weight per rooted explant in mg						
1087	0.1	0.7	0.8	1.0	0.9	0.5
4331	0.1	0.1	2.9	2.5	2.8	1.6
Ma 63/1889	0.8	0.9	3.0	2.7	2.4	1.7

	Heller minor		Knop major
1 =	0	+	0
2 =	½	+	0
3 =	0	+	½
4 =	½	+	½
5 =	1	+	1
6 =	2	+	2

In the absence of Knop's major salts and in the presence of the double strength of both mineral components the rooting rate was considerably less than with the other treatments (Fig. 18). In general the clones responded similarly (Table 12) to the mineral nutrition. In comparison with a medium devoid of all minerals (Treatment 1) the minor salts of Heller slightly stimulated root formation (Treatment 2). In the absence of the major salts, however, both treatments showed a bad rooting response and finally the explants became necrotic and died off. With Knop's major salts (Treatments 3, 4, 5 and 6) root formation was stimulated and explants did not turn necrotic. The double strength of both minor and major salts proved to be supra-optimal (Treatment 6). On the whole rooting responses were best with only the major salts (Treatment 3) or with both components at half strength (Treatment 4). A solution of Knop's major and Heller's minor salts, both at half strength, was chosen for standard experiments.

After the determination of the important role of Knop's major salts, the influence of the four separate mineral salts of Knop major was investigated. In an experiment with Clone 4331 the four major salts were successively omitted, so that the following treatments (with corresponding treatment numbers) were applied:

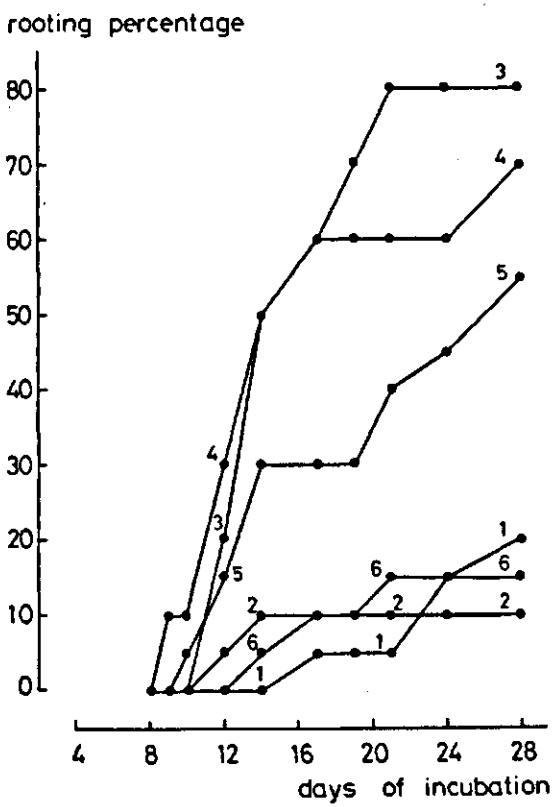


Fig. 18. The influence of various concentrations and combinations of Heller's minor salts and Knop's major salts on root formation of peduncle explants of Clone 1087. (Treatments 1-6 are explained in the text.)

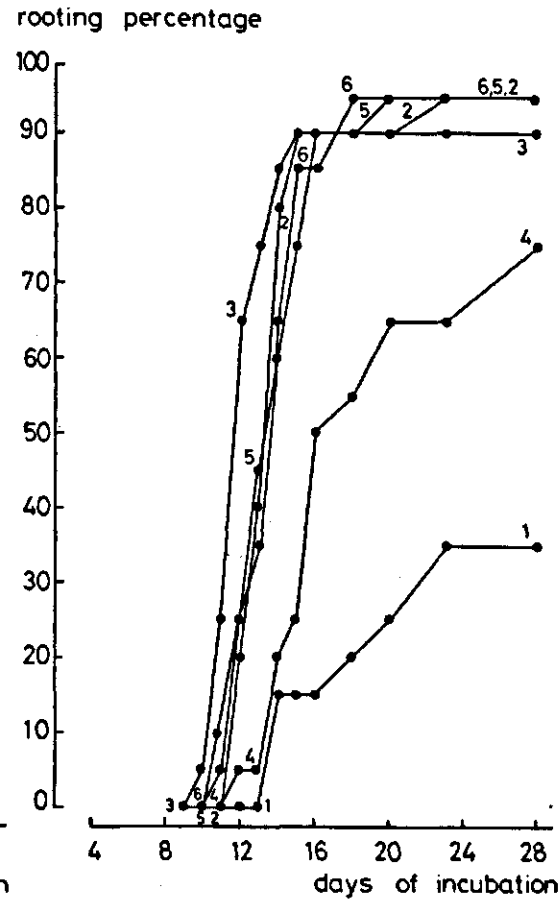


Fig. 19. The influence of Knop's major salts on root formation of peduncle explants of Clone 4331. (Treatments 1-6 are explained in the text.)

	Knop major	devoid of
1 =	0.	
2 =	½,	KNO_3
3 =	½,	$MgSO_4.7H_2O$
4 =	½,	$Ca(NO_3)_2.4H_2O$
5 =	½,	KH_2PO_4
6 =	½.	

All treatments, except 1 and 4, resulted in a high rooting rate (Fig. 19). Table 13 demonstrates that the omission of $Ca(NO_3)_2.4H_2O$ was much more harmful than the omission of any of the other three components of Knop major. The Treatments 2, 3, 5 and 6, which refer to the presence of $Ca(NO_3)_2.4H_2O$, gave a good rooting response.

The effect of the mineral nutrition (minor and major salts) on root formation differs from species to species. In general it has been shown that an exogenous supply

Table 13. The influence of Knop's major salts on root formation of peduncle explants of Clone 4331. (Treatments 1-6 are explained in the text.)

	1	2	3	4	5	6
rooting percentage	35	95	90	75	95	95
number of roots per rooted explant	2.4	21.0	26.5	5.3	13.6	25.2
average root length in mm	1.4	4.2	5.0	2.3	4.6	4.1
dry root weight per rooted explant in mg	0.0	2.2	3.7	0.2	1.3	2.9

of minerals to the culture medium is not essential for rooting, probably because (ex)plants contain a sufficient amount of endogenous minerals. In the absence of minerals petiole segments of *Lunaria annua* (Pierik, 1972), leaf midrib fragments of *Gerbera jamesonii* (Pierik & Segers, 1973) and stem explants of *Pisum sativum* (Leroux, 1973), *Helianthus tuberosus* (Tripathi, 1974) and *Rhododendron* (Pierik, 1969; Pierik & Steegmans, 1975c) initiated roots in vitro, although sometimes the number of the root initials was very limited. The same result was also found for peduncle explants of pyrethrum. Just one example can be mentioned in which rooting did not occur at all on a medium devoid of mineral salts, namely with rhizome explants of *Helianthus tuberosus* (Tripathi, 1974).

The role of the minor salts in rhizogenesis has been investigated sporadically. Torrey (1956a) observed that fragments of pea roots required certain micro-elements (Zn, Mn, Cu, Mo and B) for lateral root formation. With stem segments of *Pisum sativum*, Leroux (1973) noticed a detrimental effect; the number of adventitious roots produced was less with only minor salts than on a medium devoid of all minerals.

The most extensive study on root formation as affected by the mineral nutrition was carried out by Tripathi (1974) with rhizome explants of *Helianthus tuberosus*. In the presence of only minor salts hardly any root formation was obtained. When the minor salts together with major salts were added to the culture medium, less roots were regenerated than in the presence of only the major salts. A separate addition of the micro-elements (Fe, Cu, Zn, Mn, Al, Ni, B and J) together with the major salts, however, revealed that each micro-element at a certain concentration enhanced root formation. In pyrethrum, the organogenesis of roots on peduncle explants is hardly influenced by the minor salts.

On the other hand, the major salts strongly enhanced root formation in pyrethrum. A favourable effect of major salts upon rooting has also been found for leaf midrib fragments of *Gerbera jamesonii* (Pierik & Segers, 1973) and an almost essential role was reported for rhizome explants of *Helianthus tuberosus* (Tripathi, 1968, 1974; Tripathi & Gautheret, 1969; Gautheret, 1969c). The major salts did not markedly influence root formation on stem segments of *Pisum sativum* (Leroux, 1969c, 1973) and *Rhododendron* (Pierik, 1969; Pierik & Steegmans, 1975c), epicotyl explants of *Phaseolus vulgaris* (Olieman-van der Meer & Pierik, unpublished data) and petiole sections of *Lunaria annua* (Pierik, 1972).

Tripathi (1974) demonstrated that rhizogenesis of rhizome explants of *Helianthus tuberosus* was completely inhibited in the absence of the macro-elements Ca and N, and the significance of these elements was fully confirmed in the experiments with

pyrethrum. Tripathi further reported a slight influence of the macro-elements K, P, Mg and S, which result is also in conformity with the pyrethrum experiments.

3.4.2.3 Sugars

First six sugars were compared to find out which was the most suitable for root formation of peduncle explants of Clone 4331. All sugars were applied at a concentration of 2% to the culture medium.

Adventitious roots only developed fast in the presence of glucose, maltose and sucrose (Fig. 20). Table 14 shows that an adequate root production was achieved with

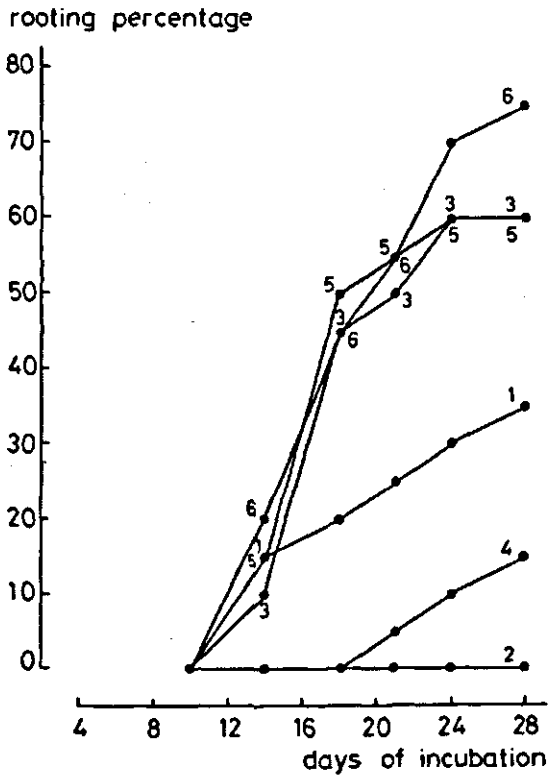


Fig. 20. The influence of various sugars (at 2%) on root formation of peduncle explants of Clone 4331. 1 = fructose, 2 = galactose, 3 = glucose, 4 = lactose, 5 = maltose and 6 = sucrose.

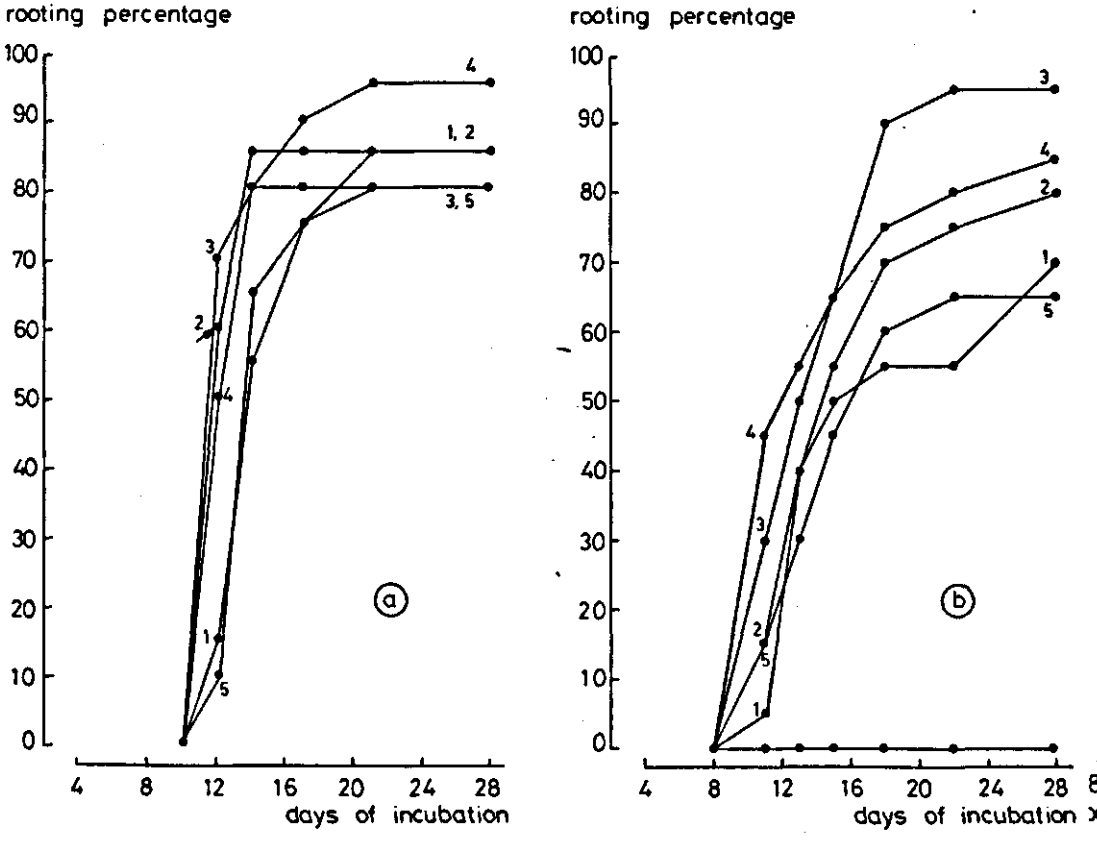
Table 14. The influence of various sugars (at 2%) on root formation of peduncle explants of Clone 4331.

	fructose	galactose	glucose	lactose	maltose	sucrose
rooting percentage	35	0	60	15	60	75
number of roots per rooted explant	5.9	0	15.4	1.0	19.2	15.3
dry root weight per rooted explant in mg	0.4	0	1.0	0.5	1.2	1.1

glucose, maltose and sucrose. The formation of adventitious roots was inadequate with fructose and lactose, while in the presence of galactose roots were not initiated at all and explants died off.

Subsequently, in the next experiments with the three clones, glucose and sucrose were added at different concentrations to the medium. Root formation was completely inhibited when sugar was absent. Usually root formation was retarded at glucose and sucrose concentrations of 1 and 5% (Figs 21a and 21b). Obviously (Table 15) the addition of sugar to the medium was of essential significance for rooting. In the absence of sugar rhizogenesis was completely inhibited and the explants became necrotic and died off within 4 weeks after incubation. The results were not very decisive on the optimum sugar concentrations and varied from clone to clone. In the range of 2-4% glucose and sucrose mostly good rooting responses were observed.

In the following two experiments with Clones 1087 and 4331, a direct comparison of the effect of glucose and sucrose, applied at various concentrations was made. A delayed rooting rate was obtained at glucose and sucrose 5% (Fig. 22). Table 16 shows that glucose and sucrose concentrations of 1 and 5% are suboptimal and supra-optimal, respectively. Sucrose at 3% has to be preferred to glucose at 3%; in standard experiments a sucrose concentration of 2% was chosen.



Figs 21a-b. The influence of various concentrations (0-5%) of glucose (a) and sucrose (b) on root formation of peduncle explants of Clone 4331.

Table 15. The influence of various concentrations (0-5%) of glucose and sucrose on root formation of peduncle explants.

Clone	Glucose						Sucrose					
	0	1	2	3	4	5	0	1	2	3	4	5
rooting percentage												
1087	0[1]	60	40	45	25	25	0	65	70	55	45	30
4331	-[1]	85	85	80	95	80	0	70	80	95	85	65
Ma 63/1889	0	75	70	75	70	40	0	40	50	40	25	30
number of roots per rooted explant												
1087	0[1]	45.0	25.6	30.7	32.0	2.0	0	9.4	12.8	10.1	25.6	12.6
4331	-[1]	32.2	47.7	42.9	32.1	21.9	0	7.9	7.6	7.6	10.4	12.0
Ma 63/1889	0	13.9	17.4	17.5	18.9	24.3	0	14.2	12.8	9.0	6.5	23.0
average root length in mm												
Ma 63/1889	0	5.9	5.2	4.8	5.5	4.5	0	6.4	8.5	9.8	7.7	5.3
dry root weight per rooted explant in mg												
1087	0[1]	3.0	2.3	3.2	2.1	0.2	0	1.0	1.6	4.1	3.1	1.9
4331	-[1]	1.8	5.2	6.1	5.5	3.0	0	0.5	0.5	0.6	0.8	0.9
Ma 63/1889	0	1.7	2.5	2.9	3.4	4.7	0	1.3	2.6	2.4	1.1	3.0

1. Treatment not included.

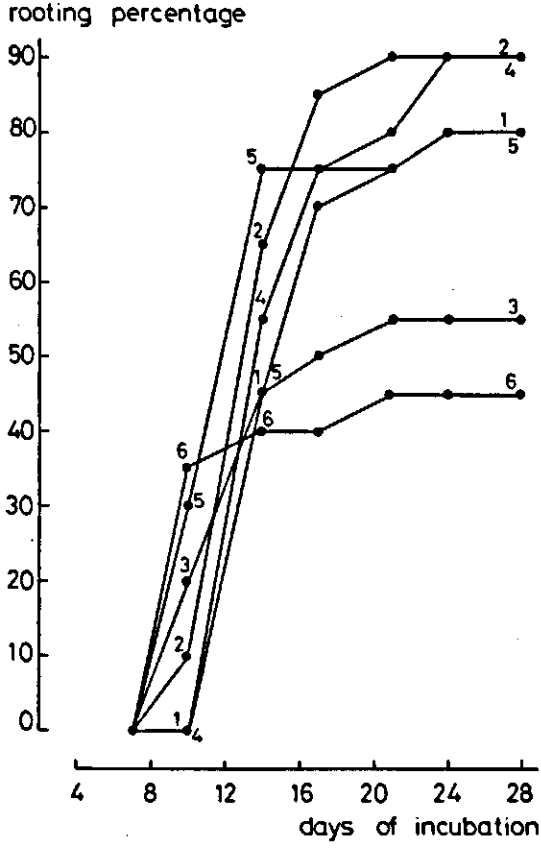


Fig. 22. The influence of various concentrations of glucose and sucrose on root formation of peduncle explants of Clone 1087. Treatments 1, 2 and 3 represent glucose 1, 3 and 5% and treatments 4, 5 and 6 sucrose 1, 3 and 5%.

Table 16. The influence of various concentrations (1-5%) of glucose and sucrose on root formation of peduncle explants.

Clone	Glucose			Sucrose		
	1	3	5	1	3	5
rooting percentage						
1087	80	90	55	90	80	45
4331	100	95	95	95	95	95
number of roots per rooted explant						
1087	4.0	4.3	7.3	4.8	16.6	8.0
4331	17.4	20.2	15.6	11.7	25.0	14.0
average root length in mm						
4331	5.2	8.0	5.7	6.3	5.6	4.2
dry root weight per rooted explant in mg						
1087	0.2	0.4	0.5	0.3	0.9	0.8
4331	0.9	3.2	2.3	1.0	2.8	1.5

Table 17. The influence of sucrose (at 2%), applied during different periods, on root formation of peduncle explants of Clone 4331.

	Last 2 weeks	First 2 weeks	4 weeks
rooting percentage	0	95	90
number of roots per rooted explant	0	6.7	15.3
average root length in mm	0	3.9	8.0
dry root weight per rooted explant in mg	0	0.3	3.2

Finally it was determined whether sucrose at 2% is essential during the whole process of root formation of peduncle explants of Clone 4331. When sucrose was added for 4 weeks or during the first 2 weeks of incubation then adventitious roots were initiated with almost the same rooting rate. The data of Table 17 indicate that the addition of sucrose is required during the whole process of root formation. When after 2 weeks culture on a medium devoid of sugar the explants were transferred to a medium supplemented with sucrose, the ability to regenerate roots was lost and explants became necrotic and died off. A culture in the presence of sucrose for the first 2 weeks resulted in a high rooting percentage, but the other rooting parameters were much less in comparison with the treatment in which sucrose was always present. Evidently, sucrose is essential for both the initiation of roots and the development of the root initials.

The absolute requirement of sugar for adventitious root formation has been confirmed by many authors: Gautheret (1969c) and Tripathi (1974) with rhizome explants of *Helianthus tuberosus*, Pierik (1969) and Pierik & Steegmans (1975c) for *Rhododendron* stem segments, Olieman-van der Meer et al. (1971) using epicotyl segments of *Phaseolus vulgaris*, Pierik (1972) with petiole explants of *Lunaria annua* and Pierik & Segers (1973) with leaf midrib fragments of *Gerbera jamesonii*. Leroux (1973) and Fellenberg (1967) reported an essential and a stimulating effect of sugar on adventitious root formation of stem segments and epicotyl explants of *Pisum sativum*, respectively. A beneficial

effect of sugar on root organogenesis was also demonstrated by Nanda et al. (1968) with stem segments of *Populus nigra*.

Since rooting is inhibited by light (Section 3.4.3.2), cultures were always placed in darkness. However, in darkness no photosynthesis takes place and therefore an exogenous supply of sugar becomes essential for rooting.

The influence of the kind of sugar on root regeneration was investigated by Spanjersberg & Gautheret (1963ab), Paupardin (1966) and Gautheret (1969c), who reported rhizogenesis on rhizome segments of *Helianthus tuberosus* with fructose, glucose, sucrose and xylose, but no or hardly any root formation with galactose, lactose, maltose, mannose, raffinose and ribose. Leroux (1973) found an optimum root formation on stem segments of *Pisum sativum* after the addition of sucrose. Fructose, glucose, lactose and ribose were less efficient and no or hardly any rhizogenesis occurred when galactose, maltose, mannose, raffinose or xylose were added to the culture medium. These results correspond well with those from pyrethrum experiments, where also glucose and sucrose were found to be the most efficient sugars.

The importance of the presence of sugar during the whole process of root formation, as found in pyrethrum, was also confirmed by Gautheret (1969c) using rhizome explants of *Helianthus tuberosus*.

The decreasing rooting potential at high sugar concentrations can be explained by a too high osmotic pressure (Pierik, 1969; Pierik & Steegmans, 1975c). In the presence of 2% glucose, the osmotic pressure of the medium was 2.5 atm. When the osmotic pressure was increased from 2.5 to 6 atm. by a physiological inactive sugar such as mannitol, then root formation of *Rhododendron* stem segments decreased (4% sucrose corresponds with approximately 4 atm.).

3.4.2.4 Auxins

The following auxins were examined with respect to the influence on root formation: 2,4-dichlorophenoxyacetic acid (2,4-D), β-indoleacetic acid (IAA), β-indolebutyric acid (IBA) and α-naphthaleneacetic acid (NAA). In four experiments the effect of various concentrations of these auxins on root formation of Clones 1087 and 4331 was analysed.

Peduncle explants of Clone 1087 only rooted at 2,4-D 10^{-7} and 10^{-6} g/ml and yielded about the same rooting rate at both concentrations. Clone 4331 showed the highest rooting rate at the concentrations 10^{-6} and 10^{-5} g/ml of IAA (Fig. 23), IBA and NAA. In the absence of an auxin, roots never developed and the explants became necrotic and died off (Table 18). IAA induced root formation in a much wider range of concentrations than 2,4-D, IBA and NAA. Intensive rhizogenesis occurred at 10^{-6} and 10^{-5} g/ml of IAA, IBA and NAA, whereas 2,4-D showed only a weak tendency to form roots.

In the next experiments with the three clones, IAA, IBA and NAA were compared directly at the concentrations 10^{-7}, 10^{-6} and 10^{-5} g/ml. At 10^{-5} g/ml of IAA, IBA and NAA, rate of rooting was highest for all clones (Fig. 24). Table 19 indicates that with respect to root initiation the optimum concentration was 10^{-5} for all three auxins used. The dry root weight per rooted explant, however, was higher at lower concentrations, viz. 10^{-7} or 10^{-6}, which was due to the stronger elongation of the roots at these

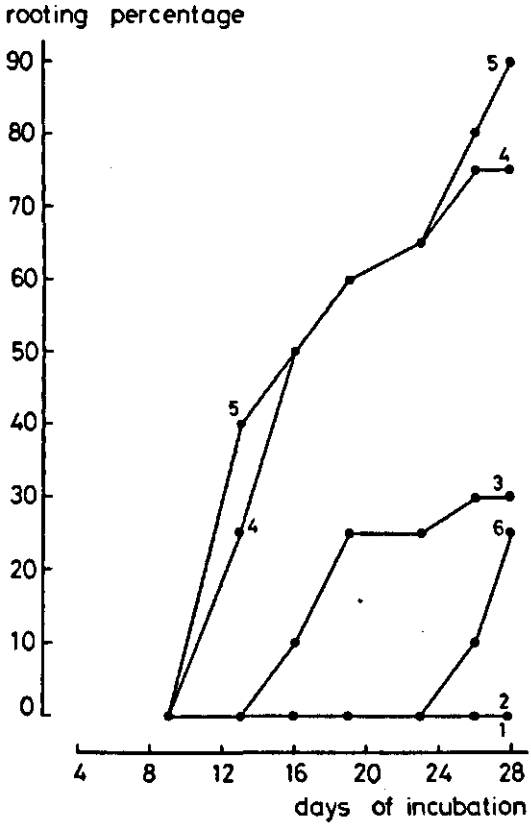


Fig. 23. The influence of various concentrations of IAA on root formation of peduncle explants of Clone 4331. Treatments 1-6 represent 0, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} g/ml, respectively.

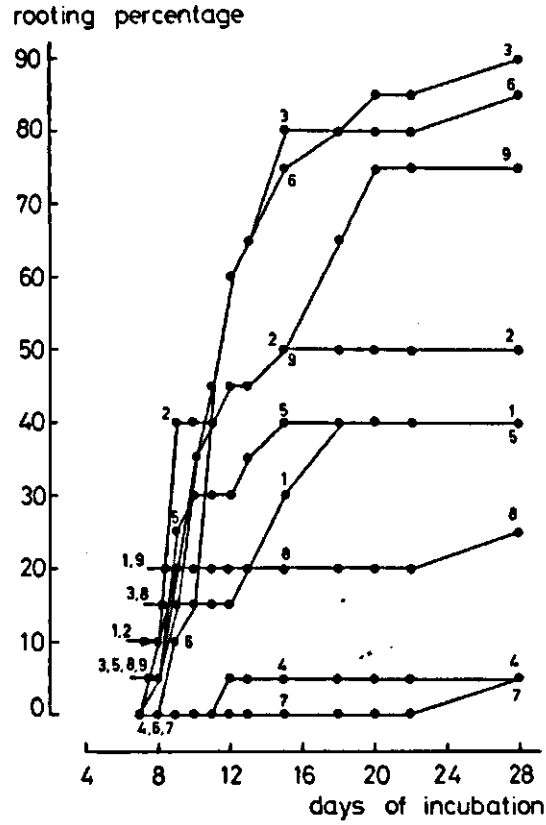


Fig. 24. The influence of various concentrations of IAA, IBA and NAA on root formation of peduncle explants of Clone 1087. Treatments 1, 2 and 3; 4, 5 and 6; 7, 8 and 9 represent 10^{-7}, 10^{-6} and 10^{-5} g/ml of IAA, IBA and NAA, respectively.

concentrations. IAA yielded better results than NAA, and IBA was still better than IAA. IBA at 10^{-5} g/ml gave the best rooting response and this auxin and this concentration were also chosen for the standard experiments.

In view of the poor root elongation at an auxin concentration of 10^{-5} g/ml, the question arose whether root elongation could be stimulated when, after incubation for 0, 3, 6, 9 or 12 d on a medium containing IBA 10^{-5} g/ml, the explants were transferred to a medium without IBA. In this experiment with Clone 4331, initially the rooting rate was highest in those treatments where the explants were transferred 6 and 9 d after incubation, but gradually the rooting rate of all the treatments did not differ markedly and rooting percentages were almost equal 39 d after incubation.

As found previously, roots were not initiated in the absence of auxin (Table 20). The presence of auxin during the first 3 d of incubation was sufficient for the initiation of a considerable number of roots. The maximum number of roots per rooted explant was already reached after an application of auxin for 6 d. The average root length decreased with an increasing application time and the highest dry root weight was measured after an auxin treatment for 6 d. After transfer to a medium without auxin the roots grew into the medium. This was hardly ever noticed on a medium containing auxin.

Table 18. The influence of various concentrations (0-10^{-4} g/ml) of 2,4-D, IAA, IBA and NAA on root formation of peduncle explants.

Clone	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
rooting percentage						
1087 2,4-D	0	0	35	30	0	0
4331 IAA	0	0	30	75	90	25
4331 IBA	0	0	0	50	85	10
4331 NAA	0	0	0	80	90	0
number of roots per rooted explant						
1087 2,4-D	0	0	5.0	6.5	0	0
4331 IAA	0	0	1.2	10.2	14.6	5.2
4331 IBA	0	0	0	4.7	15.4	3.0
4331 NAA	0	0	0	4.3	16.6	0
dry root weight per rooted explant in mg						
1087 2,4-D	0	0	0.6	0.4	0	0
4331 IAA	0	0	0.2	0.7	0.7	0.0
4331 IBA	0	0	0	0.8	0.8	0.2
4331 NAA	0	0	0	1.7	1.1	0

Table 19. The influence of various concentrations (10^{-7}-10^{-5} g/ml) of IAA, IBA and NAA on root formation of peduncle explants.

Clone	IAA			IBA			NAA		
	10^{-7}	10^{-6}	10^{-5}	10^{-7}	10^{-6}	10^{-5}	10^{-7}	10^{-6}	10^{-5}
rooting percentage									
1087	40	50	90	5	40	85	5	25	75
4331	70	85	70	0	85	75	0	90	90
Ma 63/1889	45	100	100	0	20	100	0	30	90
number of roots per rooted explant									
1087	3.8	7.7	23.2	1.0	9.5	31.9	1.0	5.0	14.9
4331	3.2	7.7	13.1	0	4.7	15.3	0	3.5	10.5
Ma 63/1889	3.3	8.2	25.5	0	5.0	34.0	0	4.0	11.7
average root length in mm									
1087	28.5	9.1	4.4	8.0	6.4	3.9	5.0	7.0	2.3
Ma 63/1889	29.8	8.4	5.2	0	18.6	4.7	0	13.1	2.8
dry root weight per rooted explant in mg									
1087	3.5	1.4	1.8	0.1	1.8	2.4	0.1	0.8	1.0
4331	2.6	2.8	1.7	0	3.2	2.0	0	3.2	1.7
Ma 63/1889	4.8	2.5	3.3	0	3.7	4.4	0	2.2	1.3

Table 20. The influence of various incubation periods (0-12 d) on a medium containing IBA 10^{-5} g/ml, before transfer to an auxin-free medium, on root formation of peduncle explants of Clone 4331, 39 d after incubation.

	0	3	6	9	12	Continuously IBA 10^{-5} g/ml
rooting percentage	0	85	95	95	95	85
number of roots per rooted explant	0	12.9	18.7	18.3	20.0	17.8
average root length in mm	0	20.8	16.4	11.5	11.4	7.5
dry root weight per rooted explant in mg	0	5.7	7.4	5.0	4.8	4.4

Apparently different optima exist for root initiation and root elongation; root initiation occurs at high auxin concentrations, whereas the elongation of the root initials is stimulated after transfer to an auxin-free medium.

In the next experiment, explants of Clone 4331 were incubated for 0, 3, 6, 9 or 12 d on a medium without auxin, and subsequently transferred to media containing auxin.

The explants started rooting in the same sequence as they were incubated on media containing auxin (Fig. 25). After incubation for 0 (IBA 10^{-5} continuously) and 3 d on an auxin-free medium, good rooting responses were obtained. With an increasing incubation time on auxin-free media the formation of roots decreased (Table 21). Finally, adventitious roots were not formed on a medium in which auxin was absent during the whole incubation period. This result shows that auxin has to be supplied almost from the very beginning, otherwise root formation will be strongly or even completely inhibited.

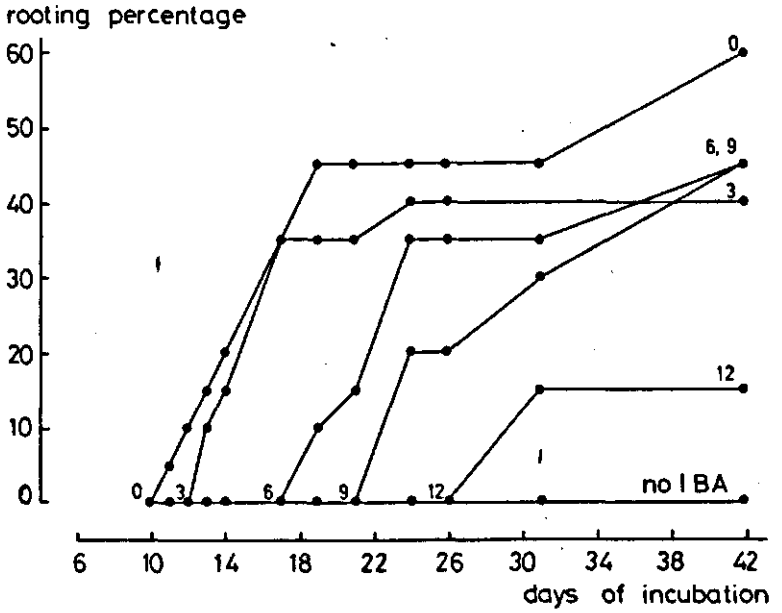


Fig. 25. The influence of incubation periods for 0, 3, 6, 9 and 12 d on an auxin-free medium, before transfer to a medium containing IBA 10^{-5} g/ml, on root formation of peduncle explants of Clone 4331. Treatment 'No IBA' represents the continuous absence of auxin.

Table 21. The influence of various incubation periods (0-12 d) on an auxin-free medium, before transfer to a medium containing IBA 10^{-5} g/ml, on root formation of peduncle explants of Clone 4331, 42 d after incubation.

	0	3	6	9	12	No IBA
rooting percentage	60	40	45	45	15	0
number of roots per rooted explant	14.7	13.6	11.6	5.2	8.3	0
average root length in mm	8.5	8.8	7.1	4.7	3.0	0
dry root weight per rooted explant in mg	3.7	6.2	2.7	1.3	0.9	0

Numerous publications have dealt with the essential or stimulating role that an exogenous auxin supply plays in the formation in vitro of adventitious roots on different explants of various plant species: shoot tips of *Asparagus officinalis* (Galston, 1948; Gorter, 1965; Andreassen & Ellison, 1967), stem explants of two species of *Parthenocissus* (Leroux, 1965) and of the three species of *Salix* (Leroux, 1966), rhizome fragments and stem segments of *Helianthus tuberosus* (Paupardin, 1966; Gautheret, 1969c; Tripathi, 1974), stem segments of *Populus nigra* (Nanda et al., 1968) and *Rhododendron* (Pierik, 1969; Olieman-van der Meer et al., 1971; Pierik & Steegmans, 1975c), epicotyl explants of *Phaseolus vulgaris* (Olieman-van der Meer et al., 1971), petiole segments of *Lunaria annua* (Pierik, 1972), leaf midrib explants of *Gerbera jamesonii* (Pierik & Segers, 1973) and stem segments of *Pisum sativum* (Leroux, 1973).

The superiority of IBA over IAA, NAA and 2,4-D, which was found for pyrethrum, cannot be considered as a general rule. The influence of different concentrations of different auxins on root formation greatly varies with the kind of explant and the plant species used.

The finding with pyrethrum that the optimum auxin concentration for root initiation is different from that for elongation of the root initials confirms results of several authors (Meyer & Anderson, 1952; Torrey, 1956b; Thimann, 1969). They established that the effect of auxins on root initiation should be clearly distinguished from their effect on root elongation. In general the concentrations required for the former are much higher than for the latter. The experiments with pyrethrum demonstrated that auxin at a high concentration has to be present to evoke root initiation, while subsequent root elongation is promoted by transfer to a medium in which auxin is absent. Though roots, after initiation on an auxin-containing medium, rapidly elongate on an auxin-free medium, it does not necessarily follow that auxins are not required for root elongation. The explants may contain natural endogenous auxins or synthetic auxins taken up from the previous medium, to which they were applied exogenously.

Auxin has to be present from the beginning of the culture in vitro; a delay of the auxin supply causes an inhibition of rhizogenesis of peduncle explants of pyrethrum. This was also observed by Leroux (1973) with stem segments of *Pisum sativum*, whereas Gautheret (1969c) using rhizome explants of *Helianthus tuberosus* noticed an optimum rooting when the auxin application was delayed by 9 d.

The presence of auxin during the first 3 and 6 d of the culture in vitro, resulted in initiation and elongation of a high number of adventitious roots on peduncle explants of pyrethrum. Gautheret (1969c) observed that an application of auxin for 4 h, at the beginning of culture, resulted in appreciable rhizogenetic properties on rhizome explants of *Helianthus tuberosus*, but the rooting intensity increased with an increase in the duration of application.

3.4.2.5 Cytokinins

The effect of the cytokinins BA (6-benzylamino purine) and kinetin (6-furfurylamino purine) on adventitious root formation was analysed with Clone 4331. These growth regulators were applied at the concentrations 0, 10^{-8}, 10^{-7} or 10^{-6} g/ml to the culture

medium together with IBA at 10^{-5} g/ml.

The rooting rate was already reduced by a concentration of BA and kinetin (Fig. 26) as low as 10^{-8} g/ml. Table 22 shows that root formation decreases by raising the cytokinin concentration. At BA and kinetin 10^{-6} g/ml root formation was completely suppressed. Root formation was strongly delayed and adventitious roots still emerged several months after incubation at a concentration of 10^{-7} g/ml of both cytokinins. BA and kinetin at

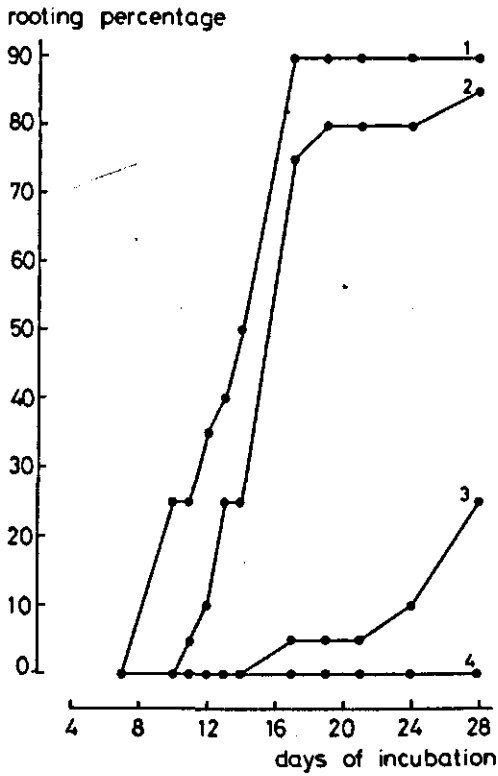


Fig. 26. The influence of various concentrations of kinetin on root formation of peduncle explants of Clone 4331. Treatments 1-4 represent 0, 10^{-8}, 10^{-7} and 10^{-6} g/ml, respectively.

Table 22. The influence of various concentrations (0-10^{-6} g/ml) of BA and kinetin on root formation of peduncle explants of Clone 4331. (Rooting percentages between brackets were determined 19 weeks after incubation.)

	0	10^{-8}	10^{-7}	10^{-6}
rooting percentage				
BA	90	90	5(25)	0
kinetin	90	85	25(50)	0
number of roots per rooted explant				
BA	43.0	16.6	1.0	0
kinetin	36.3	24.0	1.8	0
average root length in mm				
BA	5.3	6.2	3.0	0
kinetin	4.1	5.0	4.0	0
dry root weight per rooted explant in mg				
BA	3.9	1.9	0.1	0
kinetin	2.3	2.1	0.1	0

10^{-8} g/ml, however, slightly seemed to promote the elongation of roots.

In the absence of an auxin the addition of a cytokinin generally does not lead to the formation of (lateral) roots, as has been demonstrated in vitro by Torrey (1958) with isolated roots of *Convolvulus arvensis* and by Gautheret (1969c) using rhizome explants of *Helianthus tuberosus*. For that reason mostly cytokinin is added simultaneously with an auxin to the culture medium. It is quite common that high cytokinin concentrations antagonize auxin-induced root formation in tissue cultures, as has been observed with tobacco callus (Skoog & Miller, 1957), rhizome explants of *Helianthus tuberosus* (Gautheret, 1966a, 1969c), stem segments of *Pisum sativum* (Leroux, 1969ab, 1973), leaf midrib fragments of *Gerbera jamesonii* (Pierik & Segers, 1973) and *Rhododendron* stem segments (Pierik & Steegmans, 1975c).

Adversely, low cytokinin concentrations can promote (lateral) root formation, as reported for tobacco callus (Skoog & Miller, 1957), isolated roots of *Convolvulus arvensis* (Torrey, 1958) and pea (Torrey, 1962), carrot callus tissue (Pilet, 1961), leaf fragments of *Cichorium intybus* (Toponi, 1963) and stem segments of *Pisum sativum* (Leroux, 1973) and *Rhododendron* (Pierik & Steegmans, 1975c).

The findings with pyrethrum closely correspond with those reported in literature. The indication that root elongation is stimulated by a low cytokinin concentration, suggests that the promotive effect on rooting found in other studies may be due to the same phenomenon.

3.4.2.6 Gibberellic acid

Gibberellic acid (GA_3) was added at the concentrations 0, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} g/ml to the culture medium, in the presence of IBA at 10^{-5} g/ml, to determine its influence on rooting of explants of Clone 4331.

About the same rooting rate was observed at GA_3 concentrations of 0, 10^{-8}, 10^{-7} and 10^{-6}, whereas GA_3 at 10^{-5} and 10^{-4} g/ml strongly delayed rhizogenesis (Fig. 27). The application of GA_3 at 10^{-8}, 10^{-7} and 10^{-6} g/ml slightly stimulated the elongation of the initiated roots (Table 23). The other parameters for rooting did not differ markedly at GA_3 0, 10^{-8} and 10^{-7}, while root formation was slightly inhibited by GA_3 at 10^{-6} and a strong detrimental effect was caused by GA_3 at 10^{-5} and 10^{-4} g/ml.

Spanjersberg & Gautheret (1964) and Gautheret (1966a, 1969c) reported that GA_3, when applied alone, was not able to initiate roots on rhizome explants of *Helianthus tuberosus*. In most studies gibberellic acid is added simultaneously with an auxin to the

Table 23. The influence of various concentrations (0–10^{-4} g/ml) of GA_3 on root formation of peduncle explants of Clone 4331.

	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
rooting percentage	100	85	90	95	70	55
number of roots per rooted explant	32.7	29.1	30.2	24.9	4.8	3.6
average root length in mm	5.0	5.9	5.8	5.8	1.7	3.1
dry root weight per rooted explant in mg	2.3	2.3	2.0	1.7	0.1	0.1

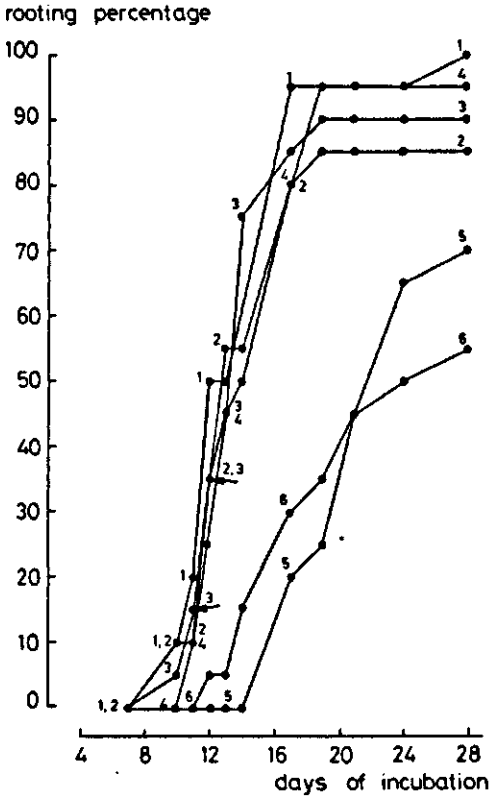


Fig. 27. The influence of various concentrations of GA_3 on root formation of peduncle explants of Clone 4331. Treatments 1-6 represent 0, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} g/ml, respectively.

culture medium; the effect of GA_3 on root organogenesis greatly varied with the plant species.

Most frequently GA_3 had an inhibitory effect on auxin-induced root formation, as has been reported for stem segments of *Populus nigra* (Nanda et al., 1968) and *Rhododendron* (Pierik & Steegmans, 1975c). Leroux (1967, 1968a, 1973) with *Pisum sativum* noticed that GA_3, when applied to the plants from which the segments were taken, stimulated auxin-induced adventitious root formation of stem segments but, when applied directly to the stem segments, GA_3 inhibited the formation of roots. Spanjersberg & Gautheret (1964) and Gautheret (1966a, 1969c) observed that in the presence of NAA, GA_3 did promote the production of a high number of roots on rhizome explants of *Helianthus tuberosus* when the cultures were kept in darkness, whereas in light adventitious root formation was suppressed, especially at high GA_3 concentrations.

In pyrethrum the effect of GA_3 varied with the concentration used and also depended on the stage in root formation. High GA_3 concentrations considerably diminished both the initiation and elongation of adventitious roots, whereas low concentrations did not influence root initiation and had a slightly favourable effect on root elongation.

3.4.3 Climatic factors

3.4.3.1 Temperature

Explants of the three clones were exposed to constant temperatures of 9, 13, 17, 21 or 25°C in the phytotron. This experiment showed that the regeneration of roots was accelerated by raising the temperature from 9 to 25°C. However, after an incubation for 4 weeks, in the range 13-25°C rooting percentages did not differ much, while at 9°C the development of roots was still inhibited (Fig. 28). In spite of the rapid development of adventitious roots at high temperatures, the highest number of roots after 4 weeks was obtained at low temperatures: 9°C (Clones 1087 and 4331) and 13°C (Clone Ma 63/1889) (Table 24).

In Clone Ma 63/1889, the number of roots after incubation for 4 weeks at 9°C was very low. Moreover, 10 explants (50%) of this treatment did not show macroscopically visible roots after 4 weeks incubation. When these 10 'unrooted' explants were transferred from 9 to 20°C, one day later 6 of these explants had developed macroscopically visible roots and after 4 days adventitious roots had emerged from all the explants. After 2 weeks at 20°C these explants had developed 22.9 roots per rooted explant, the average root length was 6.0 mm and the dry root weight per rooted explant 2.9 mg! As anatomical observations (Section 3.3) have shown that adventitious roots did not appear earlier than about 10 days after incubation at 20°C, the roots on explants of Clone Ma 63/1889 must have been initiated at 9°C. Consequently, root initials almost fail to elongate at this temperature. Hence the temperature optima for the initiation and for the elongation of adventitious roots are different, being low for initiation and higher for elongation.

The question arose whether a more effective root formation can be obtained when explants are first grown at a low temperature and subsequently at a higher temperature,

Table 24. The influence of constant temperatures (9-25°C) on root formation of peduncle explants.

Clone	9	13	17	21	25
rooting percentage					
1087	85	85	95	85	60
4331	30	60	70	90	60
Ma 63/1889	50	85	90	80	80
number of roots per rooted explant					
1087	43.7	11.0	14.8	9.8	3.5
4331	43.7	17.0	15.8	13.8	9.1
Ma 63/1889	5.4	22.1	16.3	14.9	11.2
average root length in mm					
4331	1.0	4.5	9.4	9.5	7.7
Ma 63/1889	2.0	6.1	6.3	6.6	6.0
dry root weight per rooted explant in mg					
1087	0.1	0.3	0.9	0.7	0.6
4331	0.1	0.8	2.1	2.7	1.5
Ma 63/1889	0.2	2.1	2.6	3.0	2.1

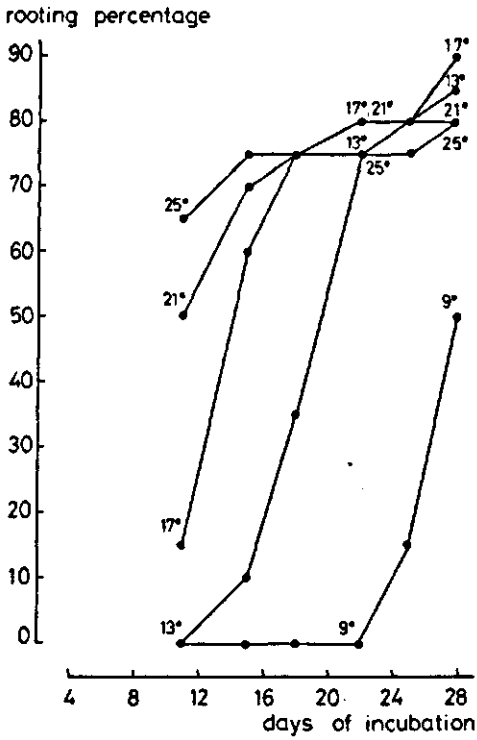


Fig. 28. The influence of constant temperatures (9-25°C) on root formation of peduncle explants of Clone Ma 63/1889.

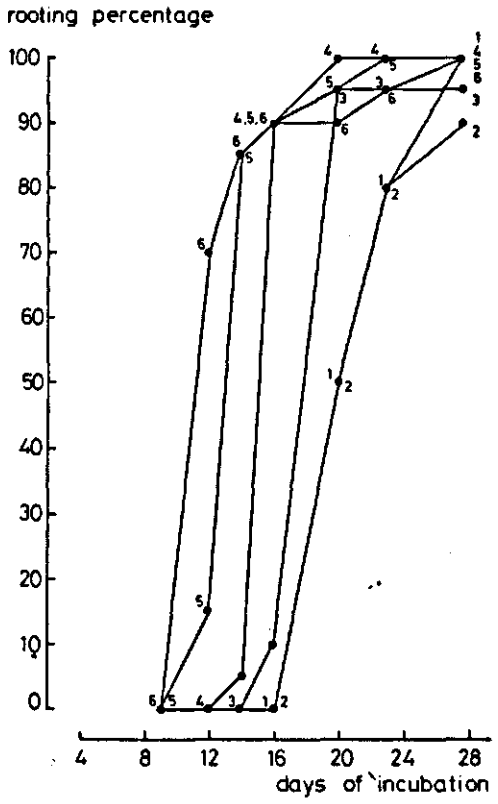


Fig. 29. The influence of various temperature combinations on root formation of peduncle explants of Clone 4331.
1 = 28(d 13°C)/0(d 21°C), 2 = 20/8,
3 = 15/13, 4 = 10/18, 5 = 5/23 and 6 = 0/28.

instead of growing them at a constant low or high temperature. To answer this question explants from Clone 4331 were submitted to 13°C for various periods, before being transferred to 21°C.

Fig. 29 shows that rooting speed decreased as the period during which the explants were incubated at 13°C increased. Table 25 shows that incubation for 10 and 15 d at 13°C before transfer to 21°C results in the best rooting responses. The anatomical observations (Section 3.3) demonstrated that at a constant temperature of 20°C in general adventitious roots were initiated during the first 2 weeks of incubation and elongation of the initiated roots occurred during the subsequent 2 weeks of incubation. Thus 10-15 d at 13°C are sufficient for an adequate initiation of adventitious roots.

The next question was: which initial temperature is the most favourable? This was examined in an experiment in which explants of Clone 4331 were exposed to 9, 13, 17 or 21°C for 2 weeks before transfer to 21°C for the next 2 weeks.

Fig. 30 illustrates that again rooting rate was optimum when the explants were grown continuously at 21°C and that the development of the roots was retarded progressively as the temperature during the first 2 weeks decreased. The highest number

Table 25. The influence of various temperature combinations (d 13°C/d 21°C) on root formation of peduncle explants of Clone 4331.

	28/0	20/8	15/13	10/18	5/23	0/28
rooting percentage	100	90	95	100	100	100
number of roots per rooted explant	21.7	32.1	36.3	32.9	26.6	19.0
dry root weight per rooted explant in mg	1.0	2.7	3.6	4.1	3.7	3.3

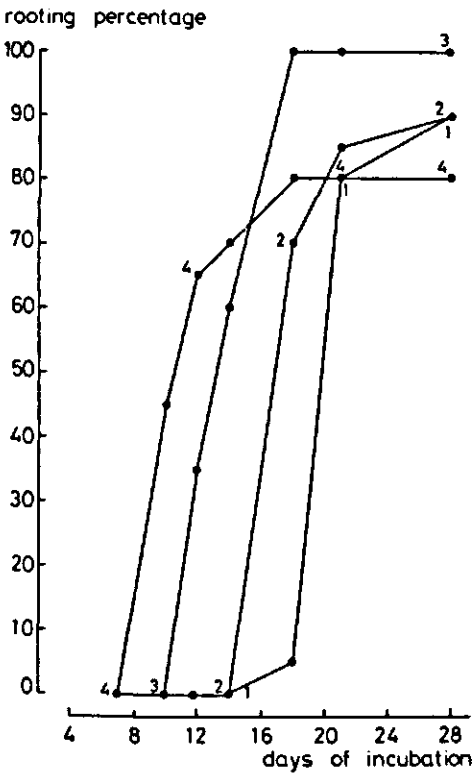


Fig. 30. The influence of various temperature combinations on root formation of peduncle explants of Clone 4331. 1 = 2 weeks 9° + 2 weeks 21°C, 2 = 2 weeks 13° + 2 weeks 21°C, 3 = 2 weeks 17° + 2 weeks 21°C and 4 = 4 weeks 21°C.

of roots was initiated at an initial temperature of 9 and 13°C, followed by 21°C during the last 2 weeks (Table 26). Numbers of roots as well as dry root weights were high when 2 weeks at 13°C were followed by 2 weeks at 21°C.

The experiments described in this section clearly demonstrated that a constant temperature, whether low or high, does not lead to an optimum root formation, but that the temperature has to be adapted to the stage reached in root formation. Because peduncle explants of various experiments and at different stages of root formation were incubated simultaneously in one growth chamber, a constant temperature of 20°C was always maintained in standard experiments.

Most publications gave a constant high temperature as optimum for rooting. An optimum of 25°C has been found for root formation of shoot tips of *Asparagus officinalis*

Table 26. The influence of various temperature combinations on root formation of peduncle explants of Clone 4331.

	2 weeks 9+ 2 weeks 21	2 weeks 13+ 2 weeks 21	2 weeks 17+ 2 weeks 21	4 weeks 21
rooting percentage	90	90	100	80
number of roots per rooted explant	31.9	30.8	25.4	21.5
dry root weight per rooted explant in mg	2.4	3.6	3.9	3.8

(Gorter, 1965), rhizome explants of *Helianthus tuberosus* (Gautheret, 1969c), stem segments of *Rhododendron* (Pierik, 1969; Pierik & Steegmans, 1975c) and *Pisum sativum* (Leroux, 1973) and leaf midrib fragments of *Gerbera jamesonii* (Pierik & Segers, 1973).

The findings with pyrethrum showed that, when fluctuating temperatures are considered, also a low temperature can play a specific role in the process of root formation. This has also been observed in the most detailed study about the influence of temperature on adventitious root formation of rhizome explants of *Helianthus tuberosus* (Gautheret, 1961, 1966b, 1968ab, 1969abc; Spanjersberg & Gautheret, 1963ab; Tripathi, 1974). They found that the formation of cambia was enhanced by a high temperature and that the organization of root primordia from these cambia was stimulated by a lower temperature. In fact the high temperature was involved in a stage prior to rhizogenesis and did not act directly upon root formation.

Adventitious root formation of petiole segments of *Lunaria annua* (Pierik, 1972) was inhibited at 25°C in continuous light. This inhibition could be removed by a cold treatment at 5°C. But in darkness there was no evidence that 5°C was more effective than 25°C.

Leroux (1973) reported that a temperature of 5-10°C to which plants of *Pisum sativum* were exposed, exerted a delayed positive effect on root formation of isolated stem segments at 25°C. Furthermore, he proved that 5°C also exerted a direct positive effect; when the segments were exposed for the first 4 d of incubation to this low temperature, root formation was subsequently enhanced at 25°C, compared with a constant temperature of 25°C. He attributed this stimulation of root formation at low temperatures to the increase in the amount of soluble sugars within the (ex)plant.

It can be concluded that the effect of the temperature greatly varies from species to species and also according to the stage reached in the process of adventitious root formation.

3.4.3.2 Light

Stock plants of Clone 4331 were grown in the greenhouse under natural daylight conditions. To determine whether light may exert a delayed effect on root formation, peduncles of Clone 4331, which were still attached to the stock plants, were etiolated with black plastic cloth for 0, 2, 4, 6, 8 or 10 d before isolation of the peduncles and incubation of the peduncle explants in vitro. Peduncles which were etiolated for 10 d showed a wider diameter and a lighter green colour than unetiolated ones.

Table 27. The influence of various etiolation periods (0-10 d) of peduncles on root formation of peduncle explants of Clone 4331.

	0	2	4	6	8	10
rooting percentage	100	90	95	85	100	100
number of roots per rooted explant	9.3	11.5	9.4	11.1	14.9	14.9
average root length in mm	11.3	10.9	10.5	9.3	9.2	7.9
dry root weight per rooted explant in mg	2.8	3.0	3.1	3.2	4.1	4.2

Table 28. The influence of continuous light or darkness on root formation of peduncle explants of Clones 1087, 4331 and Ma 63/1889, 29 days after incubation.

Clone	Light	Darkness
rooting percentage		
1087	25	40
4331	90	75
Ma 63/1889	40	70
number of roots per rooted explant		
1087	5.4	19.0
4331	15.1	29.1
Ma 63/1889	12.8	25.3
average root length in mm		
1087	2.1	4.9
4331	2.8	6.3
Ma 63/1889	3.0	4.9
dry root weight per rooted explant in mg		
1087	0.4	1.2
4331	1.5	3.1
Ma 63/1889	2.5	3.1

The rooting rate increased with a more prolonged period of etiolation, but did not markedly differ in the various treatments 4 weeks after incubation. The number of roots increased and the average root length decreased by prolonging the etiolation period (Table 27). Dry root weights were highest after etiolation for 8 and 10 d. Thus it was shown that light has a delayed inhibiting effect on adventitious root formation.

Thereafter, the direct influence of continuous light and darkness on adventitious root formation of the three clones was investigated at 21°C in the phytotron. Fig. 31 and Table 28 illustrate that, although substantial differences exist among the clones, the general pattern is similar and a direct inhibiting effect of light on root formation can be observed.

The favourable effect of darkness in comparison with light on adventitious root formation was confirmed in two other experiments with Clone 4331. In the first experiment explants of the peduncle were exposed to various 16-h photoperiods, before exposure to darkness. When the explants were exposed to more photoperiods, rooting rate slightly decreased. Moreover, all other rooting parameters improved when the photoperiods were fewer and 0 (darkness) and 4 photoperiods yielded the best rooting responses (Table 29).

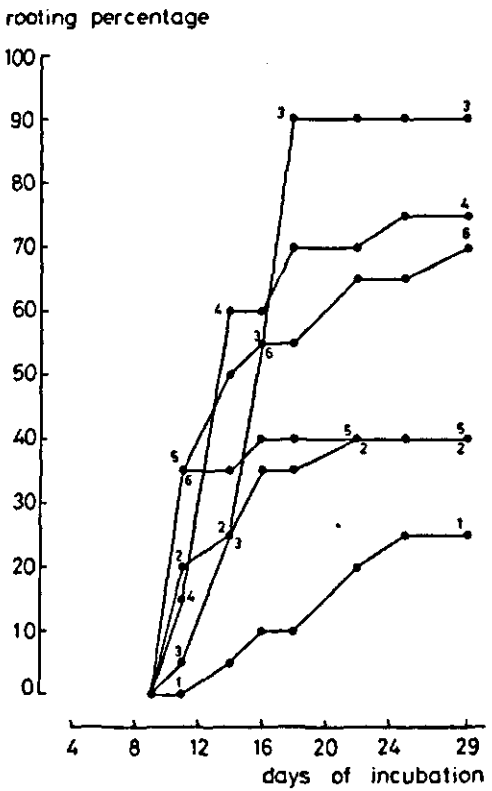


Fig. 31. The influence of continuous light or darkness on root formation of peduncle explants of Clones 1087 (1 = light, 2 = darkness), 4331 (3 = light, 4 = darkness) and Ma 63/1889 (5 = light, 6 = darkness).

Table 29. The influence of various 16-h photoperiods (0-20), before exposure to darkness, on root formation of peduncle explants of Clone 4331.

	0	4	8	12	16	20
rooting percentage	100	100	100	100	95	90
average number of roots per rooted explant	15.6	13.7	12.2	13.0	11.8	10.8
average root length in mm	6.4	8.2	6.7	5.7	4,5	4.6
dry root weight per rooted explant in mg	3.4	3.4	2.7	2.7	2.3	2.2

Table 30. The influence of various darkness periods (20-0 d), before exposure to 16-h photoperiods, on root formation of peduncle explants of Clone 4331, 5 weeks after incubation.

	20	16	12	8	4	0
rooting percentage	80	95	85	80	95	45
number of roots per rooted explant	18.9	14.8	13.4	20.3	14.7	14.0
average root length in mm	7.2	6.1	5.3	3.8	3.9	4.0
dry root weight per rooted explant in mg	4.3	3.3	3.0	3.7	2.2	2.8

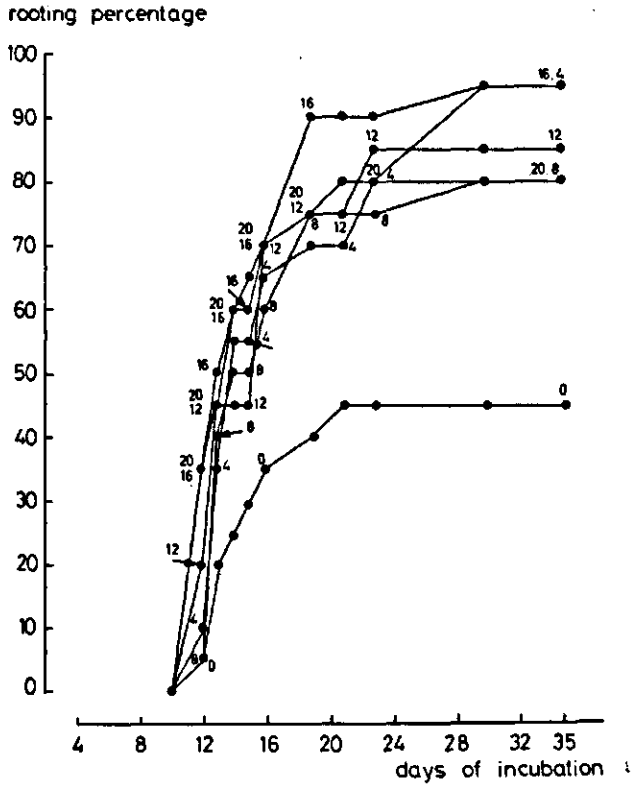


Fig. 32. The influence of darkness periods for 20, 16, 12, 8, 4 and 0 d, before exposure to 16-h photoperiods, on root formation of peduncle explants of Clone 4331.

In the second experiment explants of Clone 4331 were exposed for different periods to darkness, before exposure to 16-h photoperiods. All treatments showed about the same rooting rate, with the exception of continuous exposure to 16-h photoperiods (0 d of darkness), which treatment delayed the organogenesis of roots (Fig. 32). Some variation can be observed among the numbers of roots per rooted explant; the average root length, however, diminished as the explants became exposed earlier to 16-h photoperiods (Table 30). Again it was demonstrated that exposure for a long period to darkness (20 d) strongly favours adventitious root formation. In standard experiments darkness was always maintained.

As has been reported in literature and found for pyrethrum, light inhibited or decreased the formation of (lateral) roots, as demonstrated with: shoot tips of *Asparagus officinalis* (Galston, 1948; Gorter, 1965), isolated roots (Torrey, 1952; Furuya & Torrey, 1964) and stem segments (Leroux, 1967, 1968ab, 1969ab, 1973) of *Pisum sativum*, isolated roots of *Convolvulus arvensis* (Torrey, 1958), stem explants of *Populus nigra* (Nanda et al., 1968) and *Rhododendron* (Pierik, 1969; Olieman-van der Meer et al., 1971; Pierik & Steegmans, 1975c), epicotyl segments of *Phaseolus vulgaris* (Olieman-van der Meer et al., 1971), petiole pieces of *Lunaria annua* (Pierik, 1972) and leaf midrib fragments of *Gerbera jamesonii* (Pierik & Segers, 1973).

In experiments with *Helianthus tuberosus* (Gautheret, 1961, 1966a, 1969c; Spanjersberg & Gautheret, 1963ab; Letouzé & Beauchesne, 1969; Rücker & Paupardin, 1969;

Tripathi, 1974) light inhibited root formation of rhizome explants of the variety 'Violet commun' and stem segments of the variety 'Violet de Rennes', whereas a promoting effect of light on rooting was observed on rhizome explants of the variety 'Violet de Rennes'. With stem segments of different species of both *Parthenocissus* (Leroux, 1965) and *Salix* (Leroux, 1966) it was also found that light inhibited or stimulated rhizogenesis, depending on the species.

A promotive effect of light on root formation in isolated tissues was reported for tobacco pith cultures (Weiss & Jaffe, 1969) and excised cotyledons of *Sinapis alba* (Lovell & Moore, 1969).

The inhibiting effect of light in one group of plants and the promoting effect in another group, makes it very difficult to elucidate the action of light. Four possible explanations were suggested by Olieman-van der Meer et al. (1971).

3.5 DISCUSSION AND CONCLUSIONS

In this chapter factors were investigated that are involved in the process of adventitious root formation of peduncle explants cultivated in vitro.

Through this procedure it could be established that adventitious root formation is regulated by a complex system involving a number of limiting factors. These are related to properties of the stock plant and its explant, the nutritional/hormonal composition of the culture medium and the climatic conditions (Section 3.4).

The anatomical data (Section 3.3) indicated that during the process of adventitious root formation, the initiation of adventitious roots and the development of the root initials take place during the first and last 2 weeks of incubation of peduncle explants, respectively. Both stages do not respond similarly to the growth conditions, but show their own optima.

According to this study some new growth conditions can be mentioned, that differ from the standard growth conditions (Section 3.2), but with which root formation may be further optimalized. They can be summarized as follows: The upper portions, with a length of 1.5 cm, of peduncles in a young flowering stage, have to be wounded over the whole length (by excision of the cortex at one side of the explant), placed horizontally with the wounded side on the medium, which is composed of pyrex-distilled water, supplemented with agar 0.8%, the major salts of Knop at half strength and sucrose at 2%. The presence of IBA at 10^{-5} g/ml is required for the first week to ensure an optimum root initiation, after which period the explants have to be transferred to an auxin-free medium for an optimum elongation of the root initials. The explants have to be exposed to darkness; during the first 2 weeks to about 13°C and during the subsequent weeks of incubation to approximately 20°C. When all these conditions are maintained, a good initiation as well as elongation of adventitious roots can be expected 4 weeks after incubation of peduncle explants in vitro.

Though the exact role of the various factors involved in root formation has not yet been completely elucidated, these investigations clearly demonstrated that in addition to physiological studies, supplementary anatomical observations are essential for a better understanding of the exact role of separate factors on both root initiation and

elongation. As pointed out by Pierik (1969) in this way it becomes possible to discriminate between factors affecting root initiation or root elongation. This distinction would be impossible on the basis of data only about the physiological aspect of root formation. As a consequence of this approach, optimum growth conditions can be selected and maintained during the different stages in the process of root formation.

In Chapter 4 it is evaluated whether this knowledge, obtained through a culture of peduncle explants in vitro, leads to an optimalization of root formation of shoot cuttings in vivo.

4 Root formation of shoot cuttings in vivo

4.1 INTRODUCTION

In practice pyrethrum is propagated vegetatively by splits. This method results in a low multiplication rate as one stock-plant of 1-2 years old can be divided into only 5-10 splits. Studies on alternative asexual propagation methods, for instance from shoot cuttings in vivo, like is described in this chapter, and by the cultivation of capitulum explants in vitro (Chapter 5), have a direct bearing on the current attempts of the pyrethrum industry to improve the rate of multiplication of selected, high yielding clones.

An additional problem is that the flower yield strongly decreases when plants are attacked by the root-knot nematode *Meloidogyne hapla* (Bullock, 1961; Robinson, 1963; Parlevliet & Brewer, 1970, 1971; Parlevliet, 1971). Splitting infested stock-plants results in a contaminated clone, whereas with vegetative propagation from shoot cuttings planting material would be free of nematodes, even from infested stock-plants.

A prerequisite for the production of a plant from a shoot cutting is the formation of adventitious roots at the basal stem portion of the cutting. The influence of various factors on the process of adventitious root formation of peduncle explants in vitro has already been described in Chapter 3. Although the circumstances for root formation in vivo and in vitro are different (Section 3.1), this 'in-vitro' knowledge may lead to the optimalization of adventitious root formation of shoot cuttings in vivo. Section 4.3 deals with anatomical observations and Section 4.4 with the effect of various factors on root formation of shoot cuttings.

4.2 MATERIAL AND METHODS

From the rosettes of 1-2 year-old stock-plants of Clones 1087, 4331 and Ma 63/1889, cultivated in the greenhouse (Section 2.2), vegetative shoots with 4-5 developing leaves and with a length of about 10 cm were excised. Apart from the basal wound, the cuttings were wounded by removing the lower emplanted leaves. In this way a basal stem portion was obtained, with a length of about 1.5 cm, suitable for the application of auxins and insertion into the rooting substrate (Fig. 33, left). The cuttings were immersed in a 0.2% solution of the fungicide benomyl (methyl ester of 1-(butylcarbamoyl)-2-benzimidazole carbamic acid) and their basal stem portions were dipped in a powder of the auxin IAA at 1% (on talc basis). The cuttings were planted in a sieved substrate of equal volumes of leaf mould and sand in 4 cm square 'Jiffy' peat-pots. The leaf mould was made up as follows: foliage debris was collected and left for one year, mixed with 200 dm^3 manure per m^3 and left to decompose for another year, then mixed with 6 kg $CaCO_3$ per m^3

Fig. 33. From left to right:

- prepared shoot cutting just before insertion; the basal stem portion with a length of about 1.5 cm was obtained by removal of the lower emplanted leaves.
- rooted shoot cutting in 'Jiffy' pot, 4 weeks after insertion,
- rooted shoot cutting after removal of the 'Jiffy' pot, 4 weeks after insertion.

and ground, subsequently left again for one year, after which about 300 dm^3 of 'Trio' potsoil 17 + 6 kg $CaCO_3$ per m^3 were added; finally the mixture was ground again and treated with methyl bromide. The 'Jiffy' pots were embedded in a leaf mould layer in propagation boxes (Fig. 34) in a growth chamber (Fig. 35) at 15°C and 80-90% relative humidity. The growth chamber was illuminated by fluorescent light (Philips TL MF 140 W/ 33RS) supplemented with incandescent lamps (Philinea) at a daylength of 14 h and a light intensity of 25 000 lx, measured just above the propagation boxes. In standard experiments, in unheated boxes, a soil temperature of 17°C was maintained. The substrate could be heated up to 30°C with heating coils, which were buried in sand below the leaf mould layer. The first 2 weeks after insertion, the propagation boxes were covered with thin polyethylene foil (Fig. 35) to keep the relative humidity at almost 100% and thereby to reduce the evaporation of the unrooted cuttings.

As was done with peduncle explants in vitro (Chapter 3), the experiments were terminated 4 weeks after insertion of the shoot cuttings. The cuttings were lifted (Fig. 33, middle) and after removal of the 'Jiffy' pots (Fig. 33, right), the percentage of the cuttings which produced main and lateral roots, the average number of main roots per rooted cutting and of lateral roots per cutting with lateral root formation, and the average length (in mm) per main and lateral root, were estimated. By measuring the dry root weight (in 0.1 g after drying for 4 h at 80°C) additional information was obtained about root formation, which occurs at the basal part of the cutting. Sometimes the

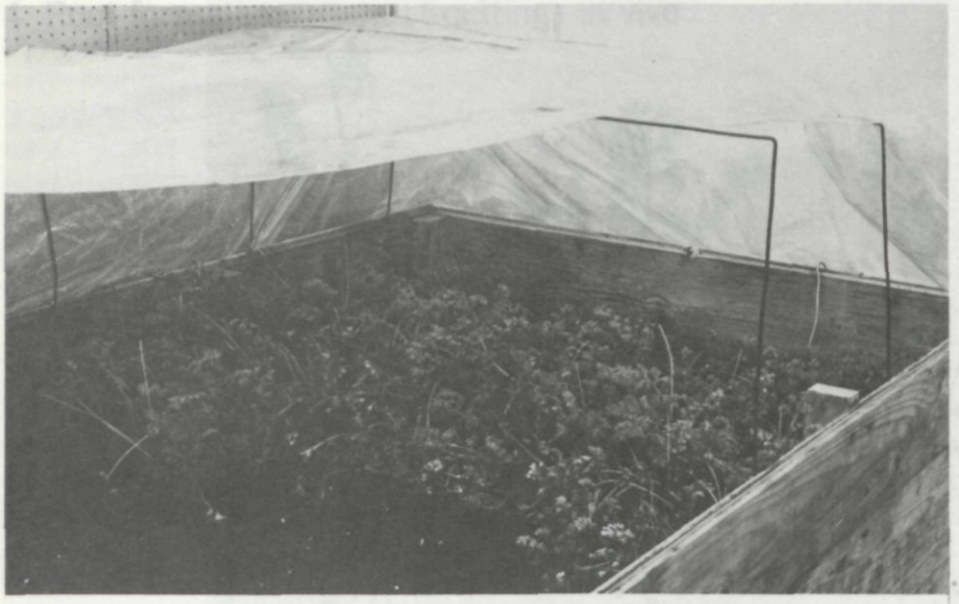


Fig. 34. Propagation box; shoot cuttings planted in 'Jiffy' pots and embedded in a leaf mould layer.

Fig. 35. Growth chamber provided with two propagation boxes; the propagation box at the right is covered with polyethylene foil.

number of developing shoots was also determined to obtain information about the development of the upper part of the cutting.

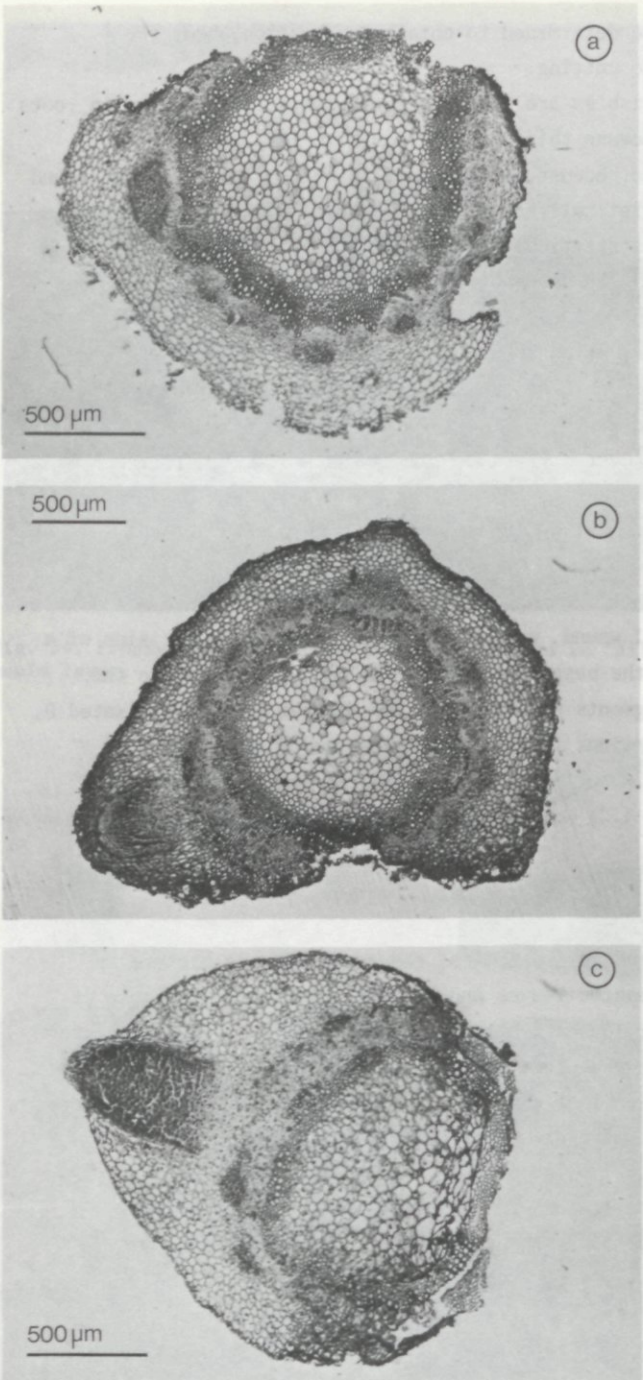
The most important rooting variables are those referring to formation of main roots (percentage, number and length), because this is a prerequisite for lateral root formation. For that reason, but also because lateral root formation was sometimes found to be very sporadic, data were statistically analysed for variables which concern main root formation. The results of the statistical analysis are presented in the form of P values (for effects of interest) and LSD values (for pairwise comparisons of a number of treatments). These parameters are defined as follows:
P = probability of obtaining an observation about the effect of interest as extreme as or more extreme than the one actually obtained in the experiment, where the probability is calculated under the null hypothesis (here always indicating 'no effect').
LSD = abbreviation of 'least significant difference', i.e. the smallest difference (in absolute value) that would lead to rejecting the null hypothesis of zero difference against the alternative hypothesis that the difference is not zero with a level α significance test (α was always chosen to be 0.05).

For anatomical observations shoot cuttings of Clone 1087 with long internodes were selected, which apart from the basal wound, were additionally wounded by excision of a strip of the cortex at one side of the basal internode, and then inserted into the substrate. Basal internodal stem segments with a length of about 1.5 cm were fixated 0, 4, 8, 11, 14, 16 or 18 d after insertion. The plant material was further prepared according to the methods described in Section 3.2.2. Unless otherwise stated, the standard growth conditions (Section 4.2) were maintained in all rooting experiments.

4.3 ANATOMICAL OBSERVATIONS

When nodal stem segments are used for anatomical observations, the picture is confused by connections between emplanted leaves and vascular bundles and because of injuries due to removal of the lower leaves. Therefore shoot cuttings with basal internodes with a length of about 1.5 cm were used, while a strip of the cortex was excised for a better comparison with the anatomical observations on root formation of peduncle explants, which were wounded in a similar way (Section 3.3).

Transverse sections of the basal internodal stem segment of a shoot cutting (Fig. 36a) and of a peduncle explant (Fig. 12a), just before insertion and incubation, respectively, look morphologically similar, though there are some differences:
- A basal stem section of a shoot cutting is almost cylindrical in outline and the cortex consists of chlorenchymous tissue, while a section of the stem on top of the peduncle has a corrugated outline because in the cortex loose chlorenchymous tissue alternates with the tight collenchymous tissue of the ribs.
- In a peduncle segment a fascicular cambium gives rise to the formation of secondary phloem and xylem. In a basal stem portion of a shoot cutting, secondary phloem and xylem also originate from an interfascicular cambium.
- In the vascular bundle of a peduncle segment parenchymous pericambium cells occur, whereas sklerenchymous fibres can be observed in the fascicular pericycle in a basal



Figs 36a-c. Transverse sections of basal internodal stem segments of shoot cuttings:

a. Note the wounded side, just before insertion.
b. A root primordium (left from the wounded side) initiated in the interfascicular pericycle brings about a collapse of cells of the endodermis and the cortex, 11 d after insertion.
c. An adventitious root (opposite the wounded side) penetrates the epidermis, 18 d after insertion.

stem portion of a shoot cutting.

In Figs 36b and 36c it can be observed that adventitious root formation of shoot cuttings progresses similarly to that for peduncle explants (Section 3.3). Main roots were initiated in the interfascicular pericycle, usually within 2 weeks after insertion, and during the subsequent 2 weeks the root initials elongated and emerged. In contrast with peduncle segments, rather often lateral roots branched off from main roots, initiated and elongated on basal stem portions of shoot cuttings.

4.4 FACTORS INFLUENCING ROOT FORMATION

4.4.1 Plant factors

4.4.1.1 Genotype

In most experiments described in Section 4.4 shoot cuttings of Clones 1087, 4331 and Ma 63/1889 were used simultaneously. Although sometimes significant differences existed between clones, in general the clones reacted similarly and gave good rooting responses under various conditions.

4.4.1.2 Wounding

In Section 4.2 it was described that initially, apart from the basal wound, the basal stem portions of shoot cuttings were wounded by removal of the lower emplanted leaves. Adventitious root formation of peduncle explants in vitro was positively affected by the excision of a strip of the cortex at one side and over the whole length of the explant (Section 3.4.1.5).

The effect of an additional wound on root formation of shoot cuttings was examined in an experiment with 100 shoot cuttings per treatment of Clones 1087, 4331 and Ma 63/1889. An additional wound cut was made by excision of a strip of the cortex at one side of the base of the cutting over a length of about 1.5 cm.

A statistical analysis was not carried out because the data (Table 31) indicated that no significant wounding effects are to be expected. Evidently, the excision of a strip of the cortex is not an important procedure in the preparation of shoot cuttings.

Table 31. The influence of wounding on root formation and shoot development of shoot cuttings of Clones 1087, 4331 and Ma 63/1889.

Clone		Main root formation			Lateral root formation			Dry root weight	Shoot number
		%	number	length	%	number	length		
1087	unwounded	54	9.1	33.0	25	7.0	5.2	6.1	135
	wounded	62	8.5	28.5	24	7.3	10.4	6.1	127
4331	unwounded	41	6.3	24.4	13	2.0	4.8	4.5	104
	wounded	36	7.4	25.4	12	3.6	4.6	4.4	103
Ma 63/1889	unwounded	71	12.0	28.3	43	9.3	8.2	11.0	106
	wounded	73	10.2	31.0	43	8.9	8.5	8.7	106

However, the initial injuries which are made by preparing shoot cuttings may stimulate root formation. The effect of these initial injuries on rooting could not be determined, because all shoot cuttings were initially wounded and unwounded shoot cuttings were not available.

4.4.1.3 Shoot tip

The effect of the terminal shoot tip on root formation and shoot development was determined in an experiment with 60 shoot cuttings per treatment of Clones 1087, 4331 and Ma 63/1889.

The data of Table 32 indicate significant promotive effects (averaged over clones) of shoot tip removal on percentage of main root formation and average number of main roots. P_{stcl} showed that there may be differences between clones with respect to the effect of the shoot tip on the percentage of main root formation and not on the other variables. The LSD value for the rooting percentage suggested that the effect of the shoot tip is large for Clones 1087 and Ma 63/1889 and negligible for Clone 4331. The variables referring to main root length and lateral root formation were not strongly affected by the shoot tip. On the other hand the dry root weights were much higher in the absence of the shoot tip, which effect, however, was not analysed statistically, because there were no replicates. The shoot number comprises the number of both shoots arising from terminal shoot tips and from buds in the axils of leaves. Thus in those treatments where the shoot tips were not removed, the shoot number has to be reduced by 60 to find the number of developing axillary shoots. In the presence of the shoot tip, axillary shoots rarely developed, whereas high numbers of axillary shoots developed

Table 32. The influence of the shoot tip on root formation and shoot development of shoot cuttings of Clones 1087, 4331 and Ma 63/1889 (+ and − represent the presence and absence of the shoot tip, respectively).

Clone		Main root formation			Lateral root formation			Dry root weight	Shoot number
		%	number	length	%	number	length		
1087	+	25	4.5	23.6	10	6.1	4.7	0.7	61
	−	53	9.5	31.2	12	3.2	7.1	2.3	75
4331	+	63	11.4	34.1	22	10.3	9.9	5.4	65
	−	67	15.8	41.5	18	11.7	5.7	10.9	63
Ma 63/1889	+	67	9.6	30.4	27	7.8	11.2	2.8	62
	−	93	13.2	28.9	27	7.8	8.4	6.3	88
P_{st}		0.00	0.03	0.35					
P_{cl}		0.17	0.10	0.09					
P_{stcl}		0.07	0.93	0.65					
LSD		16.8	6.6	18.6					

st = Difference between presence and absence of the shoot tip, averaged over clones.
cl = Difference between clones, averaged over presence and absence of the shoot tip.
stcl = Differences between clones of the difference between presence and absence of the shoot tip.
LSD for difference between presence and absence of the shoot tip per clone.

after shoot tip removal. The number of excised terminal shoots was just compensated or overcompensated by the number of developing axillary shoots.

4.4.2 Nutritional/hormonal factors

4.4.2.1 Substrate

In the first experiment, with 50 shoot cuttings of Clone 1087 per treatment, the influence of various substrates on rooting was analysed. In addition to the substrates sand, leaf mould and leaf mould/sand (Section 4.2), vermiculite, two grades of perlite, three grades of 'Trio' potsoil, 'Asef' potsoil, peat litter and peat mould were used as rooting substrates.

Table 33 shows that vermiculite and sand are unsuitable rooting substrates. The data of both media were illustrated separately, because a statistical analysis was carried out using exclusively the data of the other 10 rooting substrates. There are significant differences between substrates with respect to percentage of main root formation and elongation of main roots. As illustrated by the LSD values, the substrates leaf mould, peat litter and peat mould, have to be regarded as unsuitable for rooting. Good rooting responses were obtained in the other substrates, especially with perlite (fine and coarse grained).

In the second experiment, with 50 shoot cuttings per treatment, the influence of four substrates on root formation of shoot cuttings of Clones 1087, 4331 and Ma 63/1889 was investigated.

Table 34 illustrates that for the variables, which refer to main root formation, there are significant differences between substrates and between clones. These variables

Table 33. The influence of various substrates on root formation of shoot cuttings of Clone 1087.

Substrate	Main root formation			Lateral root formation			Dry root weight
	%	number	length	%	number	length	
vermiculite	2	2.0	12.0	2	6.0	4.0	0.4
sand	36	12.5	28.5	10	7.0	8.5	4.3
leaf mould	74	12.7	47.3	34	15.9	16.5	9.1
leaf mould/sand	84	18.4	47.4	46	28.5	13.0	16.3
perlite (fine grained)	94	17.7	45.1	50	24.3	13.5	16.6
perlite (coarse grained)	96	16.9	41.8	62	18.6	13.5	15.0
'Trio' potsoil 17	84	17.5	47.4	44	22.5	16.5	14.7
'Trio' potsoil 26	84	15.3	45.0	48	22.6	15.1	13.3
'Trio' potsoil 35	82	18.0	47.9	40	15.8	14.6	16.7
'Asef' potsoil	86	18.6	46.7	36	24.3	15.2	14.4
peat litter	72	17.2	39.7	58	34.0	14.1	13.6
peat mould	82	17.8	29.9	64	30.3	12.5	13.1
P_{su}	0.03	0.73	0.00				0.54
LSD	13.7	6.2	6.8				13.7

su = Difference between substrates.
LSD for difference between two substrates.

Table 34. The influence of various substrates on root formation of shoot cuttings of Clones 1087, 4331 and Ma 63/1889.

Clone	Substrate	Main root formation			Lateral root formation			Dry root weight
		%	number	length	%	number	length	
1087	sand	26	8.2	20.4	4	1.2	17.0	1.9
	leaf mould	52	6.4	38.2	28	7.2	8.0	2.2
	leaf mould/sand	80	10.6	35.8	46	11.7	9.3	5.1
	perlite (fine grained)	74	8.8	33.3	20	14.6	8.5	3.0
4331	sand	12	4.6	16.4	4	2.2	4.0	0.7
	leaf mould	40	7.7	35.1	18	2.9	10.0	2.3
	leaf mould/sand	56	12.1	33.8	18	7.0	10.0	4.2
	perlite (fine grained)	46	5.1	26.2	10	6.9	8.5	1.7
Ma 63/1889	sand	30	13.0	26.0	16	6.3	7.9	2.7
	leaf mould	82	11.0	35.6	50	13.4	9.6	5.7
	leaf mould/sand	84	15.0	30.7	44	12.5	9.6	6.5
	perlite (fine grained)	92	9.5	31.1	62	8.9	9.9	4.8
P_{su}		0.00	0.02	0.00				
P_{cl}		0.00	0.00	0.29				
P_{sucl}		0.08	0.58	0.66				
LSD[1]		9.2	3.2	6.1				
LSD[2]		7.9	2.8	5.3				

su = Difference between substrates, averaged over clones.
cl = Difference between clones, averaged over substrates.
sucl = Differences between substrates of the differences between clones.
1. LSD for difference between two substrates, averaged over clones.
2. LSD for difference between two clones, averaged over substrates.

did not show significant interactions between substrates and clones, so that the effect of the substrate was almost independent of the clone. The data confirmed the results of the first experiment, where sand and leaf mould were found to be unsuitable and perlite and a mixture of leaf mould and sand to be suitable rooting substrates. Leaf mould/sand yielded the best rooting response and this substrate was also used in standard experiments.

4.4.2.2 Auxins

In an experiment with Clone Ma 63/1889 various concentrations of IAA, IBA and NAA (% on talc basis) were tested. Per treatment 50 shoot cuttings were used, whereas the control group consisted of 150 cuttings.

Significant differences between the various auxin treatments were found for the average number of main roots and the dry root weight (Table 35). These variables and the percentage of main root formation were increased considerably by a treatment with IAA at 1% and, to a lesser extent, by IBA at 1%. The concentrations of NAA tested were much less effective than those of IAA and IBA. Main root elongation and variables referring to lateral root formation were slightly enhanced by the application of auxin. The optimum IAA or IBA concentration for main root elongation (0.5%) was lower than for an optimum initiation of main roots at high percentages (1%).

Table 35. The influence of various concentrations of IAA, IBA and NAA on root formation of shoot cuttings of Clone Ma 63/1889.

Auxin %	Main root formation			Lateral root formation			Dry root weight
	%	number	length	%	number	length	
Control	61	4.2	53.8	47	33.7	11.8	8.2[3]
IAA 0.25	64	4.2	49.4	56	35.6	13.8	9.0
0.5	78	6.0	59.1	70	47.6	12.6	16.0
1.0	86	14.4	56.7	82	47.6	11.4	25.0
IBA 0.25	72	5.9	55.3	62	47.9	13.2	15.0
0.5	80	5.3	59.0	70	44.5	13.8	16.0
1.0	86	7.6	58.0	82	47.6	12.4	21.1
NAA 0.05	62	4.3	55.6	60	34.0	13.2	10.2
0.1	78	3.9	57.7	64	38.6	13.9	12.8
0.2	70	4.8	54.5	66	37.2	12.2	11.7
P_a	0.07	0.00	0.58				0.00
LSD[1]	22.3	2.7	9.5				11.7
LSD[2]	18.3	2.2	7.8				9.6

a = Difference between various concentrations of IAA, IBA and NAA. (Control is designated as auxin concentration 0.)
1. LSD for difference between two auxin concentrations. (Control excluded.)
2. LSD for difference between control and auxin treatment.
3. Dry root weight of 50 shoot cuttings.

Main root initiation may further be improved at higher auxin concentrations. This was examined in three experiments with 50 shoot cuttings per treatment of Clone Ma 63/1889. Various concentrations of IAA, IBA and NAA were applied as powders (basal stem portions dipped in auxin powders on talc basis) as well as liquid solutions (basal stem portions immersed in auxin solutions for 24 h).

The variables referring to main root formation were statistically analysed and almost always auxin application had a significant positive effect (Table 36). Because the auxin NAA had a less favourable effect on main root initiation than IAA and IBA, the influence of NAA is not further discussed.

The optimum concentration of IAA and IBA varied with the variable involved. In general high percentages of shoot cuttings initiated high numbers of main roots at high auxin concentrations, whereas the length of the initiated main roots and lateral root formation were enhanced by low auxin concentrations. The dry root weights increased substantially after the application of both IAA and IBA, but tended to decrease again at the highest concentrations used. In comparison with the control treatment the shoot number was higher at low concentrations of IAA, applied in powder or solution, and slightly higher at low concentrations of IBA, applied in powder. The shoot number progressively decreased at increasing concentrations of IAA and IBA. It is impossible to indicate an auxin concentration that is optimum for all the variables. On the whole a good rooting response and shoot development can be observed after application of powders of IAA or IBA at a concentration of 1%.

To determine whether these findings are also valid for Clones 1087 and 4331, the effect of IAA and IBA at various concentrations (% on talc basis) on root formation and

Table 36. The influence of various concentrations of powders (%) and solutions (mg/l) of IAA, IBA and NAA on root formation and shoot development of shoot cuttings of Clone Ma 63/1889.

Auxin conc.		Main root formation			Lateral root formation			Dry root weight	Shoot number
		%	number	length	%	number	length		
Control		80	9.3	33.7	40	11.8	9.8	6.4	113
IAA	0.1%	86	11.0	28.5	30	18.6	7.5	5.4	156
	0.25	96	10.9	33.3	64	13.9	8.0	8.0	121
	0.5	100	12.5	33.4	60	13.9	8.0	10.3	133
	1.0	100	17.4	37.7	54	27.4	7.8	15.0	136
	2.5	98	27.7	33.5	28	13.1	7.8	16.2	126
	5.0	94	38.7	25.9	4	6.0	2.0	16.1	62
	10 mg/l	88	11.4	37.2	60	22.2	9.5	10.8	154
	25	96	17.2	43.6	56	30.0	8.4	19.9	155
	50	100	26.0	35.9	12	9.5	4.9	17.1	99
	100	100	48.3	27.7	4	3.0	3.0	23.7	50
	250	96	56.4	22.3	2	5.0	1.0	23.0	25
P_a[1]		0.00	0.00	0.00					
LSD[1]		9.5	7.1	5.6					
Control		80	8.6	29.7	28	15.7	8.0	3.9	102
IBA	0.1%	82	9.3	34.4	42	11.3	13.5	5.9	112
	0.25	88	12.9	41.7	56	33.6	12.7	10.8	117
	0.5	96	12.7	37.3	46	20.5	10.6	9.9	113
	1.0	98	18.9	39.3	20	18.6	8.4	16.9	118
	2.5	98	25.0	35.1	14	20.3	10.0	14.7	60
	5.0	90	23.7	30.7	6	13.0	4.0	12.0	50
	10 mg/l	88	14.2	39.6	34	25.5	11.3	11.4	100
	25	90	18.5	36.0	16	8.5	6.5	10.2	66
	50	94	24.1	34.5	26	37.0	14.2	14.9	56
	100	90	24.9	35.9	4	7.0	4.0	14.0	30
	250	84	23.3	28.9	4	4.0	4.0	7.5	16
P_a[2]		0.07	0.00	0.03					
LSD[2]		13.1	5.0	7.7					
Control		56	6.2	27.0	14	11.6	14.3	1.8	104
NAA	0.1%	64	5.2	34.5	32	11.3	16.9	2.5	101
	0.25	76	5.7	29.4	26	10.5	10.5	2.4	82
	0.5	70	5.7	26.7	22	7.8	10.6	2.1	80
	1.0	84	5.3	23.7	22	8.5	15.4	3.1	57
	2.5	78	5.0	15.4	14	4.6	7.8	2.7	45
	5.0	62	5.3	6.9	0	0	0	0.7	39
	10 mg/l	46	5.3	23.0	10	3.3	5.1	1.0	77
	25	40	5.1	31.0	12	4.3	7.0	1.2	63
	50	48	4.2	21.0	8	6.7	4.3	1.2	54
	100	56	5.5	25.7	14	5.1	10.3	2.0	46
	250	58	3.7	15.7	10	6.2	7.0	0.8	30
P_a[3]		0.00	0.80	0.00					
LSD[3]		22.1	2.4	7.3					

a = Difference between various concentrations of powders and solutions of IAA (1), IBA (2) and NAA (3). (Control is designated as auxin concentration 0.)
LSD for difference between two concentrations of IAA (1), IBA (2) and NAA (3).

shoot development of shoot cuttings of both clones was investigated. Per hormone treatment 30 shoot cuttings were used, whereas the control treatments consisted of 60 shoot cuttings.

For an unknown reason low rooting responses were obtained in this experiment (Table 37). The auxin concentration (averaged over auxins and clones) had a significant positive effect on percentage of main roots formed and the shoot number. In addition this last variable was influenced by clones (averaged over auxins and concentrations). Of all the calculated interactions between auxins, concentrations and clones, the interaction between concentrations and clones is presented, because only this interaction was found to influence significantly the number of shoots. In comparison with the control treatment the application of IAA or IBA at 1 and 2.5% resulted in higher rooting percentages and numbers of main roots. Main root elongation of Clone 1087 seemed to be enhanced by IAA and IBA at 0.5%, whereas main root elongation of Clone 4331 was

Table 37. The influence of various concentrations of IAA and IBA on root formation and shoot development of shoot cuttings of Clones 1087 and 4331.

Clone	Auxin	Main root formation			Lateral root formation			Dry root weight	Shoot number
	%	%	number	length	%	number	length		
1087	Control	18	3.1	26.3	7	3.3	5.6	0.3[2]	42[2]
	IAA 0.5	40	7.6	35.6	17	2.8	10.5	0.7	37
	1.0	53	12.6	23.7	10	2.2	2.5	1.3	31
	2.5	53	9.9	32.8	17	8.4	10.6	1.5	34
	5.0	37	9.2	24.8	10	6.0	8.0	0.4	34
	IBA 0.5	50	4.8	32.7	27	4.3	14.2	1.5	38
	1.0	37	10.9	31.0	7	1.7	5.0	0.9	33
	2.5	33	8.8	27.4	13	6.2	11.7	0.5	36
	5.0	10	5.0	8.9	0	0	0	0.3	30
4331	Control	10	4.9	29.0	10	7.8	11.7	0.4[2]	31[2]
	IAA 0.5	10	7.0	20.0	7	10.0	8.3	0.5	30
	1.0	30	6.5	24.4	10	6.0	6.7	1.1	30
	2.5	27	6.3	21.0	7	2.5	3.3	0.6	31
	5.0	27	13.6	21.0	3	3.3	3.3	0.9	32
	IBA 0.5	23	6.3	25.6	10	6.7	6.7	0.4	30
	1.0	23	13.4	21.5	7	5.0	3.3	2.0	30
	2.5	13	11.5	21.7	0	0	0	0.9	31
	5.0	13	6.8	26.2	0	0	0	0.4	30
P_a		0.24	0.86	0.92					0.27
P_{co}		0.04	0.17	0.54					0.02
P_{cl}		0.19	0.76	0.58					0.02
P_{cocl}		0.44	0.93	0.37					0.01
LSD[1]		15.1	5.8	10.4					1.1

a = Difference between IAA and IBA, averaged over concentrations and clones.
co = Difference between various auxin concentrations, averaged over auxins and clones. (Control is designated as auxin concentration 0.)
cl = Difference between clones, averaged over auxins and concentrations.
cocl = Differences between concentrations of the difference between clones.
1. LSD for difference between two concentrations, averaged over auxins and clones.
2. Dry root weights and shoot numbers of 30 shoot cuttings.

inhibited at all auxin concentrations. The influence of auxins on lateral root formation was slight and not clear, with the exception of IBA at 2.5 and 5%, at which concentrations lateral root formation was almost completely suppressed. After the application of auxins higher dry root weights were measured than in the control treatments, with optima at about 1% of IAA and IBA. The shoot number of Clone 1087 decreased with an increasing auxin concentration, while in Clone 4331 about the same number of shoots developed in all treatments.

Hence both auxins yielded about the same rooting response. As was found in preceding experiments, optimum concentrations are approximately 1% of IAA or IBA (on talc basis); the former was chosen as standard treatment.

4.4.3 Climatic factors

4.4.3.1 Constant temperature

In the phytotron in two experiments the influence of constant air temperatures of 9, 13, 17, 21 or 25°C on root formation of shoot cuttings of Clones 1087 and Ma 63/1889 was examined. Per treatment 60 shoot cuttings were exposed to a daylength of 16 h.

Because there were no replicates, the data of Table 38 could not be analysed statistically. However, shoot cuttings of both clones responded similarly to temperature. Shoot cuttings of Clone 1087 yielded high rooting percentages at 13 and 17°C, while shoot cuttings of Clone Ma 63/1889 regenerated roots at high percentages at all temperatures. On shoot cuttings of both clones the highest number of main roots was initiated at 13°C; an optimum elongation of the initiated main roots, however, was observed at 21 and 25°C. In view of the poor elongation of main roots initiated at 9°C on shoot cuttings of Clone 1087, probably a part of the initiated roots did not become macroscopically visible, as occurred in vitro (Section 3.4.3.1). Hence, a higher percentage of shoot cuttings of Clone 1087 may have initiated a higher number of main roots than is expressed in Table 38. Because of the poor main root elongation, lateral roots did not form at 9 (and 13°C); an optimum lateral root formation was realized at 21

Table 38. The influence of constant temperatures (9-25°C) on root formation of shoot cuttings of Clones 1087 and Ma 63/1889.

Clone	Temperature (°C)	Main root formation			Lateral root formation			Dry root weight
		%	number	length	%	number	length	
1087	9	50	14.4	1.9	0	0	0	1.6
	13	78	21.6	12.2	0	0	0	7.8
	17	82	17.3	20.0	27	8.8	5.4	17.6
	21	62	10.7	26.4	50	10.8	8.9	7.9
	25	43	9.7	26.2	38	11.4	9.6	4.7
Ma 63/1889	9	85	14.5	8.8	0	0	0	1.8
	13	91	20.2	18.4	0	0	0	5.8
	17	90	14.5	19.2	23	9.6	11.5	4.5
	21	83	17.3	24.3	35	10.3	13.1	4.6
	25	80	7.1	22.7	55	18.4	12.2	2.9

Table 39. The influence of constant soil temperatures (17-30°C) on root formation of shoot cuttings of Clone 1087.

Soil temperature (°C)	Main root formation			Lateral root formation			Dry root weight
	%	number	length	%	number	length	
17	81	25.0	39.0	32	22.2	12.4	28.0
22	96	15.6	49.4	69	34.8	13.5	38.8
26	82	15.8	44.3	67	43.6	15.9	40.6
30	43	4.3	17.8	10	6.6	9.2	1.7
P_t	0.00	0.02	0.00				0.02
LSD	18.3	10.7	11.0				5.7

t = Difference between soil temperatures.
LSD for difference between two soil temperatures.

and 25°C. The highest dry root weights were obtained at 13, 17 and 21°C.

Subsequently, in a growth chamber at a constant air temperature of 15°C, the influence of constant soil temperatures of 17, 22, 26 or 30°C on root formation of shoot cuttings of Clone 1087 was examined. The effect of the temperature on root formation was investigated in four replicates, to make sure that a statistical analysis could be carried out. Per temperature 100 shoot cuttings were inserted into the substrate.

The variables referring to main root formation and dry root weight were significantly influenced by temperature. A soil temperature of 30°C always yielded the worst results (Table 39). At 17, 22 and 26°C rooting percentages were high. The number of main roots increased with decreasing temperature to an optimum at 17°C, and the elongation of the initiated main roots was improved at higher temperatures of 22 and 26°C. An optimum lateral root formation and the highest dry root weights could also be observed at soil temperatures of 22 and 26°C.

From both experiments it can be concluded that the initiation of main roots is favoured by a low temperature and that a high temperature is suitable for main root elongation and lateral root formation. Anatomical observations (Section 4.3) have shown that main roots are usually initiated during the first 2 weeks after insertion and that the root initials elongate and lateral roots are formed during the subsequent 2 weeks. Therefore, the question arises whether the process of root formation can be stimulated by a fluctuating soil temperature.

4.4.3.2 Fluctuating temperature

The effect of a temperature combination was examined in an experiment with Clones 1087, 4331 and Ma 63/1889. During the first 2 weeks a low soil temperature of 17°C was maintained and during the subsequent period a high soil temperature of 25°C. This temperature combination (17/25) was compared with the combination 25/17 and constant temperatures of 17 and 25°C. Per clone and at each temperature combination 60 shoot cuttings were inserted. The numbers of Table 40 are the average of data scored 3, 3½ and 4 weeks after insertion. A statistical analysis was not carried out, because there

Table 40. The influence of various soil temperature combinations on root formation of shoot cuttings of Clones 1087, 4331 and Ma 63/1889.

Clone	Soil temperature (°C)	Main root formation			Lateral root formation			Dry root weight
		%	number	length	%	number	length	
1087	17	86.7	19.1	20.9	1.7	5.0	5.0	8.0
	17/25	78.3	15.2	28.3	30.0	13.7	8.0	7.0
	25/17	60.0	14.9	30.8	21.7	19.3	14.5	7.1
	25	65.0	10.5	38.2	48.3	20.2	13.8	9.8
4331	17	35.0	16.0	14.8	1.7	3.3	1.7	2.5
	17/25	63.3	12.0	29.0	21.7	7.2	4.6	4.4
	25/17	20.0	7.3	19.1	3.3	2.5	2.8	0.6
	25	16.7	3.4	30.7	5.0	5.3	6.7	0.9
Ma 63/1889	17	98.3	29.3	21.3	15.0	9.7	6.9	22.2
	17/25	98.3	21.8	24.3	20.0	13.8	7.0	13.5
	25/17	95.0	14.1	22.7	58.3	12.5	8.8	10.7
	25	85.0	17.9	35.2	43.3	12.9	12.1	13.5

were no replicates.

High rooting percentages could be observed at all temperature combinations, with the exception of Clone 4331, especially at 25/17 and 25°C (Table 40). Rooting percentages and numbers of main roots were highest at 17 and 17/25 and in general main root elongation and subsequent lateral root formation were favoured at 25 and 17/25°C. The unsuitability of the temperature combination 25/17 can be seen from the dry root weights. The highest dry root weights were measured at 17 and 17/25°C.

It can be concluded that the initiation of main roots and the elongation of root initials (and lateral root formation) are stimulated by low and high temperatures, respectively. A good rooting response is obtained at a fluctuating temperature: a low temperature for the first 2 weeks and a high temperature for the subsequent 2 weeks.

4.5 DISCUSSION AND CONCLUSIONS

The experiments in Section 4.4 showed that adventitious root formation on basal stem portions of shoot cuttings as well as the development of the upper part of shoot cuttings can be affected in various ways.

Anatomical observations (Section 4.3) have revealed that the process of adventitious root formation progresses similarly as has been reported for peduncle explants (Section 3.3). Main roots are usually initiated during the first 2 weeks after insertion of the shoot cuttings into the rooting substrate and main root elongation (and lateral root formation) occur during the subsequent 2 weeks.

Consequently, a variation of a factor just before insertion or during the first 2 weeks after insertion directly affects main root initiation. Such a variation has a delayed effect on subsequent main root elongation and lateral root formation, which are directly regulated by factors varied during the next 2 weeks.

The influence of auxin and temperature on adventitious root formation of shoot cuttings was identical to the effect of these factors on root formation of peduncle explants.

In conformity with investigations in vitro (Section 3.4.2.4) the application of exogenous auxins was favourable for adventitious root formation of shoot cuttings. The initiation of main roots was enhanced by high auxin concentrations and subsequent main root elongation (and lateral root formation) by lower auxin concentrations. The auxins IAA and IBA were more suitable for root formation than NAA; optimum concentration of IAA or IBA was approximately 1% (on talc basis).

Furthermore, in agreement with experiments in vitro (Section 3.4.3.1), it has been shown that main root initiation is promoted by low temperatures and root elongation (and lateral root formation) by higher temperatures. Of the temperature combinations tested, the best was 17° for the first 2 weeks after insertion followed by 25°C for the next 2 weeks; at 17° shoot cuttings at high percentages initiated high numbers of main roots, which elongated and branched rapidly at 25°C.

In contrast with experiments in vitro (Section 3.4.1.5), however, excision of a strip of the cortex hardly influenced root formation of shoot cuttings, probably because this is an additional wound cut.

The shoot tip turned out to be of great importance and adventitious root formation was substantially promoted by its removal. This may be due to endogenous substances, like auxin, which are synthesized by shoots developing from axillary buds (Meyer & Anderson, 1952).

Good rooting responses were achieved in the substrates leaf mould/sand and perlite. Since a substrate of leaf mould/sand is prepared according to a special procedure (Section 4.2) and is not widely available, the manufactured product perlite, which is used all over the world, has to be preferred as rooting substrate.

Some of the factors affecting root formation also demonstrated an effect on the development of shoots on the upper part of shoot cuttings.

In the presence of the shoot tip axillary shoots rarely developed, a phenomenon called 'apical dominance'. Breaking of apical dominance and the development of axillary buds was considerably stimulated by shoot tip removal.

Axillary shoot development was also promoted by an application with exogenous auxins at low concentrations, whereas it was inhibited by higher auxin concentrations, more so by the application of auxins in solutions than in powders. Axillary shoot development is probably inhibited by auxin solutions (applied for 24 h) because auxins are directly accessible to the shoot cutting and then a higher auxin concentration may build up within the shoot cutting. When cuttings are dipped in auxin powders, much auxin may get lost as a result of insertion of the shoot cuttings into the rooting substrate and rinsing off after watering and, moreover, auxins have to be dissolved before they are accessible to the shoot cutting.

Both root formation and shoot development, processes which are essential for vegetative development of shoot cuttings, are influenced by an application with exogenous auxins. In general root formation is stimulated by higher auxin concentrations than shoot development. A compromise can be reached at 1% of IAA or IBA (on talc basis), at which concentration rooting response and shoot development are good.

When shoot cuttings are struck under the standard conditions (Section 4.2) and, in addition, the terminal shoot tip is removed and the basal stem portions of the cuttings

are exposed to a fluctuating soil temperature (initially a low temperature, which is gradually raised), it is expected that shoot cuttings of all clones produce high numbers of roots quite rapidly and at high percentages. On an average 25-100 shoot cuttings can be obtained from one stock-plant of 1-2 years old, instead of 5-10 splits, which means that the multiplication rate can be increased considerably.

To be assured of the availability of vegetative shoots throughout the season, the plants should be kept in a vegetative state, which can be achieved at high temperatures in the lowlands of tropical countries (Chapter 2).

5 Shoot formation of capitulum explants in vitro and plantlet production by root formation of detached shoots

5.1 INTRODUCTION

Vegetative propagation can be achieved in several ways, for instance through the formation of adventitious shoots. By definition such shoots do not arise from pre-existing (terminal or axillary) meristems, but originate, like adventitious roots (Section 3.3), from a more or less differentiated tissue, which after dedifferentiation, gives rise to the formation of a shoot meristem and a shoot primordium (Venverloo, 1973). The development of a shoot primordium leads to the formation of an adventitious shoot and when, ultimately, adventitious roots are initiated on the basal end of a detached shoot, a complete plantlet is obtained.

Adventitious shoots can emerge in vivo from different plant parts, like roots, stems and leaves. Broertjes et al. (1968) listed over 350 species, reported in the literature, which can be propagated through adventitious shoot formation on detached leaves. However, attempts to produce adventitious shoots on detached leaves of pyrethrum in vivo failed.

Although shoots do not regenerate in vitro so commonly as adventitious roots, this phenomenon has been reported in literature with various plant parts cultivated in vitro. Since cultures in vitro are increasingly used for the rapid vegetative propagation of many plant species, it seemed worthwhile to examine the regeneration ability of pyrethrum explants excised from various plant parts.

It has been shown (Chapter 3) that peduncle explants cultivated in vitro rapidly produced high numbers of adventitious roots at high percentages. Therefore the ability of this plant material to regenerate shoots was first examined. Even though shoots were initiated on peduncle explants of Clone 4331, a satisfactory development of the shoot initials was not realized.

Thereafter attempts were made to propagate pyrethrum vegetatively through flower (head) explants cultivated in vitro, as has been described for several plant species e.g. *Nemesia strumosa* and *Kalanchoë globulifera* (La Rue, 1942), *Phlox drummondii* (Konar & Konar, 1966), *Kalanchoë pinnata* (Mohan Ram & Wadhi, 1966, 1968), *Browallia demissa* (Johri & Ganapathy, 1967), *Chrysanthemum morifolium* (Hill, 1968; Matsubara et al., 1971; Roest & Bokelmann, 1975), *Ranunculus sceleratus* (Konar & Nataraja, 1969), *Brassica oleraceae* (Pow, 1969), *Beta vulgaris* (Margara, 1970), *Lilium* (Bigot, 1970), *Orchidaceae* (Intuwong & Sagawa, 1973), *Gerbera jamesonii* (Pierik et al., 1973, 1975), *Freesia* (Pierik & Steegmans, 1975a), *Hyacinthus orientalis* (Saniewski, 1975) and *Dahlia* (Asahira et al., 1975). In preliminary experiments it was found that shoot formation could be obtained through the culture in vitro of sections of the capitulum (flower head) of pyrethrum (Roest & Bokelmann, 1973).

Section 5.3 deals with anatomical observations on the origin, initiation and development of the shoots. In Section 5.4 the influence of various factors in this process of shoot formation is examined. The production of plantlets by root formation of detached shoots is described in Section 5.5 and finally in Section 5.6 is investigated whether other plant species can be propagated vegetatively in vitro.

5.2 MATERIAL AND METHODS

5.2.1 Material

Clone 4331 is one of the most important clones in Kenya, because of its very high flower yields. Therefore this clone was chosen and stock plants were grown in the greenhouse. In winter at low temperatures flower heads were initiated and in spring and summer, with a rising temperature, the initiated flower heads developed rapidly and plants started flowering (Section 2.2). Capitula in a young flowering stage (stages 10, 11 and 12, Fig. 40) were detached, each with the top portion of the peduncle (Fig. 37a). Flower heads were sterilized in a 5% calcium hypochlorite filtered solution for 30 min

Figs 37a-d. Procedure of plantlet production from a capitulum explant of Clone 4331 cultivated in vitro:

a. Capitulum in a young flowering stage.
b. Prepared capitulum explant, just before incubation.
c. Some small shoots and one tall shoot initiated and developed on a capitulum explant, 5 weeks after incubation.
d. Plantlet production by root formation of a detached shoot, 3 weeks after transfer to soil.

and subsequently rinsed several times with sterilized tap water for 30 min. In an inoculation room under a stereomicroscope, the capitulum was divided aseptically and as accurately as possible into 2 equal sections. In each section the upper bracts of the involucrum were almost completely cut off and the florets were excised above the bottom half of the ovary (Fig. 37b). Finally, the explants were placed in a vertical position, with the portion of the peduncle in the medium, and distributed systematically and in a balanced way over the various treatments.

5.2.2 Methods

The basic culture medium was composed of pyrex-distilled water, 'Difco' Bacto-agar 0.6%, Knop's major and Heller's minor salts (both at half strength), sucrose 0.5% and 6-benzylamino purine (BA) at 10^{-6} g/ml. The pH of the medium was adjusted to 5.8 before autoclaving. Per treatment 20 explants were exposed in a growth chamber to a photoperiod of 14 h (fluorescent tubes (Philips TL MF 140 W/33RS) supplemented with incandescent light (Philinea)) and a temperature of 18° during the day and 14°C during the night.

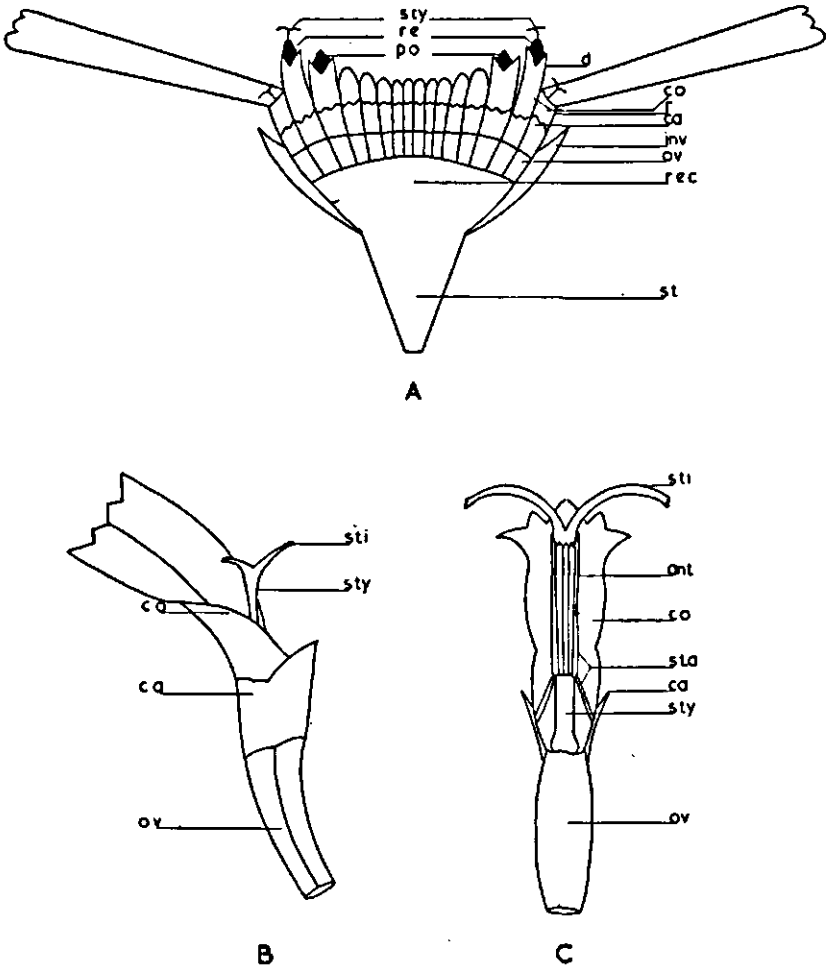
The formation of the first shoots was observed about 3 weeks after incubation. The development of the shoots varied with the treatment and was almost complete 1½-3 months after incubation (Section 5.3). In this final stage the experiments were terminated and the percentage of shoots formed was measured over 20 explants per treatment. Mostly the mean number of shoots per shoot-forming explant was also calculated and the length of these shoots was measured. According to length, shoots were divided into small shoots (< 0.2 cm), the number if which was estimated, intermediate shoots (0.2-0.6 cm), which were counted rather precisely, and tall shoots (≥ 0.6 cm), which number could be counted very exactly.

For anatomical observations (Section 5.3) capitulum explants of Clone 4331 were incubated in vitro under the standard conditions described in this section, which are suitable for shoot development. The plant material was fixated 0, 3, 6, 9, 12, 14, 16, 19 or 23 d after incubation. For a detailed description how the plant material was prepared further, see Section 3.2, where more information is also presented about the sterile-culture techniques.

5.3 ANATOMICAL OBSERVATIONS

Figs 38a-c show the morphology of an inflorescence, a ray and a disc floret. The capitulum is borne on the peduncle and built up as follows: on top of a slightly convex receptacle, sheathed by green involucral bracts, white petalled ray florets are situated on the margin and yellow disc florets occupy the centre. The monosexual ray florets have a strap-shaped corolla, the base of the corolla is tubular and envelopes a bi-lobed cylindrical style. The style is emplanted in the centre of the floret on top of the inferior ovary. A small green, irregularly shaped calyx is attached to a pentagonal ovary. The bisexual disc florets have a tubular yellow corolla and five stamens in addition to the style.

The capitulum explants were prepared by cutting off the florets above the bottom

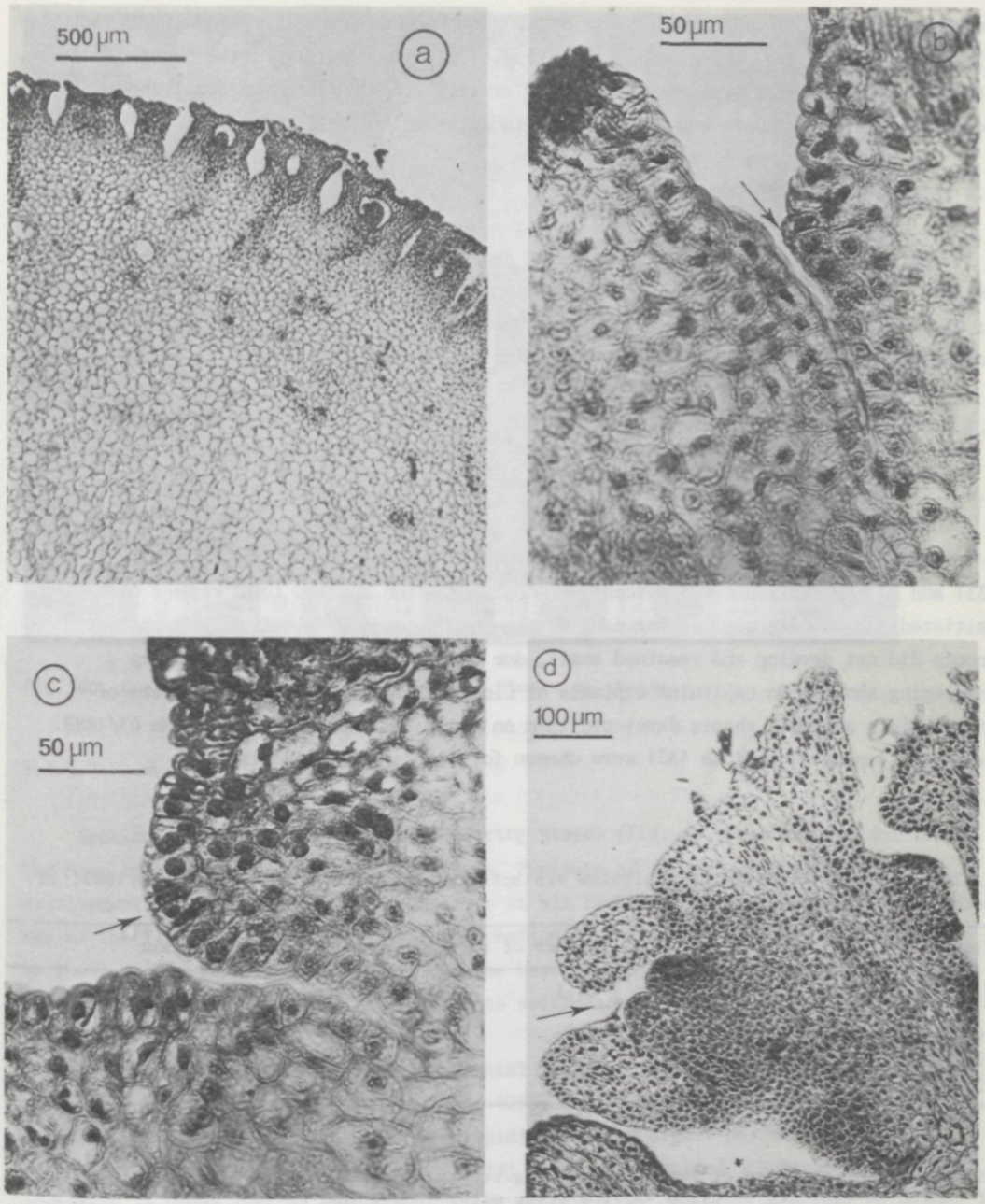


Figs 38a-c. The morphology of an inflorescence (a), a ray floret (b) and a disc floret (c) (Brewer, 1968):

ca: calyx	po: pollen	st: stalk
co: corolla	r: ray floret	sta: stamens
d: disc floret	re: rest anther	sti: stigma
inv: involucre	rec: receptacle	sty: style
ov: ovary		

half of the ovary (Fig. 39a). Microscopical observations showed the first cell divisions in the epidermal cell layer of the bottom halves of the ovaries of disc florets, 6 d after incubation (Fig. 39b). An initiated shoot meristem was observed after an incubation for 9 d (Fig. 39c) and a shoot primordium was initiated 15 d after incubation (Fig. 39d). Most shoots originated adventitiously from the epidermal cell wall of the ovaries, as was described for *Dahlia* (Asahira et al., 1975). In pyrethrum sometimes shoots were noticed emerging directly from the receptacle and occasionally meristematic activity was observed in the axil of an involucral bract. Therefore some of the shoots may be of an axillary origin.

A variable number of shoots (1-50, with an average of about 25 per section)



Figs 39a-d.

a. Receptacle bearing the bottom halves of the ovaries of disc florets, just before incubation.
b. Cell division in the epidermal cell layer of the ovary of a disc floret (arrow), 6 d after incubation.
c. Initiated shoot meristem (arrow), 9 d after incubation.
d. Initiated shoot primordium (arrow), 15 d after incubation.

developed after an incubation for 3 weeks. During further incubation a few (tall) shoots elongated to a length of at least 0.6 cm (Fig. 37c); the majority of the shoots, however, attained a length of only a few millimeters or less (intermediate and small shoots). The development of the shoots was almost complete after an incubation period for 1½-3 months.

5.4 FACTORS INFLUENCING SHOOT FORMATION

The factors involved in the process of shoot formation can be distinguished into factors which are associated with the (ex)plant, the nutritional/hormonal composition of the medium and the climatic conditions. Unless otherwise stated, the standard growth conditions (Section 5.2) have been maintained in all experiments.

5.4.1 Plant factors

5.4.1.1 Genotype

The formation of shoots was investigated with capitulum explants of Clones 1087, 4331 and Ma 63/1889. Both the percentage shoot formation and the total number of initiated shoots were similar for each of the three clones (Table 41). Most initiated shoots did not develop and remained small, due undoubtedly to competition among developing shoots. On capitulum explants of Clones 1087 and 4331 higher numbers of intermediate and tall shoots developed than on capitulum explants of Clone Ma 63/1889. Capitulum explants of Clone 4331 were chosen for standard experiments.

Table 41. Shoot formation of capitulum explants of Clones 1087, 4331 and Ma 63/1889, 12 weeks after incubation.

Clone	Percentage shoot formation	Number of shoots per shoot-forming explant			
		tall	intermediate	small	total
1087	95	1.3	3.3	15.6	20.2
4331	100	1.0	2.3	18.3	21.6
Ma 63/1889	100	0.0	0.2	18.3	18.5

Table 42. The influence of various flowering stages of the capitulum on shoot formation of capitulum explants of Clone 4331, 8 weeks after incubation.

Flowering stage	Percentage shoot formation	Number of shoots per shoot-forming explant			
		tall	intermediate	small	total
0	0	0	0	0	0
5	0	0	0	0	0
8	40	0.8	4.5	0.8	6.1
10	50	1.2	5.0	4.3	10.5
12	90	1.5	6.2	4.3	12.0
13	85	3.5	6.8	2.3	12.6

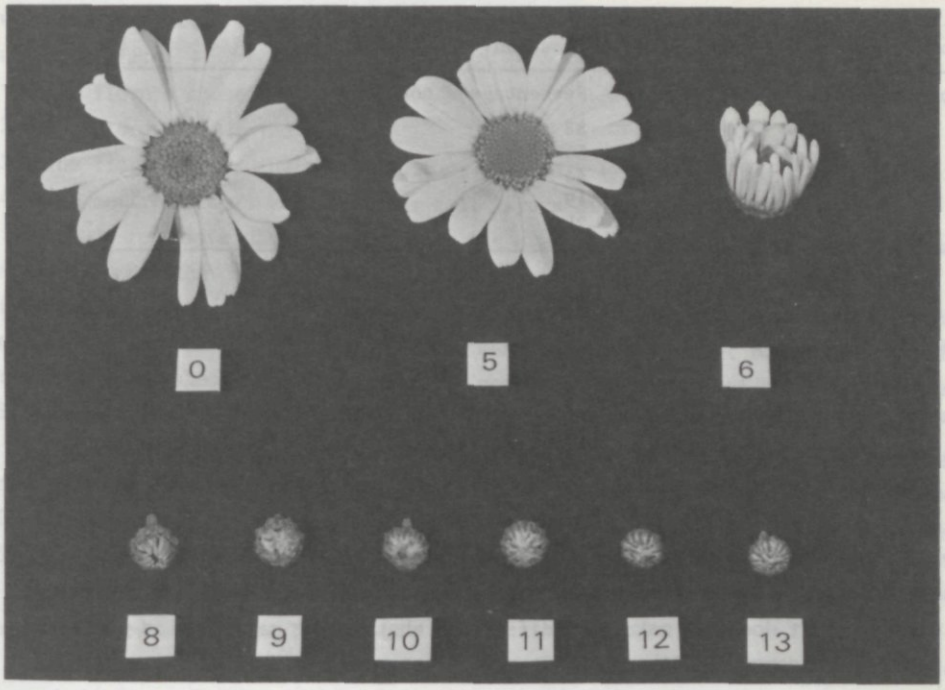


Fig. 40. Capitula of Clone 4331 in various flowering stages.

5.4.1.2 Flowering stage

Sections of capitula in various flowering stages (Fig. 40) were used to determine the most suitable stage for shoot formation. Explants of flower heads in the oldest developmental stages did not produce shoots at all (Table 42; stages 0 and 5). As the age of the flower head decreased, shoot formation increased and explants from capitula in the youngest flowering stages yielded the best initiation and development of adventitious shoots. In standard experiments sections were excised from flower heads in stages 10-12.

The significance of a young developmental stage of the explant on shoot formation was also found by Mohan Ram & Wadhi (1966, 1968) who reported that young flower buds of *Kalanchoë pinnata* developed shoots more rapidly and at a higher percentage than those of an older developmental stage, and by Bajaj (1972) who noticed a better shoot formation on young excised leaves of *Torenia fournieri* as compared with older leaves. In contrast Pierik et al. (1975) found no differences in shoot development between wide open and older flower heads of *Gerbera jamesonii*, while closed flower heads and very young flower heads were inferior. For a more detailed discussion about the influence of the developmental stage on organogenesis see Section 3.4.1.2.

Table 43. The influence of various explant sizes on shoot formation of capitulum explants of Clone 4331, 8 weeks after incubation.

Sections per capitulum	Percentage shoot formation
2	88
3	56
4	44
5	19
6	25

5.4.1.3 Explant size

As intact flower heads yielded only a very limited number of shoots, flower heads were sectioned into 2, 3, 4, 5 or 6 segments. Per treatment 16 explants were used to determine the explant size that would result in the best initiation and development of shoots.

Table 43 shows that the percentage shoot formation increased with an increasing explant size. However, when the percentage shoot formation was calculated per capitulum, it became clear that it does not make much difference whether the capitulum is sectioned into 2, 3, 4, 5 or 6 segments. Although it is not illustrated in Table 43, it was found that sectioning of the capitula into 2 segments resulted in the best development of the initiated shoots, and this preparation method was also chosen in standard experiments.

In conformity with pyrethrum it was found that the number of regenerated bulblets on bulb scale segments of *Hyacinthus orientalis* (Pierik & Woets, 1971; Pierik & Ruibing, 1973; Pierik & Post, 1975) increased almost linearly by increasing the length or width of the explants, while also the development of the bulblets was promoted with an increasing explant size. An optimum development of shoots on capitula of *Gerbera jamesonii* (Pierik et al., 1973, 1975) was achieved after division into 4 or 6 explants; shoot development decreased when the capitula were cut into 8, 10 or 12 explants. With pedicel explants of *Chrysanthemum morifolium* (Roest & Bokelmann, 1975) a favourable effect of an increasing explant length on shoot formation was observed.

Probably explants derived after sectioning the flower head into 2 segments yield a better shoot formation than smaller explants because the total amount of endogenous nutritional/hormonal substances is larger within the bigger explants. Flower heads that were not sectioned, however, regenerated a smaller number of shoots than explants that were obtained after sectioning the capitulum into 2 segments. This result may be attributed to the additional wound cut that is made by sectioning the capitulum. Such a wound may assure a better aeration or uptake of exogenous applied nutritional/hormonal substances from the medium (Section 3.4.1.5) and so stimulate shoot regeneration.

5.4.1.4 Wounding

In a preliminary experiment it was observed that shoot formation did not occur when the capitulum was just sectioned and the sections were incubated without further wounding. To determine the effect of additional wounding on shoot formation, various

wounding procedures were compared. The following parts of the flower head (Figs 38a-c) were cut off:

1 = The florets above the calyx.

2 = The florets above the ovary.

3 = The florets above the bottom half of the ovary (Fig. 39a).

4 = The entire florets and the upper cell layers of the receptacle.

Shoot formation did not occur when the ovaries were not wounded at all (Treatments 1 and 2) or when the ovaries were completely excised (Treatment 4), whereas 40% of the flower heads produced shoots when the ovaries of the florets were wounded according to the standard preparation procedure (Treatment 3). The important role of the ovaries in the process of shoot formation was fully confirmed by anatomical observations (Section 5.3).

A stimulating effect of wounding on shoot formation was also reported for leaf explants of *Dendrophthoe falcata* (Nag & Johri, 1970) and of *Torenia fournieri* (Bajaj, 1972). The effect of wounding on the regeneration of roots in vitro was already discussed in Section 3.4.1.5.

5.4.2 Nutritional/hormonal factors

5.4.2.1 Minerals

The influence of mineral nutrition was examined in an experiment with various combinations of the minor salts of Heller and the major salts of Knop (both at half strength) in the culture medium.

As can be observed in Table 44 addition of minerals to the culture medium is not necessary for shoot initiation. With the minor salts according to Heller the development of the shoot was not stimulated, but the addition of the major salts according to Knop seemed to be important for shoot development. In standard experiments the major salts of Knop and the minor salts of Heller (both at half strength) were added to the culture medium.

With the exception of callus tissue of *Anthurium andreanum* (Pierik, 1976) where NH_4NO_3 at high concentrations had found to repress and at low concentrations to be

Table 44. The influence of various combinations of Heller's minor salts and Knop's major salts on shoot formation of capitulum explants of Clone 4331, 6 weeks after incubation.

	Percentage shoot formation	Number of shoots per shoot-forming explant			
		tall	intermediate	small	total
devoid of minerals	95	0.0	0.6	16.4	17.0
Heller minor ½	85	0.0	0.2	16.2	16.4
Knop major ½	85	0.4	1.5	16.9	18.8
Heller minor ½ + Knop major ½	95	0.3	2.7	16.1	19.1

promotive for shoot formation, salts containing NO_3 or NH_4 ions have been reported to stimulate shoot formation, e.g. stem segments of tobacco (Miller & Skoog, 1953), leaf discs of *Cardamine pratensis* (Paulet & Nitsch, 1959), peduncle explants of *Brassica oleraceae* (Margara, 1969) and pedicel segments of *Chrysanthemum morifolium* (Roest & Bokelmann, 1975).

The important role of nitrogen in root regeneration was already pointed out in Section 3.4.2.2 and whether nitrogen is also involved in shoot formation of pyrethrum should be investigated.

5.4.2.2 Sugars

To examine the effect of sugar on shoot formation, sucrose was added at various concentrations to the culture medium. In the absence of sucrose shoots were not formed (Table 45) and explants became necrotic and died off. In the range of 0.5-2.5% sucrose all treatments yielded the maximum percentage shoot formation, but the total number and length of the shoots diminished as the sucrose concentration increased. In the concentration range tested optimum results were obtained after the addition of 0.5% sucrose to the medium (Fig. 41).

Although vegetative shoots were usually produced, the development of 'floral primordia', which look like 'disc florets', occurred 6-7 weeks after incubation. At the end of the experiment the number of these primordia was positively correlated with the sucrose concentration. These 'disc florets' with 5-10 stamens and an equal number of

Table 45. The influence of various concentrations (0-2.5%) of sucrose on shoot formation of capitulum explants of Clone 4331, 10 weeks after incubation.

Sucrose %	Percentage shoot formation	Number per shoot-forming explant of				
		vegetative shoots				disc florets
		tall	intermediate	small	total	
0.0	0	0	0	0	0	0
0.5	100	6.6	11.6	10.3	28.5	0.0
1.0	100	3.1	3.9	21.1	28.1	0.1
1.5	100	1.1	1.6	22.6	25.3	1.3
2.0	100	0.4	0.8	26.7	27.9	3.6
2.5	100	0.4	1.4	20.0	21.8	4.4

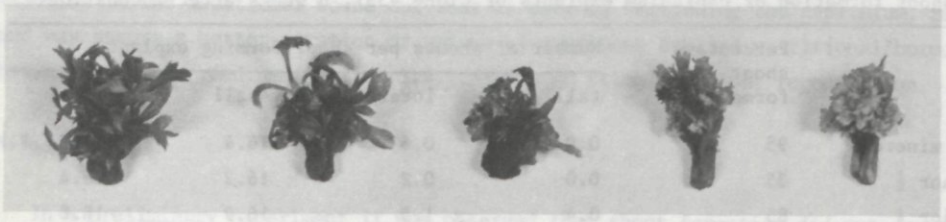


Fig. 41. The influence of various concentrations of sucrose on shoot formation of capitulum explants of Clone 4331, 10 weeks after incubation. From left to right: 0.5, 1.0, 1.5, 2.0 and 2.5% sucrose.

Fig. 42. 'Disc florets' initiated and developed on a capitulum explant of Clone 4331 at 2.5% sucrose, 10 weeks after incubation.

Table 46. The influence of various concentrations (0.5-2.5%) of sucrose and glucose on shoot formation of capitulum explants of Clone 4331, 8 weeks after incubation.

Sugar %	Percentage shoot formation	Number per shoot-forming explant of				
		vegetative shoots				disc florets
		tall	intermediate	small	total	
Sucrose 0.5	100	4.4	9.4	16.3	30.1	0.0
1.5	100	0.4	0.9	22.5	23.8	1.8
2.5	100	0.0	2.2	19.6	21.8	3.9
Glucose 0.5	100	5.0	9.4	15.0	29.4	0.0
1.5	100	0.1	0.7	24.2	25.0	1.6
2.5	95	0.0	0.8	22.1	22.9	2.7

corolla teeth, can be observed in more detail in Fig. 42.

In a second experiment sucrose and glucose were compared at various concentrations. Table 46 shows that the influence of the two sugars did not differ markedly. Both sugars, at all concentrations tested, yielded almost maximum percentages of shoot formation. Sucrose and glucose at 0.5% favoured the development of a high number of initiated vegetative shoots. Moreover a low sugar concentration is desirable, because the formation of 'disc florets' is prevented. Unlike vegetative shoots, they cannot be used for a direct vegetative propagation.

There are numerous publications on the essential role of sugar in shoot organogenesis. Mostly a high sugar concentration has been reported to be optimum for shoot formation. However, low sugar concentrations have been shown to be optimum for shoot formation in pyrethrum and shoot development of capitulum explants of *Gerbera jamesonii* (Pierik et al., 1973, 1975).

At high sugar concentrations floral primordia were formed in pyrethrum explants. Such a determining influence of the sugar level on the orientation of the buds towards the vegetative or the flowering state was also reported for stem segments of *Nicotiana tabacum* (Chouard & Aghion, 1961; Aghion-Prat, 1965), petiole or root tissue (Paulet & Nitsch, 1964) and peduncle explants (Margara, 1965) of *Cichorium intybus*, stem segments of *Plumbago indica* (Nitsch & Nitsch, 1967ab) and peduncle explants of *Lunaria annua* (Pierik, 1967).

5.4.2.3 Auxin

In preliminary experiments it was observed that shoot formation did not occur when only IAA, at various concentrations, was added to the medium.
Therefore the influence of various IAA concentrations was investigated in the presence of the cytokinin BA at 10^{-6} g/ml in the medium.

In combination with BA, all concentrations of IAA inhibited shoot formation (Table 47), this inhibition being more pronounced at higher IAA concentrations.

Sometimes without cytokinin in the medium, exogenously applied auxin stimulated shoot formation, as has been reported for flower buds of *Kalanchoë pinnata* (Mohan Ram & Wadhi, 1968), leaf discs of *Streptocarpus* (Appelgren & Heide, 1972) and bulb scale segments of *Hyacinthus orientalis* (Pierik & Steegmans, 1975b).

In general it can be stated that the combination of a low auxin concentration with a high cytokinin concentration is favourable for shoot formation and that a high auxin concentration combined with a low cytokinin concentration has a detrimental effect on shoot formation, which for instance was found for tobacco tissue (Miller & Skoog, 1953; Skoog & Miller, 1957), leaf explants of *Cichorium intybus* (Toponi, 1963; Bouriquet & Vasseur, 1966), stem segments of *Plumbago indica* (Nitsch & Nitsch, 1967ab), leaf discs of *Begonia* (Ringe & Nitsch, 1968), endosperm cultures of *Scurrula pulverulenta* (Bhojwani & Johri, 1970), excised leaves of *Atropa belladonna* (Zenkteler, 1971), pedicel explants of *Chrysanthemum morifolium* (Roest & Bokelmann, 1975) and rachis explants of *Solanum tuberosum* (Roest & Bokelmann, 1976).

Table 47. The influence of various concentrations (0–10^{-4} g/ml) of IAA on shoot formation of capitulum explants of Clone 4331, 6 weeks after incubation.

IAA g/ml	Percentage shoot formation	Number of shoots per shoot-forming explant			
		tall	intermediate	small	total
0	95	0.3	4.2	13.5	18.0
10^{-8}	85	0.0	2.0	19.0	21.0
10^{-7}	60	0.0	1.7	8.3	10.0
10^{-6}	25	0.6	0.4	2.0	3.0
10^{-5}	10	0.0	0.0	1.0	1.0
10^{-4}	5	0.0	0.0	2.0	2.0

Table 48. The influence of various concentrations (0-10^{-4} g/ml) of BA on shoot formation of capitulum explants of Clone 4331, 6½ weeks after incubation.

BA g/ml	Percentage shoot formation	Number of shoots per shoot-forming explant			
		tall	intermediate	small	total
0	0	0	0	0	0
10^{-8}	0	0	0	0	0
10^{-7}	75	0.0	1.3	3.7	5.0
10^{-6}	90	1.0	4.0	12.0	17.0
10^{-5}	90	0.1	18.0	23.6	41.7
10^{-4}	80	0.1	13.5	17.8	31.4

5.4.2.4 Cytokinin

The cytokinin BA was added at various concentrations to the medium to determine its effect on shoot formation. Table 48 illustrates that BA was required for shoot formation. For an adequate shoot production BA concentrations of 10^{-8} and 10^{-7} g/ml were too low and suboptimal, respectively. High numbers of shoots were initiated and developed at BA 10^{-6}, 10^{-5} and 10^{-4} g/ml. Although at BA 10^{-6} the highest number of tall shoots was measured, BA at 10^{-5} g/ml has to be preferred, because at this concentration a very high number of intermediate shoots has developed. In standard experiments BA was applied at 10^{-6} g/ml to the culture medium.

The finding in pyrethrum that the addition of cytokinin is essential for shoot formation, has also been reported for many plant species cultivated in vitro e.g. for tobacco tissue (Miller & Skoog, 1953; Skoog & Miller, 1957), stem segments of *Plumbago indica* (Nitsch & Nitsch, 1967ab), peduncle explants of *Brassica oleraceae* (Margara, 1969), excised leaves of *Dendropthoe falcata* (Nag & Johri, 1970), pedicel explants of *Chrysanthemum morifolium* (Roest & Bokelmann, 1975) and rachis explants of *Solanum tuberosum* (Roest & Bokelmann, 1976).

As already pointed out in Section 5.4.2.3 shoot formation is often optimum when an auxin is added simultaneously with a cytokinin to the culture medium.

5.4.2.5 Gibberellic acid

In previous experiments it was noticed that although a high number of shoots is usually initiated, the development of the shoot initials is generally very poor. In the following two experiments, with 10 explants per treatment, it was investigated whether the addition of GA_3, in the presence of BA at 10^{-6} g/ml, would enhance the development of the initiated shoots.

In the first experiment autoclaved GA_3 was applied at various concentrations to the culture medium. The percentage of shoot formation was hardly influenced by GA_3 (Table 49). At a concentration of GA_3 at 10^{-4} g/ml the initiation and development of shoots were suppressed. In comparison with the other treatments shoot development was stimulated by GA_3 at 10^{-5} g/ml.

In the second experiment autoclaved GA_3 at 10^{-5} g/ml was added to the culture

Table 49. The influence of various concentrations (0-10^{-4} g/ml) of GA_3 on shoot formation of capitulum explants of Clone 4331, 7 weeks after incubation.

GA_3 g/ml	Percentage shoot formation	Number of shoots per shoot-forming explant			
		tall	intermediate	small	total
0	90	2.4	7.5	10.0	19.9
10^{-6}	100	3.3	7.9	9.3	20.5
10^{-5}	100	4.0	12.5	2.5	19.0
10^{-4}	80	0.0	1.1	8.3	9.4

Table 50. The influence of GA_3 at 10^{-5} g/ml applied at various periods (0-4½ weeks after incubation) on shoot formation of capitulum explants of Clone 4331, 7 weeks after incubation.

Application time (weeks after incubation)	Percentage shoot formation	Number of shoots per shoot-forming explant			
		tall	intermediate	small	total
0	100	4.0	12.5	2.5	19.0
1½	90	3.8	11.9	10.9	26.6
3	90	3.3	9.4	10.6	23.3
4½	70	1.2	12.5	12.5	26.2

medium 0, 1½, 3 or 4½ weeks after incubation, to determine at which time after incubation, addition of GA_3 would result in the best development of the initiated shoots.

All treatments showed more or less the same number of initiated shoots (Table 50). Addition of GA_3 4½ weeks after incubation, however, had an unfavourable effect on the percentage shoot formation and the development of the initiated shoots in comparison with earlier applications. Though differences were small between the treatments with additions of GA_3 0, 1½ or 3 weeks after incubation, the development of the shoots was stimulated when GA_3 was applied during the first weeks after incubation.

The inhibiting effect of gibberellic acid, especially at high concentrations, was also found for shoot regeneration on leaf discs of *Begonia* (Schraudolf & Reinert, 1959), stem segments of tobacco (Aghion-Prat, 1965), leaf fragments of *Cichorium intybus* (Bouriquet & Vasseur, 1966), peduncle explants of *Lunaria annua* (Pierik, 1967), stem segments of *Plumbago indica* (Nitsch & Nitsch, 1967ab), leaf discs of *Streptocarpus* (Appelgren & Heide, 1972) and bulb scale segments of *Hyacinthus orientalis* (Pierik & Steegmans, 1975b).

Gibberellic acid, mostly at low concentrations, has also been reported to be optimum for shoot formation on leaf discs of *Cardamine pratensis* (Paulet & Nitsch, 1959) and *Cichorium intybus* (Bouriquet & Vasseur, 1966), peduncle segments of *Brassica oleraceae* (Margara, 1969) and rachis explants of *Solanum tuberosum* (Roest & Bokelmann, 1976).

Table 51. The influence of constant temperatures (9-25°C) on shoot formation of capitulum explants of Clone 4331, 6 weeks after incubation.

Temperature °C	Percentage shoot formation	Number of shoots per shoot-forming explant			
		tall	intermediate	small	total
9	80	0.0	0.1	6.9	7.0
13	95	0.1	4.4	17.1	21.6
17	95	0.7	3.8	17.9	22.4
21	80	0.6	4.3	8.8	13.7
25	85	0.6	3.3	24.3	28.2

5.4.3 Climatic factors

5.4.3.1 Temperature

In continuous light in the phytotron, the effect of various constant temperatures on shoot formation was examined. All treatments showed about the same percentage shoot formation (Table 51). The total number of initiated shoots as well as the development of these shoots was suboptimal at 9°C. Presumably, the low total number of shoots initiated at 21°C was due to some experimental error. This also resulted in a low number of small shoots, unsuitable for plantlet production. The numbers of intermediate and tall shoots, which can be used for vegetative propagation, were about the same at 13, 17, 21 and 25°C. Apparently, almost independent of the number of initiated shoots, a certain number of shoots developed, which can be ascribed to competition among developing shoots. Because there was a decrease in the number of tall shoots at 13°C, a temperature in the range 17-25°C is best for an optimum development of initiated shoots.

Mostly a high temperature has been reported to be optimum for shoot formation. This may be due to a fast development of shoots at a high temperature, as has been found for leaf discs of *Streptocarpus* (Appelgren & Heide, 1972), bulb scale segments of *Hyacinthus orientalis* (Pierik & Ruibing, 1973) and capitulum explants of *Gerbera jamesonii* (Pierik et al., 1975).

A low temperature, however, was described to be promotive for shoot initiation on leaf discs of *Streptocarpus* (Appelgren & Heide, 1972) and bulb scale segments of *Hyacinthus orientalis* (Pierik & Ruibing, 1973).

5.4.3.2 Light

In preliminary experiments a period of continuous light just after incubation was unfavourable for shoot formation. Thus the influence of various periods of light and darkness was examined at 21°C in the phytotron.

As shown in Table 52 all treatments yielded high percentages of shoot formation as well as almost equal total numbers of initiated shoots. The development of the shoot initials depended on the treatment; a period of darkness in the beginning of the experiment promoted shoot development. This promotion became more pronounced as the

Table 52. The influence of various periods of light and darkness on shoot formation of *capitulum explants of Clone 4331, 4 weeks after incubation.*

	Percentage shoot formation	Number of shoots per shoot-forming explant			
		tall	intermediate	small	total
4 weeks light	90	0.3	1.7	18.0	20.0
1 week darkness + 3 weeks light	85	0.4	3.3	20.7	24.4
2 weeks darkness + 2 weeks light	90	0.4	5.6	17.0	23.0
4 weeks darkness	95	0.4	8.7	15.0	24.1

duration of the darkness period increased and the best shoot development was observed after 4 weeks of darkness. When an etiolated pale yellow coloured shoot, resulting from incubation in darkness, was exposed to light, it turned green within a few days (Fig. 43).

The influence of light and darkness on shoot formation greatly differed from species to species. By growing explants first in darkness and subsequently in light the formation af adventitious shoots was also promoted in leaf explants of *Cichorium intybus* (Legrand, 1972) and flower buds of *Freesia* (Pierik & Steegmans, 1975a), while a period of darkness also enhanced shoot development on capitulum explants of *Gerbera jamesonii* (Pierik et al., 1973, 1975). Adversely, an immediate exposure to light was favourable for shoot formation on stem segments of *Plumbago indica* (Nitsch & Nitsch, 1967ab), peduncle explants of *Brassica oleraceae* (Margara, 1969) and in callus tissue of *Anthurium andreanum* (Pierik et al., 1974). Finally, no effect of light or darkness was

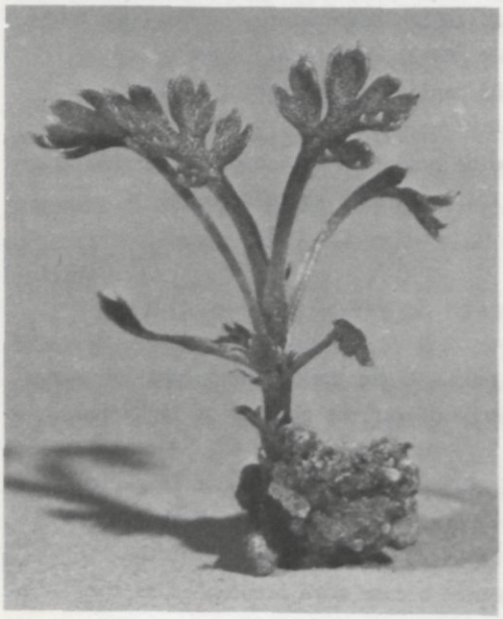


Fig. 43. Shoot formation of a capitulum explant of Clone 4331, 9 weeks after incubation. The explant was exposed to darkness for 8½ weeks, after which period the etiolated shoot turned green within a few days in light.

found on shoot regeneration of bulb scale segments of *Hyacinthus orientalis* (Pierik & Ruibing, 1973).

5.5 PLANTLET PRODUCTION BY ROOT FORMATION OF DETACHED SHOOTS

At the termination of the experiments 1½-3 months after the incubation of the capitulum explants, tall shoots with a length of at least 0.6 cm (Fig. 37c), were detached from the explant. The detached shoots were subcultured in vitro on a medium containing auxin for root formation and subsequent plantlet production. However, adventitious root formation was never observed.

A good rooting was achieved after treating the basal end of the excised shoot with 1% IAA (on talc basis) and subsequently transferring the shoot to an unsterilized soil mixture of leaf mould and sand. By maintaining a relative humidity of approximately 100%, adventitious roots were initiated at the basal end of the shoot, within 3 weeks after transfer to soil (Fig. 37d). Thus plantlets were obtained from most shoots, about 3 months after the incubation of the capitulum explants in vitro.

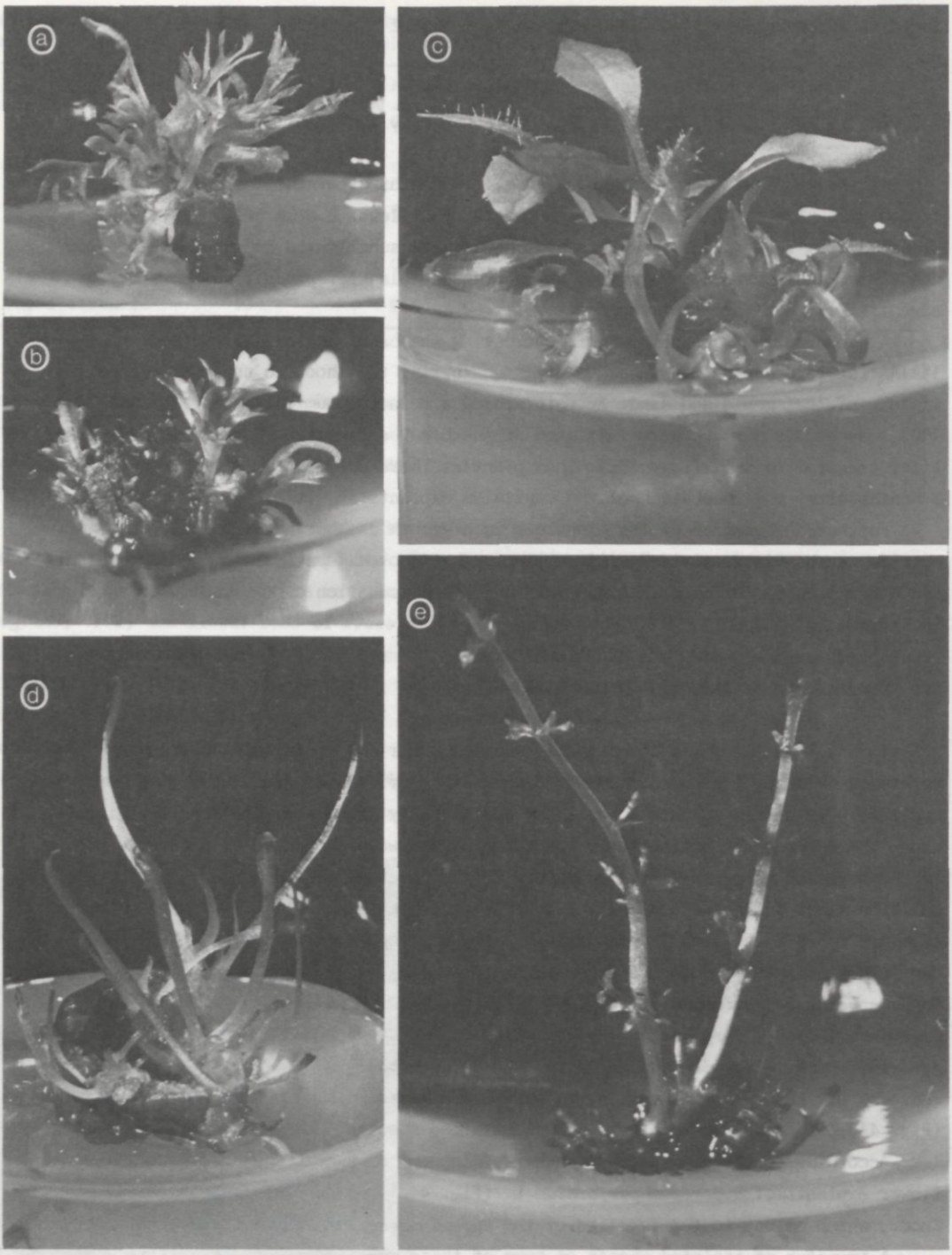
Further cultivation of the plantlets in a growth chamber and, subsequently, outside on the field, yielded uniform plants that looked identical to the stock plants and started to flower about half a year after the incubation of the capitulum sections in vitro.

5.6 VEGETATIVE PROPAGATION OF OTHER PLANT SPECIES

In this section it is investigated whether other *Compositae* can be propagated vegetatively according to the same procedure as described for pyrethrum. Therefore capitula were sectioned into 2 segments, additionally wounded by excising the florets above the bottom half of the ovary and the explants were incubated on a culture medium in the presence of pyrex-distilled water, 'Difco' Bacto-agar 0.6%, Knop's major and Heller's minor salts (both at half strength), sucrose 1% and BA at 10^{-6} g/ml. After exposure to 20°C and a daylength of 14 h shoot development was also observed on capitulum explants of *Anthemis arvensis* L. (Fig. 44a), *Calendula officinalis* L., *Chrysanthemum leucanthemum* L. (Fig. 44b), *Chrysanthemum morifolium* Ram., *Chrysanthemum parthenium* Bernh., *Chrysanthemum roseum* Web. et M., *Chrysanthemum segetum* L., *Gerbera jamesonii* Hook., *Helenium autumnale* L., *Hieracium umbellatum* L. (Fig. 44c), *Hypochaeris radicata* L., *Leontodon autumnalis* L., *Matricaria maritima* L., *Taraxacum officinale* Web. (Fig. 44d), *Tagetes patula* L. and of unidentified species of *Gazanea* and *Rudbeckia*.

According to the same procedure the development of shoots could also be achieved on flower explants of *Hypericum perforatum* L. (Fig. 44e) and *Anemone japonica* Sieb. et Zucc., which belong to the families of the *Hypericaceae* and *Ranunculaceae*, respectively.

A transfer of isolated shoots of some of these species, to an unsterilized soil mixture of leaf mould and sand, lead to the production of adventitious roots and, consequently, plantlets were produced of *Chrysanthemum leucanthemum* L., *Chrysanthemum morifolium* Ram., *Chrysanthemum parthenium* Bernh., *Hieracium umbellatum* L., *Hypericum perforatum* L., *Matricaria maritima* L., *Gazanea* sp. and *Rudbeckia* sp.



Figs 44a-e. Shoot development on flower(head) explants of other plant species cultivated in vitro:

a. *Anthemis arvensis* L.
b. *Chrysanthemum leucanthemum* L.
c. *Hiëracium umbellatum* L.
d. *Taraxacum officinale* Web.
e. *Hypericum perforatum* L.

Because it was not examined anatomically whether shoots developed adventitiously or axillarily on explants of the plant species mentioned before, their origin remains obscure.

5.7 DISCUSSION AND CONCLUSIONS

Anatomical observations (Section 5.3) have shown that almost all shoots developing on capitulum explants of pyrethrum do not arise from pre-existing shoot initials, but that they emerge from shoot meristems formed adventitiously in the epidermal cell layer of the ovaries of florets. Several successive stages had to be passed before an adventitious shoot has been produced, such as the initiation of a shoot meristem and the subsequent development (elongation) of the initiated shoot.

The experimental results (Section 5.4) have demonstrated that many factors are involved in shoot organogenesis; the optimum conditions for the separate stages in the process of adventitious shoot formation were different.

Since all three clones tested, namely 1087, 4331 and Ma 63/1889, initiated and developed adventitious shoots, shoot formation on capitulum explants of pyrethrum seems to be a rather common phenomenon. However, it has to be emphasized that despite the initiation of high numbers of shoots (on average approximately 25), the development of the small shoot initials into intermediate and tall shoots (which can be used for vegetative propagation) was commonly found to be too variable and too sporadic (a few shoots per explant).

It has already been shown that the development of initiated shoots can be stimulated by the addition of GA_3 at 10^{-5} g/ml to the medium (Section 5.4.2.5). Nevertheless, additional research has to be concentrated on factors which can effect a better development of the shoots.

Low sugar concentrations were optimum for shoot formation in pyrethrum (Section 5.4.2.2) and shoot development on capitulum explants of *Gerbera jamesonii* (Pierik et al., 1973, 1975). In both plant species an optimum low sugar concentration coincides with a stimulating effect of a period of darkness during the first weeks of incubation (Section 5.4.3.2). The coincidence of the inhibiting effect of a high sugar concentration and of a period of light during the first weeks of incubation, may be due to the synthesis of a supra-optimum amount of carbohydrates in light.

Plantlets were produced by root formation of detached tall shoots (Section 5.5), which had previously been developed on a medium containing BA at 10^{-6} g/ml. Optimum shoot formation on capitulum explants, however, was realized in the presence of BA at 10^{-5} g/ml (Section 5.4.2.4). Difficulties may arise with respect to root formation of isolated shoots, previously grown on a medium supplemented with BA at 10^{-5}, because it was found in Section 3.4.2.5 that peduncle explants regenerated hardly any roots at BA 10^{-7} and no roots at all at higher BA concentrations in the medium. Root formation of shoots, which had developed at a BA concentration of 10^{-5} in the medium, may be prevented or greatly reduced because of an excess of the endogenous BA content in the shoot. As long as it is uncertain whether good rooting can be achieved with shoots, cultivated previously at BA 10^{-5}, a concentration of BA 10^{-6} g/ml is recommended for

shoot formation to ensure a reliable subsequent root formation of isolated shoots.

Shoot development was also observed on capitulum explants of many other species of the *Compositae*, and on flower explants of two species belonging to other families, cultivated in vitro. In some of these species, by root formation of isolated shoots, plantlets were produced (Section 5.6).

Apart from the aspect of vegetative propagation, the method in vitro may also be of significance to keep disease-free plant material in stock for a long period, as has been found in *Chrysanthemum morifolium* (Roest & Bokelmann, 1975).

Moreover, an adventitious bud technique in vitro may be a powerful tool in mutation breeding. In vivo it has been demonstrated in a number of ornamentals, like *Streptocarpus* (Broertjes, 1969), *Achimenes* (Broertjes, 1972a), *Saintpaulia* (Broertjes, 1972b), *Kalanchoë* (Broertjes & Leffring, 1972) and *Begonia* (Doorenbos & Karper, 1975), that after irradiation of detached leaves and through subsequent adventitious shoot formation and plantlet production, solid (non-chimeral) mutants can be obtained. This finding is based on the phenomenon that (the apex of) adventitious shoots, formed at the base of the petiole, ultimately originate(s) from a single (epidermal) cell. Recently, after irradiation of pedicel explants and through adventitious shoot formation in vitro, solid mutants have been produced in *Chrysanthemem morifolium* (Broertjes et al., 1976).

After irradiation of capitulum explants of pyrethrum and by the regeneration of adventitious shoots in vitro, solid mutants might be produced and chimera formation would be avoided or at least greatly reduced. Mutation breeding may be a useful approach because Clone 4331 is available, which has very high flower yields, but is characterized by an undesirable ratio of the six pyrethrin constituents.

Summary

Chapter 1 describes why flowering and vegetative propagation of pyrethrum (*Chrysanthemum cinerariaefolium* Vis.) were studied.

Flowering (Chapter 2), root formation of peduncle explants (Chapter 3) and of shoot cuttings (Chapter 4), as well as shoot formation of capitulum explants (Chapter 5), are processes which are controlled by various factors. These factors are related to properties of the (ex)plant, the nutritional/hormonal composition of the medium or substrate and the climatic conditions to which the (ex)plant is exposed. Anatomical observations have revealed that in these processes successive stages can be distinguished, such as *initiation* and *development*, before flower heads, roots or shoots are produced. Some factors had an effect during initiation, which differed from that during the stage of development. Hence, an optimum course of these processes can only occur when specific conditions are provided in every stage.

Chapter 2 The effect of temperature and photoperiod on the flowering behaviour of plants of Clone Ma 63/1889 was examined.

Of the various temperatures studied, 9, 13, 17, 21 and 25°C, high numbers of plants initiated high numbers of flower heads after being at 9°C for at least 6 weeks. Both development of initiated flower heads and vegetative development of the plants were stimulated at 17, 21 and 25°C.

For an adequate flowering, which is a prerequisite for the commercial production of natural insecticide (pyrethrins) from the flower heads, plants have to be cultivated at low temperatures in the highlands of tropical countries. For an optimum vegetative development of plants, desirable for vegetative propagation by splits or shoot cuttings, cultivation at higher temperatures in the lowlands is to be preferred.

Flowering was affected by the photoperiod as well and pyrethrum has to be regarded as a quantitative short-day plant. This finding, however, is not of direct practical importance for the cultivation of pyrethrum in tropical countries, where throughout the year the photoperiod amounts to approximately 12 h.

Chapter 3 Anatomical and physiological aspects of adventitious root formation were studied by the cultivation of peduncle explants in vitro.

Anatomical observations have shown that adventitious roots were initiated in the interfascicular pericycle during the first two weeks of incubation, whereas root initials developed during the subsequent two weeks. When optimum conditions were maintained during these separate stages, peduncle explants of Clones 1087, 4331 and Ma 63/1889 yielded good rooting responses.

Chapter 4 Subsequently, it was examined whether vegetative propagation can be realized by adventitious root formation of shoot cuttings in vivo.

Anatomical observations showed that the process of adventitious root formation on basal stem portions of shoot cuttings progressed in an almost identical way to that of peduncle explants. With the information obtained from experiments with peduncle explants in vitro (Chapter 3), good rooting responses of shoot cuttings of Clones 1087, 4331 and Ma 63/1889 were achieved.

One vegetative stock-plant of 1-2 years old can be divided into 5-10 splits, instead of 25-100 shoot cuttings. Hence, vegetative propagation by root formation of shoot cuttings can considerably improve the rate of multiplication and may further lead to the buildup of a healthy clone of selected, high yielding plants.

Chapter 5 Finally, it was investigated whether plantlets can be obtained through adventitious shoot formation on capitulum explants cultivated in vitro.

Anatomical observations have revealed that adventitious shoots were initiated in the epidermal cell layer of the ovaries of disc florets during the first weeks after incubation, whereas the initiated shoots developed during the subsequent weeks. By root formation of detached shoots plantlets were produced of Clones 1087, 4331 and Ma 63/1889.

This method of vegetative propagation in vitro is not yet available for large-scale multiplication because despite the initiation of numerous shoots, their development into intermediate and tall shoots, which are used for plantlet production, was still inadequate.

Initially the technique in vitro may be applied for a fast propagation of selected genotypes, whereas for further clonal multiplication a vegetative propagation in vivo by splits or shoot cuttings has presumably to be preferred.

The development of shoots was also achieved on capitulum explants of other *Compositae* cultivated in vitro. The simple procedure of plantlet production seems to become attractive for a fast vegetative propagation of various *Compositae*.

Apart from propagation, this technique in vitro may be useful for the maintenance of selected, high yielding and healthy plant material in stock, while it may also be of significance for mutation breeding of pyrethrum.

Samenvatting

Pyrethrum (*Chrysanthemum cinerariaefolium* Vis., behorende tot de familie van de *Compositae*) wordt geteeld vanwege de in de bloemhoofdjes voorkomende insekticiden, welke gezamenlijk worden aangeduid als pyrethrinen. Gunstige eigenschappen van deze pyrethrinen zijn:

- de geringe giftigheid voor mensen en zoogdieren,
- de snelle afbraak in het milieu,
- er vindt nauwelijks opbouw plaats van resistentie in insektenpopulaties.

Vanwege deze eigenschappen is de vraag naar dit natuurlijk insekticide de laatste jaren geleidelijk toegenomen. Voor de produktie van pyrethrinen uit de bloemhoofdjes is bloei essentieel. Om die reden is de invloed van klimatologische omstandigheden op de bloei onderzocht (hoofdstuk 2).

Pyrethrum wordt vooral geteeld in Oostafrikaanse landen, waarvan Kenya de grootste producent is van pyrethrinen met 60% van de wereldproduktie. In Kenya komt men onder meer aan de toegenomen vraag naar pyrethrinen tegemoet door veredeling, selectie op planten met een hoge opbrengst aan pyrethrinen en vegetatieve vermeerdering van geselecteerde planten. In de praktijk worden de planten klonaal vermeerderd door scheuren, aan welke methode evenwel twee nadelen verbonden zijn:

- een moederplant levert maar een beperkt aantal nakomelingen,
- het wortelknobbelaaltje *Meloidogyne hapla*, dat algemeen wordt aangetroffen en na infectie van worteldelen een sterke vermindering van de bloemopbrengst veroorzaakt, wordt van geïnfecteerde moederplanten over de scheurlingen verdeeld.

Het is wenselijk dat alternatieve methoden van vegetatieve vermeerdering worden ontwikkeld, welke deze nadelen niet vertonen (hoofdstukken 3, 4 en 5). Onderzoek naar een vegetatieve vermeerdering door middel van scheutstekken verdient hierbij hoge prioriteit, omdat via een stekmethode de vermeerderingssnelheid aanzienlijk verhoogd kan worden en uitgaande van geïnfecteerde moederplanten gezonde nakomelingen worden verkregen, omdat bij scheutstekken de (geïnfecteerde) worteldelen worden weggesneden.

Voor een vegetatieve vermeerdering via scheutstekken is het noodzakelijk dat adventieve wortels worden gevormd aan de basis van de stekken. Om inzicht te krijgen in de factoren welke van invloed zijn op de wortelvorming werd gebruik gemaakt van een cultuur in vitro, waarbij bloemsteel-explantaten onder steriele omstandigheden op een kunstmatige voedingsbodem in de cultuurbuis werden opgekweekt (hoofdstuk 3). Vervolgens werd onderzocht of met behulp van deze kennis een goede adventieve wortelvorming van scheutstekken gerealiseerd kon worden (hoofdstuk 4). Tenslotte werd vastgesteld of een vegetatieve vermeerdering kon worden verkregen via de vorming van adventieve scheutjes aan in vitro gekweekte bloemhoofd-explantaten (hoofdstuk 5).

De vorming van bloemhoofdjes, wortels en scheuten zijn processen welke moeten worden onderscheiden in een aantal opeenvolgende stadia, zoals een stadium van initiatie (aanleg) en ontwikkeling (uitgroei). Deze processen worden beïnvloed door factoren welke samenhangen met de plant of het explantaat, de samenstelling van het substraat of de voedingsbodem en de klimatologische omstandigheden. Een aantal van deze factoren vertoonde een effect gedurende het stadium van initiatie welke duidelijk afweek van dat gedurende het stadium van ontwikkeling. Voor een optimaal verloop van deze processen is het dus noodzakelijk dat in beide stadia de omstandigheden optimaal zijn.

De resultaten zullen vervolgens per hoofdstuk gedetailleerd worden beschreven.

In hoofdstuk 2 wordt de invloed besproken van temperatuur en daglengte op de bloei van planten van kloon Ma 63/1889.

Wanneer planten werden geplaatst bij temperaturen van 9, 13, 17, 21 of 25°C, kwamen de meeste planten in bloei en werd het grootste aantal bloemhoofdjes geïnitieerd bij 9°C, welke temperatuur gedurende tenminste 6 weken moest worden gehandhaafd. Zowel de ontwikkeling van de geïnitieerde bloemhoofdjes als een vegetatieve ontwikkeling van de planten werden gestimuleerd bij 17, 21 en 25°C.

Een goede bloei, essentieel voor de commerciële produktie van pyrethrinen, kan dus alleen verwacht worden bij de lage temperaturen in het hoogland van tropische gebieden. Voor een optimale vegetatieve ontwikkeling van de planten, welke de voorkeur verdient wanneer de planten vegetatief worden vermeerderd door scheurlingen of scheutstekken, moet pyrethrum echter worden geteeld bij de hogere temperaturen in het laagland.

De bloei werd ook beïnvloed door de daglengte en pyrethrum moet beschouwd worden als een kwantitatieve korte-dag-plant. Dit effect van de daglengte is niet van directe praktische betekenis voor de teelt van pyrethrum in tropische gebieden, waar de daglengte het gehele jaar door ongeveer 12 uur bedraagt.

In hoofdstuk 3 wordt het proces van adventieve wortelvorming anatomisch en fysiologisch onderzocht aan in vitro gekweekte bloemsteel-explantaten.

Anatomisch onderzoek toonde aan dat adventieve wortels voornamelijk werden geïnitieerd gedurende de eerste twee weken van incubatie in het interfasciculaire pericambium, terwijl de geïnitieerde wortels tot ontwikkeling kwamen gedurende de daaropvolgende twee weken. Wanneer optimale omstandigheden werden gehandhaafd gedurende beide stadia, werd een goede beworteling verkregen aan bloemsteel-explantaten van de klonen 1087, 4331 en Ma 63/1889.

Hoofdstuk 4 beschrijft onderzoek betreffende adventieve wortelvorming van scheutstekken waardoor een vegetatieve vermeerdering in vivo kan worden gerealiseerd.

Anatomisch onderzoek heeft uitgewezen dat het proces van adventieve wortelvorming aan basale stengeldelen van scheutstekken op een vrijwel identieke wijze verloopt als is waargenomen bij bloemsteel-explantaten. Door gebruik te maken van de kennis verkregen via de in vitro cultuur van bloemsteel-explantaten (hoofdstuk 3), werd een goede wortelvorming gerealiseerd aan scheutstekken van de klonen 1087, 4331 en Ma 63/1889.

Een vegetatief ontwikkelde moederplant van 1-2 jaar oud kan worden verdeeld in 5-10

scheurlingen, terwijl 25-100 scheutstekken van een dergelijke plant kunnen worden verkregen. Een vegetatieve vermeerdering in vivo via scheutstekken kan de vermeerderingssnelheid dan ook aanzienlijk verhogen en tevens resulteren in de opbouw van een gezonde kloon, bestaande uit geselecteerd, hoogwaardig plantmateriaal.

Hoofdstuk 5 behandelt onderzoek betreffende vegetatieve vermeerdering via de vorming van adventieve scheuten aan in vitro gekweekte bloemhoofd-explantaten.

Anatomisch onderzoek heeft aangetoond dat adventieve scheutjes gedurende de eerste weken na incubatie werden geïnitieerd in de epidermis van de vruchtbeginsels van buisbloemen en dat de scheutjes tot ontwikkeling kwamen gedurende de daaropvolgende weken. Na wortelvorming van geïsoleerde scheutjes werden plantjes verkregen van de klonen 1087, 4331 en Ma 63/1889.

Deze in vitro methode van vegetatieve vermeerdering kan nog niet op grote schaal worden toegepast omdat, ondanks de initiatie van een groot aantal scheutjes, de ontwikkeling hiervan tot middelgrote en grote scheutjes (welke voor de vegetatieve vermeerdering worden gebruikt) nog onvoldoende is. Aanvullend onderzoek zal noodzakelijk zijn om de scheutontwikkeling te verbeteren.

In de praktijk zal de in vitro techniek aanvankelijk van betekenis kunnen zijn voor een snelle vermeerdering van geselecteerde genotypen, terwijl een verdere klonale vermeerdering vermoedelijk het beste gerealiseerd kan worden via scheurlingen of scheutstekken in vivo.

Scheutvorming werd ook waargenomen aan in vitro gekweekte bloemhoofd-explantaten van andere *Compositae*. De eenvoudige wijze waarop na beworteling van geïsoleerde scheutjes plantjes worden geproduceerd, lijkt ook perspectief te bieden voor een snelle vegetatieve vermeerdering van diverse composieten.

Tenslotte kan een in vitro vermeerderingsmethode nuttig zijn voor het opslaan van geselecteerd, gezond en hoogwaardig plantmateriaal in de cultuurbuis en bovendien belangrijk zijn voor de mutatieveredeling van pyrethrum.

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