ADVENTITIOUS SHOOT FORMATION ON LEAF CUTTINGS IN VIVO, A TOOL IN HORTICULTURE

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STELLINGEN

1. Voor het induceren van adventieve scheuten is het wegnemen van remmende factoren in de aangrenzende weefsels van groter belang dan het geven van de juiste prikkels ter plaatse.

   Dit proefschrift.

2. Het niet uitgroeien van de adventieve knoppen aan de bases van bladstekken van 'Elatior'-begonia's (*Begonia x hiemalis*) tijdens een lange periode korte dag wordt bepaald door de als moeder gebruikte ouder van deze soorthybride, namelijk de knolbegonia (*Begonia x tuberhybrida*).

   Dit proefschrift.

3. De bladponsmethode die door Horsch et al. werd geïntroduceerd als eenvoudig systeem voor in vitro transformatie met *Agrobacterium tumefaciens*, kan nog worden verbeterd door explantaten te gebruiken uit metabolisch minder actief weefsel.

   Horsch, R.B., J.E. Fry, N.L. Hoffmann, M. Walbroth, D. Eichholtz, S.G. Rogers en R.T. Fraley, 1985. Science 227, 1229-1231.

4. Het beeld dat Broertjes en Keen geven van het ontstaan van adventieve scheuten, namelijk dat dit terug te voeren is tot één cel, is te algemeen.

   Broertjes, C. en A. Keen, 1980. Euphytica 29, 73-87.

5. In tegenstelling tot de gangbare opvattingen groeien zeer jonge, hartvormige embryo's van komkommer in vitro gemakkelijker uit tot plant dan embryo's in gevorderde ontwikkelingsstadia. Deze ervaring is van belang voor het onderzoek naar de mogelijkheden van kunstmatig zaad.

   Custers, J.B.M., 1981. Cucurbit Genetics Coop. Rpt. 4, 48-49.

6. Typische problemen bij de snelle vermeerdering in vitro, zoals infectie, glazigheid, rejuveneren en verbruinen van het medium, zullen sneller worden opgelost door eerst aandacht te schenken aan automatisering en robotisering van de werkzaamheden.

7. De bruikbaarheid van somaklonale variatie wordt bepaald door de mogelijkheid om ongewenste mutaties te beperken of te elimineren.

8. Gedreven door de angst een markt te missen gaan verschillende genetische manipulatiebedrijven er toe over bij koolzaad (*Brassica napus*) hetzelfde cytoplasmatype te gebruiken. Dit leidt tot ongewenste genetische verenging en vergroot de kans op snelle uitbreiding van (nieuwe) ziekten met een cytoplasmatisch-genetische basis.

9. Wanneer op congressen uitsluitend posterpresentaties worden toegelaten, is de kans op een bevredigende informatie-overdracht groter dan wanneer het programma voor een belangrijk deel wordt gevuld met korte lezingen.

10. Een ver doorgevoerde verzelfstandiging van het landbouwkundig onderzoek zal versnippering van onderzoekprogramma's in de hand werken.

11. Leren lezen en schrijven is een recht, geen voorrecht.

Jan B.M. Custers
Adventitious shoot formation on leaf cuttings in vivo, a tool in horticulture.

7 november 1986, Wageningen.

VOORWOORD

Bij het verschijnen van dit proefschrift maak ik graag van de gelegenheid gebruik om iedereen te bedanken die er op één of andere manier aan heeft meegewerkt. Allereerst moeten mijn beide promotoren, prof.dr.ir. J. Doorenbos en prof.dr.ir. R.L.M. Pierik, worden genoemd. Hun opbouwende commentaren hebben vooral een duidelijke invloed gehad op de eindversie van dit proefschrift. De directie van het het Instituut voor de Veredeling van Tuinbouwgewassen ben ik erkentelijk voor haar aanmoediging dit werkstuk weer ter hand te nemen en af te ronden. Vooral Hans Dons heeft mij in het laatste anderhalf jaar met wijze raad bijgestaan. Jan Bergervoet maakte het mij mogelijk regelmatig tijd aan het schrijven van dit proefschrift te besteden door zeer zelfstandig mijn huidige onderzoek te behartigen.

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Aan de uiteindelijke totstandkoming van het manuscript hielpen mee Gerrit Berends die behulpzaam was bij de Engelse tekst, José Zeevat-van Homelen die al het typwerk uitvoerde en Arie Bergwerff en Reijer Jansen die op vakkundige wijze het teken- en fotowerk verzorgden.

CONTENTS

GENERAL INTRODUCTION

TOWARDS A MORE GENERAL APPLICATION OF IN VIVO ADVENTITIOUS SHOOT FORMATION

Adventitious shoots develop from secondary meristems, i.e. from fully differentiated tissue, which has resumed meristematic activity (Hartmann and Kester, 1983; Haccius and Hausner, 1975). This process is also called shoot regeneration. Conditions which give rise to the initiation of adventitious shoots generally are removal of all existing buds from a plant or isolation and culture of plant parts without buds, for instance leaf and root cuttings.

The regeneration of shoots in vivo has been studied by numerous scientists for many years. Early publications were mainly descriptive (e.g. Regel, 1896; Rechinger, 1894; Hartsema, 1926; Prévot, 1948) and gave several explanations for the process of regeneration (e.g. Sachs, 1880; Rechinger, 1894; Kupfer, 1907; Haberlandt, 1913; Simon, 1920; Prévot, 1939). Later papers reported on studies dealing with physiological aspects of regeneration (e.g. Rünger, 1959; Heide, 1964, 1965a, b; Harris and Hart, 1964; Pierik, 1967, Appelgren and Heide, 1972; Miedema, 1973). Little is recorded about the practical application of adventitious shoot formation in vivo, only in more recent publications this aspect is getting some attention (e.g. Marston, 1962; Heide, 1964; Miedema, 1967; Broertjes et al., 1968). In practical horticulture, however, adventitious shoot formation in vivo is hardly used (cf. Hartmann and Kester, 1983). This obvious neglect is accentuated by the increasing number of positive results of application of shoot regeneration in vitro (Murashige, 1974). In the early seventies this raised the question whether adventitious shoot formation in vivo could be improved and applied more generally in horticulture both for vegetative propagation (Van Bragt, 1974) and for rapid production of solid, i.e. chimaera-free, mutants (Broertjes et al., 1968).

The general aim of the present research was to ascertain whether adventitious shoot formation in vivo, especially on

leaf cuttings, could be applied in horticulture on a large scale. Would it be applicable to important species, especially those in which conventional techniques permit only slow increases in number? Would it yield a high rate of multiplication? Would it produce uniform plants of a selected genotype, without the occurrence of variants or genetically aberrant types? Would it, eventually, become a commercially successful application? Before the latter questions could be answered, however, at first it should be demonstrated that shoots could regenerate in vivo with relative ease. This was the primary objective of the experiments described in this thesis.

ACTUAL STATE OF IN VIVO ADVENTITIOUS SHOOT FORMATION ON LEAF CUTTINGS, IN HORTICULTURAL PRACTICE AND IN SCIENTIFIC RESEARCH

In commercial horticulture of the early seventies no special cultural methods were used in propagation by leaf cuttings of plants like *Begonia rex*, *B. cheimantha*, ('Gloire de Lorraine'-begonias), *Saintpaulia ionantha*, *Streptocarpus* spp., *Pavonia intermedia*, and with flower bulb crops like *Hyacinthus orientalis* and *Lilium* spp. (cf. Hartmann and Kester, 1983). With 'Gloire de Lorraine'-begonias for instance, it was known that shoot regeneration was improved by short day conditions (Rünger, 1957a; Heide, 1964, 1965a), but application of artificial short day treatments was not necessary as autumn and winter were practically the only seasons for commercial propagation. In the same begonias cytokinins could promote adventitious shoot formation (Heide, 1965b), but for the same reason as above this did not lead to practical application. Also in adventitious shoot formation used for rapid production of chimaera-free mutants no specific aids were used (see Broertjes, 1969, 1972a, b; Broertjes and Alkema, 1970; Broertjes and Leffring, 1972; Doorenbos and Karper, 1975).

While in actual practice leaf cuttings were still handled in a classical way, fundamental research had already yielded valuable information on factors influencing regeneration on these cuttings. The genotype was shown to have a clear effect (e.g. Heide 1965b; Bigot, 1966; Broertjes and Leffring, 1972) and breeding for easy propagation by leaf cuttings could be carried out with success (Karper, 1971). The physiological condition of the stock plants which supplied the leaf cuttings appeared to be important, but the effects differed depending on the species used (e.g. Prévot, 1939; Wellensiek, 1961; Heide, 1965b; Pierik,

1967; Broertjes en Leffring, 1972). The environmental factors light and temperature had very pronounced effects in most species studied (e.g. Rünger, 1959; Heide, 1964, 1965a; Appelgren and Heide, 1972; Broertjes and Leffring, 1972; Cohl and Moser, 1976a; Von Hentig, 1976).

The largest effects were obtained with application of plant growth regulators. Cytokinins were often found to stimulate regeneration of shoots on leaf cuttings (Plummer and Leopold, 1957; Schraudolf and Reinert, 1959; Heide, 1965b; Bigot, 1966; Prévot, 1967; Bigot and Chlyah, 1970). Heide (1972), in a review article, mentioned three types of operation of cytokinin and auxin relevant to in vivo organogenesis:

1. Determination of the type of adventitious organs by the ratio cytokinin/auxin. This reaction pattern fully agrees with the concept of Skoog and Miller (1957), that the cytokinin/auxin ratio determines the type of organ formation; a high ratio promotes shoot regeneration and a low one root regeneration. Heide, who observed this reaction pattern in his own work with 'Gloire de Lorraine'-begonias (Heide, 1965b) judged it the most common regulatory mechanism of organogenesis.
2. Stimulation of shoot regeneration by low auxin concentrations, whereas high concentrations result in root formation and inhibition of shoot formation (see Chouard, 1938; Mayer, 1956; Stichel, 1959; Wirth, 1960). Heide regarded stimulation of adventitious shoot formation by low auxin concentration as fully compatible with the concept of Skoog and Miller (1957). He assumed that the plant material in question has a high level of endogenous cytokinin and that auxin acts as the limiting factor (Appelgren and Heide, 1972). Dore (1965), however, considered the auxin model to be more general than the cytokinin/auxin interaction model. He stated that the type of organ formation is determined by the concentration of auxin alone, with cytokinin affecting only the number of organs to be formed. Also later on it was regularly found in studies in vitro that auxin was the major factor in regulating bud regeneration (Pierik and Steegmans, 1975; Hussey, 1976, 1982; Van Aartrijk and Blom-Barnhoorn, 1981; Van Aartrijk et al., 1985).
3. Stimulation of root formation as well as bud formation by auxin, cytokinin being inhibitory to both, in those cases where root formation is a prerequisite for shoot regeneration (Harris and Hart, 1964; Pierik, 1967). Heide regarded this reaction pattern as exceptional.

It was suggested that developments and experiences in adventitious shoot regeneration in vitro may give important indications for the improvement of regeneration in vivo (Van Bragt, 1974). The in vitro culture techniques used for shoot regeneration generally are characterized by aseptic conditions, defined nutrient media, small explants with a large wound surface, and relatively low doses of regulators. These traits, however, are not easily applicable to the in vivo conditions. Also with regard to the actual basis of regeneration of adventitious shoots and regulation mechanisms behind it, cultures of small pieces of tissue in vitro, in the early seventies, gave little more information on the role of the regulators in the process of regeneration than leaf cuttings in vivo did. In contrast to data from in vivo conditions, however, those in vitro generally confirm the cytokinin/auxin interaction hypothesis of Skoog and Miller (1957). Notwithstanding several species are known in which a high cytokinin/auxin ratio or the ommission of auxin from the medium failed to induce shoot regeneration in vitro (see Reinert, 1973; Murashige, 1974; Street, 1977; Gresshoff, 1978).

SCOPE OF THE INVESTIGATIONS

Commercial application of in vivo propagation by leaf cuttings is the overall goal of the present research. At first sight regulators, especially cytokinins, offer the best prospects of reaching that aim. As, however, extensive information about the occurrence of adventitious shoot formation in vivo in horticultural plants and about general methods to induce it is still lacking, experiments were started at that very beginning.

The following research was done:

Chapter 1 describes experiments carried out to answer the questions (1) how many horticultural plants may regenerate shoots on leaf cuttings? and (2) may cytokinin induce regeneration of shoots? It was thought to be easy to obtain shoots by application of exogenous cytokinin, but the reverse appeared to be true.

Chapter 2 describes experiments with model species like *Nicotiana alata* and *Lunaria annua* for testing the reliability of the procedures used for the application of cytokinin. The results presented evidence that one species can exhibit two types of shoot regeneration in reaction to regulators.

Chapters 3, 4 and 5 describe experiments with chrysanthemum (*Chrysanthemum morifolium*) cvs. Bravo and Super Yellow as models of a genotype with moderate and one with no adventitious shoot formation respectively. Good shoot formation could be induced by a combination of methods very much like those used for in vitro induction of adventitious shoots. The low rate of success and the complexity of the procedure, however, would not make it a successful commercial method.
Chapters 6 and 7 describe experiments with the so-called 'T-group' of 'Elatior'-begonias (*Begonia x hiemalis*). These experiments were started when the initial goal, a system for many important horticultural plants, had been given up. Generally, begonias are easily propagated by leaf cuttings. Heide (1964, 1965a, b) presented a clear picture of the relationships between the various factors involved in shoot regeneration in 'Gloire de Lorraine'-begonias. In practice it was assumed that the same conclusions held true also for other *Begonia* hybrids (Karper, 1971; Goldschmidt, 1974). The aim of our study was to check whether this opinion was correct. The results showed that commercial leaf cutting propagation in 'Elatior'-begonias can be drastically improved.

The investigations described in this thesis were carried out from October 1972 till the end of December 1975. During the following two years the results were analysed. It should be kept in mind that the statement of the problem is typically a product of the early seventies. The experiments have been designed and carried out on the basis of knowledge available at that time.

CHAPTER 1

REGENERATION OF LEAF CUTTINGS OF HORTICULTURAL PLANTS IN VIVO, WITH OR WITHOUT EXOGENOUS CYTOKININ

INTRODUCTION

In horticulture only a small number of species is propagated by leaf cuttings (Wellensiek and Doorenbos, 1956; Hartmann and Kester, 1983). The question arose whether this method of propagation could be applied to a larger number of species, especially those which are difficult to propagate vegetatively so far. This raised two sub-questions, (1) which horticultural species and cultivars can spontaneously form shoots on leaf cuttings?, and (2) might a simple application of cytokinin stimulate the formation of adventitious shoots, especially with species which are reluctant or unable to regenerate shoots spontaneously?

The most extensive survey of regeneration on leaf cuttings was made by Hagemann (1932). He listed over 300 species with the capacity to regenerate shoots, partly found by his own experiments and partly deduced from literature. In a more recent review covering over 100 publications Broertjes et al. (1968) added about 60 species to this list. These authors concluded that successful regeneration of leaf cuttings is relatively widespread among Angiosperms, especially among Dicotyledons. They also concluded, however, that it cannot be considered as a systematic characteristic.

Both the surveys list only very few important horticultural species which are normally propagated vegetatively, e.g. *Begonia* spp., *Saintpaulia ionantha* and hyacinth. So in a large number the ability to regenerate shoots on leaf cuttings in vivo has not yet been demonstrated, e.g. in rose, carnation, chrysanthemum, tulip, *Freesia*, *Gerbera*, *Cyclamen*, *Rhododendron*, *Clematis*, *Cotoneaster*, apple, pear, strawberry and many others. Therefore, we judged it wise to examine first the ability for shoot formation in a number of these plants.

The first part of this chapter describes an experiment in which shoot regeneration on leaf cuttings was studied in various plant species. Not only economically the most important vegetatively propagated plants, but several trees, shrubs, per-

ennials, and ornamental plants, and also some sexually propagated plants were included. The aim was te get an idea of the frequency of occurrence of adventitious shoot formation in higher plants. In all the species included the ability for spontaneous shoot formation was studied. This implied shoot formation on untreated leaf cuttings as well as that on cuttings pretreated with auxin. Separately, in a restricted number of the species leaf cuttings were also treated with cytokinin.

The second part of this chapter describes experiments in which different ways of applying cytokinin were studied to see if shoot regeneration would depend on the method of application.

MATERIAL AND METHODS

Origin of the material. The leaf cuttings used were from different origin:

1. From perennials, shrubs and trees grown in the Botanic Gardens of the Agricultural University in Wageningen (AU) and in the gardens of the commercial nurseries Darthuizer in Leersum and Schiphorst in Wageningen collecting was done from early August to mid October 1972;
2. From perennials and shrubs propagated in a greenhouse of the Department of Horticulture (AU) collecting was done in winter and spring of 1973;
3. From typical glasshouse crops grown at the Research Station for Floriculture at Aalsmeer, the Institute for Horticultural Plant Breeding in Wageningen, the Department of Horticulture (AU), and at various commercial holdings collecting was done in different seasons of 1973.

The leaf cuttings were excised by a cut through the base of the petiole or the leaf blade. So they were made as long as possible.

Leaf cuttings from 172 species and cultivars altogether were planted, each taxon represented by at least 20 cuttings. In 40 species leaf cuttings were soon lost, partly by wilting and partly by rotting. This left 132 taxa for study. Fourteen species were already known to be capable of regenerating shoots, as they were in the list of Broertjes et al. (1968).

For the investigation of the methods of application of cytokinin leaf cuttings were used from *Gerbera jamesonii* 'Wageningen Rood', *Buddleia davidii* 'Cardinal' and *Capsicum annuum* 'Verbeterde Westlandse'. All the leaf cuttings were from young

just full-grown leaves. These experiments were carried out during spring.

Growing conditions of the leaf cuttings. The leaf cuttings were planted in a greenhouse at the Department of Horticulture (AU) in a mixture of sand and peat (1:2) in benches covered with Dutch lights, which in case of strong solar radiation were shaded with cheesecloth. After rooting the cuttings were transplanted in 9 cm plastic pots containing a sieved garden compost of clay, peat and manure, and then placed on greenhouse tables. The temperature in the greenhouse was set at 19°C D/17°C N. On sunny days temperature in the greenhouse regularly reached 30°C and more, but in the shaded benches it did not rise above about 21°C.

Plant growth regulator treatments. In the experiments on the frequency of spontaneous adventitious shoot formation, the bases of 10 leaf cuttings of each taxon were dipped in a mixture of NAA 0.2% or IBA 1% in talc to facilitate rooting. It was supposed that rooting could be a prerequisite for shoot regeneration. Ten cuttings were planted untreated. The effect of cytokinin application was studied in a part of the species and cultivars: in 47 taxa 10 cuttings were dipped in a mixture of BA 2% + adenine 2% in talc just prior to planting, and in 36 taxa 10 cuttings, which had rooted well after six weeks, were treated with a mixture of BA 2% + adenine 2% in lanolin paste. This mixture was applied to a wound made on the petiole or at the base of the leaf blade. Adenine was used to improve the cytokinin effect (cf. Nitsch and Nitsch, 1967).

In the experiments on the methods of application of cytokinin, a talc mixture with 0.5, 1 or 2% of both BA and adenine was applied to the leaf cuttings just before planting. Two weeks after transplanting, other samples of cuttings were treated with cytokinin in the following ways: (1) 0.05 ml of a lanolin mixture with 1 or 2% of both BA and adenine was spread on a wound on the petiole or at the base of the leaf blade, (2) 0.2 ml of an aqueous solution with 2, 10 or 50 mgl^{-1} of both BA and adenine was applied in a cotton plug covering this wound, (3) 5 ml of the same solutions was applied per pot as a root drench, and (4) about 0.5 ml of the same solutions was sprayed per leaf blade. Procedure (1) was also carried out 4, 6 and 8 weeks after transplanting. In another sample it was repeated every two weeks. Each treatment involved 10 cuttings of each of the species.

Observations. Root and shoot regeneration were determined regularly. The criterion for a shoot was the presence of at least one visible leaflet.

RESULTS

Frequency of occurrence of adventitious shoot formation. Spontaneous shoot formation was observed in 30 of the 132 species and cultivars investigated. They are listed in Table 1 according to family and in alphabetical order. For five families (indicated in the Table by +) this is the first time that shoot regeneration is described. Totally 13 species (also indicated by +) were not represented in earlier lists (cf. Broertjes et al., 1968). Roots always appeared first and then the shoots, except in hyacinth 'Anne Marie' which first formed shoots and after that roots, and hyacinth 'Jan Bos' which formed both almost simultaneously. In all species shoots developed on the control as well as on the auxin-treated leaf cuttings, except in the two *Rubus* cultivars which only formed shoots on the auxin-treated cuttings and always on the roots. This is category III after Winkler (1903). In two cases only one adventitious shoot was observed, viz. in *Hibiscus syriacus* (after 4 months) and in *Viburnum burkwoodii* (after 14 months).

The 102 species and cultivars which did not show regeneration of shoots are listed in Table 2. In spite of the auxin pretreatment, several species and cultivars rooted very poorly (< 25% after 6 weeks, indicated in the table by ±) or even not at all (indicated by -). As for the shrubs and trees, this was probably due to the fact that the leaves were collected in late summer and early autumn and may have been senescent.

Two species, *Iberis sempervirens* and *Capsicum annuum* showed no shoot regeneration, although this has been reported elsewhere (cf. Broertjes et al., 1968).

Cytokinin treatment of the leaf cuttings. Cytokinin application just prior to planting reduced or prevented rooting of the leaf cuttings, yet most cuttings survived for a very long period. A beneficial effect as a result of cytokinin application on shoot formation was only observed in four species, viz. *Rudbeckia laciniata* 'Goldquelle', *Ruta graveolens*, *Gratiola officinalis* and *Verbascum* hybr. 'Olympicum'. All these species also regenerated shoots both in the control and in the auxin treatment (Table 1). After cytokinin treatment more shoots were formed, but shoot formation did not occur earlier. In *Ruta graveolens*

Table 1. Survey of species and cultivars which showed spontaneous shoot regeneration on leaf cuttings. Totally 132 species and cultivars were tested each comprising 20 cuttings. Families and species for which shoot regeneration is described for the first time (cf. the list by Broertjes et al., 1968) are indicated by +.

Family name		Species	Cultivar
+ Boraginaceae	+	*Cynoglossum nervosum*	
+ Buddleiaceae	+	*Buddleia davidii*	an unnamed hybrid
+ Caprifoliaeae	+	*Viburnum burkwoodii*	
Compositae	+	*Chrysanthemum morifolium*	'Bravo'
	+	*Coreopsis lanceolata*	'Zonnekind'
		Rudbeckia laciniata	'Goldquelle'
			'Herbstwald'
Cruciferae		*Brassica oleracea* var. *botrytis*	'Raket'
		Brassica oleracea var. *gemmifera*	'Thor'
		Brassica oleracea var. *laciniata*	'Verdura'
		Raphanus sativus	'Novired'
			'Novitas'
Gesneriaceae	+	*Ramonda myconi*	
Liliaceae		*Hyacinthus orientalis*	'Anne Marie'
			'Jan Bos'
+ Malvaceae	+	*Hibiscus syriacus*	'Duc de Brabant'
	+	*Lavatera olbea*	'Rosea'
+ Ranunculaceae	+	*Anemone hybrida*	'Elegans'
Rosaceae	+	*Rubus laciniatus*	'Thornless Evergreen'
	+	*Rubus tridel*	'Benenden'
	+	*Waldsteinia ternata*	
Rutaceae		*Ruta graveolens*	
Scrophulariaceae		*Gratiola officinalis*	
		Linaria cymbalaria	
		Mimulus hybr.	'Sunset'
	+	*Verbascum* hybr.	'Olympicum'
Solanaceae		*Lycopersicon esculentum*	'Moneymaker'
		Petunia hybr.	
		Solanum dulcamara	
		Solanum nigrum	

Table 2. Survey of 102 species and cultivars which did not show shoot regeneration on leaf cuttings. Totally 132 species and cultivars were tested, each comprising 20 cuttings. Extent of root formation is indicated by +: 25-100% of the cuttings with roots, ±:less than 25% of the cuttings with roots, and -: no root formation, after six weeks.

Family name	Species	Cultivar	Rooting
Aceraceae	*Acer campestre*		+
Actinidiaceae	*Actinidia kolomikta*		+
	Actinidia polygama		+
Apocynaceae	*Vinca major*		+
Aquifoliaceae	*Ilex altaclarensis*		-
	Ilex aquifolium		-
Araceae	*Anthurium andraeanum*		-
	Anthurium scherzerianum		-
Berberidaceae	*Mahonia aquifolium*	'Atropurpurea'	-
	Mahonia pinnata		-
Bromeliaceae	*Aechmea fasciata*		-
	Vriesea splendens	'Major'	±
Buddleiaceae	*Buddleia alternifolia*		+
	Buddleia davidii	'African Queen'	+
		'Cardinal'	+
		'Empire Blue'	+
		'Purple Prince'	+
Buxaceae	*Pachysandra terminalis*		+
Caprifoliaceae	*Macrodiervilla middendorffiana*		+
	Lonicera japonica var. *halliana*		+
	Symphoricarpus occidentalis		+
	Viburnum carlcephalum		+
	Viburnum dentatum		+
	Viburnum lantana	'Aureum'	+

Table 2. Continued.

Family name	Species	Cultivar	Rooting
	Viburnum rhytidophyllum	'Holland'	+
	Weigelia florida	'Venusta'	+
	Weigelia hybr.	'Ideal'	+
Caryophyllaceae	*Dianthus barbatus*		+
	Dianthus caryophyllus	'Alice'	+
		'Orchid Beauty'	+
		'White Sim'	+
Chenopodiaceae	*Beta vulgaris* var. *rubra*	'Egyptische Platronde'	±
	Spinacea oleracea	'Maveto'	±
Compositae	*Ageratum houstonianum*		+
	Chrysanthemum morifolium	'Super Yellow'	+
	Doronicum orientale	'Magnificum'	+
	Gerbera jamesonii	'Wageningen Rood'	+
	Leucanthemum maximum		+
	Zinnia elegans		+
Cruciferae	*Iberis sempervirens*	'Snowflake'	+
Cucurbitaceae	*Cucumis melo*	'Ogen'	+
	Cucumis sativus	'Sporu'	+
		'Toska'	+
Elaeagnaceae	*Elaeagnus ebbingei*		+
	Elaeagnus umbellata		±
Ericaceae	*Rhododendron catawbiense*	'Album'	-
	Rhododendron catawbiense var. *compactum*		-
	Rhododendron simsii		±
Iridaceae	*Freesia hybrida*	'Aurora'	+
		'Ballerina'	+
	Gladiolus tubergenii	'Fair lady'	±
	Iris hollandica	'Ideal'	+
		'Wedgwood'	+
Juglandaceae	*Pterocarya rhoifolia*		+

Table 2. Continued.

Family name	Species	Cultivar	Rooting
Labiatae	*Glechoma hederacea*		+
	Salvia splendens	'Vuur van St. Jan'	+
Lauraceae	*Laurus nobilis*		+
Liliaceae	*Allium porrum*	'Herfstreus'	±
	Tulipa gesneriana	'Christmas Marvel'	-
		'Paul Richter'	-
Magnoliaceae	*Magnolia acuminata*		-
Malvaceae	*Hibiscus rosa-sinensis*		+
Oleaceae	*Forsythia intermedia*		±
Papilionaceae	*Phaseolus vulgaris*	'Prelude'	+
Plumbaginaceae	*Ceratostigma plumbaginoides*		+
Polygonaceae	*Polygonum compactum*		+
Primulaceae	*Primula malacoides*		±
Rosaceae	*Chaenomeles cathayensis*		±
	Chaenomeles superba	'Nicoline'	±
	Cotoneaster salicifolia	'Herbstfeuer'	+
		'Scarlet Leader'	+
	Cotoneaster watereri	'Cornubia'	+
	Fragaria ananassa	'Gorella'	±
		'Senga Sengana'	+
	Malus pumila	'Cox's Orange Pippin'	+
		'Golden Delicious'	±
		'Jonathan'	+
	Pyrus communis var. *sativa*	'Beurré Hardy'	+
		'Doyenné du Comice'	+

Table 2. Continued.

Family name	Species	Cultivar	Rooting
	Prunus laurocerasus	'Herbergii'	+
		'Otto Luyken'	+
	Prunus padus	'Colorata'	+
	Prunus virginiana	'Schubert'	-
	Rosa odorata	'Peer Gynt'	+
	Rosa multiflora	'Friedrich Heyer'	+
	Rosa polyantha	'Korona'	+
		'Lilli Marlene'	+
Rubiaceae	*Bouvardia longiflora*	'Albatros'	+
	Coffea arabica		+
Saxifragaceae	*Deutzia scabra*	'Plena'	+
	Deutzia schneideriana		+
	Heuchera pruhoniciaria		+
	Hydrangea sargentiana		±
	Tiarella cordifolia		+
Solanaceae	*Capsicum annuum*	'Verbeterde Westlandse'	+
	Physalis alkekengi	'Franchetii'	+
Styracaceae	*Pterostyrax hispida*		+
Umbelliferae	*Daucus carota*	'Amsterdamse Bak'	+
	Apium graveolens		±
Valerianaceae	*Centranthus ruber*		+
Verbenaceae	*Callicarpa bodinieri* var. *giraldii*	'Profusion'	±
Vitaceae	*Ampelopsis brevipedunculata*	'Variegata'	+

shoots developed before the roots. The other species listed in Table 1 did not show an increase in shoot regeneration after cytokinin treatment. No shoot regeneration was induced in species listed in Table 2.

Cytokinin application after rooting strongly increased shoot formation in *Ruta graveolens* and *Verbascum* hybr. 'Olympicum'. The cuttings from the other species did not show any effect, i.e. no improvement of shoot regeneration as far as species of Table 1 were concerned, and no shoot formation induction in species listed in Table 2. *Rudbeckia laciniata* 'Goldquelle' and *Gratiola officinalis* cuttings were not treated, as they already showed initial bud regeneration six weeks after planting.

Different methods of application of cytokinin. Shoot formation was not observed at all in *Gerbera jamesonii* 'Wageningen Rood', *Buddleia davidii* 'Cardinal' and *Capsicum annuum* 'Verbeterde Westlandse' leaf cuttings after application of cytokinin in various ways. With *Gerbera* and *Buddleia* only a green, compact callus-like tissue developed on the wound, to which the cytokinin was applied. With *Capsicum* only warty knots arose on the petiole and the leaf blade near the wound.

DISCUSSION

Of the 132 species and cultivars examined for their ability to regenerate shoots spontaneously on leaf cuttings, 30 exhibited a positive reaction. For 13 species it was the first time that shoot regeneration was found. This indicates that in Angiosperms the ability to form shoots on leaf cuttings has a rather low frequency of occurrence. Also later on, only incidentally new species with the ability to form shoots on leaf cuttings have been reported (Custers, 1978; Miedema, 1980).

Fourteen species were included which were already known to be capable of regenerating shoots (cf. Broertjes et al., 1968). Twelve of these also formed shoots in the present experiments. This shows that shoot regeneration in these species is readily reproducible. In the two species which failed to form shoots it is likely that other cultivars were used than in the original studies.

Application of cytokinin improved shoot regeneration only in four species, which also regenerated well in the control. This probably means that cytokinin only had a quantitative and not a qualitative effect, i.e. it only increased an already existing capacity for shoot regeneration.

After application of cytokinin by various methods, leaf cuttings of *Gerbera jamesonii*, *Buddleia davidii*, and *Capsicum annuum* did not develop any shoots. This means that not the slightest sign of induction of shoot regeneration by cytokinin was found. Also in other studies, with species which rarely formed adventitious shoots on leaf cuttings, no improvement whatsoever of regeneration could be attained by various cytokinin treatments (Van Harten, 1978; Miedema, 1980).

The results obtained with the cytokinin treatments are rather in contradiction with literature (cf. Heide, 1972). Therefore, it seems worthwhile to check in model species the activity of the regulators and the effectiveness of the methods of application used.

CHAPTER 2

EFFECTS OF CYTOKININ AND AUXIN ON IN VIVO REGENERATION OF LEAF CUTTINGS OF *BRASSICA OLERACEA*, *LUNARIA ANNUA*, *NICOTIANA ALATA* AND *RUTA GRAVEOLENS*

INTRODUCTION

Literature on regeneration generally supports the view that cytokinin may induce regeneration of shoots (Heide, 1972; Murashige, 1974). In an experiment with leaf cuttings in vivo from various horticultural plants, however, the only positive effect of cytokinin application was promotion of shoot regeneration in a few plants, which also spontaneously formed shoots (Chapter 1). This raised doubts about the activity of the regulator used and about the effectiveness of the procedures. Therefore it was decided to test the effects of cytokinin application on leaf cutting regeneration in some model species.

Nicotiana alata Link and Otto was chosen as related to *N. tabacum* L., in the pith tissue cultures of which Skoog and Miller (1957) found that the cytokinin/auxin ratio determines the type of organ formation. Leaf cuttings of *N. alata* in vivo appeared to have a greater chance to form shoots after pretreatment with auxin (Nettancourt et al., 1971). *Lunaria annua* L. was chosen because cotyledon cuttings exhibited the same reaction pattern to regulators as Harris and Hart (1964) described for *Peperomia sandersii* A. DC, viz. stimulation of root as well as bud formation by auxin, cytokinin being inhibitory to both (Pierik, 1967). *Ruta graveolens* L. was chosen because of its typical response to cytokinin in an earlier experiment, viz. regeneration of numerous shoots, whereas roots remained absent for a long time (Chapter 1). *Brassica oleracea* L. was chosen as a representative of the species in Chapter 1, which spontaneously formed shoots on leaf cuttings, but did not exhibit increase in shoot formation after treatment with cytokinin.

Besides cytokinin, also auxin and combinations of both regulators were applied to obtain a more complete picture of the regeneration patterns. Both application of cytokinin just prior to planting and application a short time after root

formation of the cuttings were studied. In the latter case, attention was given also to the effect of a wound made at the place of application. Adenine was applied regularly in combination with cytokinin, as Nitsch and Nitsch (1967) established that the cytokinin effect only became clearly manifest when adenine was added.

MATERIAL AND METHODS

Stock plants. The species were *Brassica oleracea* L. var. *gemmifera* (DC.) Schulz 'Thor' (Brussels sprouts), *Brassica oleracea* L. var. *laciniata* (L.) Schulz 'Verdura' (curly kale), *Lunaria annua* L., *Nicotiana alata* Link and Otto 'Fleur de Tabac', and *Ruta graveolens* L. Plants were grown from seeds, except *R. graveolens*, which was propagated vegetatively from shoot cuttings. They were grown in a greenhouse, in which temperature was set at 19 °C D/17 °C N, in 6 cm plastic pots with a mixture of garden peat and loamy soil, from September to April. Additional light, by Philips HPLR 400 W lamps giving about 15 Wm^{-2} at plant level, was given from November to March.

Leaf cuttings. The leaf cuttings were collected when the plants were 6-7 weeks old (12 weeks in the case of *R. graveolens*) and had formed 5-6 leaves. Only the almost and just fully developed leaves (with *L. annua* the first pair of leaves) were used. The leaves were cut as close as possible to the stem of the plant and planted in cutting benches with a mixture of sand and peat (1:2). After rooting the cuttings were transplanted in a fertile horticultural soil in 9 cm pots. The leaf cutting conditions were the same as for the stock plants.

Plant growth regulators. Combinations of 6-benzylaminopurine (BA) and indole-3-acetic acid (IAA) or 1-naphthalene-acetic acid (NAA) were applied prior to planting (unrooted leaf cuttings). BA alone or in combination with adenine was applied shortly after root formation (rooted leaf cuttings).

Generally the unrooted leaf cuttings were soaked, for 24 h, with the basal 1.5 cm of the petioles in aqueous solutions of the regulators. This was done under continuous light from Philips TL 57 fluorescent tubes giving about 30 Wm^{-2} at level of the cuttings. This method of application, however, damaged the petiole bases of *R. graveolens* cuttings, and in that case the regulators were applied in a talc mixture.

For rooted leaf cuttings the lanolin method of application

was used. A wound was made in the middle or at the basal part of the petiole of leaf cuttings which had been rooted at 23 °C for 2-3 weeks and then were transplanted into pots. This wound was greased with the regulator-lanolin mixture.

Observations. Root and shoot formation were determined periodically. Shoot formation was observed under a magnifying glass. The criterion for a shoot was the presence of at least one visible leaflet.

The number of cuttings per treatment was 20. The results presented are confirmed by the data of at least two experiments. For means the standard deviation was calculated and differences were tested for significance ($P = 0.05$) by Student's *t* test. Mean numbers of shoots were calculated for the cuttings with shoots only.

RESULTS

Various combinations of cytokinin and auxin were used in the different species. Only the most striking results are presented.

Brassica oleracea. Results of application of BA and NAA to unrooted leaf cuttings are presented in Table 1. After two weeks almost all cuttings had developed roots, irrespective of the concentration or combination used. Observation of the root system, however, showed that root mass was about 1.3 times over the control in all cases where NAA 0.1 mgl^{-1} was used, and even 1.5 times in the case of NAA 1 mgl^{-1}. BA had hardly any influence on root mass. Leaf cuttings of Brussels sprouts regenerated shoots much better than those of curly kale. After six and a half weeks it was observed in Brussels sprouts that BA decreased the percentage of shoot formation, whereas NAA increased it. In general, combinations of BA and NAA gave intermediate results. In curly kale relatively low percentages of shoot formation were obtained in treatments with NAA 1 mgl^{-1}, whereas in the other treatments shoots were rarely formed. After 11 weeks the numbers of cuttings showing shoot regeneration had slightly increased. The overall reaction pattern was comparable with that at six and a half weeks. After week 11 the pattern of shoot formation hardly changed anymore, neither in Brussels sprouts nor in curly kale. The number of shoots formed per cutting was not significantly different between the treatments. The average numbers ranged from 2.3 - 4.0 shoots.

Table 1. Effects of plant growth regulators on regeneration of leaf cuttings of *Brassica oleracea* var. *gemmifera* 'Thor' (Brussels sprouts) and *Brassica oleracea* var. *laciniata* 'Verdura' (curly kale). BA and NAA at different concentrations were applied by 24 h soaking just prior to planting. Each treatment comprised 20 leaf cuttings. R: percentage of root formation, S: percentage of shoot formation.

BA mgl^{-1}	NAA mgl^{-1}	Brussels sprouts R (%) 2 wks	Brussels sprouts S (%) after 6.5	Brussels sprouts S (%) after 11 wks	Curly kale R (%) 2 wks	Curly kale S (%) after 6.5	Curly kale S (%) after 11 wks
0	0	85	35	60	65	0	0
0.1	0	100	15	45	85	0	0
1	0	95	15	25	80	0	0
0	0.1	100	40	55	80	0	0
0.1	0.1	100	25	35	100	6	6
1	0.1	100	15	40	95	10	10
0	1	100	74	79	100	25	30
0.1	1	100	60	85	85	21	26
1	1	100	10	20	100	15	20

Table 2. Effects of cytokinin BA on shoot regeneration of leaf cuttings of *Brassica oleracea* var. *gemmifera* 'Thor' (Brussels sprouts) and *Brassica oleracea* var. *laciniata* 'Verdura' (curly kale). After root formation different concentrations of BA mixed in lanolin were applied to a wound in the middle of the petiole. Each treatment comprised 20 cuttings. The data were collected after 11 weeks, when percentages of shoot formation did not change anymore. S: percentage of shoot formation, MS: mean number of shoots, calculated for the cuttings with shoots.

Wound	BA (%)	Brussels sprouts S (%)	Brussels sprouts MS	Curly kale S (%)	Curly kale MS
-	-	35	1.8*	0	-
+	0	35	2.0	5	2*
+	0.5	25	1.6	0	-
+	2	25	2.4	5	1

* Means were not different at P = 0.05

Rooted leaf cuttings were treated four and a half weeks after planting. BA in lanolin was applied on a wound in the petiole just above the soil. Table 2 presents the results. The BA treatment affected neither the percentage of shoot formation nor the number of shoots. The shoots always developed from the petiole base and not from the wound where BA was applied. BA application to the petiole base did not improve shoot formation either.

Lunaria annua. Table 3 presents results of application of BA and IAA to unrooted leaf cuttings. Increasing the concentration of BA, alone or in combination with IAA, decreased both rooting percentage and percentage of shoot formation, but did not affect the number of shoots formed. After week 8 the percentages of shoot formation gradually increased to 100% in all treatments. If NAA was used instead of IAA (data not presented), rooting was promoted and shoots arose earlier as compared with the control, but also NAA did not influence the number of shoots. The shoots always developed from the lower part of the petiole near the places where the roots emerged.

Table 3. Effects of plant growth regulators on regeneration of leaf cuttings of *Lunaria annua*. BA and IAA at different concentrations were applied by 24 h soaking just prior to planting. Each treatment comprised 20 leaf cuttings. R: percentage of root formation, S: percentage of shoot formation, MS: mean number of shoots, calculated for the cuttings with shoots.

BA mgl^{-1}	IAA mgl^{-1}	R (%) 2 wks	S (%) 8 wks	MS 8 wks
0	0	80	80	3.0 *
0.1	0	75	80	3.3
1	0	50	45	4.0
0	0.1	100	95	3.4
0.1	0.1	70	85	4.3
1	0.1	45	50	4.3
0	1	100	85	3.3
0.1	1	75	60	4.4
1	1	60	40	3.9

* Means were not different at P = 0.05.

Rooted leaf cuttings were treated with a mixture of BA + adenine which was applied to a wound about 1 mm deep and 0.5-1 cm long at the side of the lower part of the petiole. When making this wound 10-30% of the roots were cut away. Table 4 presents the results of an experiment in which the cuttings were treated at different times after planting. BA + adenine hastened shoot formation and increased the number of shoots, but wounding alone also contributed considerably to this. The time of the treatment had no effect. The increase in the number of shoots as a result of wounding was in one case significant at P = 0.05, in the other cases it was not. The same was true for the treatment with cytokinin. Higher concentrations, 1 or 2%, for both BA and adenine did not further increase the number of shoots produced (data not presented). As a result of the cytokinin treatment, shoots arose not only from the lower part of the petiole, but also from the root necks which were markedly swollen.

Table 4. Effects of wounding and application of cytokinin on shoot regeneration of leaf cuttings of *Lunaria annua*. At different times after planting rooted cuttings were wounded at the lower petiole end and treated with BA 0.5% + adenine 0.5% in lanolin. Each treatment comprised 20 cuttings. S: percentage of shoot formation, MS: mean number of shoots, calculated for the cuttings with shoots.

Wound	BA + ad.	Days from planting	S (%) after		MS
			6	8 wks	8 wks
-	-		50	90	3.3 a*
+	-	22	95	95	4.6 ab
+	+	22	95	100	9.0 d
+	-	28	70	95	6.1 bcd
+	+	28	80	100	9.1 d
+	-	34	60	95	5.3 abc
+	+	34	50	100	7.9 cd

* Means designated by the same letter are not significantly different from each other at P = 0.05.

Nicotiana alata. Unrooted tobacco leaf cuttings were not found to be easy experimental material. They showed early senescence and leaf blade tissue in contact with soil readily decayed. Results of an experiment using this material are shown in Table 5. A BA concentration of 20 mgl^{-1}, applied alone as well as in combination with NAA, was detrimental to rooting. BA at 0.2 mgl^{-1}, alone or in combination with NAA, and NAA alone improved rooting. After week 5 the percentages of rooting did not change any more, and all leaf cuttings which had not rooted at that time gradually died. In general, shoot formation was affected in a similar way as root formation, except in the treatment with NAA 20 mgl^{-1}, which combined good rooting with moderate shoot formation. After 12 weeks all the remaining leaf cuttings showed shoot formation. In the treatments in which BA at 0.2 mgl^{-1} was combined with NAA shoot number was significantly higher than in the control.

Table 5. Effects of growth regulators on regeneration of leaf cuttings of *Nicotiana alata* 'Fleur de Tabac'. BA and NAA at different concentrations were applied by 24 h soaking just prior to planting. Each treatment comprised 20 leaf cuttings. R: percentage of root formation, S: percentage of shoot formation, MS: mean number of shoots, calculated for the cuttings with shoots.

BA mgl^{-1}	NAA mgl^{-1}	R (%) after		S (%) after		MS
		1.5	5 wks	6.5	8 wks	12 wks*
0	0	35	80	0	20	5.8
0.2	0	60	100	20	85	7.4
20	0	0	15	0	0	2.0
0	0.2	70	100	45	90	7.8
0.2	0.2	90	100	15	70	11.1**
20	0.2	0	40	0	0	2.2
0	20	100	100	25	50	8.6
0.2	20	100	100	25	75	10.7**
20	20	0	10	0	0	2.0

* Only the cuttings with roots after five weeks had formed shoots.

** Means significantly higher than the control mean at P = 0.05.

Table 6. Effects of wounding and application of cytokinin on shoot regeneration of leaf cuttings of *Nicotiana alata* 'Fleur de Tabac'. After root formation a mixture of BA 2% + adenine 2% in lanolin was applied to a wound on the petiole base. All the cuttings were given NAA 2 mgl^{-1} as a 24 h soak just prior to planting. Each treatment comprised 20 cuttings. S: percentage of shoot formation, MS: mean number of shoots, calculated for the cuttings with shoots.

Wound	BA + ad.	S (%) after		MS after	
		6.5	8 wks	6.5	8 wks
-	-	30	80	2.0 a	3.8 a
+	-	35	90	1.7 a	4.2 a
+	+	95	100	6.4 b	10.9 b

* Within each column means designated by the same letter are not significantly different from each other at P = 0.05.

Results of application of BA 2% + adenine 2% after root formation, to a wound on the lowest part of the petiole between the roots, are presented in Table 6. The leaf cuttings used for this experiment were pretreated with NAA 2 mgl^{-1} (as a 24 h soak) to stimulate rooting and diminish loss of vitality. The BA + adenine treatment hastened regeneration of shoots and significantly increased their number.

The same mixture of BA 2% + adenine 2% applied to a wound on the midrib about 2 cm above the leaf blade base had much less effect. First a greenish callus developed. Six weeks after the treatment sometimes a few small shoots were visible, but at that moment shoots from the bases of the same cuttings were already 2.5 cm long. A wound alone had no effect, neither on the base (see Table 6) nor on the midrib of the leaf cuttings.

Ruta graveolens. In this species it was shown that an increase in the concentration of BA, applied to unrooted leaf cuttings, decreased the percentage of rooting but increased the percentage of shoot formation and the mean number of shoots (Figure 1). Application of adenine together with BA was found to counteract the suppression of rooting by BA and to increase the initial percentage of shoot formation. Both effects, however, were visible only for a short period of time. NAA 0.1 and 1% in

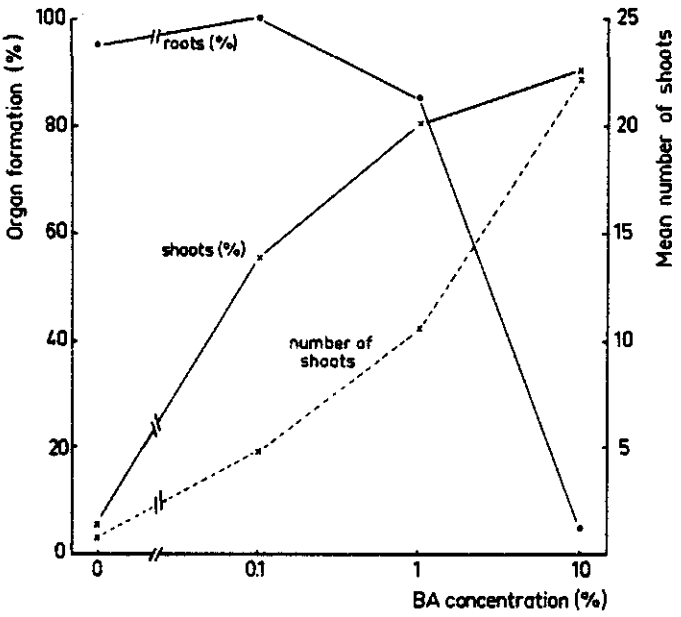


Figure 1. Effects of cytokinin application on regeneration of leaf cuttings of *Ruta graveolens*. BA at different concentrations was applied as a talc dip just prior to planting. Each treatment comprised 20 leaf cuttings. The data were collected after seven and a half weeks.

talc (data not presented in detail) were found to stimulate rooting, but did not affect shoot formation (10 and 0% respectively after seven and a half weeks).

Rooted leaf cuttings were treated with BA 0.5, 1 or 2% in lanolin applied to the bases of the leaf cuttings. These treatments promoted shoot formation from the petiole bases. When applied in the middle of the petiole, shoot formation also occurred at that place. With increasing concentrations more callus-like tissue and more shoots were formed. Wounding alone seemed slightly promotive to shoot regeneration, but this was only the case with a wound on the petiole base.

DISCUSSION

Both cytokinin and auxin clearly affected the rate of shoot formation and the number of shoots formed, either positively or negatively. Especially cytokinin application a short time after root formation of the cuttings generally was very promotive to shoot regeneration. Thus the chemicals used were active and the procedures effective. Doubts on this point (Chapter 1) were unfounded.

Wounding alone strongly promoted shoot formation of *Lunaria annua* leaf cuttings. Recently a similar influence was found in studies on in vitro regeneration of *Lilium* bulb-scale explants, and this influence was supposed to be related to ethylene biosynthesis (Van Aartrijk and Blom-Barnhoorn, 1983, 1984). Indeed ethylene was found to play a role in regeneration processes (Imaseki, 1985). Exogenously applied it promoted shoot regeneration, but this action appeared to depend on the presence of other regulators in the medium and also on the plant species used (Cornejo-Martin et al., 1979; Van Aartrijk et al., 1985; Pérez-Bermúdez et al., 1985). The obviously secondary role of ethylene in shoot regeneration might explain why wounding did not promote shoot regeneration in all species in the present study.

In analogy to the results of Nitsch and Nitsch (1967) it was expected that addition of adenine to the cytokinin would be beneficial. Actually such favourable effects were found, both on root and shoot formation (not presented in detail), but generally they were rather slight and temporary, and they depended on the species used.

The reaction patterns of regeneration in the four species studied were different from each other. They showed similarities with reaction patterns found earlier (see review by Heide, 1972), but there were also contradictions with earlier findings.

The leaf cuttings of *Brassica oleracea* var. *gemmifera* 'Thor' and *Brassica oleracea* var. *laciniata* 'Verdura' showed a regeneration pattern which closely resembled that of *Peperomia sandersii* (Harris and Hart, 1964), in which cytokinin was shown to be inhibitory to both root and shoot formation, whereas auxin was stimulating. With the 'Verdura' leaf cuttings a pretreatment with auxin even seemed to be essential for shoot regeneration. Possibly, 'Verdura' needs a greater number of roots or a greater root activity for shoot regeneration than 'Thor'. Cytokinin treatment of the rooted leaf cuttings had no adverse

effect on shoot regeneration. Harris and Hart (1964) found the same. So cytokinin inhibits shoot formation only through inhibition of root initiation.

Unrooted leaf cuttings of *Lunaria annua* showed rather the same regeneration pattern as found by Pierik (1967) with cotyledon cuttings. This is the reaction pattern as in *Peperomia sandersii* (Harris and Hart, 1964). In rooted leaf cuttings, however, cytokinin treatment improved shoot formation. This effect fits in with the reaction pattern of the tobacco pith cultures of Skoog and Miller (1957).

Regulator treatments of unrooted leaf cuttings of *Nicotiana alata* showed a rather complex reaction pattern. The following physiological processes may have been concerned in it: (1) inhibition of both root and shoot formation by high concentration of cytokinin and the reverse by low concentration of auxin; this is the reaction type as described by Harris and Hart (1964), (2) stimulation of rooting and inhibition of shoot formation by high concentration of auxin, and the reverse, at least with respect to the number of shoots, by low concentration of cytokinin in combination with auxin; this is the reaction type as described by Skoog and Miller (1957), (3) counteraction of senescing of the leaf tissue by low concentration of cytokinin (cf. Mothes, 1960), which enhances the chance of rooting and thereby the chance of shoot formation, and (4) stimulation of root formation by low concentration of auxin and thereby maintenance of vitality and a higher chance of shoot formation. The latter two types of regulator actions influence shoot regeneration only indirectly. Cytokinin treatment of the rooted cuttings showed only one effect, namely improvement of shoot regeneration, which is typical of the pattern described by Skoog and Miller (1957).

The leaf cuttings of *Ruta graveolens*, whether unrooted or rooted, only showed the Skoog and Miller (1957) reaction type. The favourable effect of high concentration of cytokinin on shoot regeneration was also evident without previous initiation of roots.

In the sequence from *Brassica oleracea* via *Lunaria annua* and *Nicotiana alata* to *Ruta graveolens*, the regeneration reaction of the leaf cuttings to regulator application shifted from exactly the model of Harris and Hart (1964) to exactly the model of Skoog and Miller (1957). With *Lunaria annua* and *Nicotiana alata* reaction types according to both models were found. With *Lunaria annua* the Harris and Hart model was found in the unrooted leaf cuttings, and the Skoog and Miller model in the

rooted leaf cuttings. With *Nicotiana alata* both models occurred in the unrooted leaf cuttings. It is for the first time that the reaction types according to both models were found together in the same plant material.

In the same series as above the capacity of shoot regeneration seems to increase. Promotion of shoot formation by cytokinin was found for *Lunaria annua*, *Nicotiana alata* and *Ruta graveolens*, but not for *Brassica oleracea* leaf cuttings. This may mean that the promoting effect of cytokinin on shoot regeneration only occurs in plants which readily form shoots spontaneously. This was also the impression obtained from the results in Chapter 1.

As an explanation of the different results after cytokinin treatment it can be postulated that a positive reaction of the leaf cuttings to cytokinin is dependent on a responsive physiological condition. It seems that in some cases such favourable condition is passed on by the stock plant, as for instance with *Ruta graveolens*. In other cases it is brought about by the roots which are initiated, as for instance with *Lunaria annua* and *Nicotiana alata*.

In conclusion, exogenous application of cytokinin and auxin appears a suitable aid to attain shoot regeneration on leaf cuttings from various plants. Adenine is probably less suitable. A positive effect can be obtained by extra wounding but this is strongly dependent on the species. In plants in which cytokinin and auxin do not induce or improve shoot regeneration it would be interesting to ascertain whether the leaf cuttings or the stock plants could be physiologically conditioned to such an extent that they become responsive to regulator treatments.

CHAPTER 3

IN VIVO SHOOT REGENERATION OF LEAF CUTTINGS OF CHRYSANTHEMUM (*CHRYSANTHEMUM MORIFOLIUM* RAM.)
I. SPONTANEOUS REGENERATION

INTRODUCTION

Most species tested did not regenerate shoots on leaf cuttings in vivo, neither spontaneously nor after application of cytokinin (Chapter 1). Hence it was decided first to concentrate on one single species, the cultivated chrysanthemum (*Chrysanthemum morifolium* Ram.). This species was chosen, as (1) adventitious shoots are of practical importance for obtaining chimaera-free mutants, (2) in vitro regeneration of chrysanthemum was studied elsewhere (Roest and Bokelmann, 1975), and (3) chrysanthemum is generally easy to handle as experimental material. Moreover, genotypes are available which readily form adventitious shoots as well as genotypes which do not (Chapter 1).

Before studying the effect of plant growth regulators on adventitious shoot formation it seemed worthwhile to characterize the pattern of spontaneous regeneration. In this chapter experiments are described on the influences of light and temperature and of the physiological condition of the plant material on the regeneration of the cultivar Bravo.

'Bravo' occasionally regenerates shoots in vivo (Chapter 1; Broertjes et al., 1976). In *Begonia*, short day conditions clearly affect regeneration (e.g. Heide 1965a; Cohl and Moser, 1976a; Powell and Bunt, 1980). Since chrysanthemum, like the *Begonia* spp. in question, is a short day plant, here too effects may be expected. Heide (1964), Appelgren and Heide (1972), Broertjes and Leffring (1972), Miedema (1973) and others described important temperature influences on the regeneration of shoots, but the effects depended on the species used. Heide (1965b) and Pierik (1967) found strong effects of the age of the cuttings. It seemed worthwhile to investigate these factors also in chrysanthemum.

MATERIAL AND METHODS

Plant material. In all experiments the cultivar Bravo, primarily grown as a pot plant was used. Sometimes 'Super Yellow', used for year round cut flower production was also investigated. To obtain disease- and pest-free material, freshly rooted shoot cuttings were regularly ordered from commercial practice (Messrs. Fides International, De Lier). These cuttings were propagated two or three times for use as stock plants.

The stock plants were grown in a greenhouse under long day conditions (LD) in 14 cm plastic pots containing a compost of clay, peat and manure. Fertilizers supplying N, P, K, Ca, Mg, S and micro elements were incorporated in the compost. Replenishment of the nutrients was done by application of a mineral solution every two or three weeks. Temperature in the greenhouse was set at 17 °C D and 15 °C N. During summer the greenhouse was shaded with whitewash, but nevertheless temperature regularly reached 30 °C and more on sunny days. During natural short day (SD) daylength was extended with MLL Philips lamps giving about 22 Wm^{-2} at plant level. To promote plant growth these lamps burnt 16 h per day during December and January.

The stock plant age at which the leaf cuttings were collected ranged from two and a half months in mid summer to about four months in mid winter. At harvest the plants had about 20 full-grown leaves.

Stage of development of the stock plants. Stock plants in different stages of development were raised by choosing different ways of propagation and by application of daylength treatments. Physiologically young vegetative stock plants were produced by the standard way of propagating chrysanthemums, the cutting-from-cutting method. To increase uniformity and minimize transfer of information from the former generation, shortly after the cuttings had rooted, the shoot tips were removed above the fourth leaf from the base, and only the lateral shoot in each upper axil was allowed to develop.

The generative stage of development was induced by transferring the plants to SD. The length of the SD period determined the number of buds which were induced to flower. Besides this usual way of inducing flowering, plants were produced with so-called crown buds. Crown buds are inflorescence buds initiated in LD, which may show some flower initiation, but fail to reach anthesis when left on the plant (e.g. Schwabe, 1950; Furuta, 1954). Such buds are generally formed by chrysanthemum

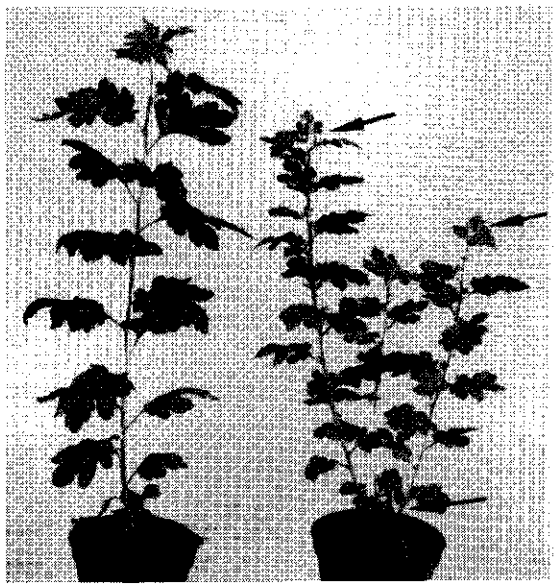


Figure 1. Different stages of development of *Chrysanthemum* 'Bravo' plants, at the left a normal vegetative plant, at the right a crown bud plant (for details see text). Arrows point to already completed and beginning crown bud formation.

plants which are in a certain state of aging after having formed numerous vegetative leaves (e.g. Furuta and Kiplinger, 1955; Cockshull, 1976; Cockshull and Kofranek, 1985). Physiologically young plants with crown buds (hereafter indicated as crown bud plants) were raised by using shoot cuttings from old plants which were just starting crown bud development. These cuttings completed the formation of the crown buds shortly after rooting and then developed 2-4 relatively slow growing side-shoots, which themselves again formed crown buds fairly soon (Figure 1).

Leaf cuttings. The leaves were excised by a cut through the lowest part of the petiole, yielding a cutting consisting of leaf blade and entire petiole. They were planted in a horticultural compost (composition as for the stock plants, but previously ground), each in a 6.5 cm pot to minimize damage to the roots from examining and repotting. The pots were placed in benches 1.3 m in width and 2-6 m in length and covered with

Dutch lights which were regularly opened for ventilation. Temperature in the benches was controlled, by heating (electric wires at the bottom) and cooling (lower temperature in the surrounding greenhouse air, whitewash on the glass outside the greenhouse and, if necessary, moistened cheesecloth on the benches), at a standard temperature of 20 °C with a maximum fluctuation of 1.2 °C. LD conditions were maintained continuously as described for the plant material.

After sufficient root formation, mostly after eight weeks, the cuttings were transferred to 9 cm pots and placed in the same greenhouse as used for the stock plants. After 2-5 weeks they were repotted again, but now with the petiole base just above soil level to facilitate examination of shoots regenerating on or near that base.

Age of the leaves. The leaves were numbered from the top of the stock plants downwards, the young 3-4 cm long leaf at the top was designated as position 1, the next as position 2, etc. The leaves at positions 3-4 were just full-grown leaves. Per treatment at least 50 leaf cuttings were used, all originating from positions 2, 3 and 4. They were spread over 5 different places in the bench to minimize location effects. In the case of stock plant treatments mostly 20 cuttings were used per treatment. Treatments of the stock plants were expected to affect the rate of leaf formation. Therefore at the beginning of the treatments the leaves at position 2 were marked to allow collecting of leaf cuttings of the same chronological age later on.

Temperature. Different temperature regimes (9, 13, 17, 21 and 25 °C) were applied under natural day length conditions in the glasshouses of the phytotron (Doorenbos, 1964) or under LD conditions in the phytotron climate rooms which were illuminated by TL 57 Philips fluorescent tubes giving about 35 Wm^{-2} at cutting level. Under both conditions the leaf cuttings were shaded with cheesecloth, which reduced the radiation to about 40%. In the text actual temperature will only be given when the mean is deviating more than 0.7 °C from the temperature adjusted.

Light intensity. Different light intensities were given by covering the stock plants or the leaf cuttings with cheesecloth or lace curtain of different mesh.

Daylength. Daylength treatments (8 or 16 h) were given in the climate rooms of the phytotron or in a greenhouse. In the

greenhouse experiments were carried out only from October till March at a temperature of 20°C D/18 °C N. For SD treatment the plants or cuttings were covered with a tent of black cloth, 1.5 m high and equipped with a ventilator, from 4.30 p.m. till 8.30 a.m., whereas for LD treatment they received daylength extension by 40 W incandescent lamps from 4.30 p.m. till 0.30 a.m. For both SD and LD material natural daylight was supplemented, from 8.30 a.m. till 4.30 p.m., whith MLL 160 W Philips lamps giving about 22 W m^{-2} at plant or cutting level.

Observations. Progress of root formation and beginning of shoot formation were determined by regular examination of the cuttings. The number of shoots developed per cutting was recorded 4-5 weeks after appearance of the first adventitious buds. The criteria of a shoot were the presence of at least two green leaves and some growth. Average numbers of shoots were calculated for the cuttings which had formed shoots.

The results presented are confirmed by the data of at least two experiments. For means the standard deviation was calculated and differences were tested for significance ($P = 0.05$) by Student's *t* test.

RESULTS

General observations. At 20 °C the leaf cuttings readily formed roots within two to four weeks. After that, the leaf blades started to grow strongly, became very thick and sometimes reached up to 15 cm in length. Nevertheless, they were bright green and flexible and looked full of vital strength. During this period which lasted two or three months, no adventitious shoots were detected. Shoot formation started only four or five months after planting of the cuttings, when the luxurious growth had declined and the leaf blades had become stiff, brittle, dark green and later dull and greyish. The first formed adventitious buds emerged from the petiole base and the roots, especially at places where the tissue had been damaged accidentally and some callus had formed. Cuttings which reacted positively, generally formed a rather constant number of buds, about two to five, of which on average two to three developed into shoots and plantlets (Figure 2).

Figure 2. Twenty two weeks old leaf cutting of *Chrysanthemum* 'Bravo' about four weeks after initiation of adventitious shoot formation.

It was striking that shoot formation was observed on 'Bravo' leaf cuttings only. Apparently 'Super Yellow' was not able to regenerate shoots under the conditions provided.

The ability of the cuttings to regenerate shoots seemingly depended strongly on the seasonal conditions. This was concluded from results of a preliminary experiment under the greenhouse conditions with five groups of leaf cuttings planted at five weeks intervals, the first group starting on April 28 (Figure 3). The formation of adventitious shoots started in the autumn in each of the first four groups. Large differences were found in the numbers of cuttings with shoots. The frequency of shoot regeneration ultimately obtained increased from very low (5%) in the first group planted in April to very high (85%) in the fourth group planted in August. Good shoot regeneration was also found in the last group of leaf cuttings which were planted on September 15. Shoot formation in this last group, however, was about ten weeks later than in the fourth group. The results suggest that shoot regeneration strongly depends on the period of the year in which the cuttings are planted. Possibly the

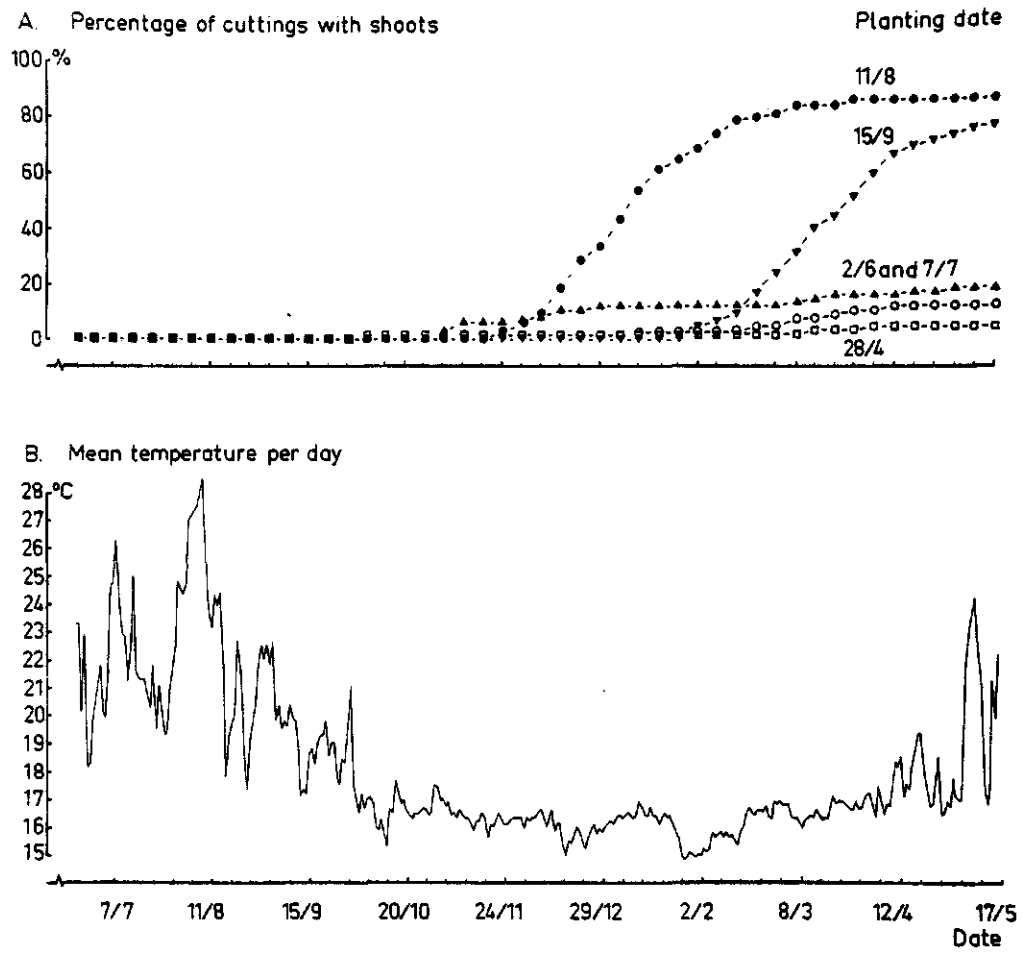


Figure 3. Shoot regeneration of *Chrysanthemum* 'Bravo' leaf cuttings as influenced by planting date. A. Time course of shoot formation in five groups of leaf cuttings each planted at five weeks intervals. Each group consisted of 60 or 80 cuttings which, after rooting at 20 °C for eight weeks, were transferred into a greenhouse. B. Mean greenhouse temperature as calculated from measurements taken every two hours.

cuttings are more inclined to regenerate shoots when collected at the end of the summer. From the course of the temperature during the experiment (Figure 3B) one might also suppose that a too long period of high temperature could impede shoot regen-

eration. The same might be the case for the factor high light intensity (not presented in detail). The winter period with moderately high temperatures and low light intensity might have caused the delay in shoot formation in the fifth group as compared with the fourth. These suppositions led to further investigation of the influences of temperature and light.

Effect of temperature. From early March on leaf cuttings were rooted for eight weeks in the phytotron glasshouses at 9, 13, 17, 21 and 25 °C. After potting they were kept in the same glasshouses until the end of August. The actual temperatures during that period were on the average 10.7, 13.6, 18.3, 22.0 and 25.0 °C. To continue the LD conditions, the cuttings were kept in the climate rooms of the phytotron from September onwards. Root formation in this experiment was strongly promoted at higher temperatures (Table 1), but after eight weeks also at the lowest temperature 100% root formation was obtained. Shoot formation showed the opposite temperature effect (Figure 4). It was almost inhibited at 21 and 25 °C, nearly 100% at 13 °C, and clearly delayed at 9 °C. So the results suggest an optimum temperature for shoot regeneration. This was confirmed by an experiment in which leaf cuttings were rooted at the same temperature (20 °C) for eight weeks and thereafter grown at the different temperatures (climate rooms). Again shoot regeneration was promoted at lower temperatures with an optimum (96%) at 13 °C.

Table 1. Root formation of *Chrysanthemum* 'Bravo' leaf cuttings at different temperatures. Each treatment comprised 100 cuttings. They are the total of the cuttings of the experiments mentioned in Figures 4 and 5.

Temperature (°C)	Percentage of root formation after		
	2	4	6 wks
9	0	50	94
13	0	98	100
17	80	100	100
21	88	100	100
25	92	100	100

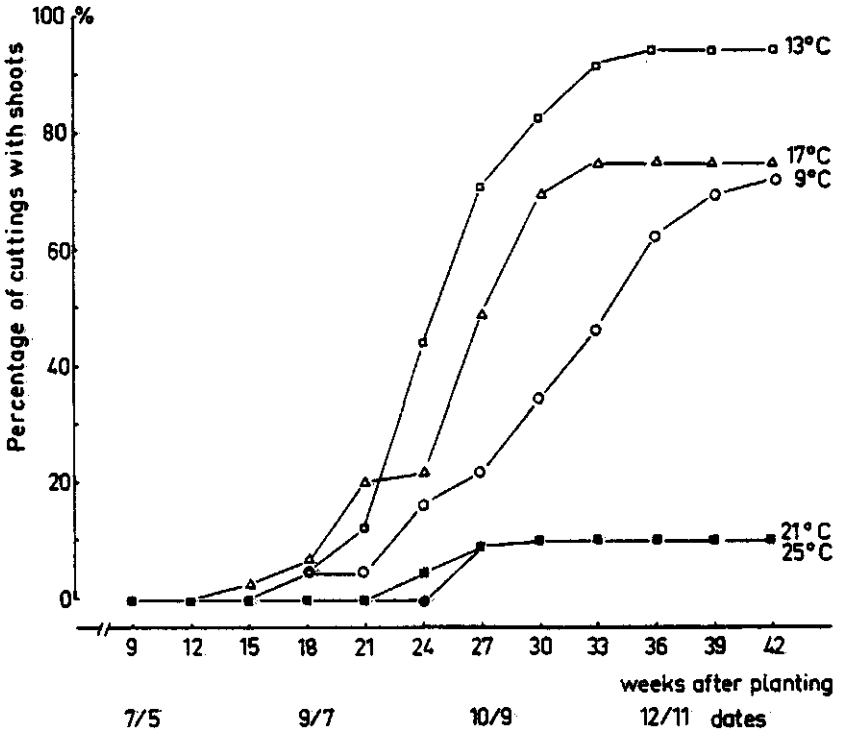


Figure 4. Time course of adventitious shoot formation on *Chrysanthemum* 'Bravo' leaf cuttings grown from planting at different temperatures. Each treatment comprised 50 cuttings from positions 2, 3 and 4.

To determine the effect of the temperature during the rooting period on the regeneration of shoots, leaf cuttings were rooted at different temperatures and after potting transferred to the greenhouse. Lower temperatures during rooting resulted in increasing shoot formation (Figure 5). Remarkably, shoot formation started earlier than when the same temperatures were given continuously (Figure 4). Since the lowest temperature (9 °C) gave the best shoot regeneration, the question arose whether rooting at even lower temperatures would further improve it. Therefore, leaf cuttings were grown for 5, 10, 15 and 20 weeks at 5 and 9 °C. Improved shoot regeneration was obtained only after pretreatment for 15 and 20 weeks at 9 °C and

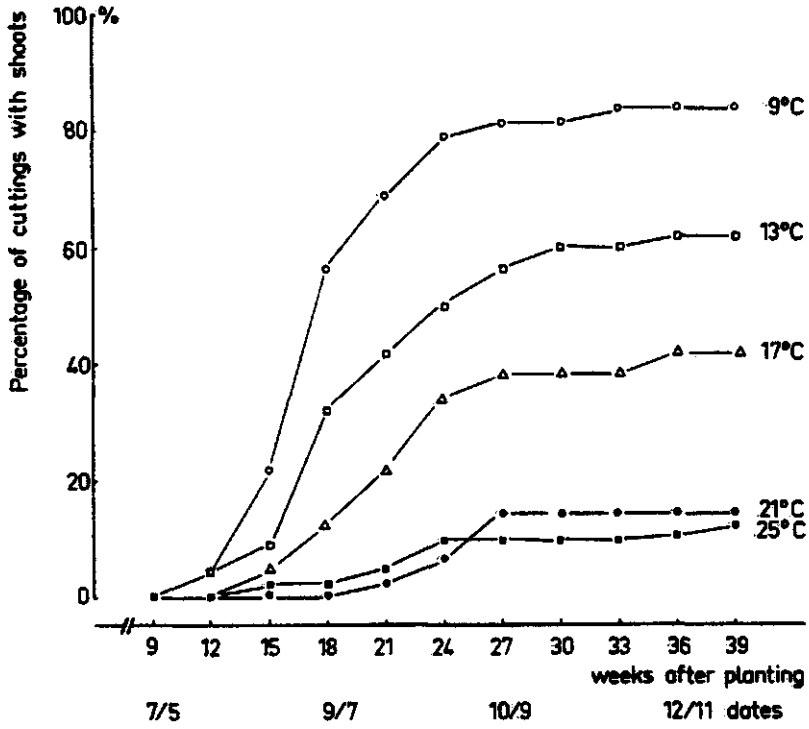


Figure 5. Time course of adventitious shoot formation on *Chrysanthemum* 'Bravo' leaf cuttings rooted for eight weeks at different temperatures and subsequently grown under standard conditions in a greenhouse. Each treatment comprised 50 cuttings from positions 2, 3 and 4. The results of root formation are presented in Table 1.

for 20 weeks at 5 °C (68, 80, and 64% respectively versus 16% in the control). At the end of the long, low temperature treatments root formation had already reached or was approaching 100%, whereas after the shorter periods only a part of the cuttings (0-85%) had rooted. It was hypothesized, therefore, that low temperature could promote shoot regeneration only if sufficient roots were present.

The beneficial effects of low temperatures in the previous experiments seemed to be restricted mainly to the early induc-

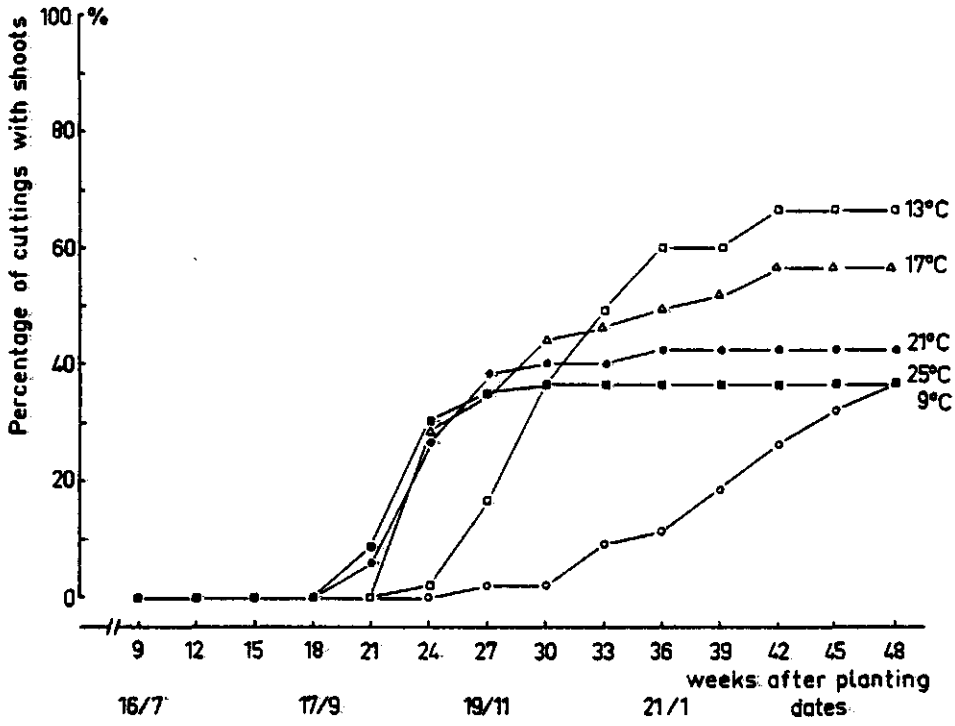


Figure 6. Time course of adventitious shoot formation on *Chrysanthemum* 'Bravo' leaf cuttings rooted at 20 °C for six weeks, then kept at 9 °C for ten weeks and thereafter transferred to different temperatures. Each treatment comprised 50 cuttings from positions 3, 4 and 5.

tive stages of shoot regeneration. There were indications that these temperatures delayed later stages of shoot formation (see Figure 3, cuttings planted on September 15). This latter temperature effect was studied in more detail. Leaf cuttings from positions 3, 4 and 5 were rooted for six weeks at 20°C and then placed in the phytotron glasshouse at 9 °C (actual mean temperature 10.7) for ten weeks. Next, the cuttings were distributed over the rooms at 9, 13, 17, 21 and 25 °C. As shown in Figure 6, shoot formation started earlier as the temperature was higher, which confirms that shoot realization is delayed by low temperature. Eventually, however, higher percentages

of shoot formation were reached with lower temperatures than with higher ones, except at 9 °C with a very low rate of shoot formation. At the high temperatures (21 and 25 °C) much higher percentages of shoot formation were reached than when these temperatures were applied during the whole life of the cuttings (Figure 4, 10% at both temperatures). This indicates that indeed a certain degree of induction had been achieved by the low temperature pretreatment. As compared with the experiment in which the cuttings were rooted at different temperatures (Figure 5), however, overall shoot formation percentages in this experiment were rather low. This may indicate that the induction at low temperature is less effective after previous rooting at high temperature of 20 °C. Another explanation may be the use of older leaves.

In three experiments, besides the percentages of shoot formation, the numbers of adventitious shoots were determined (Table 2). With different temperatures given continuously during the whole life of the leaf cuttings the mean number of shoots increased with decreasing temperature with an optimum at 13 °C. A similar temperature effect is shown in Figure 4.

Table 2. Mean numbers of adventitious shoots on *Chrysanthemum* 'Bravo' leaf cuttings grown at different temperatures during the whole life of the cuttings (L), during the rooting period (R), and during the period of shoot development (S). For detailed descriptions see legends of Figures 4, 5 and 6 respectively.

Temperature (°C)	Mean number of shoots*		
	Period of temperature treatment		
	L	R	S
9	3.1 ab	3.3 b	2.4 a
13	4.3 c	2.7 ab	2.6 a
17	3.7 bc	2.5 a	3.1 a
21	1.8 a	2.1 a	3.1 a
25	2.0 a	2.0 a	3.2 a

* Means were calculated for the cuttings with shoots only. In the same column means designated by the same letter are not significantly different from each other at P = 0.05.

The numbers of shoots obtained with different temperatures during the rooting period exhibited the same reaction picture as illustrated in Figure 5, 9 °C being the best pretreatment. With different temperatures during the period of shoot development (cf. Figure 6) the mean number of shoots at the different temperatures did not differ significantly. On the average the numbers were rather high. This might reflect the promotive effect on shoot formation of the 9 °C pretreatment, which was applied in all the treatments.

Effect of light intensity and daylength. 'Bravo' leaf cuttings, rooted at 20 °C for six weeks, were placed under different light intensities in the phytotron rooms at 13 and 17 °C. Table 3 presents the results. The shaded cuttings (9 and 18 Wm^{-2}) reached much higher percentages of shoot formation than the unshaded ones (36 Wm^{-2}). Likely this has to do with the effects of light intensity on growth and longevity of the cuttings. With increasing light intensity the leaves became larger (leaf blade length about 15 cm at 36 Wm^{-2}, about 9 cm at 9 Wm^{-2}), thicker and more brittle and formed more anthocyanin. After about 15 weeks the unshaded leaf cuttings became very dull and necrotic spots developed on the leaf blades. They were senescing much faster than the shaded cuttings. The percentage of

Table 3. Shoot formation on *Chrysanthemum* 'Bravo' leaf cuttings grown under different light intensities (Philips TL 57 fluorescent tubes) at two temperatures after rooting at 20 °C for six weeks. Each treatment comprised 50 cuttings from positions 2, 3 and 4.

Treatment		Percentage of shoot formation after		
Temperature (°C)	Light intensity ($W\ m^{-2}$)	18	27	36 wks
13	9	0	72	88
	18	0	74	92
	36	0	38	50
17	9	12	58	72
	18	8	60	78
	36	4	28	38

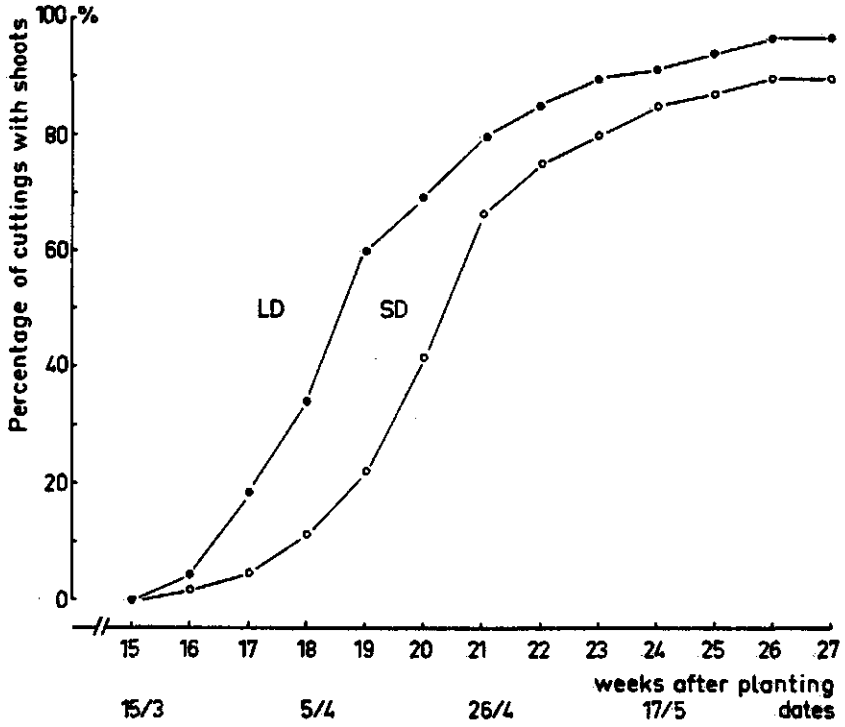


Figure 7. Time course of adventitious shoot formation on *Chrysanthemum* 'Bravo' leaf cuttings rooted at 20 °C for eight weeks and then grown under different day-lengths (LD = 16 h and SD = 8 h light). Each treatment comprised 70 cuttings from positions 1 and 2.

shoot formation was higher at 13 °C than at 17 °C, which is in agreement with results shown in the preceding section. Leaf blade senescence was more intense at 17 °C than at 13 °C.

Leaf cuttings of positions 1 and 2, collected at the end of November and rooted for eight weeks, were subsequently kept under LD and SD in the greenhouse. Starting at about the same time, shoot formation was slightly faster in LD than in SD (Figure 7), but at the end of the experiment there was only a little difference in percentage of shoot formation between the two treatments. The shoots appearing during the first one or two months under SD were very small and without extension growth. They formed a rosette of 3 to 6 bract-like leaves, then

Table 4. Effects of stage of development of the stock plant and leaf age on regeneration of leaf cuttings of *Chrysanthemum* 'Bravo'. For details of the stock plant stage see Figure 1. Each leaf position comprised 11 cuttings. The cuttings of two successive positions were pooled.

Stage of development of the stock plant	Leaf age (position)	Percentage of root formation after			Percentage of shoot formation after		
		2	3.5	5 wks	21	27	33 wks
Vegetative	1 + 2	14	55	100	9	91	95
	3 + 4	23	64	96	9	50	67
	5 + 6	23	46	91	0	27	41
	7 + 8	9	41	77	0	18	50
Crown bud producing	1 + 2	0	64	91	0	18	50
	3 + 4	0	46	91	0	0	9
	5 + 6	0	23	50	0	9	9
	7 + 8	0	23	64	0	0	0

developed a small flower bud, elongated and started flowering. The shoots which emerged from about week 20 onwards formed normal leaves first, elongated for a short time, and then entered the generative stage.

Effect of stock plant condition. Stock plants were grown at different temperatures (9, 13, 17, 21 and 25 °C climate rooms for five weeks), at different light intensities (4.5, 9, 18 and 36 Wm^{-2} for five weeks) and under different SD regimes (0, 2, 4 and 8 SD; 0, 4, 8, 16, 24, 32 and 40 SD). None of these treatments had any effect on shoot regeneration of the leaf cuttings. Important effects of the condition of the experimental material, however, were found in experiments with leaf cuttings of different ages (positions 1-8) from normal vegetative stock plants and from so-called crown bud plants (Table 4). In both types of stock plants the younger leaf cuttings rooted earlier, developed shoots earlier and yielded higher percentages of shoot formation than older leaves. Both rooting and shoot formation were much better in leaf cuttings from vegetative plants than in those from crown bud plants. Within the treatments no stock plant effect was found. Observation of the root system during the period of shoot formation showed that

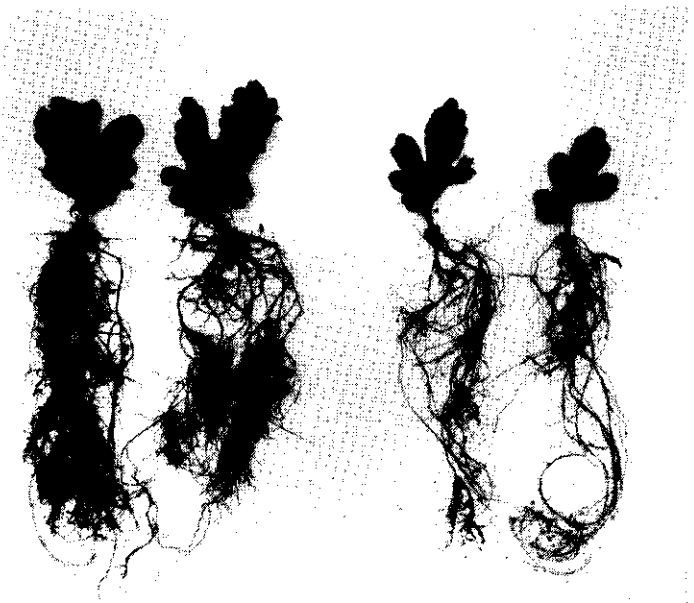


Figure 8. Different root masses on *Chrysanthemum* 'Bravo' leaf cuttings, at the left from vegetative plants, at the right from crown bud plants. Photograph taken 24 weeks after planting.

the root mass of the cuttings from crown bud plants was much smaller than that of the cuttings from vegetative plants (Figure 8). Supposedly, this had a causal relation with the difference in shoot formation.

DISCUSSION

In this study leaf cuttings of chrysanthemum 'Bravo' always formed roots before shoot formation. Apparently the roots were a prerequisite for shoot formation; the presence of a large root mass increased the chance of obtaining shoots. In this respect the regeneration characteristic of chrysanthemum closely resembles that of *Peperomia sandersii* (Harris and Hart, 1964).

The most important result from this study is that low to moderately high temperature is very important for a good shoot regeneration on leaf cuttings of chrysanthemum. The highest

percentages of shoot formation were generally obtained at 13 and 17°C. Pretreatment at lower temperature (9 °C) was favourable. Apparently, this pretreatment was effective only when the roots were present, and its effectiveness seemed to be higher if low temperature was also given during rooting. Comparable effects of low temperature have been found in *Begonia* and *Streptocarpus* spp. (Rünger, 1959; Heide 1964, 1965b; Chlyah and Tran Thanh Van, 1971; Appelgren and Heide 1972). With *Begonia x cheimantha*, however, Heide (1965b) found that the leaf cuttings were most sensitive to the low temperature when they had not yet formed roots.

It was shown that high temperatures (21 and 25 °C) promoted the realization of shoot formation, whereas low temperatures retarded it. This points to two part-processes, which follow one another, (1) induction of shoot regeneration, which proceeds better at low temperature, and (2) realization of shoot regeneration, which is more rapid at high temperature. Appelgren and Heide (1972) found a similar dual temperature effect on shoot regeneration in *Streptocarpus* 'Constant Nymph'.

Temperature treatment of the stock plants of chrysanthemum had no effect on shoot regeneration. This is in contrast to the situation with *Begonia x cheimantha* and *Streptocarpus* 'Constant Nymph', where temperature treatment of the stock plants had the same effect as temperature treatment of the cuttings (Heide, 1964; Appelgren and Heide, 1972).

During the period of root and shoot formation of the leaf cuttings temperature was obviously the most important environmental factor. The effects of the other factors studied, light intensity and daylength, were much less pronounced. High light intensity inhibited shoot regeneration, probably because it caused overproduction and accumulation of assimilates and impaired the vitality of the cuttings in that way.

Under SD conditions, it was found that the initially formed shoots were rosette-like and flowered early. An explanation for this might be that the leaf blade picked up the SD signal and a flowering stimulus was passed on directly to the apices of the young adventitious shoots. In chrysanthemum plants SD induces flattening of the apex and inhibits elongation growth (Popham and Chan, 1952). This might explain the lower rate of shoot formation under SD as compared with LD in the present experiments. Suppression of vegetative growth and induction of flowering under SD were also found by Rünger (1957b) with *Begonia x cheimantha* leaf cuttings. Besides these effects, Heide (1964,

1965a) observed in the same begonia that SD improved shoot regeneration by a higher rate of success and more shoots. This effect was not found in the chrysanthemum of the present experiments nor in other species (Harris and Hart, 1964; Van Harten, 1978). As described in this study, gradually the appearance of the rosette-like shoots diminished, and normal shoots developed instead. Apparently, at that time, as a result of aging or loss of functioning, the leaf blade was no longer capable to receive the SD signal.

The environmental factors studied did not have any effect when applied to the stock plants. Important differences in regeneration capacity, however, were found between cuttings from vegetative plants and those form crown bud plants. The latter reached a much lower percentage of shoot formation than the former. The smaller root mass, perhaps as a result of the smaller leaf blade, of the leaf cuttings from the crown bud plants might be responsible for this. A smaller root mass may mean a lower synthesis of cytokinin. As is generally accepted this hormone plays an important role in the regeneration of shoots.

Leaf age also appeared to have a large effect. The younger the leaf the higher was the capacity to form adventitious shoots. The explanation for this should probably be sought in the more rapid rooting of the younger leaves. Other results, however, are not in agreement with this explanation, for instance after low temperature a high percentage of shoot formation was preceeded by delayed rooting. Perhaps at lower temperature roots produce more cytokinin, or the cytokinin metabolism is more efficient.

The experiments described in this chapter were all done with 'Bravo' which appears to have a fairly good regeneration capacity. In some experiments 'Super Yellow' was also used, but in this cultivar shoot regeneration was never observed. This shows that in chrysanthemum, as in many other species (e.g. Heide 1965b; Broertjes and Leffring, 1972; Miedema 1973) the regeneration ability is strongly dependent on the genotype. So far, there is no plausible explanation for these large genotypical differences. In 'Super Yellow', rooting was very good, mostly even better than in 'Bravo'. So it is not likely that cytokinin synthesis is the bottleneck. Apparently this is not the only factor involved in the regeneration process.

The experiments, which dealt with the effects of various factors, did not yet give a coherent procedure for shoot regeneration of chrysanthemum 'Bravo' leaf cuttings. Such a pro-

cedure should obviously consist of the following steps. From young, well growing, vegetative plants the youngest leaves should be taken. These leaves must be rooted at a relatively low temperature, about six weeks at 13 °C. During this period shoot induction will already start. The cuttings should then be potted and grown at 9 °C under moderately high light intensity (18 Wm^{-2}) and LD. This low temperature will complete the shoot induction process within 4-8 weeks. Thereafter, the cuttings should be transferred to 17 °C to hasten the development of the shoots. This temperature will not reduce the state of induction. In this way, within 18-22 weeks approximately 80% of the cuttings will have developed 3-4 shoots each.

CHAPTER 4

IN VIVO SHOOT REGENERATION OF LEAF CUTTINGS OF CHRYSANTHEMUM (*CHRYSANTHEMUM MORIFOLIUM* RAM.) II. EFFECTS OF CYTOKININS AND AUXINS

INTRODUCTION

Various authors (Heide, 1965b; Broertjes et al., 1968; Van Bragt, 1974; Broertjes and Van Harten, 1978) have suggested that adventitious shoot formation on leaf cuttings in vivo could have large potentialities for horticulture. They pointed to cytokinins as a group of plant growth regulators which were likely to induce the regeneration of shoots. Positive results of cytokinin application in vivo are hard to find, however. Especially with species which do not readily regenerate shoots spontaneously most attempts have failed (e.g. Van Harten, 1978; Miedema et al. 1980; Chapter 1 in this thesis). In the research presented here a more thorough examination was done of the capacity of cytokinin to induce shoots on leaf cuttings of chrysanthemum (*Chrysanthemum morifolium* Ram.). Also auxin was studied, because of its potential to increase the chance of shoot regeneration (Chapter 2).

Leaf cuttings of chrysanthemum cv. Bravo were found spontaneously to form shoots fairly easily, though only after a very long period, whereas those of cv. Super Yellow did not form any shoots at all (Chapter 3). It was the purpose of the present experiments to investigate (1) whether plant growth regulators applied to the leaf cuttings would result in earlier shoot regeneration in 'Bravo', and (2) whether they would be able to induce shoot regeneration in 'Super Yellow'. In the first experiments applications of cytokinin and auxin were studied separately. Different methods of application were used, and both unrooted and rooted cuttings were treated. Subsequently, experiments were done with combinations of cytokinin and auxin. Next, it was studied to what extent temperature affected the results of the regulator treatments. Heide (1965b) and Chlyah and Thran Thanh Van (1971) demonstrated with *Begonia* spp. that low temperature had similar effects as exogenously applied cytokinin. It was ascertained if this holds true in chrysanthemum.

MATERIAL AND METHODS

Plant material. The stock plants of chrysanthemum cvs. Bravo and Super Yellow were raised in the same way as described before (Chapter 3). When the leaf cuttings were collected, the plants had about 20 full-grown leaves, but sometimes also older plants with 25-30 leaves were used.

Leaf cuttings. Leaf cuttings were taken from the plants and grown as described in Chapter 3. At least 24 leaf cuttings were used per treatment, generally from positions 3, 4 and 5. They remained in the cutting benches for 10-13 weeks. Temperature in the benches was controlled at 17 °C and fluctuated not more than ± 1.5 °C, except for long periods with sunny days during summer when it rose to 20 °C and sometimes 21 °C. At the end of the cutting bench period the rooted cuttings were potted as shallow as possible to allow observation of initial shoot formation. Then they were placed on tables in the greenhouse (Chapter 3).

Plant growth regulators. Cytokinins, 6-furfurylaminopurine (K), 6-benzylaminopurine (BA) and 6-benzylamino-9-(tetrahydro-2-pyryl) purine (PBA), and auxins, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-naphthalene-acetic acid (NAA), were applied to unrooted cuttings by means of talc dip or 24 h soaking. This last treatment was performed in the phytotron (Doorenbos, 1964) at 21 °C under Philips TL 57 fluorescent tubes giving about 35 Wm^{-2} at the level of the cuttings. Relative humidity was set at 70%. It was attempted as far as possible to standardize the volume of regulator solution taken up by the individual leaf cuttings in the successive experiments. Therefore, the stock plants were not watered for two days before collecting the leaf cuttings. During the application of the regulators, in a flat tray, the leaf cuttings were supported by wire netting in such a way, that they did not cover each other. Under these conditions 'Bravo' cuttings with a leaf blade length of 8.5-10 cm took up 1.3-1.8 ml per leaf, and 'Super Yellow' cuttings with a leaf blade length of 11-12.5 cm 2.0-2.5 ml per leaf. When rooted cuttings were treated, BA mixed in lanolin was smeared on a wound about 0.5 cm long at the side of the lower end of the petiole. This regulator was also dissolved in water + Tween 20 (0.2 $ml l^{-1}$) and sprayed on the leaf blades till run off.

Temperature. Different temperature regimes (9, 13, 17, 21 and 25 °C) were applied in the climate rooms and the glasshouses of the phytotron. In the experiments performed in the glasshouses during summer the actual mean temperatures deviated not more than 1.4, 0.7, 0.9, 0.8 and 0.2 °C from the indicated values.

Observations. Root and shoot regeneration were examined regularly. Shoot formation was recorded as such when at least two leaflets had been formed and some extension growth occurred. A first flush of shoot formation starting from week 9-15 onwards was indicated as early, a second flush, similar to the shoot formation in Chapter 3, from week 15 or later onwards as late shoot regeneration. Most experiments were finished after 21-24 weeks.

High concentrations of the regulators could impair the vitality of the cuttings so severely, that after 12 weeks they still had not rooted and were nearly dead. The number of such cuttings was recorded and used as measure of the harmful side-effects of the treatments.

The results presented are confirmed by the data of at least two experiments.

RESULTS

Initial effects. In certain treatments the leaf cuttings exhibited formation of small (1-4 mm) white-pink adventitious buds with leaf-like structures already after 4-7 weeks. At that time roots had not yet been formed or root formation had just started. With increasing root formation, however, exact examination of initial bud formation was impossible without damaging the roots, so it was discontinued till repotting at the end of the cutting bench period. Remarkably, this early bud formation occurred rather erratically and shoots from these buds developed very late, sometimes only 10-15 weeks after appearance of the buds. So the initial adventitious bud formation seemed less suitable to measure the effects of the treatments, and shoot development was used instead.

Cytokinin. Results of application of different cytokinins to unrooted 'Bravo' leaf cuttings are shown in Table 1. Cytokinin inhibited rooting, particularly at higher concentrations, with an increasing effect in the series K, BA, PBA. With a talc dip

Table 1. Root formation (R) and shoot formation (S) on *Chrysanthemum* 'Bravo' leaf cuttings after treatment with different cytokinins (K, BA and PBA) at different concentrations. Application was done by talc dip (%) or by 24 h soaking (mgl^{-1}) just prior to planting. Each treatment comprised 24 cuttings from positions 3, 4 and 5. The experiment started on September 13. After 39 weeks percentages shoot formation did not change anymore.

Cytokinin		R (%)		S (%) after				
		5	10	15	21	27	33	39 wks
Talc dip, %								
	0	100	100	0	25	79	100	100
K	0.1	96	100	0	13	58	79	83
	1	75	100	0	8	46	63	71
	10	17	100	8	8	38	53	63
BA	0.1	83	100	0	0	33	58	71
	1	13	100	0	4	50	75	79
	10	0	79*	0	0	33	71	92
PBA	0.1	46	100	0	4	29	42	42
	1	0	63*	0	4	13	46	50
	10	0	17*	0	0	8	25	50
Soaking, mgl^{-1}								
	0	100	100	0	33	79	92	96
K	1	100	100	0	13	63	92	92
	5	96	100	0	13	46	83	88
	25	83	100	0	4	33	71	71
BA	1	92	100	0	8	54	83	83
	5	83	100	0	4	38	83	92
	25	71	100	0	13	50	63	67
PBA	1	71	100	0	17	50	67	83
	5	71	92	0	8	42	75	79
	25	13	88*	21	29	46	88	96

* In treatments BA 10%, PBA 1%, 10% and 25 mgl^{-1} 4, 2, 9 and 1 cuttings were lost as caused by premature collapse.

the effects were stronger than with 24 h soaking. The high cytokinin concentrations impaired leaf blade tissue, but most cuttings recovered from these initial harmful side-effects. The percentage of shoot formation was lower after cytokinin treatments than in the control; further it was lower as rooting had been more strongly inhibited. However, there were two exceptions at high concentrations of cytokinins: (1) at K 10% and PBA 25 mgl^{-1} an early flush of shoot formation occurred between weeks 12 and 15, clearly preceding the common late shoot formation, and (2) at BA 10%, PBA 1 and 10%, and PBA 5 and 25 mgl^{-1}, at a certain moment, late shoot formation recovered and eventually reached a higher percentage than at lower concentrations. Although affecting the percentages of cuttings which formed shoots, no treatments were found which significantly affected the number of shoots per cutting, the mean numbers ranged from 2.0 to 3.8.

The influence of cytokinin treatment on shoot formation was also studied with cuttings which already had formed roots. Table 2 presents the results of 'Bravo' cuttings which, after root formation for eight weeks at 20 °C, were treated with BA in lanolin paste. Wounding alone did not influence shoot formation. BA increased the rate of shoot formation, but after 24 weeks a level equal to that of the control was reached. Probably this was caused by rotting of the petiole tissue under the lanolin, which was generally observed after 20 weeks. The BA

Table 2. Shoot formation on *Chrysanthemum* 'Bravo' leaf cuttings after application of BA eight weeks after planting when roots had already been formed. BA at different concentrations mixed in lanolin was applied on a wound at the lower petiole end. Each treatment comprised 24 cuttings from positions 3, 4 and 5. The experiment started on January 22 and ended after 24 weeks.

Treatments			Percentage of shoot formation after			
wound	lanolin	BA(%)	15	18	21	24 wks
-	-	-	0	4	21	63
+	-	-	0	8	21	58
+	+	0	0	0	25	42
+	+	0.5	4	17	42	58
+	+	2	0	21	38	50

was also applied as a spray (25 and 125 mgl^{-1}) on the leaf blades of rooted cuttings, but this had no clear effect on shoot regeneration.

After the experiments with unrooted and with rooted leaf cuttings the question arose what would be the effects of BA treatments at different times between planting and 100% rooting of the cuttings. Effects of such BA treatments appeared to be better visible when an auxin pretreatment was given to the cuttings which hastened and synchronized root formation. Table 3 shows the results with 'Bravo'. After pretreatment of the cuttings with IAA 0.5% (talc dip), BA had a promotive effect on late shoot formation when it was applied at the time of 100%

Table 3. Root formation (R) and shoot formation (S) on *Chrysanthemum* 'Bravo' leaf cuttings after application of BA at different times after planting. BA 2% in lanolin was applied on a wound at the lower petiole end. Half of the cuttings were given a pretreatment with IAA 0.5% in talc just prior to planting. Each treatment comprised 24 cuttings from positions 3, 4, 5 and 6. The experiment started on September 7 and ended after 24 weeks.

IAA pre-treatment	BA treatment		R (%)		S (%) after			
	Days from planting	Perc. of rooted cuttings*	5	10	15	18	21	24 wks
-	-	-	100	100	0	0	4	17
	0	0	8	92	0	0	0	4
	6	0	17	100	0	0	0	8
	12	0	17	100	0	0	0	0
	18	17	29	96	0	0	0	8
	24	50	63	100	0	4	4	13
+	-	-	100	100	0	0	4	13
	0	0	25	100	4	4	8	8
	6	0	25	96	0	0	0	4
	12	33	75	96	0	4	4	8
	18	88	100	100	0	13	21	33
	24	100	100	100	0	13	29	38

* Percentage of root formation at time of BA treatment.

root formation or just prior to that. Earlier application retarded shoot formation as compared to the control. Some early shoot formation was seen after BA application immediately after planting. Also in this experiment the petiole wounds which were treated with lanolin + BA started to rot, and the rate of shoot formation of these cuttings decreased (not presented in Table 3).

In all the above experiments leaf cuttings of 'Super Yellow' were also investigated. After cytokinin application before planting, these cuttings also regenerated shoots, the highest percentage found was 20% after treatment with BA 1%. These shoots arose between weeks 9 and 15. In the case of rooted cuttings, not a single 'Super Yellow' leaf cutting regenerated shoots.

Auxin. In certain experiments treatments of unrooted 'Bravo' leaf cuttings with NAA 0.5-1% and IBA 1-2% in talc seemed to have a slight promoting effect on shoot regeneration, but this effect was not always observed. It was supposed that the better

Table 4. Root formation (R) and shoot formation (S) on *Chrysanthemum* 'Bravo' leaf cuttings after treatment with different auxins (NAA and IAA, 24 h soaking prior to planting) at different concentrations. The cuttings originated from positions 3, 4, 5 and 6 of crown bud plants. Each treatment comprised 40 cuttings. The experiment started on November 28. After 30 weeks shoot formation hardly increased anymore.

Auxin, mgl^{-1}		R (%)		S (%) after		
		3	5	18	24	30 wks
	0	25	63	8	8	8
NAA	5	65	100	3	8	13
	15	80	100	8	10	15
	45	93	100	3	13	28
	135	70	100	5	23	35
IAA	5	48	88	5	15	15
	15	78	98	5	13	18
	45	100	100	13	30	38
	135	100	100	10	55	65

shoot regeneration was a consequence of the improved rooting by auxin. To investigate this hypothesis leaf cuttings from stock plants in the crown bud stage were used, as they root less readily than cuttings from normal vegetative plants (cf. Table 4 and Figure 8 in Chapter 3). Table 4 shows the results of this experiment. The rate of late shoot formation increased with higher concentration of auxin, IAA particularly at 135 mgl^{-1} being more promotive than NAA. The improvement of shoot formation was well correlated with improvement of root formation, except with NAA 135 mgl^{-1} which showed a decrease in percentage of root formation after three weeks. Perhaps this high concentration of NAA was supra-optimal: both NAA 45 and 135 mgl^{-1} caused yellowish discoloration of the leaf blade veins, curling of the leaf margins, and later on development of numerous rootlets emerging along the whole length of the petioles. Summarizing, these results indicate that bad root formation may be a limiting factor to late shoot regeneration. Auxin pretreatment might improve it, but the stable NAA appeared to be less effective for this than the unstable IAA.

Combination of cytokinin and auxin. In several experiments unrooted leaf cuttings were treated with combinations of cytokinin and auxin, either applied by talc dip or by 24 h soaking. Different concentrations of the regulators were tested. The main purpose was to improve early shoot regeneration.

So far the best results were obtained in an experiment with 'Super Yellow' leaf cuttings which had been soaked in solutions of PBA + NAA. Figure 1 presents the results of this experiment. PBA inhibited rooting, particularly at higher concentrations, and NAA counteracted this inhibition. After 14 weeks almost all cuttings had roots, and only an incidental cutting had collapsed. Moderately high percentages of early shoot formation were obtained in the treatments with high concentration PBA (50 and 100 mgl^{-1}) plus high concentration NAA (5 and 50 mgl^{-1}). Other treatments yielded lower percentages of shoot formation. Seemingly, lower concentration PBA had to be combined with lower concentration NAA. With NAA alone no shoot regeneration occurred. All the shoots appeared between weeks 9 and 15, except some new shoot formation between weeks 15 and 18 in the treatments with high concentrations PBA. Obviously, high concentrations of the cytokinin retarded development of the adventitious shoots. A level of early shoot formation higher than in this experiment was not found in replications, neither using a talc dip instead of 24 h soaking nor BA and K instead of PBA.

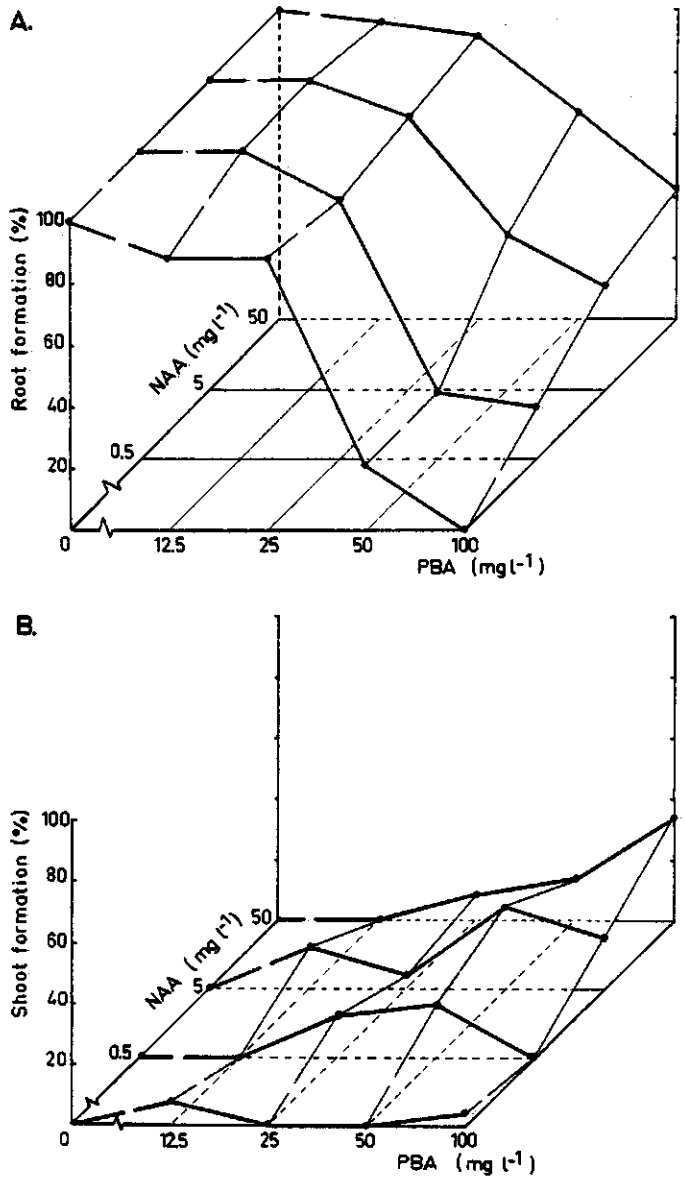


Figure 1. Root formation after ten weeks (A) and shoot formation after 18 weeks (B) on leaf cuttings of *Chrysanthemum* 'Super Yellow' after treatment with PBA and NAA at different concentrations (24 h soaking prior to planting). Each treatment comprised 24 cuttings from positions 3, 4 and 5. The experiment started on March 28.

Also lengthening the application time (48 and 72 h soaking) to enable uptake of high amounts of regulators at low concentrations did not improve the results.

With 'Bravo' leaf cuttings application of NAA together with PBA also increased the frequency of early shoot formation. In this cultivar, however, high concentrations of NAA readily impaired the vitality of the cuttings as could be concluded from discoloration of the leaf blades, lower rooting capacity and total collapse of a number of cuttings. Similar disastrous effects were found with IBA, but not when IAA was used. The highest level of early shoot formation was reached in the combination PBA 25 mgl^{-1} + IAA 50 mgl^{-1}, viz. 29% after 18 weeks when untreated control cuttings only just started bud formation (3%). A lower concentration of IAA strongly decreased the chance of early shoot formation.

Temperature influence on effectiveness of the regulator treatments. So far experiments which resulted in some degree of early shoot formation had been started between September and April. In experiments started during summer, either with 'Bravo' or with 'Super Yellow', the decrease in vitality of the leaf cuttings was such that sometimes more than 50% died, and not a single cutting regenerated shoots. The question arose whether this was due to high temperatures during growth of the stock plants. To study this 'Bravo' stock plants were grown, from June 5 till July 17, at different temperatures in the glasshouses of the phytotron (mean light intensity about 125 Wm^{-2}). Leaf cuttings from these plants were treated with PBA 25 mgl^{-1} + IAA 50 mgl^{-1} and planted under the standard conditions (17 °C ± 1.5 °C, temperature rose to 20 °C two times for half a week). The leaf blades of cuttings from the 9 and 13 °C treatments remained dark green and healthy, those from 17 °C became light green, with midribs pale yellowish, and less healthy, whereas those from 21 and 25 °C were still more discolored and also became soft. Table 5 gives the other results of this experiment.After higher growing temperature of the stock plants the rooting capacity of the leaf cuttings decreased. Extremely high percentages of premature death were found after the three highest temperatures. Early shoot regeneration occurred only after the lowest two temperatures.

These large differences between the results of low and high stock plant temperatures were not found with leaf cuttings from plants grown at the different temperatures in the climate rooms of the phytotron (light intensity 35 Wm^{-2}). In that experiment

Table 5. Root formation (R), premature collapse (C) and shoot formation (S) in *Chrysanthemum* 'Bravo' leaf cuttings originating from stock plants grown at different temperatures. The leaf cuttings were given PBA 25 mgl^{-1} + IAA 50 mgl^{-1} (24 h soak) just prior to planting. Each treatment comprised 40 cuttings from positions 4, 5, 6 and 7. The experiment started on July 17.

Stock plant temperature	R (%)		C (%)	S (%) after		
(°C)	5	10	10	15	18	21 wks
9	5	95	3	10	15	20
13	18	85	15	15	18	23
17	3	50	45	0	0	10
21	0	50	50	0	3	8
25	0	18	63	0	0	0

also cuttings from the high temperatures remained in excellent health and exhibited early shoot formation (data not presented). The results indicate that high stock plant temperature is detrimental to leaf cutting regeneration especially when it is applied under high light intensity conditions.

Table 6. Root formation (R) and shoot formation (S) on *Chrysanthemum* 'Bravo' leaf cuttings, which were treated with PBA 25 mgl^{-1} + IAA 50 mgl^{-1} (24 h soak) just prior to planting and then grown at different temperatures for 11 weeks. After the treatments the cuttings were grown in a greenhouse. Each treatment comprised 33 cuttings from positions 4, 5, 6 and 7. The experiment started on August 12.

Leaf cutting temperature	R (%)		S (%) after		
(°C)	5	10	15	18	21 wks
13	27	97	9	27	39
17	33	94	9	15	15
21	45	97	0	0	0
25	66	97	0	0	3

Apart from high temperature during stock plant growth also high temperature during leaf cutting growth might adversely affect shoot regeneration. To study this, 'Bravo' cuttings were treated with BA 25 mgl^{-1} + IAA 50 mgl^{-1} (24 h soak) on August 12 and then planted at 13, 17, 21 and 25 °C in the phytotron glasshouses. After sufficient rooting (11 weeks) they were potted up and transferred to the greenhouse. Table 6 shows the results. As expected rooting was faster at higher temperatures. Only a few cuttings collapsed. The percentage of early shoot formation (after 18 weeks) was moderately high after pretreatment at 13 °C, low after pretreatment at 17 °C, whereas no shoots formed on the cuttings which had stood at 21 and 25 °C. Similar temperature effects were obtained with 'Super Yellow' leaf cuttings, viz. 42, 29, 17 and 0% of shoot regeneration. Summarizing, the experiments showed a remarkable decrease in early shoot formation with increasing temperatures during the cutting bench period.

Since the lowest temperature (13 °C) in the above experiments gave the best shoot regeneration, the question arose whether even lower temperatures would improve it still further. Therefore, 9 °C was also included in the temperature series. This did not result in a higher level of early shoot formation than 13 °C, however. Also pretreatment of the stock plants with low temperatures (9 and 13 °C) and thereafter low cutting bench temperatures did not improve the results of early shoot formation.

DISCUSSION

At the start of the experiments the aim was the realization of earlier shoot regeneration in 'Bravo' and induction of shoot regeneration in 'Super Yellow' leaf cuttings. Both aims have been attained by application of cytokinin to the leaf cuttings just prior to planting. The cytokinin treatment inhibited rooting. Addition of auxin appeared to interact with the cytokinin. These effects are in agreement with the reaction type of Skoog and Miller (1957). By contrast, cytokinin inhibited late shoot formation in 'Bravo', whereas auxin, under favourable conditions, had the opposite effect. These effects agree with the reaction type of Harris and Hart (1964). Late shoot formation in 'Bravo' was also stimulated by cytokinin, however, particularly when applied after rooting. Such stimulation was also

found earlier with *Lunaria annua* and *Nicotiana alata* in Chapter 2. Summarizing, in 'Bravo' leaf cuttings three types of reaction to cytokinin application can be observed, in 'Super Yellow' only one.

Promotion of late shoot formation in 'Bravo' by cytokinin was only possible if the roots were present. Generally however, the improvement was rather slight. The method used was not very reliable as there were problems with the lanolin and as the cytokinin strongly inhibited shoot formation when applied a little too early. In short, in this way no better results were obtained than after suitable temperature treatments (Chapter 3).

Early shoot formation looked more promising for practical application than late shoot formation. Different combinations and concentrations of cytokinins and auxins were attempted to improve it. Apparently, the kind of cytokinin was not very crucial. Addition of auxin to the cytokinin seemed to be important, but clear optimum concentrations of the regulators could not be found. Generally, the frequencies of early shoot formation remained rather low. Probably, other factors than the concentrations of the regulators are more limiting to the improvement of early shoot regeneration.

In the experiments on early shoot formation which started in summer very much damage to the cuttings occurred, followed by premature death, and not a single cutting showed early shoot formation. Indications were found that high temperature during stock plant growth accompanied by high light intensity is responsible for that failure. Probably, it has to do with the strong metabolic activity of the leaf tissue under these environmental conditions.

Leaf cuttings which got a suitable regulator treatment exhibited early shoot formation more readily at low and moderately high temperatures (9-17 °C) than at high temperatures (21-25 °C). Similar temperature effects were found for spontaneous shoot regeneration in chrysanthemum (Chapter 3). By analogy with Heide's results (Heide, 1964, 1965b), a further improvement of shoot regeneration was expected by applying low temperature during both the stock plant and the leaf cutting period. This improvement, however, did not occur.

Summarizing, early shoot formation could be induced in both 'Bravo' and 'Super Yellow' leaf cuttings by regulator application just prior to planting, but the percentages of cuttings which regenerated shoots generally were low. Factors which typically belong to the cutting stage, such as the type and concentration of cytokinin used and the method of application,

hardly affected the frequency of shoot formation. Low temperature in the cutting benches appeared to be beneficial. One factor had a substantial effect, viz. high temperature in combination with high light intensity during the growth of the stock plants, but this effect was very negative. This indicates that the stock plant period is very important. Maybe there are also typical stock plant factors which can improve early shoot regeneration.

CHAPTER 5

IN VIVO SHOOT REGENERATION OF LEAF CUTTINGS OF CHRYSANTHEMUM (*CHRYSANTHEMUM MORIFOLIUM* RAM.)
III. INTERACTION OF STOCK PLANT CONDITION AND APPLICATION OF CYTOKININ AND AUXIN

INTRODUCTION

Treatment with cytokinin and auxin, both at a relatively high concentration, could induce adventitious bud formation on leaf cuttings of chrysanthemum (*Chrysanthemum morifolium* Ram.) cvs. Bravo and Super Yellow. The buds developed into shoots between approximately weeks 9 and 18 after planting (Chapter 4). This was called early shoot formation, to distinguish it from late shoot formation which occurs without the help of plant growth regulators (Chapter 3). It was shown that raising the stock plants at high temperature under high light intensity completely inhibited early shoot formation. Thus stock plant treatment appeared to have a strong influence, and the question arose how far it could be used to improve early shoot formation.

In the literature there are many examples of the influence of the physiological condition and pretreatment of stock plants on in vivo shoot regeneration of leaf cuttings (e.g. Prévot, 1939; Heide, 1964, 1965b; Pierik, 1967). No reports are available, however, on the effect of stock plant pretreatment on the regeneration of cuttings which also received a plant growth regulator treatment. On the other hand, in research on in vitro shoot regeneration, where regulators almost always are applied, stock plant treatments regularly are used to improve regeneration (e.g. Bonga, 1982; Evers, 1984; Read et al., 1984; George and Sherrington, 1984; Appelgren, 1985).

It was decided to compare leaf cuttings from stock plants of the two different developmental stages mentioned earlier in Chapter 3, viz. the vegetative stage and the crown bud stage. In the experiments, cuttings from crown bud plants appeared to have a much better capacity of early shoot formation than those from vegetative plants. Therefore, in further experiments it was tried to bring young vegetative stock plants in the same physiological condition as the crown bud plants. To this end

the effects of different growing conditions and regulator treatments of the stock plants were studied.

MATERIAL AND METHODS

Plant material. The chrysanthemum cvs. Bravo and Super Yellow were raised in the same way as described in Chapter 3. Normal vegetative plants were produced by the cutting-from-cutting method. Crown bud plants were raised by using cuttings from old plants which were just starting crown bud development. These cuttings completed the crown bud shortly after rooting and thereafter most of them developed three stems (cf. Figure 1, Chapter 3). For comparison, vegetative plants were also grown with three stems obtained by hand topping of young plants.

Leaf cuttings. Leaf cuttings were taken from the plants and grown at 17 °C for 10-13 weeks as described in Chapter 4. After rooting they were placed on tables in a greenhouse.

Plant growth regulators. The stock plants were treated with the cytokinin 6-benzylaminopurine (BA) and with the growth retardants succinic acid 2,2-dimethylhydrazide (B-9), 2-chloroethyl trimethylammonium chloride (CCC), and α-cyclopropyl-4-methoxy-α-(pyrimidin-5-yl) benzyl alcohol (A-Rest). All these chemicals were applied as aqueous solutions (water + Tween 20 0.2 $ml l^{-1}$) by foliar sprays at one week intervals.

The leaf cuttings were treated with a combination of cytokinin, BA or 6-benzylamino-9-(tetrahydro-2-pyryl) purine (PBA), and indole-3-acetic acid (IAA). As standard treatment for 'Bravo' cuttings BA 12.5 $mg l^{-1}$ +IAA 50 $mg l^{-1}$ or PBA 25 $mg l^{-1}$ + IAA 50 $mg l^{-1}$ applied by a 24 h soak was used, whereas 'Super Yellow' cuttings were treated with BA 1% + IAA 0.2% in talc.

Temperature, daylength and light intensity. Different temperature regimes (9, 13, 17, 21 and 25 °C), short day (SD, 8 h) and long day (LD, 16 h) conditions and different light intensities (0.6-36 Wm^{-2}) were applied in the airconditioned rooms and glasshouses of the phytotron (Doorenbos, 1964).

Observations. Progress of root formation and beginning of bud formation were determined by regular examination of the cuttings. Development of adventitious shoots with green leaflets and some extension growth was recorded and used to measure the effects of the treatments. Mean numbers of shoots were calcul-

ated for the cuttings which had formed shoots.

Results presented are confirmed by the data of at least two experiments. For means the standard deviation was calculated and differences were tested for significance (P = 0.05) by Student's *t* test.

RESULTS

Crown bud versus vegetative stock plants. In raising the stock plants it was tried to equalize the growth vigour of the vegetative plants as much as possible to that of the crown bud plants. To this end vegetative plants with three stems were raised. The sizes of three months old plants are given in Table 1. The data indicated that there were still differences in growth vigour between both plant types.

Table 2 presents shoot regeneration on leaf cuttings from vegetative plants as compared with those from crown bud plants. In both cultivars the leaf cuttings from the crown bud plants formed shoots much earlier and reached a much higher percentage of early shoot formation (from week 9 till 21) than the leaf cuttings from vegetative plants. The average numbers of shoots per leaf cutting were not different however. Within both groups of crown bud stock plants, no plant effects were found. The 'Bravo' cuttings showed a somewhat higher capacity of early shoot formation than those of 'Super Yellow'. This difference, however, may be due to the different times of starting the two

Table 1. Sizes of vegetative and crown bud plants of *Chrysanthemum* 'Bravo' and 'Super Yellow'. Both developmental stages were grown with three stems. The data are from three months old plants.

	Vegetative plants		Crown bud plants	
	'Bravo'	'Super Yellow'	'Bravo'	'Super Yellow'
Plant length (cm)	40	45	30	35
No. of leaves on longest stem	19-22	19-22	17-20	17-20
Leaf blade length (cm)	9.7-10.3	11.5-12.5	5.2-6.9	7.4-8.5

Table 2. Shoot formation on leaf cuttings from vegetative and crown bud *Chrysanthemum* plants. Two cultivars were used, 'Bravo' and 'Super Yellow'. The 'Bravo' leaf cuttings, per treatment 35 from positions 3-7, were given PBA 25 mgl^{-1} + IAA 50 mgl^{-1} by 24 h soaking, and then planted on March 13. The 'Super Yellow' leaf cuttings, per treatment 25 from positions 5-9, were given BA 1% + IAA 0.2% in talc, and then planted on April 29. S: percentage of shoot formation, MS: mean number of shoots.

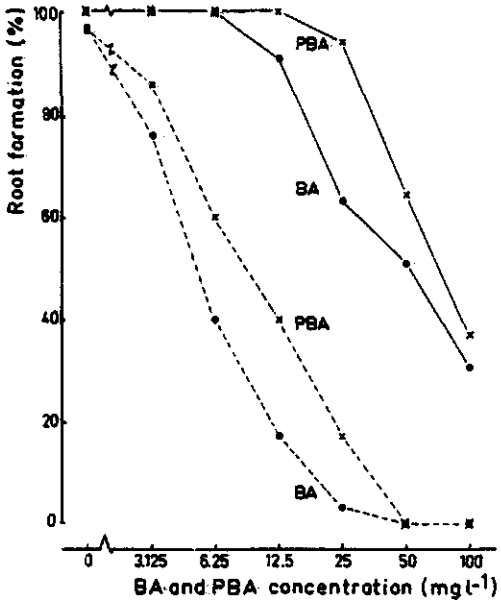
Stock plant		S (%)			MS after
Cultivar	Stage of development	9	15	21	21 wks
'Bravo'	vegetative	0	17	23	3.3 *
	crown bud	34	71	77	4.8
'Super Yellow'	vegetative	0	4	4	1.5 *
	crown bud	12	36	48	1.9

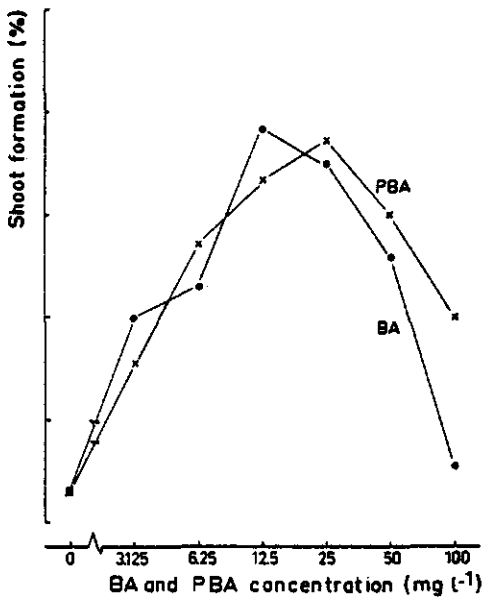
* The means were not different at P = 0.05.

experiments. Generally summer was a less suitable period.

After the favourable effects of the crown bud stock plants had been found, the question arose whether the concentrations used in the regulator treatment were indeed optimum. Figure 1 gives the results of an experiment in which different concentrations of BA and PBA were tested in combination with IAA 50 mgl^{-1}. As expected rooting was inhibited with increasing concentration of the cytokinins. The highest concentration impaired the vitality of the leaf cuttings, but almost all the cuttings survived and eventually formed roots (after 11 weeks). The early shoot formation as determined after 15 weeks was strongly dependent on the concentration of the cytokinin applied. For both BA and PBA an optimum concentration of 12.5-25 mgl^{-1} was found, at which more than 70% of the cuttings regenerated shoots. The number of shoots obtained also showed an optimum curve, but the optimum concentration range was wider.

Figure 2 gives the results of an experiment in which different concentrations of both BA and IAA were tested. IAA, particularly at the concentrations 45 and 135 mgl^{-1}, antagonized the





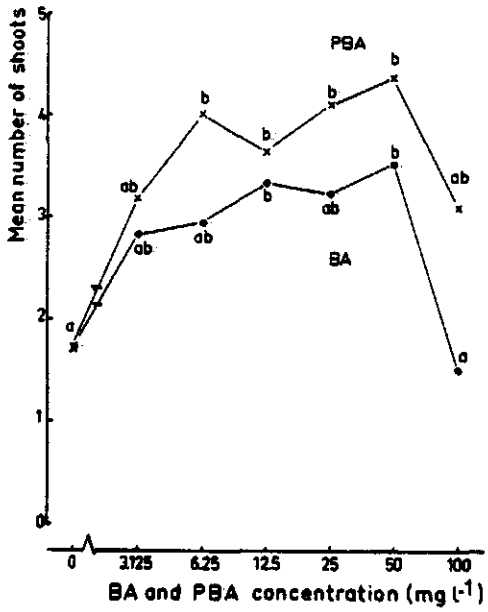


Figure 1.
Regeneration of *Chrysanthemum* 'Bravo' leaf cuttings as affected by pretreatment with different concentrations of BA and PBA in combination with IAA 50 mgl^{-1} (24 h soak just prior to planting). The cuttings, 35 per treatment, originated from positions 5-9 of crown bud plants. Root formation was recorded after three and six weeks (--- and ——), shoot formation after 15 weeks. The experiment started on February 19. Means designated by the same letter do not significantly differ at P = 0.05.

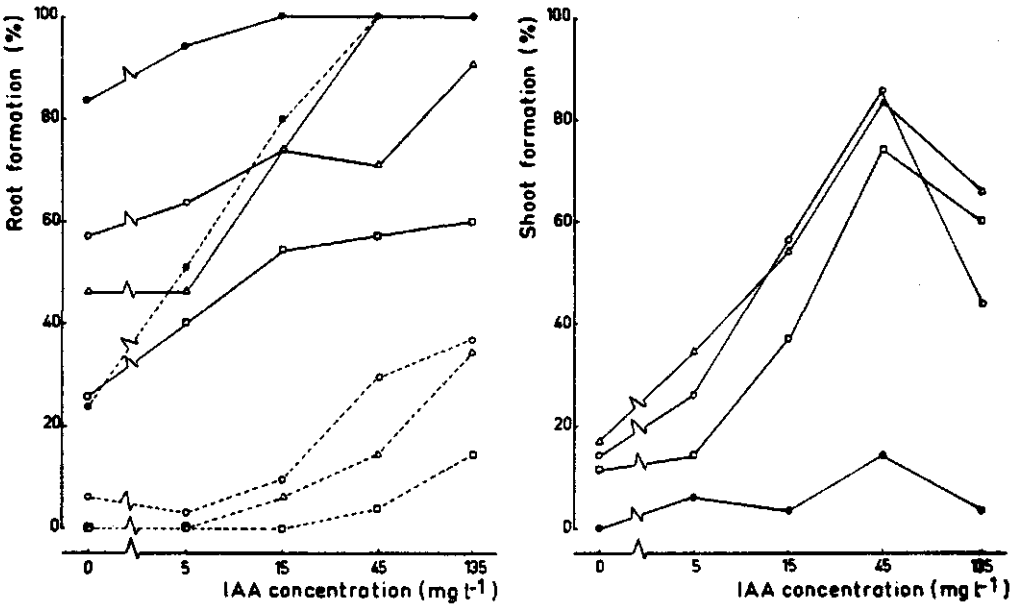


Figure 2. Regeneration of *Chrysanthemum* 'Bravo' leaf cuttings as affected by pretreatment with different concentrations of BA and IAA. The cuttings, 35 per treatment, originated from positions 5-9 of crown bud plants. BA was given at the concentrations 0 (●), 6.25 (o), 12.5 (Δ) and 25 mgl^{-1} (□), and IAA as indicated (24 h soak just prior to planting). Root formation was recorded after three and six weeks (--- and ——), shoot formation after 15 weeks. The experiment started on November 21.

inhibition of rooting by BA. The frequency of early shoot formation increased as a higher concentration IAA was applied, reaching an optimum (about 80%) in the treatments with IAA at a concentration of 45 mgl^{-1}. BA at the concentration 6.25 mgl^{-1} affected shoot regeneration similarly to BA at 12.5 mgl^{-1}, but BA at 25 mgl^{-1} appeared to be too high and reduced the frequency of early shoot formation. The IAA concentration had no pronounced effect on the number of shoots. Summarizing, it can be concluded that the cytokinin concentrations used in the standard treatment (BA 12.5 mgl^{-1} and PBA 25 mgl^{-1}) are about optimum for cv. 'Bravo' and that an addition of IAA is essential for a high frequency of early shoot regeneration.

It has been shown that leaf cuttings of crown bud stock plants of 'Bravo' had a low capacity of spontaneous so-called late shoot regeneration. Moreover the capacity decreased markedly with increasing leaf age (cf. Table 4, Chapter 3). Therefore it seemed interesting to investigate the influence of leaf age on early shoot formation. Table 3 shows the results. As expected, in untreated leaf cuttings the late shoot formation strongly decreased as older leaves were used. After treatment with cytokinin and auxin an opposite effect of leaf age was found, increasing early shoot formation with older leaves. Rooting also was faster as older leaves were used. Similar effects of leaf age on early shoot regeneration were found with 'Super Yellow'.

Table 3. The influence of leaf age on regeneration of leaf cuttings of *Chrysanthemum* 'Bravo'. The cuttings originated from crown bud plants. Each position comprised 15 cuttings, those from position 1 being the youngest with leaf blade length of about 3 cm. PBA 25 mgl^{-1} + IAA 50 mgl^{-1} or deionized water were given by 24 h soaking just prior to planting. The experiment started on January 10. R: percentage of root formation, S: percentage of shoot formation.

Cutting pretreatment	Leaf age (position)	R (%)	S (%) after		
		3.5	9	15	21 wks
deionized water	1	93	0	0	27
	2	100	0	0	33
	3	93	0	0	27
	4	93	0	0	13
	5	87	0	0	0
	6	73	0	0	7
	7	73	0	0	0
PBA 25 mgl^{-1} + IAA 50 mgl^{-1}	1	13	0	13	67
	2	33	0	7	53
	3	53	0	13	60
	4	67	0	33	60
	5	80	27	67	73
	6	67	13	60	87
	7	60	13	53	73

The favourable effects of crown bud stock plants so far were only clearly found in autumn, winter and spring. In experiments started in summer the leaf cuttings suffered much from the treatment with the regulators. They became yellowish green, the nerves even whitish green, and part of them collapsed. Various cutting bench conditions, low temperature, reduced light intensity and SD, were tried to overcome this negative aspect. Only at 13 °C a fairly good early shoot regeneration could still be attained.

Conditioning of the stock plants. The leaf cuttings from the crown bud plants had a remarkably high capacity of early shoot regeneration. The question arose whether it was possible also to get good early shoot formation on leaf cuttings from vegetative plants. Therefore, by manipulating daylength, temperature and light intensity it was tried to obtain vegetative stock plants with a physiological condition comparable to the crown bud plants.

When plants were grown under very long periods of SD (40 and 48 SD) their top parts became comparable with crown bud plants, particularly as to the presence of small leaves (blade length about 5.5 cm), in the axils of which flower buds had formed. These small leaves showed a slightly improved capacity of early shoot regeneration (up to 30%).

Normal stock plants were also grown at different temperatures (from 9-25 °C), but no temperature treatment gave an improvement of the early shoot regeneration of the leaf cuttings.

The most striking results were found with stock plants grown under various light intensities in the phytotron room at 17 °C. The lower the light intensity the higher was the shoot regeneration capacity of the leaf cuttings (Table 4). Plant growth and the production of new leaves decreased clearly with decreasing light intensity. At 0.6 Wm^{-2} the new leaves were very small and yellowish white, the leaf blade tissue soft and thin. Leaves at 6 Wm^{-2} were light green, those at the higher light intensities dark green. Rooting increased and subsequently decreased with higher light intensity, with an optimum at 12 Wm^{-2}. The leaf cuttings from plants grown at 0.6 Wm^{-2} rooted very poorly. Due to the bad development of the plants the cuttings were very tender and a large number collapsed initially during culture. Nevertheless, a very good regeneration of shoots was observed. Remarkably, this shoot regeneration was preceded, 4-6 weeks after planting, by abundant regeneration of adventitious buds, which also occurred in the 6 Wm^{-2} treatment. The level of bud

Table 4. Effects of different light intensities on growth of stock plants and on regeneration of leaf cuttings in *Chrysanthemum* 'Bravo'. The stock plants were raised at different light intensities for five weeks, ten plants per treatment. The leaf cuttings, 40 per treatment, were treated with BA 12.5 mgl^{-1} + IAA 50 mgl^{-1} by 24 h soaking just prior to planting. The experiment started on March 18. R: percentage of root formation, C: percentage of collapse and S: percentage of shoot formation.

Light intensity (Wm^{-2})	Leaves formed during the treatment		Leaf cuttings				
			R (%)	C (%)	S (%) after		
	number	length (cm)	4.5	10	12	15	18 wks
0.6	7	7.7	3	23	20	28	33
6	12	9.0	40	0	8	25	45
12	13	10.9	48	0	3	15	20
24	14	9.7	30	0	0	3	3
36	13	9.1	18	5	0	3	5

formation in both treatments was such as not seen before, 10-25 buds per leaf cutting were regularly visible, not only at the base of the petioles, but also evenly distributed over a 1-2 cm long area above the bases. With higher light intensities the number of buds decreased to 1-5 per cutting, originating only at the very base of the petiole. The frequency of early shoot formation at first increased as lower light intensity had been applied (Table 4, after 12-15 weeks), but later an optimum was found at 6 Wm^{-2}. The numbers of shoots eventually developed were not significantly affected by the stock plant treatments. Summarizing, the results obtained suggest an extremely high capacity for bud initiation in cuttings from plants temporarily grown at a very low light intensity. This capacity, however, was only partly expressed in a high degree of shoot formation.

Plant growth regulator pretreatment of the stock plants. In the preceding section the condition of normal stock plants was changed by means of changing the environment. For the same purpose, plants were sprayed with cytokinin and growth retardants.

Spraying 'Bravo' and 'Super Yellow' stock plants with cytokinin (BA 1, 5, 25 and 125 mgl^{-1} once a week for a period of 6

weeks) strongly reduced plant height and induced axillary buds to develop, but did not promote early shoot regeneration of the leaf cuttings. The cytokinin concentration required for early shoot regeneration of the leaf cuttings did not decrease either.

Spraying the stock plants with retardants had a more favourable effect. Certain treatments increased the frequency of early shoot formation. Table 5 gives the results with 'Bravo'. After treatment with B-9 the plants hardly grew anymore, the cuttings rooted poorly, many died prematurely and only low levels of shoot regeneration were found. Evidently B-9 alone or in combination with the BA + IAA treatment was harmful. After treatment with CCC and A-Rest no damage was noted. The extension growth of the plants was inhibited compared to the control

Table 5. Effects of growth retardants on growth of stock plants and on regeneration of leaf cuttings in *Chrysanthemum* 'Bravo'. The stock plants were treated with different retardants by spraying once a week for a period of three weeks. The leaf cuttings, 35 per treatment, originating from positions 4-8, were given BA 12.5 mgl^{-1} + IAA 50 mgl^{-1} by 24 h soaking just prior to planting. The experiment started on February 3. R: percentage of root formation, C: percentage of collapse and S: percentage of shoot formation.

Growth retardant, mgl^{-1}		Stock plant growth (cm)*	Leaf cuttings			
			R (%)	C (%)	S (%) after	
			5	10	12	18 wks
Control		11.7	23	0	3	17
B-9	1000	2.1	17	9	0	17
	5000	1.0	3	34	0	3
CCC	500	8.8	40	0	0	20
	2500	6.6	54	0	0	31
A-Rest	5	7.8	51	0	11	51
	25	3.3	43	0	3	37

* Increase in length during the treatment.

plants. In general leaf cuttings from these plants rooted better than in the control. In these treatments, except for CCC 500 mgl^{-1}, early shoot regeneration was slightly improved. With 'Super Yellow' only leaf cuttings from plants treated with A-Rest 25 mgl^{-1} attained better shoot formation, viz. 30% versus 18% in the control. The results suggest that it is possible to enhance the capacity of the leaf cuttings for early shoot regeneration by retardant pretreatment of the stock plants. Probably, however, not all retardants are suitable. The best concentrations and the optimum duration of the pretreatment are not yet known.

DISCUSSION

The results of this study show that early shoot formation of chrysanthemum leaf cuttings is dependent on the developmental stage and pretreatment of the stock plants. First, leaf cuttings from crown bud plants showed a much higher ability of early shoot regeneration than those from vegetative plants. Secondly, growing for long periods under SD conditions, growing under very low light intensity, and application of certain retardants, apparently changed the physiological condition of vegetative plants in a way promotive to early shoot regeneration on leaf cuttings. The most spectacular observation was the regeneration of numerous buds on the petioles of the yellowish-white and light green leaf cuttings from plants grown in almost complete darkness or dim light. It is an intriguing question whether these different conditions, which led to improvement of early shoot regeneration, have a similar physiological basis.

The leaf cuttings from the crown bud stock plants exhibited, after treatment with cytokinin and auxin a much higher capacity for early shoot regeneration than leaf cuttings from vegetative plants. The reason might be connected with the generally small size of the leaves of the crown bud plants or with the beginning of the generative development in these plants.

In the daylength experiments a slight increase in early shoot regeneration was observed only after long periods of SD given to the stock plants. Shorter periods of SD, which already effectively induce flowering, did not improve shoot regeneration. Thus the improvement of early shoot regeneration was less associated with the beginning of the generative development than with the use of the small leaves.

A potentially very high regeneration capacity was observed in the white to light green leaf cuttings from plants raised in almost complete darkness. Obviously, these leaves had a very low metabolic activity. In this, they probably resemble the small leaves in the large inflorescences of the plants after long periods of SD. Those leaves also have a low metabolic activity, as they have to compete for nutrients and carbohydrates with the young flower buds. It is not unlikely that also in the leaves of the crown bud plants and in those of the plants treated with retardants certain metabolic processes are operating at a lower level than in leaves from luxuriously growing vegetative plants.

If the above suppositions hold true, it might be concluded that the induction of early shoot regeneration succeeds better with metabolically less active leaves than with active ones. In Chapter 3 the same was observed for late shoot regeneration, which only occurred after the leaf cuttings had ceased their luxurious growth and started senescence. The obvious interpretation then is that first the metabolic activity has to be decreased before regeneration can take place. This interpretation agrees well with ideas of Tran Thanh Van (1981), viz. that the process of morphogenesis is more a matter of removing an inhibitory environment than of providing a specific stimulus.

It was very noticeable that leaf cuttings from plants grown at low light intensity already showed numerous buds relatively short time after planting. The picture was quite similar to in vitro regeneration where the full area of an explant may be covered with buds (cf. Roest and Bokelmann, 1975). In fact, we found comparable light intensity effects for in vitro adventitious bud formation on chrysanthemum pedicel explants (De Jong and Custers, 1986). In that study an increase in bud regeneration after lower stock plant light intensity was accompanied by a decrease in brown discoloration of the explants, which obviously was the result of phenolic oxidation. It is often observed in various types of cultures in vitro that there exists a strong inverse relation between regeneration and amount of phenoles or degree of release of brown exudates (e.g. George and Sherrington, 1984; Glimelius, 1984). Possibly, in the present experiments a low level of endogenous phenoles in the leaves developed under low light intensity conditions enabled them to exhibit such a high level of bud regeneration and early shoot formation.

In this study it was found that stock plant condition, besides application of cytokinin and auxin to the leaf cuttings, contributed considerably to early shoot regeneration. This leads to the conclusion that a satisfactory method can be developed giving reproduceable high percentages of shoot regeneration. The best results so far were obtained with leaf cuttings from crown bud plants. However, even more promising is raising the stock plant material at very low light intensity. A practical drawback of this procedure, however, is the soft and very tender leaf tissue, which is highly sensitive to infections. This problem may be overcome by disinfection of the cutting bench substrate. Another problem, which still exists, is that the buds, which are already visible after about one month, develop into shoots only 10-15 weeks after planting of the cuttings.

CHAPTER 6

IN VIVO REGENERATION OF LEAF CUTTINGS OF 'ELATIOR'-BEGONIAS (*BEGONIA* X *HIEMALIS* FOTSCH); THE 'T-HYBRIDS'

INTRODUCTION

In the past ten years *Begonia* in the Netherlands rose from the fourth to the first place on the list of flowering pot-plants. Turnover in 1985 was a good 30 million guilders which is more then ten times the figure for 1971 (Anon., 1974, 1977, 1986). This increase is mainly the result of the contribution of new 'Elatior'-hybrids (*Begonia x hiemalis* Fotsch) which originated from breeding programmes of Rieger at Nürtingen in Germany (Hahn, 1958, 1966) and Doorenbos and Karper at Wageningen in the Netherlands (Doorenbos, 1973a, b; Doorenbos and Karper, 1975). The cultivars from these programmes exist under the collective names 'Rieger-Begonias' and 'Tuinbouwplantenteelt- or T-Hybrids' respectively. Leaf cuttings of the 'T-hybrids' have a very high capacity of adventitious shoot regeneration, as this characteristic was especially selected for during the breeding programme (Karper, 1971). This paper deals with research on the regeneration pattern in this group of hybrids.

Successful shoot regeneration is relatively widespread in the genus *Begonia*, it has frequently been a subject of research (cf. Broertjes et al., 1968). Especially the work by Heide (1964, 1965a, b) yielded results which had practical importance. He found that shoot regeneration of leaf cuttings of 'Gloire de Lorraine'-begonias (*B. x cheimantha* Everett) was strongly improved by low temperature (15-18 °C) and short day conditions, applied either to the stock plants or to the leaf cuttings. On the other hand high temperature and long day conditions were inhibitory to shoot regeneration.

The general view was that the reaction patterns described by Heide also prevailed in 'Elatior'-begonias (Karper, 1971; Goldschmidt, 1974), but temperature seemed to be less effective than daylength (see review by Rünger, 1976). A contradiction was also found in the effects of daylength. On the one hand favourable effects on subsequent shoot regeneration of short day conditions during the stock plant period were reported (Beek, 1973; Gislerød, 1974; Hilding, 1974; Cohl and Moser,

1976a), on the other hand such effects were not found or appeared to be dependent on the season (Von Hentig, 1976; Powell and Bunt, 1979). Moreover, 2-6 weeks of short days reduced growth of the stock plants and decreased the quality of the newly formed leaves as cuttings (Von Hentig, 1976; Powell and Bunt, 1978). Conflicting results were also found with respect to short day treatment of the leaf cuttings. Promotive effects (Marijnen, 1966; Karper, 1971; Gislerød, 1974) as well as inhibiting effects (Beek and Vonk Noordegraaf, 1973; Cohl and Moser, 1976b; Powell and Bunt, 1979, 1980) have been reported.

The purpose of the present research was to study the regeneration pattern of leaf cuttings of the 'T-Hybrids', which had not been investigated extensively before. Hence it was not known if the regeneration pattern was similar to that in the other 'Elatior'-begonias, and how far it would deviate from the situation found by Heide in 'Gloire de Lorraine'-begonias. To this end the effects of daylength and temperature treatments and of leaf age were investigated. For comparison leaf cuttings of 'Riegers Schwabenland Red', which generally have a high regeneration capacity, were examined.

The second purpose was to find an explanation for the conflicting results of previous research on regeneration in 'Elatior'-begonias. To this end special attention was given to the duration of the daylength treatments.

MATERIAL AND METHODS

Plant material. Leaf cuttings from three different 'Elatior'-begonias (*Begonia x hiemalis*), 'SO_1', 'Turo' and 'Riegers Schwabenland Red', were used. The first two are 'T-Hybrids'. 'SO_1' was selected from the F_1 of the cross *B. x tuberhybrida* 'Bertinii compacta Sonnenschein' x *B. socotrana*. A yellow flowering mutant 'Tiara' derived from 'SO_1' (see Doorenbos and Karper, 1975) was introduced into commerce. 'Turo' is a mutant from a hybrid from the cross *B. x tuberhybrida* 'Bertinii compacta Leuchtfeuer' x *B. socotrana*, and was also released as a commercial cultivar (Doorenbos, 1973b). 'Riegers Schwabenland Red' (hereafter indicated as 'Schwabenland'), the most important cultivar of the 'Rieger-Begonias', originated from a cross between an unknown tuberous cultivar and *B. socotrana* (Hahn, 1966).

The stock plants were grown from adventitious shoots, each one individually potted in 11 cm pot containing peat mold en-

riched with a commercial fertilizer (Alkrisal 1 g per 1 substrate). When the plants were actively growing, this fertilizer was replenished every two weeks by addition to the irrigation water (1.5 gl^{-1}). The plants were maintained at 20 °C D/ 18 °C N under long day conditions (LD, extension of daylength to 16 h by incandescent lamps) in a greenhouse which was shaded by whitewash during spring and summer. During autumn, winter and early spring vegetative growth of the plants was stimulated by supplementary light from HPLR 400 W Philips lamps given at plant level about 10 Wm^{-2} (measured with a flat selenium photo cell). Young flower buds were regularly pinched off to stimulate vegetative growth.

The stock plant age at which the leaf cuttings were collected was 4-5 months (calculated from potting date). This is a relatively young age as compared to the situation in practice, but it was chosen to prevent the plant material from having a long history which could interfere with the factors under investigation.

Leaf cuttings. The leaf cuttings were broken off the plants at the base of the petiole. They were inserted in cutting benches of peat mold (without extra nutrients, pH 5.5) which were kept under LD and a temperature of 20 °C minimum and 22 °C maximum. During summer a higher temperature was prevented by whitewash on the greenhouse and moistened cheesecloth on the benches. As natural light intensities are low from November till March supplementary light was applied with MLL 160 W Philips lamps giving about 18 Wm^{-2} at the cutting level.

All the leaves used have been numbered consecutively from the top of the plant to the base. The youngest, but just entirely macroscopically visible leaf was designated as position 1. The leaves at positions 2, 3 and 4 were still expanding whereas those at 5, 6 and lower positions were full-grown. The leaves at positions 5-7 were the largest in size, having a longest vein of about 8, 9 and 11 cm in 'SO_1', 'Turo' and 'Schwabenland' respectively (see also Figures 1 and 4 for details on leaf blade shape). Lower leaves, about four in number, diminished in size towards the base. Those leaves had already been formed when the young stock plants were potted.

The experiments were performed with leaves from positions 3-5 (38 or more per treatment) or with the leaves from the individual positions 1-7 (16 from each position per treatment). The cuttings of each treatment were spread in small groups over the whole cutting bench to minimize location effects. During

the first four weeks in the benches a low number (at most 15%) of the cuttings became infected, and the numbers of cuttings presented were corrected accordingly.

Temperature. Different temperature regimes (13, 17, 21 and 25 °C) were applied in the glasshouses of the phytotron (Doorenbos, 1964). All the temperature experiments were performed from October untill March under natural daylength conditions. The actual mean daily temperatures deviated not more than 0.5, 0.7, 0.3 and 0.5 °C from the indicated values.

Daylength. Daylength treatments (8 and 16 h, SD and LD) of different lengths were performed in the greenhouse from October till April at 20 °C D/18 °C N. For SD treatment the plants or cuttings were placed under a tent of black cloth, 1.5 m high and equipped with a ventilator, from 4.30 p.m. till 8.30 a.m. For LD treatment the daylength was extended by 40 W incandescent lamps from 4.30 p.m. till 0.30 a.m. For both SD and LD material natural daylight was supplemented with MLL 160 W Philips lamps giving about 22 Wm^{-2} at plant or cutting level from 8.30 a.m. till 4.30 p.m.

Observations. To determine the progress of regeneration, root and bud formation were recorded after 3-5 weeks when differences were largest. Each cutting was carefully lifted and the roots were turned back to allow examination of bud initiation on the petiole base, after which it was replanted. After 8-10 weeks, when generally all cuttings had formed buds, the final examination was done. Individual buds and shoots were removed and divided into classes of different length. Mean numbers of buds and shoots were calculated for all cuttings. Shoots were regarded as such if they had a length of 1 cm or more. The percentage of cuttings with two or more shoots was determined and used as indication of usefulness of a treatment from a practical point of view.

All results presented were confirmed by the data of at least two experiments. For means the standard deviation of the mean was calculated and differences were tested for significance ($P = 0.05$) by Student's *t* test.

RESULTS

General observations. The leaf cuttings of all three cultivars readily regenerated both roots and shoots, the eventual per-

centages were normally 100%. Adventitious buds were already visible after 3-4 weeks, but generally they appeared a bit later in 'Turo' than in 'SO_1' and 'Schwabenland'. The numbers of buds were very large, roughly 10-20 in 'SO_1', 20-40 in 'Turo' and an intermediate number in 'Schwabenland'. Development of a few shoots was already visible after about 7 weeks (see Figure 1). Subsequently the number of developing shoots gradually increased, but at the end of the experiment only a small part (5-25%)of buds had actually developed into shoots.

Effects of daylength. Leaf cuttings were collected from each of the three cultivars mid November and were grown under SD conditions for 14, 28, 42 and 56 days (Table 1). The cultivars behaved rather similarly. SD did not seem to affect rooting. On the other hand, SD treatments of 14 days or more significantly increased the percentage of bud formation. A similar effect was found for the mean number of buds and shoots, except in 'SO_1'

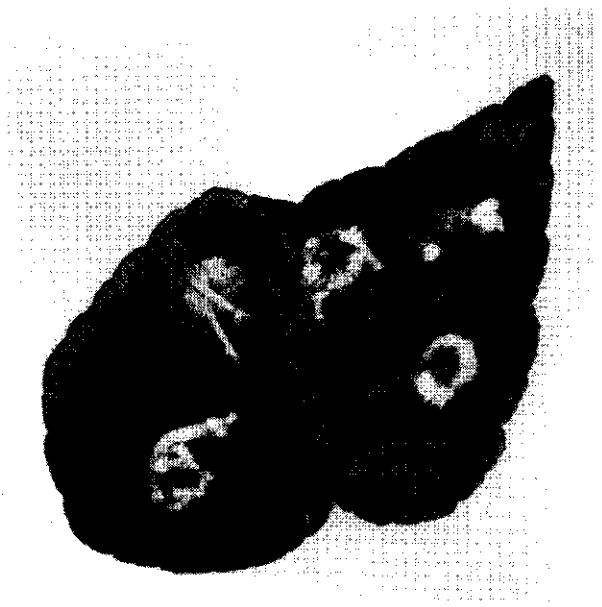


Figure 1. Adventitious bud regeneration of *Begonia* 'Turo'. Excised petiole bases showing luxurious bud regeneration and some shoot development, placed on a leaf blade used as a background. The photograph was taken seven weeks after planting.

Table 1. Regeneration of *Begonia* 'Elatior' leaf cuttings as affected by short day (SD). SD treatments of different lengths were given to the cuttings immediately after planting. Three cultivars were used, 'SO_1', 'Turo' and 'Riegers Schwabenland Red', each with at least 38 cuttings per treatment. All cuttings originated from positions 3 and 4. The experiment started on November 15. R: percentage of root formation, B: percentage of bud formation, MBS: mean number of buds and shoots, and TS: percentage of cuttings with two or more shoots.

Cultivar	SD (days)	R(%) 4 wks	B(%) 4 wks	MBS 10 wks	TS(%) after 10 wks
'SO_1'	0	100	23	11 a*	48
	14	93	57	16 b	57
	28	90	63	12 a	20
	42	92	62	12 a	23
	56	88	55	9 a	4
'Turo'	0	73	0	19 a	10
	14	89	30	27 b	35
	28	91	45	27 b	12
	42	79	39	28 b	2
	56	90	45	23 ab	0
'Schwaben-land'	0	100	25	21 a	9
	14	97	56	26 b	18
	28	98	63	26 b	3
	42	95	58	27 b	3

* Means within the same group designated by the same letter are not significantly different from each other at P = 0.05.

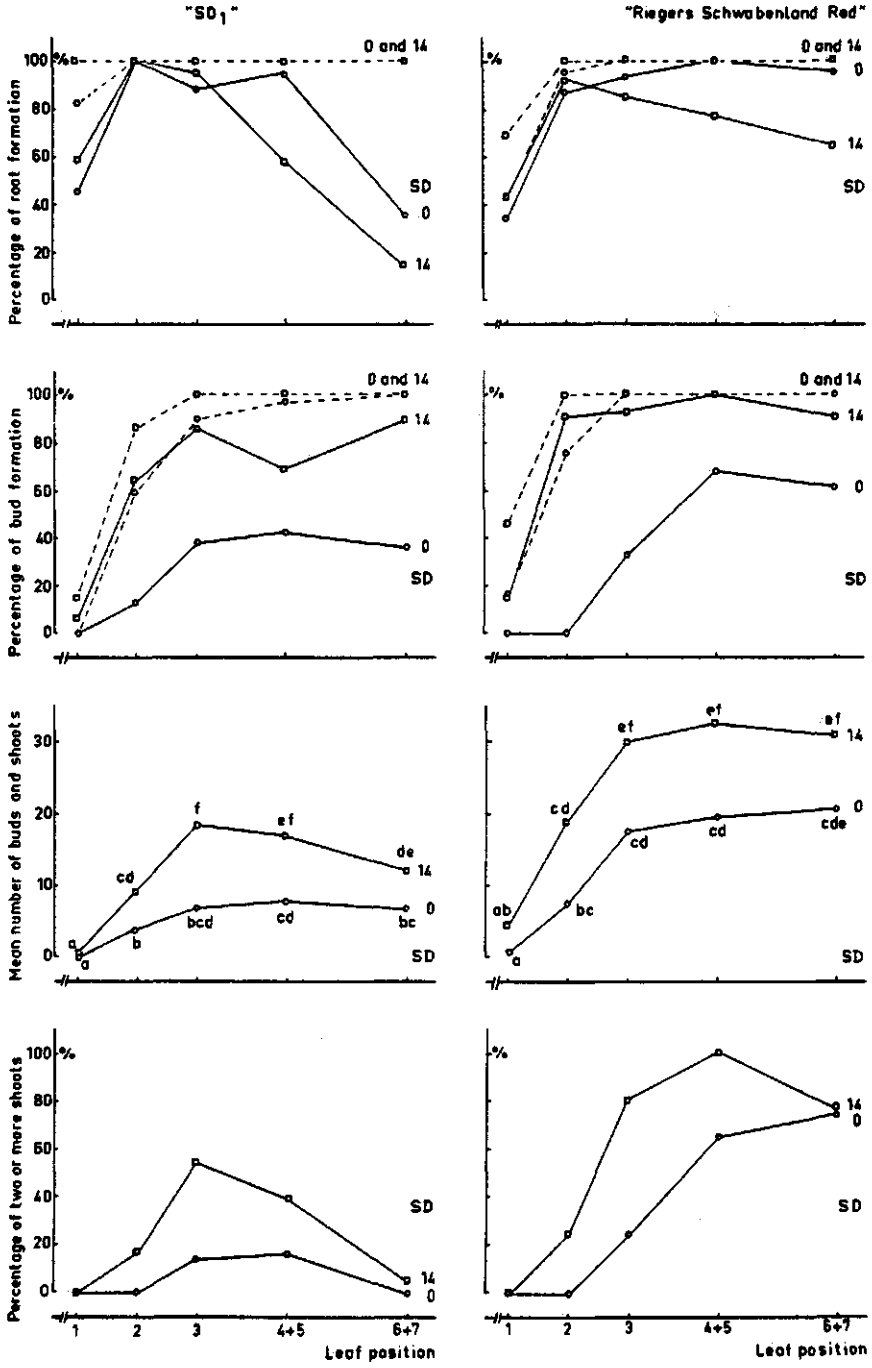
with an optimum number at 14 SD. Also the percentage of cuttings with two or more shoots increased after 14 SD, but in all three genotypes it strongly decreased after longer periods. Noticeably, hardly any shoots emerged from the soil after four or more weeks SD. In a replicated experiment shoot development was even worse under continuous SD. So as far as usefulness in practice was concerned the results showed a favourable effect of a short period of SD and an unfavourable effect of a long period.

Heide (1964, 1965a) found with 'Gloire de Lorraine'-begonias that SD treatment of the stock plants had the same promotive effect on bud regeneration as SD treatment of the leaf cuttings. This was also studied in the present 'Elatior'-begonias (Table 2). A SD treatment of four days increased the percentage

Table 2. Effects of short day (SD) treatments of *Begonia* 'Elatior' stock plants on regeneration of the leaf cuttings. SD treatments of different lengths were given just before collecting the cuttings. Three cultivars were used, 'SO_1', 'Turo' and 'Riegers Schwabenland Red', each with at least 43 cuttings per treatment. All cuttings originated from positions 3, 4 and 5. The experiment started on October 30. R: percentage of root formation, B: percentage of bud formation, MBS: mean number of buds and shoots, and TS: percentage of cuttings with two or more shoots.

Cultivar	SD (days)	R(%) 3 wks	B(%) 3 wks	MBS 8 wks	TS(%) after 8 wks
'SO_1'	0	78	43	9 a*	3
	4	61	68	14 b	14
	7	46	53	14 b	15
	14	43	58	14 b	11
	28	44	63	15 b	13
	42	42	50	13 b	17
'Turo'	0	78	17	27 a	6
	4	94	19	28 a	12
	7	88	42	26 a	18
	14	92	49	31 a	28
	28	79	58	30 a	28
	42	72	47	25 a	17
'Schwaben-land'	0	67	58	16 a	10
	4	100	91	24 b	18
	7	85	100	21 b	19
	14	72	100	20 b	24
	28	71	100	20 ab	33
	42	73	100	21 b	27

* Means within the same group designated by the same letter are not significantly different from each other at P = 0.05.


"SD 1"
"Riegers Schwabenland Red"
Percentage of root formation
Percentage of bud formation
Mean number of buds and shoots
Percentage of two or more shoots
0 and 14
0
14
SD
%
Leaf position
1
2
3
4+5
6+7
a
ab
b
bc
bcd
cd
cde
d
de
ef
f

of root formation in 'Turo' and 'Schwabenland', but not in 'SO_1'. With longer periods of SD rooting gradually decreased in all cultivars. Just as rooting bud formation was strongly increased by a short period of four SD, but now particularly in 'SO_1' and 'Schwabenland'. A similar effect was found for the number of buds and shoots and for the percentage of cuttings with two or more shoots. 'Turo' showed the same effects only after a period of 7-14 SD. In contrast to rooting, longer periods of SD did not reduce bud formation. Summarizing, SD treatment of the stock plants appeared to have rather the same effects as SD treatment of the cuttings, with the one exception that a long period did not inhibit shoot development.

The question arose as to how far the strong effect of the short period of SD would depend on the age of the leaf cuttings. To investigate this stock plants were given 0 and 14 SD and the leaves from positions 1 to 7 were recorded separately. Figure 2 gives the results of two genotypes. Root and bud initiation as well as shoot development responded in the same way to SD as in the previous experiment, at least with respect to the comparable leaf positions (cf. Table 2). Leaf position appeared to have a large effect. The youngest leaves showed a low percentage of root formation and hardly formed any shoots. With older leaves the capacity for regeneration of both roots and buds increased, reached an optimum and then more or less decreased, depending on the cultivar.

Figure 2. Regeneration of *Begonia* 'Elatior' leaf cuttings as affected by leaf age (leaf position) and short day (SD) treatment of the stock plants. Stock plants were given a period of 0 and 14 SD just preceding collecting the cuttings. Leaves from seven positions were used, each position comprising at least 16 cuttings per treatment. Those from positions 4 and 5, and those from 6 and 7 were pooled. Two genotypes were studied, 'SO_1' and 'Riegers Schwabenland Red'. Percentages of root and bud formation were recorded after four and eight weeks (—— and ---), and mean number of buds and shoots and percentage of cuttings with two or more shoots after eight weeks. Means designated by the same letter are not significantly different from each other at $P = 0.05$. The cuttings were planted on March 14.

SD treatment had a marked influence on this leaf age effect. It strongly improved both root and bud formation in the youngest leaves. With older leaves also an improvement of bud formation was found, but the SD influence on root formation gradually changed from promotive into inhibitory as leaf age increased.

Number of buds and shoots and percentage of cuttings with two or more shoots showed the same trend as percentage of bud formation. SD treatment increased the levels of both parameters. The increases were stronger with young to intermediate age leaves than with older ones, consequently optimum curves appeared. Remarkably, in 'SO_1' the optima were found at a younger leaf age than in 'Schwabenland'. In 'Turo' (results not presented) the pattern of the leaf age effects was fairly similar to that of 'Schwabenland'.

Effects of temperature. Leaf cuttings of the three cultivars were planted in the glasshouses of the phytotron at 13, 17, 21 and 25 °C, and after five weeks transferred to the standard conditions (minimum 20 °C D/ 18 °C N, LD). Both root formation and bud formation increased with higher temperature (Figure 3). The three cultivars differed in rate of organ formation, but they reacted in a similar way to the temperature treatments. The mean number of buds and shoots showed an optimum at 21 °C in all three genotypes. The percentage of cuttings with two or more shoots also increased with higher temperature. In short, the most important effects were promotion and acceleration of the whole process of regeneration with higher temperature and decrease in the number of buds and shoots at the highest temperature of 25 °C. A side-effect was observed at 13 and 17 °C, viz. swellings on the petiole bases in 'SO_1' (Figure 4) and occasionally in 'Schwabenland'. No buds developed from those swellings, which may have been part of the cause of the lower number of buds at the low temperatures.

Heide (1964) found that low temperature improved bud regeneration. So far the present experiments showed the opposite effect. To examine whether Heide's results would be repeatable for 'Elatior'-begonias, cuttings of young leaves, with a low regeneration capacity (cf. Figure 2), were placed at the different temperatures for different periods. When the temperature treatments lasted five weeks, no clear differences were observed, but after temperature treatments of two weeks a promotive effect of 13 °C on bud regeneration was demonstrated (Table 3).

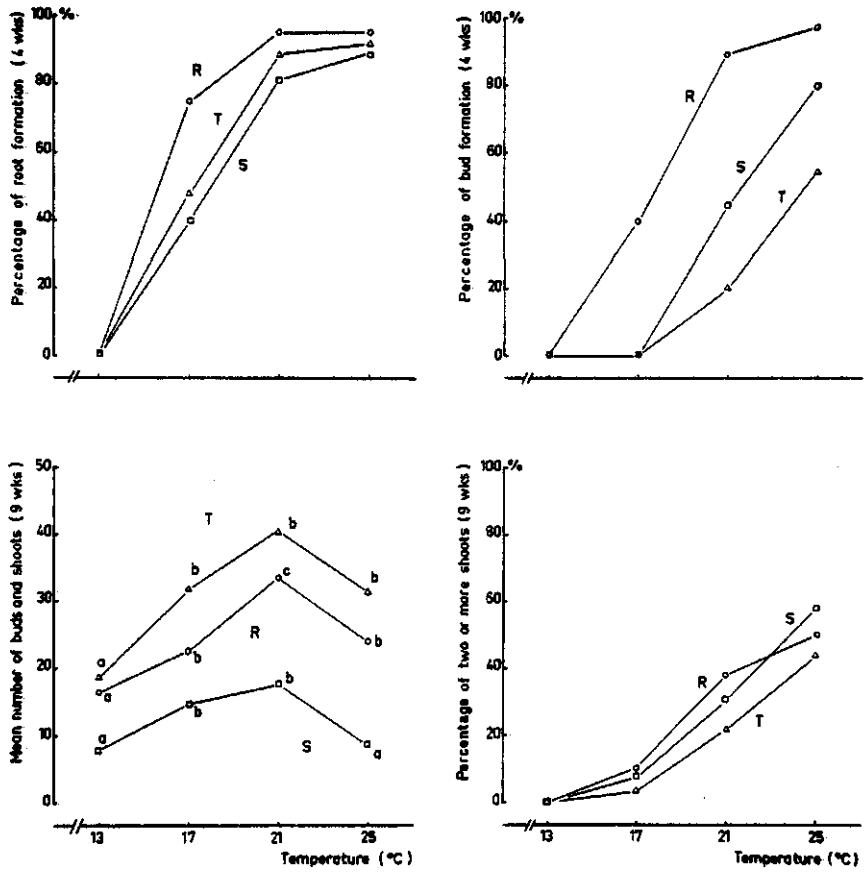


Figure 3. Regeneration of *Begonia* 'Elatior' leaf cuttings under different temperature regimes. The cuttings were kept at the indicated temperatures for five weeks. Three genotypes were tested, 'SO_1' (□, S), 'Turo' (Δ, T) and 'Riegers Schwabenland Red' (O, R), each with at least 42 cuttings per treatment. All cuttings originated from positions 3, 4 and 5. The experiment started on November 5. Means designated by the same letter do not significantly differ at P = 0.05.

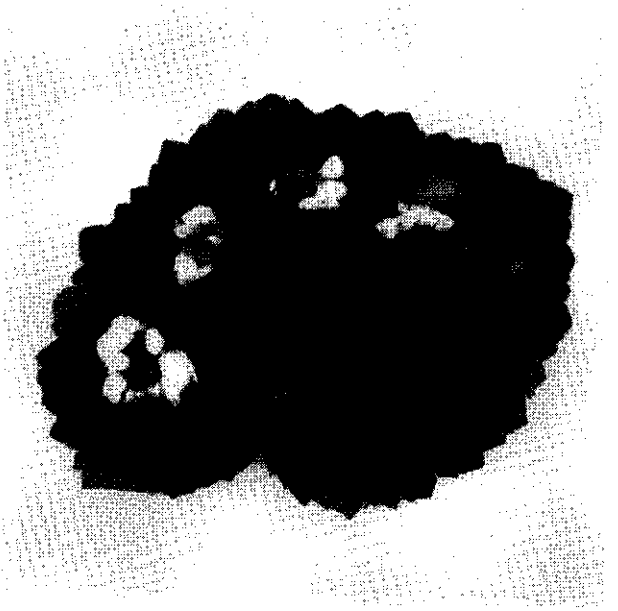


Figure 4. Adventitious bud regeneration of *Begonia* 'SO_1'. Excised petiole bases placed on a leaf blade as a background. They show normal bud regeneration (lower right) and different degrees of swellings where no buds were formed. The cuttings were grown at low temperature (13-17 °C) in SD for five weeks followed by 20 °C in LD for four weeks.

The nearly and just fully grown leaves always reached 100% of bud regeneration at the end of the experiments, irrespective of the temperature in the benches. This suggests that such leaves have already developed a high regeneration capacity on the stock plants. Therefore it was investigated whether this could be affected by a temperature pretreatment of the stock plants. Figures 5 and 6 show results with 'SO_1' and 'Turo'. At higher temperature more new leaves were formed. Therefore, to obtain a complete picture, all leaves were used except the smaller ones at the base of the plants. In 'SO_1' as well as in 'Turo', after higher stock plant temperature, regeneration of both roots and buds generally decreased, bud regeneration decreasing more rapidly than root regeneration. After eight weeks numbers of buds and shoots and shoot development showed the same temperature effect as initial bud regeneration, i.e. a decrease as a higher stock plant temperature was given. 'Schwa-

Table 3. Regeneration of young leaf cuttings of *Begonia* 'Riegers Schwabenland Red' as affected by different temperature regimes. The temperatures were given for two weeks followed by three weeks 25 °C and thereafter 20 °C. Each treatment comprised at least 16 cuttings from expanding leaves (position 2). The experiment started on March 20. R: percentage of root formation, B: percentage of bud formation, and MBS: mean number of buds and shoots.

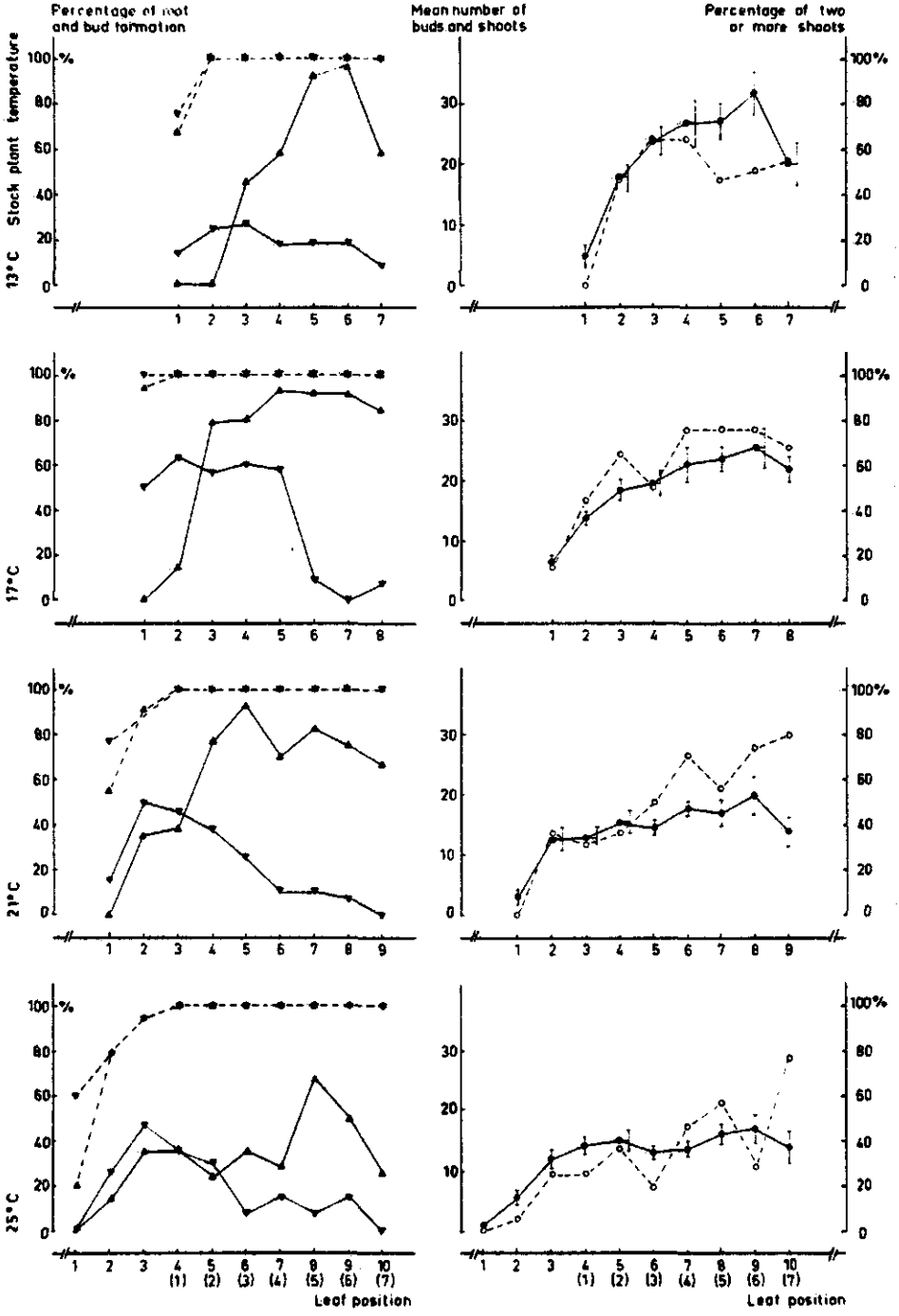
Temperature (°C)	R(%)	B(%)		MBS after
	3 wks	5	9 wks	9 wks
13	46	54	94	13 b*
17	65	41	78	9 a
21	89	47	61	6 a
25	86	50	57	5 a

* Means designated by the same letter are not significantly different from each other at P = 0.05.

benland' (results not presented) showed the same temperature effects.

Besides these temperature effects, a large influence of leaf age on regeneration was observed. In 'SO_1' (Figure 5) both initial root and initial bud formation showed an optimum curve, but the optimum for rooting was found at younger leaf ages than that for bud formation. 'Turo' showed a similar picture, but the optima were found at older leaf ages. Moreover, bud formation started later. Therefore, by comparing the curves a characteristic regeneration pattern for both genotypes can be deduced: in 'SO_1' most leaves form buds first and than roots, whereas in 'Turo' the order is reversed. From the point of view of commercial propagation, leaves of 'SO_1' can already be used at an age when those of 'Turo' are hardly productive. Compared with 'SO_1' and 'Turo', 'Schwabenland' showed an intermediate effect of leaf age.

Daylength and temperature. Heide (1964) showed with 'Gloire de Lorraine'-begonias that low temperature treatment of the stock plants had a similar promotive effect on bud formation as SD treatment. It was tested whether the same would hold true with 'Elatior'-begonias. Therefore, stock plants were grown, for short periods, at low temperature or under SD conditions. In


Percentage of root and bud formation
Mean number of buds and shoots
Percentage of two or more shoots
Stock plant temperature
13°C
17°C
21°C
25°C
%
100%
Leaf position

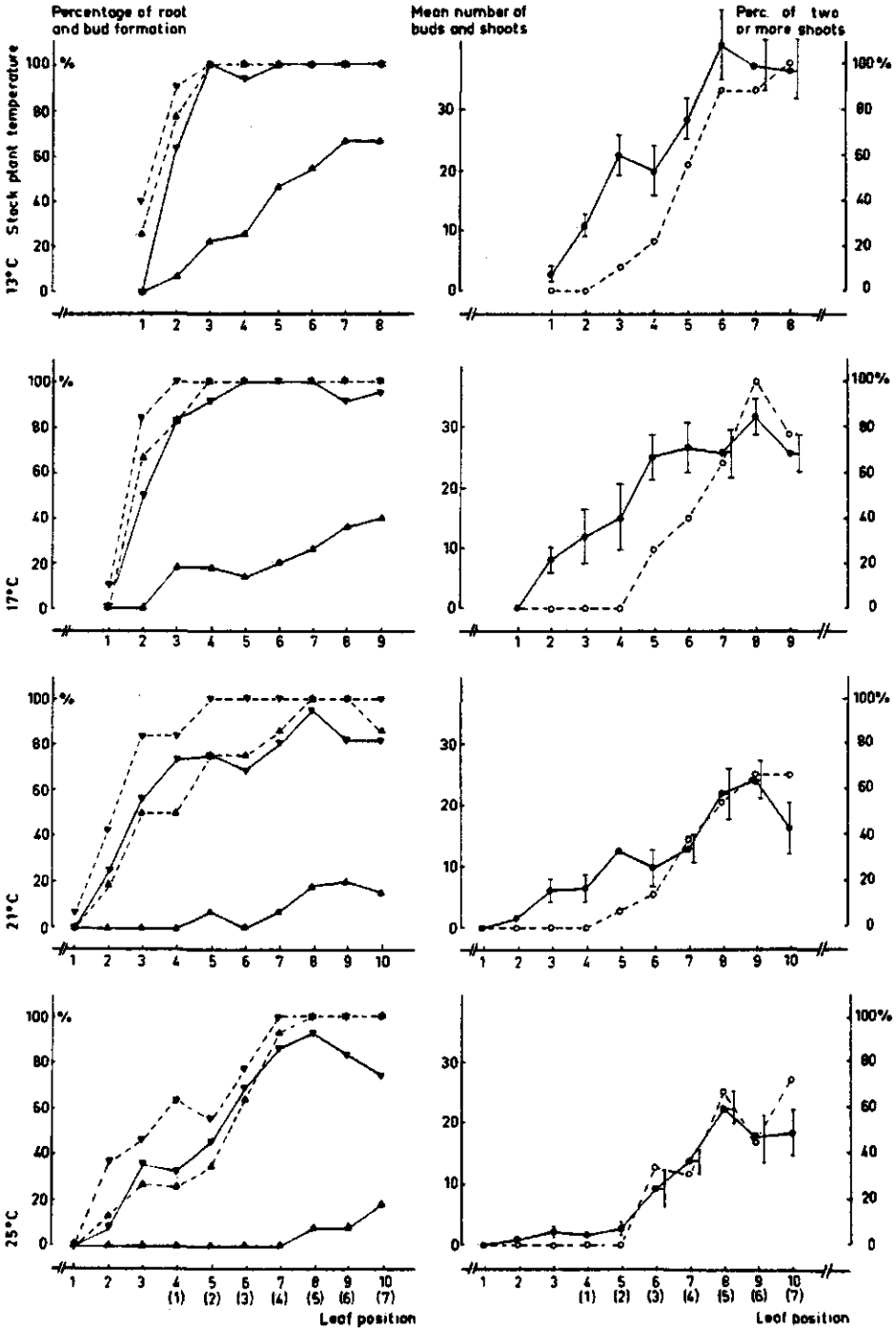
contrast to four and seven days SD, with strong promotion of bud regeneration, the same periods at 13 and 17 °C had no effect at all. This suggests that the stock plant low temperature is less effective than the SD. Probably, the effects of both factors result from different mechanisms.

DISCUSSION

The leaf cuttings of the three begonia genotypes investigated had a very high root and bud regeneration capacity and subsequently a good shoot development. A short period of SD during the leaf cutting phase promoted bud initiation considerably: the number of buds and the percentage of cuttings with two or more shoots increased. Longer periods of SD, however, counteracted shoot growth and under continuous SD hardly any shoots developed. When stock plants were pretreated by short periods of SD the same favourable effect occurred, but longer periods were not disadvantageous.

Besides daylength, temperature proved to be very important. Increasing the temperature from 13 to 25 °C in the cutting bench hastened root and bud initiation and generally resulted in a higher number of buds and shoots and a higher percentage of shoot development. Two exceptions were found, (1) decrease in the number of buds and shoots at the highest temperature (25 °C), and (2) increase in overall bud regeneration capacity when

Figure 5. Regeneration of *Begonia* 'SO_1' leaf cuttings as affected by leaf age (leaf position) and by temperature at which stock plants were grown. The stock plants were kept at the indicated temperatures for four weeks just preceding collecting the cuttings. Both leaves which developed during this temperature treatment and leaves which already existed before were used. Position numbers between brackets on the abscissa indicate the leaves present before temperature treatments. Each position comprised at least 16 cuttings per treatment. The experiment started on February 20. Percentages of root (▼) and bud (▲) formation were recorded after three and eight weeks (—— and ---), and mean number of buds and shoots (●——●) and percentage of cuttings with two or more shoots (o---o) after eight weeks. Vertical bars indicate standard errors of the means.


Percentage of root and bud formation
Mean number of buds and shoots
Perc. of two or more shoots
Stock plant temperature
13°C
17°C
21°C
25°C
%
100%
Leaf position

very young leaf cuttings, with a low regeneration capacity, were kept at low temperature (13 °C) for a short time immediately after planting. In contrast to the leaf cutting treatments, increasing temperature treatments of the stock plants generally resulted in reduced bud regeneration.

Heide (1964, 1965a) found that bud regeneration of 'Gloire de Lorraine'-begonias was strongly improved by low temperature as well as by SD, both as a stock plant and as a leaf cutting treatment. Most of the present results were not in agreement with the findings of Heide. This is probably mainly due to the fact that the leaf cuttings of the 'Gloire de Lorraine'-begonias used by Heide have a much lower regeneration capacity at the moment of planting than the genotypes investigated here. Heide used the eventual frequency of bud regeneration as the main parameter to measure the effects of the treatments. In the present research this nearly always reached 100%, so that rate of bud initiation and development of the buds into shoots had to be used as parameters.

The general regeneration pattern of the two 'T-hybrids' investigated in this research was virtually the same as found in other 'Elatior'-begonias which can be propagated from leaf cuttings on a practical scale (cf. Beek, 1973; Hilding, 1974; Rünger, 1976). Bud regeneration capacity and shoot productivity were rather similar to 'Riegers Schwabenland Red' which is generally considered to be quite well adapted to commercial propagation by leaf cuttings (cf. Cohl and Moser, 1976a). Thus the selection for a high capacity of shoot regeneration during the breeding of the 'T-Hybrids' (Karper, 1971) indeed led to the desired effect.

In this research the effects of different periods of SD applied to the cuttings were examined both with regard to bud initiation and shoot development. Very short periods of SD were beneficial for both parameters, but longer periods were negative for shoot development, although the number of buds was still promoted. This may explain the contradicting conclusions in earlier publications. One group of researchers reported on positive effects of SD treatments (e.g. Marijnen, 1966; Gislerød, 1974). They based this conclusion on the number of buds

Figure 6. Regeneration of *Begonia* 'Turo' leaf cuttings as affected by leaf age and by temperature at which stock plants were grown. The experiment started on March 21. Otherwise as Figure 5.

formed. Other researchers, however, examined the number of shoots emerging from the soil, either after application of long periods of SD (Cohl and Moser, 1976b; Powell and Bunt, 1979) or after SD treatment started only after root formation (Beek and Vonk Noordegraaf, 1973), and observed a negative SD effect.

In literature there are also different views on the usefulness of SD treatments of the stock plants in the case of 'Elatior'-begonias, some positive (Gislerød, 1974; Rünger, 1976), some negative (Von Hentig, 1976; Powell and Bunt, 1979). The results presented here demonstrate that a short period of SD given to the stock plants strongly improves shoot regeneration of the leaf cuttings

The length of the SD period required for an optimum effect on shoot regeneration was very short (4-14 SD), both when stock plants and when leaf cuttings were treated. This is the same length of SD treatment found to promote flowering in 'Elatior'-begonias (Westerhof, 1980; Powell and Bunt, 1985). It was also shown that longer periods of SD delayed flowering and arrested growth (Von Hentig, 1976; Powell and Bunt, 1978; Westerhof, 1980). Presumably, these different phenomena are based on one and the same physiological process, which might have to do with genetic information originating from *Begonia x tuberhybrida*, the female parent of 'Elatior'-begonias. In tuberous begonias, short periods of SD reduce vegetative growth and longer periods completely arrest shoot tip activity and induce tuberization (Maatsch and Rünger, 1955; Fonteno and Larson, 1982; Djurhuus, 1985). Powell and Bunt (1979) observed with 'Riegers Schwabenland Red' that young plantlets having seemingly died under SD and low temperature conditions had formed healthy subterranean tubers. The swellings on the bases of the leaf cuttings in the present experiments at 13 and 17 °C under SD conditions are probably also symptoms of tuberization.

Leaf age appeared to have a particularly strong effect on regeneration. It affected the response to daylength and temperature treatments, young leaf cuttings generally reacting contrarily to old ones. Both root and bud regeneration showed more or less optimum curves, but with the optima at different leaf ages. Moreover, the leaf age effects showed characteristic genotypical differences. It must be concluded, therefore, that the effects of temperature, daylength and possibly other factors as well, cannot be interpreted without knowledge of the effect of leaf age.

In practice stock plants are kept for one year or more. Always young nearly full-grown leaves are picked off and used as cuttings. Old leaves are far less suitable (Von Hentig, 1976, 1978). In the present research old leaves also gave good results, probably because the stock plants were still rather young. Another reason might be that the long petioles of the old leaves were left intact. Shortening the petioles appeared to have an effect similar to that of a higher leaf age (data not presented). Obviously, it was also important that most experiments were performed in a favourable season.

The high regeneration capacity of the *Begonia* genotypes used in this study enabled the determination of the influences of important conditions, daylength, temperature and leaf age, on the different aspects of regeneration. In general the regeneration process can be divided into two phases, bud initiation and shoot development. It was clearly shown that optimum conditions for these phases are different and sometimes even opposite. Applying the optimal conditions during each phase will, certainly, increase the rate of success in commercial propagation. It will also be useful to pay attention to the different parts of the regeneration process when breeding for high shoot formation capacity in new cultivars.

CHAPTER 7

IN VIVO REGENERATION OF LEAF CUTTINGS OF 'ELATIOR'-BEGONIAS (*BEGONIA X HIEMALIS* FOTSCH); EFFECTS OF CYTOKININS AND AUXINS

INTRODUCTION

It has been shown by Heide (1964, 1965a) that low temperature (15-18 °C) and short day conditions promoted shoot regeneration and inhibited root regeneration of leaf cuttings of 'Gloire de Lorraine'-begonias (*Begonia x cheimantha* Everett). He suggested that both phenomena were due to an increase in the ratio of endogenous cytokinin to auxin. The same effects were obtained after cytokinin treatments (Heide, 1965b, 1972). In contrast to this, high temperature (20-27 °C) and long day conditions exhibited opposite effects on regeneration, and the cytokinin to auxin ratio was supposed to be low. These findings were consistent with the Skoog and Miller theory (1957) which says that the type of organs regenerated in plant tissue cultures is determined by the ratio of cytokinin to auxin in the medium, a high ratio promoting shoot regeneration and a low ratio root regeneration.

The effects of temperature and daylength on regeneration of leaf cuttings of 'Elatior'-begonias (*B. x hiemalis* Fotsch) were not the same as in 'Gloire de Lorraine'-begonias. Only short day conditions, if applied for a short period, promoted shoot formation, as was described in the preceding chapter. A reason for this discrepancy could be difference in endogenous hormone levels between both groups of *Begonia* hybrids. It seemed interesting, therefore, to study the effects of application of cytokinin and auxin to 'Elatior'-begonia leaf cuttings.

In practice one regularly observes that in certain cultivars buds are initiated in abundancy but hardly any shoots emerge from the soil (Karper, pers. comm.). It will be discussed whether treatments with cytokinin and auxin may be useful to improve shoot growth.

MATERIAL AND METHODS

Plant material. Leaf cuttings of two clones, 'Riegers Schwabenland Red' and 'SO_1' were used. The origin of these clones has been described in the preceding chapter. The stock plants were propagated from adventitious shoots and grown under standard conditions (Chapter 6) to an age of 5-7 months when the leaf cuttings were collected.

Leaf cuttings. The leaf cuttings, originating from positions 3-5 and consisting of the whole petiole and leaf blade, were grown in cutting benches under long day conditions and at a minimum temperature of 20 °C as described in the preceding chapter.

Plant growth regulators. The leaf cuttings were treated with the cytokinin kinetin or 6-benzylaminopurine (BA), with the auxin indole-3-acetic acid (IAA) or 1-naphthalene-acetic acid (NAA), and with combinations of these substances at various concentrations. Generally the whole leaf cuttings were immersed for 10 min in aqueous solutions (water + Tween 20 0.2 $ml l^{-1}$) of the regulators. In some experiments the regulators were applied by 24 h soaking, but this treatment sometimes damaged the bases of the petioles.

Observations. After 2-5 weeks the leaf cuttings were carefully lifted for recording progress of root and bud regeneration. After 8-9 weeks, as a final examination, all buds and shoots (1 cm or more in length) were counted. Mean numbers of buds and shoots were calculated for all cuttings. The percentage of cuttings with two or more shoots was used as a measure of shoot development.

Per treatment at least 33 cuttings were used (not including infected cuttings, see chapter 6), spread over four different places in the bench. The results presented are confirmed by the data from at least two experiments. For means the standard deviation of the mean was calculated and differences were tested for significance ($P = 0.05$) by Student's t test.

RESULTS

Cytokinins. The effects of various concentrations of BA applied to 'SO_1' leaf cuttings are shown in Figure 1. After four weeks the percentage of root formation decreased as a higher concen-

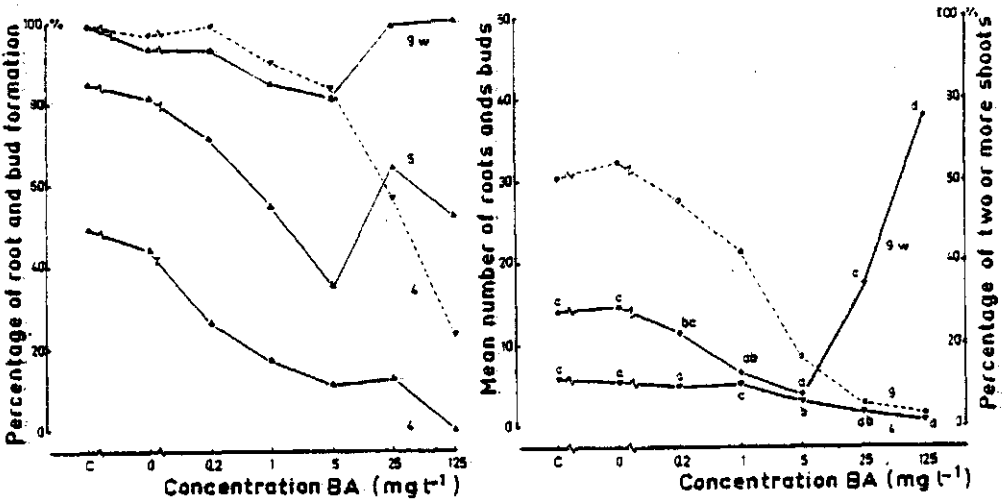


Figure 1. Regeneration of *Begonia* 'SO_1' leaf cuttings as affected by cytokinin. BA at different concentrations, in aqueous solution, was applied by 10 min immersion just prior to planting. Each treatment comprised at least 35 cuttings from positions 3-5. The experiment started on March 27. Percentages of root formation (▼---▼) were recorded after four weeks, percentages of bud formation (▲——▲) after four, five and nine weeks, mean numbers of roots (▼——▼) after four weeks, and mean numbers of buds and shoots (●——●) and percentages of cuttings with two or more shoots (o---o) after nine weeks. Means designated by the same letter do not significantly differ at P = 0.05. C = control without any treatment.

tration of BA was applied. Also the number of roots per cutting decreased. Root formation gradually recovered, however, and after seven weeks all leaf cuttings had developed roots, even at the highest BA concentration of 125 mgl^{-1}. Bud initiation was inhibited in approximately the same way. After four weeks only a very low percentage of the cuttings had formed buds at high BA concentrations. After nine weeks, this percentage had increased to about 100% over the whole range. Especially the proportionally strong increase at the highest BA concentrations was surprising. From their basal wound margins the cuttings in

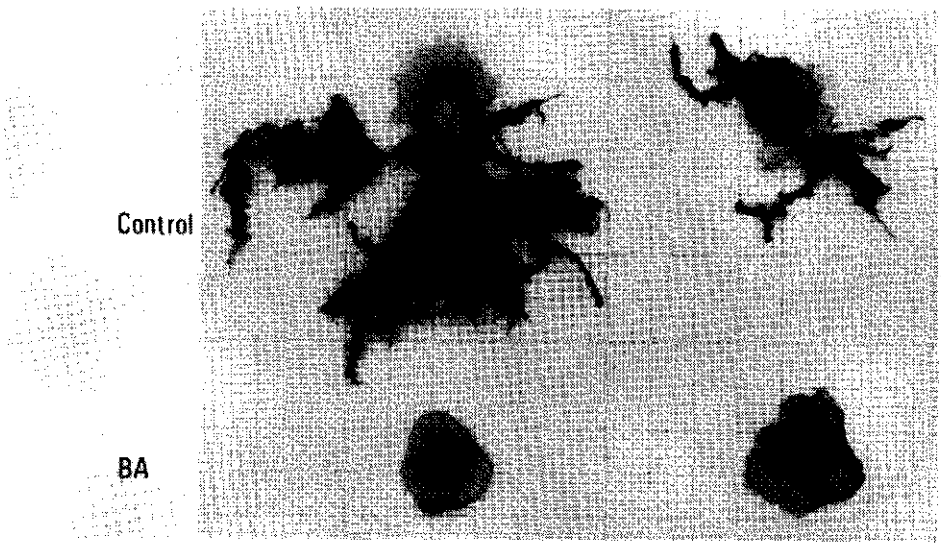


Figure 2. Regeneration on petiole bases of *Begonia* 'SO_1' leaf cuttings as affected by a high concentration of cytokinin. BA 125 mgl^{-1} was applied by 10 min immersion just prior to planting. The photograph was taken after four and a half weeks.
Left column: bases viewed from above.
Right column: bases viewed from below.
BA inhibits root as well as bud regeneration and induces formation of greyish-white protuberances.

those treatments formed firm, greyish-white protuberances (Figure 2), which eventually differentiated into buds. The numbers of buds formed in this way were significantly higher than in the control, but their development into shoots was very poor (Figure 1). Moreover, when growth occurred at all, the shoots were rather abnormal, showing swollen stem bases and very thin tips (Figure 3). To check whether the poor shoot development was due to the late initiation of the buds, the cuttings were grown four weeks longer than the control, however, normal shoot elongation did not start during this period.

In parallel batches with kinetin similar results were obtained as with BA. 'Riegers Schwabenland Red' leaf cuttings showed about the same reaction pattern after cytokinin treatments as the 'SO_1' cuttings.

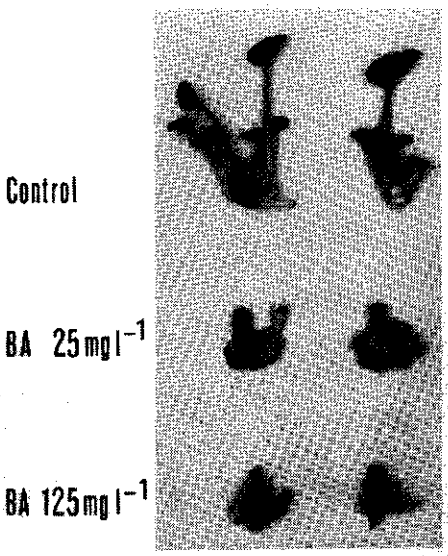


Figure 3.
Regeneration on petiole bases of *Begonia* 'SO_1' leaf cuttings as affected by cytokinin. BA in aqueous solution was applied by 10 min immersion of the cuttings. The photograph was taken after nine weeks, roots were removed. With increasing BA concentration the numbers of buds initiated increased, but it coincided with an increase in malformations and decrease in development into shoots.

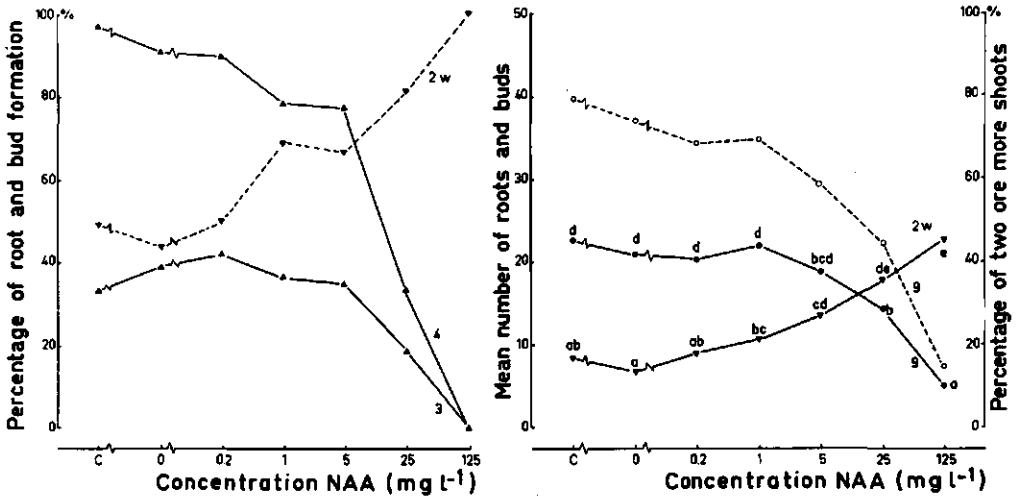


Figure 4. Regeneration of *Begonia* 'Riegers Schwabenland Red' leaf cuttings as affected by auxin. NAA at different concentrations, in aqueous solution, was applied by 10 min immersion just prior to planting. Each treatment comprised at least 33 cuttings from positions 3-5. The experiment started on October 4. Further legends as in Figure 1, except for indicated recording dates.

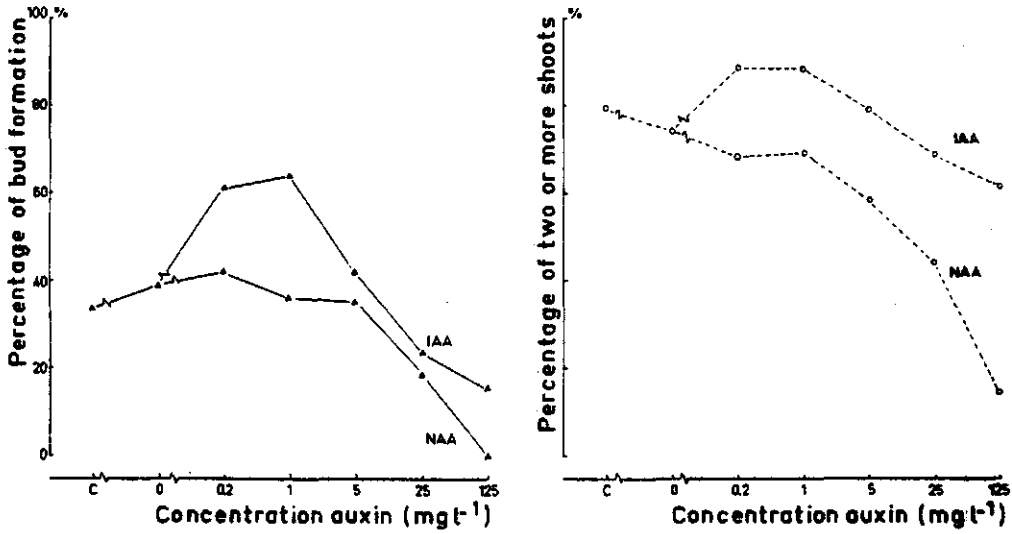


Figure 5. Regeneration of *Begonia* 'Riegers Schwabenland Red' leaf cuttings as affected by auxin. Characteristic differences between results after NAA and IAA application; ▲——▲: percentages of cuttings with bud formation after three weeks, and o---o: percentages of cuttings with two or more shoots after nine weeks.

Auxins. NAA was applied at increasing concentrations to leaf cuttings of 'Riegers Schwabenland Red'. Figure 4 shows the results. Root formation increased and bud initiation decreased as the concentration of NAA was increased. Bud regeneration, however, gradually recovered, and at the end of the experiment each treatment had reached 100%, except the highest concentration (125 mgl^{-1}) which stayed at 86%. The number of buds formed per cutting decreased with increasing concentration of NAA. This also held for the percentage of cuttings which formed two or more shoots.

Approximately the same results were attained with IAA. A remarkable difference, however, was found for the lower concentrations (0.2 and 1 mgl^{-1}, Figure 5). In contrast to NAA, the low concentrations of IAA hastened early bud initiation. This bud promoting effect was also reflected in a slight increase in the degree of shoot development.

Table 1. Bud and shoot formation of *Begonia* 'SO_1' leaf cuttings as affected by cytokinin at high concentration, whether or not combined with auxin. BA and IAA were applied by 24 h soaking just prior to planting. Times of recording results were the same as in Figure 1.

Parameter	Treatments		
	Control	BA 25 mgl^{-1}	BA 25 + IAA 5-25 mgl^{-1}
Place of bud regeneration	petiole base	petiole base	along the petiole
Time of bud regernation	3-5	5-7	4-5 weeks after planting
Mean number of buds*	5.2	16.7	24.3
Bud shape	normal	thick, puffed	normal but small and thin
Development into shoots	normal	strongly inhibited	thin shoots, poor growth

* The means were significantly different at P = 0.05.

Combination of cytokinin and auxin. Combinations of BA and IAA, both at the concentrations 0.2, 1, 5 and 25 mgl^{-1}, were applied, in this case by 24 h soaking, to leaf cuttings of 'SO_1'. BA alone showed reaction curves similar to those in the previous experiment (cf. Figure 1). The same held for IAA alone. Low concentratins of IAA improved bud initiation and shoot development in 'SO_1' in the same way as was found for 'Riegers Schwabenland Red' (cf. Figure 5).

In the combinations of the low and moderately high concentrations of BA and IAA, it was sometimes found that the regulator at low concentration slightly improved the effect of the one at the higher concentration. In general, however, results were intermediate.

The most striking results were found in the treatments with both regulators at high concentration (BA 25 mgl^{-1} + IAA 5-25 mgl^{-1}), showing a type of regeneration not noticed before (see Table 1). Numerous buds regenerated equally spread over a length of up to 3 cm on the petioles. These buds were much less abnormal than after treatment with high concentration of BA alone. They started to elongate, but unfortunately shoots were generally thin and not vigorous, perhaps partly due to the fact that the underlying petiole tissue was coloured black and had become soft by the treatment. This impaired tissue also appeared very susceptible to rotting. Thus the combination of high BA and high IAA concentrations did not seem promising for commercial propagation.

DISCUSSION

Cytokinin and auxin treatments clearly influenced regeneration of the leaf cuttings of the two 'Elatior'-begonias investigated. The effects, however, were not always in accordance with expectations based on the findings of Heide (1965b, 1972) that cytokinin improved bud regeneration and inhibited root regeneration, and that auxin had the reverse effects. Generally the reactions to the high concentrations of cytokinin and auxin were rather similar to those found by Heide, but the reactions to the low concentrations were different. In fact Heide hardly found any response to the low concentrations. It was already concluded in the preceding chapter that the leaf cuttings of the 'Gloire de Lorraine'-begonias used by Heide had a lower bud regeneration capacity than the genotypes used in this study. Obviously, this difference will have caused the different reactions to the regulator treatments.

The results found by Heide (1965b, 1972) supported the hypothesis of Skoog and Miller (1957) that a high cytokinin to auxin ratio promotes bud regeneration and a low ratio root regeneration. Contrary to Skoog and Miller, Dore (1965) stated that auxin concentration alone determines the type of organ formation, a low concentration being promotive to bud regeneration. Particularly the present reactions to low concentrations of cytokinin and auxin fit in best with the idea of Dore. Similar promotive effects of auxin on bud regeneration were found in other plants with a high regeneration capacity (Chouard, 1938; Wirth, 1960; Münzel, 1970; Appelgren and Heide, 1972;

Pierik and Steegmans, 1975; Hussey, 1982; Van Aartrijk and Blom-Barnhoorn, 1981).

Bud regeneration as a result of a high cytokinin concentration was different from regeneration in untreated cuttings. It seemed as if two types of bud regeneration occurred, a natural form and an artificial one. Exogenous cytokinin seemed to counteract the natural bud regeneration. Possibly, at the time of planting the cuttings, the endogenous hormone balance in the petiole bases was already optimal for regeneration of buds and roots, with the result that application of a small amount of cytokinin disturbed it. High concentration of cytokinin, probably 'overshadowed' the already existing hormonal information for natural regeneration, which as a consequence was rendered ineffective.

Combinations of high BA concentration with high IAA concentration led to abundant bud regeneration spread over the whole petiole. The picture closely resembled that of in vitro regeneration in the 'Elatior'-begonias (cf. Welander, 1977; Reuther, 1980). Combination of high concentrations of cytokinin and auxin also led to a typical 'in vitro type' of bud regeneration in vivo with etiolated leaf cuttings of chrysanthemum (Chapter 5).

As a consequence of the addition of IAA to a high cytokinin concentration the usual basipetal polarity of adventitious bud formation was lost. Suppression of the basipetal polarity by auxin was also found in other plants with a high bud regeneration capacity, but there it already occurred after application of auxin alone (Chouard, 1938; Wirth, 1960; Münzel, 1970; Van Aartrijk and Blom-Barnhoorn, 1981). This finding points again to an important role of auxin in the regeneration of the begonias used in this research.

In practice one often sees in certain cultivars that only few shoots are formed, although numerous buds have been initiated (Karper, pers. comm.). Apparently, the phase of bud initiation continues all the time while the phase of shoot development does not start. Cytokinin will be of little use here, because it proved to be inhibitory to normal shoot development in the present experiments, as was also noticed earlier (Sytsema, 1977; Davies and Moser, 1980). Auxin at low concentration seems more promising for solving the problem of bad shoot development. It may be useful to repeat this auxin treatment, e.g. by spraying. More important, however, is the choice of the cutting material; leaves should be taken from vigorously growing stock plants and not be too old. It will also be helpful to

start with a high temperature in the bench or to apply a short period of short day. All these treatments accelerate the start of bud initiation and consequently also its conclusion (cf. Chapter 6). Supposedly, then the transition to the next phase of shoot development will be easier.

Summarizing, the effects of the cytokinin and auxin treatments gave valuable additive information to the data on the effects of environmental factors on regeneration of 'Elatior'-begonia leaf cuttings. It can be concluded, particularly with regards to commercial propagation, that just as found for temperature and light treatments, the effects of cytokinin and auxin do not fit well into the concept of organ formation developed by Heide (1965b, 1972) for 'Gloire de Lorraine'-begonias. Differences in regeneration capacity seem to be the crucial factor. In the case of genotypes which a high bud initiation capacity application of cytokinin will not improve development of the buds into shoots.

GENERAL DISCUSSION

The aim of the research described in this thesis was to develop procedures for in vivo shoot regeneration on leaf cuttings of horticulturally important plant species. Spontaneous shoot regeneration was observed in only a few specimens from a large sample of higher plant species and cultivars (Chapter 1). This is in agreement with the findings of Broertjes et al. (1968). So a wider application of in vivo adventitious shoot formation would only be possible using special aids and treatments.

Application of cytokinin seemed the most obvious tool to induce adventitious shoot formation on leaf cuttings of species which do not form shoots spontaneously. Such a treatment, however, did not induce shoot regeneration in any of the recalcitrant species (Chapter 1). It only improved shoot regeneration in plants which already regenerated shoots very well without any help (Chapters 1 and 2). Only intensive study of chrysanthemum showed that indeed in the cv. Super Yellow cytokinin could evoke shoot regeneration in a genotype otherwise recalcitrant (Chapter 4). This result shows that cytokinin is involved in the process of shoot regeneration induction, although it certainly will not be the only factor.

It was shown that cytokinin treatment had two different effects on shoot regeneration of chrysanthemum 'Bravo' leaf cuttings; on the one hand it induced early shoot formation from week 9-15 onwards, on the other hand it improved the late shoot formation from about week 20 onwards, which also occurred without the use of the regulator (Chapter 4). This dual effect indicates that a positive reaction to cytokinin treatment also depends on a responsive physiological condition of the cuttings.

Low temperature was found to be the environmental factor which was the most consistently promotive to shoot regeneration of chrysanthemum leaf cuttings. It improved both spontaneous and regulator-aided regeneration (Chapters 3 and 4 respectively). As low temperature generally was more promotive when given before than during actual regeneration, it is assumed that mainly one of the earlier stages of the regeneration process is affected. An explanation for this phenomenon might be that cytokinin synthesis or its action is improved at lower temperatures. Low temperature, however, seemed to be promotive even when applied during rooting of the cuttings (Chapter 3), whereas cytokinin application only was promotive if roots had been formed (Chapter 4). Thus the sensitivity of the cuttings to low

temperature seemed to precede, at least partly, the sensitivity to cytokinin. This indicates that low temperature makes the cuttings more apt to respond to shoot regeneration inducing factors like cytokinins.

Early shoot formation, as induced by a combination of cytokinin and auxin, could be strongly improved by using small leaves from stock plants which were in a certain stage of generative development. The same improvement was obtained by raising the stock plants under very low light intensity, which resulted in reduction of growth vigour and development of small etiolated leaves (Chapter 5). It was suggested that this improved response of the cuttings to the treatment with the regulators was mainly due to a decrease in metabolic activity and a reduction of inhibiting factors, possibly phenolic compounds, in the tissues surrounding the place of regeneration.

The physiological condition which improved early shoot formation might be very similar to the condition which enabled late shoot formation. The latter only took place after the leaf cuttings had started senescence (Chapter 3). In young actively growing leaf cuttings, one may expect that the physiological condition of the tissue is different and is more similar to the normal situation in whole plants. Under such conditions the initiation of the regeneration process is apparently inhibited.

Pretreatment of the stock plants brought about the most drastic improvement of early shoot regeneration in chrysanthemum (Chapter 5). This indicates that stock plant conditioning is a prerequisite for a good response of the leaf cuttings to the treatment with the regulators. It is conceivable that this also holds for other plant species with a low capacity of in vivo shoot regeneration. In contrast to the in vivo shoot regeneration, however, in most of these species in vitro shoot regeneration is much easier, and in this case a stock plant pretreatment generally is no prerequisite. One might suppose, therefore, that the small size of the explants commonly used for in vitro regeneration has the same function as stock plant conditioning in the case of in vivo shoot regeneration. Small explants will not suffer any longer from inhibiting influences from surrounding tissues. If this hypothesis is true, then in vitro regeneration has great advantages over in vivo regeneration, as making small explants is much easier and more reliable than conditioning the whole stock plants.

So far the three prarameters, cytokinin application, low temperature and stock plant conditioning, have been the most

effective in increasing the chance of obtaining adventitious shoot formation on leaf cuttings. They were typically involved in the early phases of the regeneration process. On the contrary, in subsequent research with 'Eliatior'-begonia cultivars, these factors proved to be less effective and even showed opposite effects (Chapters 6 and 7). Supposedly, the stock plants of these begonias provide leaf cuttings which are already well conditioned and can be readily induced to regenerate shoots. Here the effects of the studied factors are typical of the later phases of the regeneration process, bud initiation and shoot development. Novel in the begonia experiments was the strong influence of daylength. As already stated in Chapter 6, this may be connected with the role of daylength in other important developmental processes in begonias, viz. flowering and tuberization.

The 'Eliator'-begonias studied showed a high capacity of leaf cutting regeneration, for which no special stock plant pretreatments were necessary. Induction of shoot regeneration is no problem at all and cytokinin application is even disadvantageous (Chapter 7). Therefore, there is no reason to change to the use of small explants and incubate them in vitro as was suggested above for species with low capacity of in vivo regeneration. For these begonia cultivars in vivo leaf cutting regeneration can still be recommended as a commercial propagation method.

In conclusion, by the application of special treatments rather good in vivo shoot regeneration procedures can be developed for leaf cuttings of plant species which do not regenerate well spontaneously. These treatments, however, are complicated and time consuming. As discussed, they can be replaced by well established in vitro culture systems. Consequently, as judged from the results presented in this thesis, development of in vivo shoot regeneration procedures with a general applicability in horticulture is no longer recommended for plant species which miss or have a very low capacity of in vivo regeneration. By contrast, continuation of research on in vivo shoot regeneration is very meaningful in those species which have a high natural regeneration capacity. It was shown that a commonly used practical procedure was open to improvement.

SUMMARY

Adventitious shoot formation implies the regeneration or development of shoots from fully differentiated tissue. Its application has, after the rise of in vitro culture, assumed large proportions. Then the question arose whether in vivo adventitious shoot formation could not be applied more widely in commercial horticulture. To answer this question investigations were made on the regeneration of leaf cuttings and the results are presented in this thesis.

It was found that the majority of a large number of plant species and cultivars was unable to regenerate shoots on leaf cuttings spontaneously. Various attempts to induce shoot regeneration with the cytokinin 6-benzylaminopurine (BA) were unsuccessful. BA had a positive effect, however, in a few species which regenerated shoots spontaneously (Chapter 1). These findings led to investigation of the effectiveness of plant growth regulator treatments to induce shoot regeneration in four species, *Brassica oleracea*, *Lunaria annua*, *Nicotiana alata* and *Ruta graveolens*, of which it was known that they were able to regenerate shoots, but which differed in the capacity to do so (Chapter 2). Cytokinins and auxins were applied prior to planting the cuttings as well as after roots had been formed. In all four species the treatments had marked effects. They fitted into two already known reaction patterns: that described by Skoog and Miller (1957) in which shoot regeneration is promoted at a high cytokinin/auxin ratio, and that described by Harris and Hart (1964) in which auxin is promotive for shoot regeneration. A new feature was that leaf cuttings from one and the same species showed both reaction patterns, as was found for *Lunaria annua* and *Nicotiana alata*. From these results it was concluded that regulator treatments still can be rather effective.

As was shown in Chapter 1, simple applications of cytokinin were insufficient to induce shoot regeneration on leaf cuttings of plant species with low regeneration capacity. Therefore the research was concentrated on one species, chrysanthemum (*Chrysanthemum morifolium*), of which an easy to regenerate genotype, cv. Bravo, and a difficult one, cv. Super Yellow, were used. Chapter 3 describes how shoot regeneration in 'Bravo' can be influenced by environmental factors. As a rule, at moderately high temperatures (13-17 °C) good results were obtained. Pretreatment with low temperature (9 °C) during the rooting phase had an additional favourable effect on the induction of shoot

regeneration, whereas high temperature (21 - 25 °C) during the final phase accelerated the realization of the regeneration. Daylength of 8 h (SD) initially delayed the appearance of the adventitious shoots compared with daylength of 16 h (LD), but after some time the percentage of shoot formation was the same under both conditions. The leaf cuttings could not stand a high light intensity (> approx. 30 Wm^{-2}), which accelerated senescence and decreased shoot regeneration. Young leaves provided cuttings which regenerated better than those from old leaves, and leaves from vegetative plants were better than those from so-called crown bud plants, i.e. plants which have formed flower buds in LD and which, in general, show less vigorous growth.

Shoot formation in 'Bravo' started about 18-20 weeks after planting, when the leaf cuttings began to senesce by degrees. This was called late shoot formation. The leaf cuttings from 'Super Yellow', studied under the same conditions as 'Bravo', never regenerated shoots. Cytokinin applied before planting gave shoot regeneration in both cultivars as early as nine weeks after planting; this was called early shoot formation. Application of auxin, notably indole-3-acetic-acid (IAA), together with cytokinin increased early shoot formation, yet the rate of success always remained very low under various experimental conditions. It was not possible either to establish the optimum concentrations of the regulators. However, low to moderately high temperature (9-17 °C) in the cutting benches was found to be an essential condition (Chapter 4).

In a number of experiments it was shown that the developmental stage and pretreatments of the stock plants had a pronounced effect on early shoot formation of the chrysanthemum leaf cuttings (Chapter 5). Leaf cuttings from crown bud plants gave much higher percentages of early shoot formation than those from vegetative plants: around 85% at the optimum concentrations of the regulators (BA 6.25 - 12.5 mgl^{-1} + IAA 45 mgl^{-1}). Small leaves from the inflorescences of plants which are about to start flowering also appeared to be very suitable. The most striking result was noted in leaf cuttings from plants which had stood for about a month in dim light (0.6-6 Wm^{-2}). After 4-6 weeks a few dozens of adventitious buds per cutting arose, scattered over the entire length of the petiole. The pattern of this regeneration closely resembles that of regeneration in vitro. Of these buds only a small part developed into plantlets. Moreover, many leaf cuttings were lost by rotting,

particularly because they were very etiolated and tender as a result of the stock plant treatment.

From the observation that the stock plant had such a strong influence a hypothesis was put forward regarding the process of shoot regeneration (Chapter 5 and General Discussion). In leaf cuttings from normally grown plants it is likely that regeneration inhibiting factors exist in the tissues surrounding the place of regeneration. Pretreatment of the stock plants reduces this inhibition and then the leaf cuttings can respond to shoot regeneration inducing factors like cytokinin, i.e. leaf cuttings are physiologically conditioned by the pretreatment. This hypothesis holds not only for early but also for late shoot formation where no regulators are applied just before planting the cuttings. In late shoot formation the physiological conditioning only starts when the leaf cuttings are gradually senescing. Moreover, this hypothesis explains why in vitro regeneration generally is easier than the in vivo regeneration described here: in the small explants commonly used in vitro there is only a low amount of surrounding tissue left which can inhibit shoot regeneration. Thus the small size of the in vitro explants will have the same effect as the stock plant pretreatment in the case of in vivo regeneration. From a practical point of view, however, excision of small explants is much easier than pretreatment of whole plants. Therefore, it was concluded that in plant species with a low to moderately high regeneration capacity, like in chrysanthemum studied here, in vivo shoot regeneration cannot match the results in vitro.

For practical application, in vitro shoot regeneration should be preferred to in vivo regeneration in the case of recalcitrant species. For species with a high regeneration capacity, however, it is not so clear which of the two procedures has to be chosen. Shoot regeneration in plants with such a high regeneration capacity is described in Chapters 6 and 7; it concerns certain 'Elatior'-begonia (*Begonia x hiemalis*) cultivars. In general with *Begonia* spp. SD and low temperature promote shoot regeneration, but literature on 'Elatior'-begonias contains different views on this aspect. Our experiments showed that only a short period of SD (4-14 days), applied either to the stock plants or to the cuttings, was favourable, promoting the early phase of adventitious bud initiation. A long period of SD (> 28 days) applied to the cuttings had an adverse effect on the growth of these buds; no shoots emerged from the soil. Low temperature had not the same effect as SD and shoot regeneration was generally better when high

temperatures (21-25 °C) were used in the cutting benches (Chapter 6).

The effects of regulators on regeneration in *Begonia* spp. generally fit well into the reaction pattern as described by Skoog and Miller (1957): cytokinin promotes shoot regeneration and auxin root regeneration. When applied to the 'Elatior'-begonias, however, BA had an adverse effect; the number of initiated adventitious buds was increased, but shoot growth and development were completely disturbed and inhibited. A low auxin concentration (IAA 0.2-1 mgl^{-1}) promoted adventious shoot formation. Combination of high BA concentration (25 mgl^{-1}) with high IAA concentration (5-25 mgl^{-1}) led to regeneration of many buds, distributed all along the petiole, but only a small number developed into shoots (Chapter 7).

It was supposed that with the 'Elatior'-begonias the investigated factors influence mainly the last phases of the regeneration process, bud initiation and shoot development. The earlier phase of shoot regeneration induction proceeds rapidly and presents no problems. So in this respect there is no reason to change over to in vitro procedures.

Finally, from the results presented in this thesis conclusions were drawn concerning efforts to attain a wider application of in vivo adventitious shoot formation in horticultural practice. On the one hand such efforts are very meaningful for species with a high regeneration capacity, on the other hand they are not for species with a low capacity. For the latter, in vitro procedures can be used with a much better result than the in vivo procedures.

SAMENVATTING

Adventieve scheutvorming houdt in de regeneratie of het ontstaan van scheuten uit volledig gedifferentieerd weefsel. Toepassing ervan heeft na de opkomst van de in vitro cultuur een hoge vlucht genomen. De vraag deed zich voor of adventieve scheutvorming in vivo niet méér toegepast zou kunnen worden in de tuinbouwpraktijk. Om deze vraag te beantwoorden werd onderzoek gedaan aan regeneratie van bladstekken. Dit proefschrift rapporteert de resultaten van de proeven.

Van een groot aantal onderzochte plantesoorten en cultivars bleek het merendeel niet in staat om spontaan scheuten te regenereren aan bladstekken. Diverse pogingen om de scheutvorming te induceren met het cytokinine 6-benzylaminopurine (BA) hadden geen succes. BA had wel een positief effect bij enkele soorten die spontaan scheuten regenereerden (Hoofdstuk 1). Naar aanleiding van deze bevindingen werd onderzocht hoe effectief regulatoren waren in het induceren van scheutregeneratie bij een viertal plantesoorten waarvan bekend was dat zij scheuten konden regenereren, maar die verschilden in het vermogen daartoe. Die soorten waren *Brassica oleracea*, *Lunaria annua*, *Nicotiana alata* en *Ruta graveolens* (Hoofdstuk 2). Cytokininen en auxinen werden toegediend vóór het steken van de bladstekken en nadat deze wortels hadden gevormd. In alle vier soorten hadden de behandelingen duidelijk effect. De effecten pasten in twee reeds bekende reactiepatronen: het patroon volgens Skoog en Miller (1957) met bevordering van de scheutregeneratie bij een hoge cytokinine/auxine verhouding, en het patroon volgens Harris en Hart (1964) met bevordering van de scheutregeneratie door auxine. Nieuw was dat de bladstekken van één soort beide reactiepatronen vertoonden, hetgeen werd waargenomen bij *Lunaria annua* en *Nicotiana alata*. Op grond van deze resultaten werd geconcludeerd dat behandelingen met regulatoren toch behoorlijk effectief kunnen zijn.

In Hoofdstuk 1 bleek dat een eenvoudige toediening van cytokinine niet voldoende was voor het induceren van scheutregeneratie aan bladstekken van planten met een laag regeneratievermogen. Daarom werd het onderzoek meer diepgaand voortgezet bij één gewas, namelijk chrysant (*Chrysanthemum morifolium*), waarvan een gemakkelijk te regenereren genotype, cv. Bravo, en een moeilijk te regenereren genotype, cv. Super Yellow, werden gebruikt. In Hoofdstuk 3 wordt beschreven hoe de scheutregeneratie bij 'Bravo' kan worden gestuurd door omgevingsfactoren. In het algemeen werden bij een matig hoge temperatuur (13-17 °C) goede resultaten verkregen. Een voorbehandeling met lage

temperatuur (9 °C) gedurende de bewortelingsfase was extra gunstig voor de inductie van de scheutregeneratie, terwijl een hoge temperatuur (21-25 °C) in de eindfase de realisatie van de regeneratie versnelde. Een daglengte van 8 h (KD) vertraagde aanvankelijk het zichtbaar worden van de adventieve scheuten vergeleken met een daglengte van 16 h (LD), maar na enige tijd was onder beide omstandigheden het percentage stekken dat scheuten vormde gelijk. De bladstekken konden niet tegen een hoge lichtintensiteit (> ± 30 Wm^{-2}); die versnelde de veroudering en verminderde de scheutregeneratie. Bladstekken van jong blad regenereerden beter dan die van oud blad. Blad van vegetatieve planten was geschikter dan dat van zogenaamde "crown bud" planten. Dit zijn planten die in LD bloemknoppen vormen en in het algemeen wat minder krachtig groeien.

De scheutvorming bij 'Bravo' begon pas ongeveer 18-20 weken na het steken, op het moment dat de bladstekken geleidelijk begonnen te verouderen. Dit werd de late scheutvorming genoemd. De bladstekken van 'Super Yellow' vormden onder dezelfde omstandigheden als bestudeerd voor 'Bravo' nooit scheuten. Cytokinine toegediend vóór het steken gaf bij beide cultivars al na negen weken regeneratie van scheuten; dit werd de vroege scheutvorming genoemd. Toediening van auxine, met name indol-3-azijnzuur (IAA), samen met cytokinine verhoogde de kans op vroege scheutvorming, maar toch bleef het slagingspercentage onder diverse onderzochte omstandigheden altijd zeer laag. Ook kon niet worden vastgesteld wat de optimum concentraties waren van de regulatoren. Wel werd gevonden dat een lage tot matig hoge temperatuur (9-17 °C) in de stekbak een voorwaarde was voor de vroege scheutvorming (Hoofdstuk 4).

In een aantal proeven werd gevonden, dat de ontwikkelingstoestand van de moederplant evenals moederplantvoorbehandelingen een grote invloed hadden op de vroege scheutvorming van de bladstekken van chrysant (Hoofdstuk 5). Bladstekken afkomstig van "crown bud" planten bereikten veel hogere percentages vroege scheutvorming dan die van vegetatieve planten: rond 85% slaging bij de optimum concentraties van de regulatoren (BA 6,25 -12,5 mgl^{-1} + IAA 45 mgl^{-1}). Kleine bladeren uit de bloeiwijzen van planten die op het punt stonden te gaan bloeien, bleken ook erg geschikt. Het meest opmerkelijke resultaat werd waargenomen bij bladstekken van planten die ongeveer een maand in schemerlicht (0,6-6 Wm^{-2}) hadden gestaan. Na 4-6 weken ontstonden per bladstek enkele tientallen adventieve knoppen verspreid over de hele lengte van de bladsteel. Het beeld van de regeneratie leek

sprekend op dat van regeneratie in vitro. Van deze knoppen groeide echter maar een klein deel uit tot plant. Bovendien gingen veel bladstekken verloren door verrotting, vooral omdat ze als gevolg van de moederplantbehandeling erg geëtioleerd en teer waren.

Na het waarnemen van de ingrijpende moederplanteffecten werd een hypothese opgesteld betreffende de gang van zaken bij het proces van scheutregeneratie (Hoofdstuk 5 en Algemene Discussie). In bladstekken van normaal opgekweekte planten is de situatie ongunstig voor de regeneratie. In de weefsels rond de plaats van regeneratie zijn remmende factoren aanwezig. Voorbehandeling van de moederplanten vermindert deze remming en de bladstekken zijn vervolgens in staat om te reageren op scheutregeneratie inducerende factoren zoals cytokinine. De bladstekken worden als het ware fysiologisch geconditioneerd door de voorbehandeling. Deze veronderstelling gaat niet alleen op voor de vroege scheutvorming maar ook voor de late, waarbij geen regulatoren worden toegediend vóór het steken. Bij de late scheutvorming begint de fysiologische conditionering pas wanneer het bladstek zelf langzaam gaat verouderen. De hypothese verklaart ook waarom scheutregeneratie in vitro in het algemeen gemakkelijker gaat dan de hier beschreven regeneratie in vivo. Immers kleine explantaten, die normaal worden gebruikt in vitro, bezitten maar weinig informatie afkomstig van de moederplant. Er is ook maar weinig aangrenzend weefsel dat de scheutregeneratie kan tegengaan. In feite zal de geringe grootte van een explantaat in vitro dus hetzelfde effect hebben als de moederplantvoorbehandeling bij de regeneratie in vivo. Praktisch gezien is het maken van kleine explantaten echter veel gemakkelijker dan het voorbehandelen van hele planten. Daarom werd geconcludeerd, dat bij planten met een laag regeneratievermogen, zoals bij de bestudeerde chrysanten, de scheutregeneratie in vivo die in vitro niet zal kunnen evenaren.

Voor praktische toepassing verdient bij moeilijk te regenereren planten de scheutregeneratie in vitro de voorkeur boven die in vivo. Bij planten met een hoog regeneratievermogen is het niet zo duidelijk welk van beide systemen moet worden gekozen. De Hoofdstukken 6 en 7 beschrijven de adventieve scheutvorming op bladstekken van enkele 'Elatior'-begonia's (*Begonia x hiemalis*) met een hoog regeneratievermogen. In het algemeen zijn bij *Begonia* spp. KD en lage temperatuur gunstig voor de scheutregeneratie, maar bij 'Elatior'-begonia's is de literatuur wat dit betreft niet eenduidig. De proeven toonden aan

dat alleen een zeer korte periode (4-14 dagen) KD gunstig was, zowel gegeven tijdens de moederplant- als tijdens de bladstekfase. Zij bevorderde de aanleg van de adventieve knoppen. Een lange periode (> 28 dagen) KD gedurende de bladstekfase was nadelig voor de uitgroei van deze knoppen; er kwamen geen scheuten boven de grond. Lage temperatuur had niet dezelfde effecten als KD. De regeneratie verliep in het algemeen beter bij hoge temperatuur (21-25 °C) in de stekbak (Hoofdstuk 6).

De effecten van regulatoren op de regeneratie bij *Begonia* spp. passen over het algemeen goed in het reactiepatroon volgens Skoog en Miller (1957): cytokinine bevordert de scheutregeneratie en auxine de wortelregeneratie. Bij de 'Elatior'-begonia's had toediening van BA echter een nadelige invloed. Het vergrootte wel het aantal aangelegde adventieve knoppen, maar de scheutuitgroei werd volledig verstoord en geremd. Een lage concentratie auxine (IAA 0,2 - 1 mgl^{-1}) was in het algemeen gunstig voor de adventieve scheutvorming. Combinatie van een hoge concentratie BA (25 mgl^{-1}) met een hoge concentratie IAA (5-25 mgl^{-1}) leidde tot regeneratie van veel adventieve knoppen verspreid over de hele bladsteel, maar slechts een klein deel ervan groeide uit tot scheut (Hoofdstuk 7).

Verondersteld werd dat de onderzochte factoren bij de 'Elatior'-begonia's vooral de laatste fasen van het regeneratieproces beïnvloeden, die van de knopaanleg en van de scheutuitgroei. De eerdere fase van scheutregeneratie-inductie verloopt bij deze groep van begonia's snel en probleemloos. Wat dat betreft is er dus geen reden om over te stappen naar in vitro procedures.

Uiteindelijk werd uit de resultaten van dit proefschrift een tweeledige conclusie getrokken, namelijk dat het wel zinvol is zich onderzoeksinspanning te getroosten voor uitbreiding in de tuinbouw van het gebruik van adventieve scheutvorming in vivo bij gewassen met een hoog regeneratievermogen, terwijl dat zeker niet het geval is bij gewassen met een laag regeneratievermogen. Voor die laatste groep van gewassen kan met veel meer succes in vitro cultuur worden toegepast.

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CURRICULUM VITAE

Jan B.M. Custers werd op 21 mei 1947 geboren te Bergen (L). In 1965 behaalde hij het gymnasium-α diploma aan het Gabriël-college te Mook. In datzelfde jaar begon hij met de studie aan de Landbouwhogeschool te Wageningen. In juni 1972 werd het ingenieursexamen afgelegd met als hoofdvak tuinbouwplantenteelt en als bijvakken plantenveredeling, plantensystematiek en entomologie. Van 1 november 1972 tot en met 31 december 1975 werd een promotieonderzoek verricht bij de vakgroep Tuinbouwplantenteelt, hetgeen resulteerde in dit proefschrift. Vanaf 1 januari 1976 is hij in dienst van het Instituut voor de Veredeling van Tuinbouwgewassen en geeft daar leiding aan het weefselkweekonderzoek.