STUDIES ON ADVENTITIOUS BUD AND FLOWER DIFFERENTIATION IN *TORENIA* STEM SEGMENTS CULTURED *IN VITRO*

SHIZUFUMI TANIMOTO

1972

STUDIES ON ADVENTITIOUS BUD AND FLOWER DIFFERENTIATION IN *TORENIA* STEM SEGMENTS CULTURED *IN VITRO*

SHIZUFUMI TANIMOTO

1982

Submitted in partial fulfillment of the requirements for the degree of Doctor of Science, in Doctoral Program in Biological Sciences, University of Tsukuba.

TABLE OF CONTENTS

Abbreviations

AA-P: 4-*n*-amylamino-2-methylpyrrolo[2,3-*d*]pyrimidine

ABA: abscisic acid

Act.D: actinomycin-D

BA: N^6-benzyladenine

BA-P: 4-*sec*-butylamino-2-methylpyrrolo[2,3-*d*]pyrimidine

CB-P: 4-cyclobutylamino-2-methylpyrrolo[2,3-*d*]pyrimidine

CHI: cycloheximide

CH-P: 4-cyclohexylamino-2-methylpyrrolo[2,3-*d*]pyrimidine

CP-P: 4-cyclopentylamino-2-methylpyrrolo[2,3-*d*]pyrimidine

GA_3: gibberellic acid

HE-P: 4-(2-hydroxyethylamino)-2-methylpyrrolo[2,3-*d*]pyrimidine

IAA: indole-3-acetic acid

MS medium: Murashige and Skoog's medium

MZ: meristematic zone

NAA: naphthaleneacetic acid

4PU: *N*-phenyl-*N*-(4-pyridyl)urea

4PU-Cl: *N*-phenyl-*N*-(2-chloro-4-pyridyl)urea

RNA: ribonucleic acid

SDS: sodium dodecyl sulfate

TCA: trichloroacetic acid

Z: zeatin

STUDIES ON ADVENTITIOUS BUD AND FLOWER DIFFERENTIATION IN *TORENIA* STEM SEGMENTS CULTURED *IN VITRO*

Historical Background of the Present Study

1. *In vitro* adventitious bud differentiation in higher plants

Since the pioneering work by Haberlandt (1902), technologies associated with plant tissue culture have been developed in this century. This relatively new area of research has been recognized as a valuable means of investigating morphogenetic events in higher plants.

As early as 1939, White was successful to demonstrate *in vitro* organogenesis from callus tissues of *Nicotiana* hybrids. Furthermore, Skoog (1944) found that auxin inhibited bud formation but stimulated adventitious root formation in tobacco callus tissues. The inhibitory effect of auxin on adventitious bud differentiation could partially be removed by a simultaneous addition of adenine sulfate (Skoog and Tsui, 1948, 1951).

Following the discovery of cytokinin (Miller *et al.*, 1956), extensive studies on the hormonal control of organogenesis were carried out by Skoog and his associates. In 1957, Skoog and Miller reported that the adventitious

organ differentiation in tobacco pith callus was controlled by a balance between auxin and cytokinin; a relatively low level of auxin to cytokinin stimulated bud differentiation, while the reverse proportion favored root formation. This auxin-cytokinin-mediated organogenesis in tissues and organs of a number of plant species has been the subject of extensive studies by many research workers (review by Murashige, 1974, 1978; Vasil and Vasil, 1980).

Although the high ratio of cytokinin to auxin causes bud differentiation in many plant species, the application of cytokinin alone may induce adventitious buds in excised organ fragments of several plant species including Petunia inflata (Rao et al., 1973), Limnophila chinensis (Sangwan et al., 1976), Picea abies (Arnold and Eriksson, 1978, 1979), Torenia fournieri (Kamada and Harada, 1979), Perilla frutescens (Tanimoto and Harada, 1980), and Rudbeckia bicolor (Tanimoto and Harada, 1982). Contrary instance can be seen in the case of cultured explants of Brassica oleracea, where auxin alone can induce bud formation (Margara, 1977). Such results intimate a possibility that the endogenous auxin-cytokinin balance seems to play a key role on the events of organogenesis. In this point of view, several experiments were carried out using various kinds of available chemicals. The application of auxin-antagonists promoted bud formation in tobacco callus (Feng

and Linck, 1970). Also morphactin which is an auxin transport inhibitor, stimulated the differentiation of adventitious buds in leaf discs of Begonia (Schott and Schraudolf, 1967), tobacco callus (Ogura, 1975) and excised leaves of Heloniopsis orientalis (Kato, 1978). Skoog et al. (1973) reported that a cytokinin-antagonist inhibited cytokinin-induced bud formation in tobacco callus. Furthermore, a significant increase in the activity of peroxidase prior to bud initiation was noted in tobacco callus cultures (Rücker and Rodola, 1971; Thorpe and Gasper, 1978) and leaf explants of endive (Legrand, 1974).

In addition to auxin and cytokinin, effects of other phytohormones on bud formation have been investigated. Murashige (1961) demonstrated that bud formation in tobacco callus was suppressed by gibberellic acid (GA_3). Similar results were obtained in tobacco stem segments (Wardell and Skoog, 1969a), stem segments of Plumbago indica (Nitsch and Nitsch, 1967a), Begonia leaf explants (Bigot and Nitsch, 1968) and tobacco callus (Thorpe and Meier, 1973). In Brassica oleracea, however, auxin-induced bud formation was stimulated or inhibited by GA_3 depending on the concentration (Margara, 1977). Moreover, GA_3 was known to promote bud initiation in callus tissues derived from hypocotyls of Ranunclus seleratus (Konar and Konar, 1973). The suppression of bud formation by GA_3 was not reversed

by some growth retardants and anti-gibberellins (Murashige, 1965), but could be partially overcome by simultaneous addition of abscisic acid (ABA) (Thorpe and Meier, 1973). ABA stimulated bud formation in Begonia leaf explants (Heide, 1968) and sweet potato tuber explants (Yamaguchi and Nakajima, 1974). In tobacco callus, ABA had no effect on shoot formation (Thorpe and Meier, 1973). Bouriquet (1972) and Lefebvre (1972) demonstrated that application of exogenous ethylene to root fragments of endive promoted bud differentiation.

In addition to phytohormones, other chemicals have been shown to affect organogenetic responses. Rücker and Paupardin (1969) reported that various phenolic acids stimulated root formation in Jerusalem artichoke. They suggested that phenolic acids exert influence on IAA-oxidase activity. Lee and Skoog (1965) described monohydroxy-substituted phenols were effective in inducing bud formation. Fridborg and Eriksson (1975) observed that activated charcol stimulated root and bud formation in Allium cepa, suggesting the removal of inhibitory phenols from medium by charcol. Traumatic acid enhanced bud formation in explants of cauliflower heads (Margara, 1977). This chemical isolated by English et al. (1939) was one of the plant wound hormones. Kochhar et al. (1971b) demonstrated that some chelating agents caused bud formation in haploid tobacco callus,

but not in diploid. Such selective effects were also observed in a tobacco smoke component (benzanthracene) which induced bud formation in haploid tobacco callus cultured on a medium lacking growth regulators (Kochhar et al., 1971a; Kochhar and Sabharwal, 1977).

Effects of various amino acids on organogenesis have also been examined. In the presence of auxin and cytokinin, tyrosine and phenylalanine stimulated both root and bud formation in tobacco callus (Skoog and Miller, 1957). Kamada and Harada (1979) reported the stimulative effects of glutamic acid and aspartic acid on bud formation in Torenia stem segments.

The chemicals mentioned above seem to affect bud differentiation probably through the changes in the endogenous levels of auxin and/or cytokinin. In fact, several amino acids including glutamic acid and aspartic acid raised the endogenous levels of cytokinin in crown gall tissues of Vinca rosea (Peterson and Miller, 1976).

There have been several reports demonstrating biochemical analysis of adventitious bud differentiation in tissue culture. Increase of starch contents in bud-forming tissues was observed in tobacco callus (Thorpe and Murashige, 1968). The starch accumulation in callus occurred prior to the initiation of bud primordia (Thorpe and Murashige, 1970; Thorpe and Meier, 1972). Changes in the activities

of various enzymes associated with starch metabolism during the initial stage of bud formation were investigated by Thorpe and his co-workers (Ross and Thorpe, 1973; Thorpe and Laishley, 1973; Thorpe and Meier, 1974, 1975). Thorpe (1978, 1980) postulated that the role of the starch was to supply energy and regulate osmotic pressure for bud initiation.

The content of RNA and protein increased during bud differentiation in tobacco callus (Thorpe and Murashige, 1970; Thorpe and Meier, 1974). The increased proteins seemed to be localized in meristematically divided cells which eventually resulted in the formation of bud primordia (Ross et al., 1973). Syōno (1965) reported that bud formation in carrot callus tissues was inhibited by chloramphenicol, without imparing callus growth. He proposed the presence of specific proteins for bud formation. Sekiya and Yamada (1974) demonstrated that tobacco callus tissue synthesized certain specific proteins when it was cultured under bud-forming conditions. Recently Hasegawa et al. (1979) and Yasuda et al. (1980) reported that bud-forming cultures of Douglas fir cotyledons produced specific proteins at low molecular weight during the first 2 to 4 days of culture.

Despite the efforts of several biochemical studies on plant morphogenesis, little is known about the regulation of specific protein synthesis by phytohormones.

2. In vitro flower formation

Flower initiation in higher plants marks the transition from vegetative growth to reproductive development, which would be the most crucial events in the plant life cycle. Numerous studies have been accumulated on the physiology of flowering (review by Searle, 1965; Evans, 1971; Zeevaart, 1976, 1978). According to the classical theories, some environmental factors such as photoperiod and temperature would be responsible for the production of certain flowering hormones; florigen, in the case of photo-induced flowering (Chailakhyan, 1937), and vernalin, in the case of thermo-induced flowering (Melchers, 1939). Unfortunately, attempts to isolate these flowering hormones were so far unsuccessful. Several known chemicals may play a key role on plant flowering. One of the reasons for the failure in isolating flowering hormone(s) would be a lack of an appropriate assay system for the detection of flower inducing substances. Thus, the development of sensitive assay systems and re-examinations of various factors influencing flower induction would be urgently needed. In this respect, in vitro culture of plant tissues and organs can provide useful means for the research of the physiology of flowering.

In 1955, Skoog observed for the first time in vitro floral bud formation in tobacco tissue culture. Later,

many investigators reported *in vitro* floral bud formation using various explants taken from different parts of many plant species including the followings: stems (Chouard and Aghion, 1961) and floral stalks (Aghion, 1962) of tobacco, roots (Paulet and Nitsch, 1964) and floral stalks (Harada, 1966) of *Cichorium intybus*, petioles of *Lunaria annua* (Pierik, 1966), stems of *Phlox drummondii* (Konar and Konar, 1966), leaves of *Streptocarpus nobilis* (Rossini and Nitsch, 1966), stems of *Plumbago indica* (Nitsch and Nitsch, 1967b), floral stalks of some *Begonia* varieties (Ringe and Nitsch, 1968), inflorescences of *Muzus pumilus* (Raste and Ganapathy, 1970), hypocotyls of *Crepis capillaris* (Jayakar, 1970), thin cell layers of tobacco stems (Tran Thanh Van, 1973a) and *Nautilocalyx lynchei* (Tran Thanh Van, 1973b), stems and leaves of *Torenia fournieri* (Chlyah, 1973a), stems (Chailakhyan *et al.*, 1974, 1975) and epidermal cell layers (Tran Thanh Van, 1978, 1980; Trinh and Tran Thanh Van, 1981; Kamate *et al.*, 1981) of some *Nicotiana* varieties, roots formed from epidermal tissues of tobacco floral stalks (Trinh, 1978), leaves of *Perilla frutescens* (Tanimoto and Harada, 1980), isolated somatic embryos of ginseng (Chang and Hsing, 1980), and stems and leaves of *Rudbeckia bicolor* (Tanimoto and Harada, 1982).

In long-day plants, *Cichorium* (Harada, 1966; Margara, 1974), flower bud formation in explants of floral stalk

and root was induced or stimulated under strictly long-day conditions. Similarly, internodal segments of Plumbago, short-day plant, produced flower only under short-day photoperiod (Nitsch and Nitsch, 1967b). These results proved that the induction of in vitro flowering could be regulated by photoperiodism. In stem and leaf segments of Rudbeckia, a long-day plant, bolting of neo-formed bud was caused by application of GA_3 under both short and long-day conditions, but floral bud differentiation in the apices of elongated shoots occurred only under long-day photoperiod and in the presence of GA_3 (Tanimoto and Harada, 1982).

Margara and Bounioles (1967) and Cousson and Tran Thanh Van (1981) examined the effects of liquid medium on in vitro flowering, and they suggested the importance of changes in pH during the induction of floral buds.

Using tobacco stem segments cultured on a medium lacking growth regulators, Chouard and Aghion (1961) demonstrated the presence of a gradient of flower-forming capacity along the stem; it being greater in explants taken from higher portions of stems than from the lower portions. This kind of gradient has also been reported by Wardell and Skoog (1969a) and Hillson and LaMotte (1977) in tobacco stem segments, and by Tanimoto and Harada (1980) in Perilla leaf discs. Aghion-Prat (1965) also showed that the

flowering gradient apparently existed in floral bud formation on crown gall tissues developed on the stems of tobacco plants. He suggested that the gradient was presumably due to a gradient of endogenous levels of phytohormones. Epidermal explants taken from various parts of tobacco stems clearly showed the existence of similar gradients (Tran Thanh Van, 1973a). Remarkably high contents of peroxidase was found in the most basal region of tobacco stem than in its floral branch (Thorpe et al., 1978). Addition of RNA base analogues to a culture medium partially removed the floral gradient in tobacco stem segments (Wardell and Skoog, 1969b). Wardell and Skoog (1973) found that apical stem segments of tobacco contained 10 times more DNA than basal segments.

Physiological states of mother plants from which explants were taken, also affected in vitro flowering capacity. Flower buds could be differentiated in tobacco epidermal layers, only when explants were excised from mother plants where the terminal bud was in the green-fruits stage (Tran Thanh Van et al., 1974). Ringe and Nitsch (1968) observed that flower buds were differentiated from excised floral stalks of Begonia, but they were not formed in petiole segments. In Crepis capillaris, leaf discs taken from vegetative plants initiated only adventitious buds, but ones taken from reproductive mother plants formed floral

buds (Brossard, 1979). Although many workers observed in vitro flowering using explants excised from reproductive mother plants, systematic examinations on physiological states of explants were rather limited. Wardell (1975, 1977) showed qualitative differences in DNA between stems of flowering and vegetative tobacco plants. A purified DNA solution prepared from flowering tobacco plants induced flowers in vegetative plants of the same species (Wardell, 1976, 1977).

The effects of nutrient factors on in vitro flowering also was examined by many investigators. A high concentration of sucrose or glucose promoted in vitro flowering of Cichorium (Harada, 1966; Margara, 1974), Plumbago (Nitsch and Nitsch, 1967b) and Nicotiana (Chailakhyan et al., 1974; Tran Thanh Van, 1977).

Regarding the effects of various growth regulators added to culture medium, many published results seem to be inconsistent or difficult to explain. For example, floral buds were formed on tobacco stem segments cultured on a medium without growth regulators (Chouard and Aghion, 1961), while flower formation from epidermal explants of the same species required both auxin and cytokinin (Tran Thanh Van et al., 1974). Auxin inhibited in vitro flowering of Plumbago (Nitsch and Nitsch, 1967b) and Torenia (Chlyah, 1973b), but stimulated it in Streptocarpus (Rossini and

Nitsch, 1966) and Begonia (Ringe and Nitsch, 1968). Addition of adenine sulfate stimulated floral bud formation in Begonia (Ringe and Nitsch, 1968) and Plumbago (Nitsch and Nitsch, 1967b). Cytokinin promoted in vitro flowering in Streptocarpus (Rossini and Nitsch, 1966) and Muzus (Raste and Ganapathy, 1970), while inhibited that in Torenia (Chlyah, 1973b). Paulet (1979) reported that endogenous levels of cytokinins in explants prepared from the roots of Cichorium, cold-requiring plant, increased during cold-treatment. Reasons for these differences in the responses of the explants to growth regulators are not well understood at present.

3. *In vitro* morphogenetic studies in *Torenia*

In 1903, Winkler reported for the first time that flowers were formed on midribs of excised leaf-cuttings of *Torenia asiatica* L. Working with *T. fournieri* Lind., Chlyah and Tran Thanh Van (1971) also observed flower formation in excised leaves grown on vermiculite. They reported that the short-day condition (9 hr light/15 hr dark) was favorable for flowering. Using excised leaves of *T. fournieri* cultured on a nutrient medium containing auxin and cytokinin, Bajaj (1972) observed the formation of vegetative and floral buds.

Working with excised stem and leaf fragments of *T. fournieri*, Chlyah reported that floral bud formation was delayed by the treatment with high temperature (36°C) (1973a), and that both auxin and cytokinin strongly inhibited flowering of explants excised from young vegetative mother plants (1973b).

Histological studies on adventitious bud and root formation in *T. fournieri* stem segments showed that buds were formed from epidermal cells and roots from endogenous perivascular tissues (Chlyah, 1974a). Since *Torenia* stem epidermis could easily be stripped off the stem segments, Chlyah (1974b) directly observed the process of meristematic cell divisions in the epidermis, and he reported the

distribution pattern of organized cell divisions (Chlyah et al., 1975). Regarding the distribution of dividing cells in the epidermis of explants, a large number of meristematic cells were seen toward the base and on both sides of the medial zone. Chlyah (1978) also studied the relationship between the cell division and DNA synthesis. Most of cells in epidermis synthesized their DNA between 20 and 48 hr after the start of culture, and the first cell division took place at the 20 hr stage.

Chlyah (1974c) examined the organogenetic capacities of tissues excised from stem segments. The isolated epidermis did not show any morphogenetic response. Suprisingly enough, adventitious buds were formed in the epidermis when it was placed on the surface of original stem segments. Tissues such as subepidermal parenchyma or stem segments without epidermis formed only adventitious roots.

References

Aghion, D. (1962) Conditions expérimentales conduisant à l'initiation et au développement de fleurs à partir de la culture stérile de fragments de tige de tabac. C. R. Acad. Sic., Paris, 255; 993-995.

Aghion-Prat, D. (1965) Floral meristem-organizing gradient in tobacco stems. Nature 207; 1211.

Arnold, S. von and T. Eriksson (1978) Induction of adventitious buds on embryos of Norway spruce in vitro. Physiol. Plant. 44; 283-287.

Arnold, S. von and T. Eriksson (1979) Induction of adventitious buds on buds of Norway spruce (Picea abies) grown in vitro. Physiol. Plant. 45; 29-34.

Bajaj, Y. P. S. (1972) Effects of some growth regulators on bud formation by excised leaves of Torenia fournieri. Z. Pflanzenphysiol. 66; 284-287.

Bigot, C. and J. P. Nitsch (1968) Effet du moment de l'application d'acide gibberellique ou de 2,6-diaminopurine sur la neoformation des bourgeons chez Begonia rex Putz. C. R. Acad. Sci., Paris, 267; 619-621.

Bouriquet, R. (1972) Action de l'ethylene sur le bourgeonnement et la floraison in vitro de fragments de racines d'endive. C. R. Acad. Sci., Paris, 275; 33-34.

Brossard, D. (1979) Néoformation de bourgeons végétatifs

et inflorescentiels à partir de disques foliaires du Crepis capillaris L. Wallr. cultivés in vitro. Z. Pflanzenphysiol. 93; 69-81.

Chailakhyan, M. Kh. (1937) Concerning the hormonal nature of plant development processes. C. R. (Dokl.) Acad. Sci. URSS, 16; 227-230.

Chailakhyan, M. Kh., N. P. Aksenova, T. N. Konstantinova and T. V. Bavrina (1974) Use of tobacco stem calluses for the investigation of some regularities of plant flowering. Phytomorphology 24; 86-96.

Chailakhyan, M. Kh., N. P. Aksenova, T. N. Konstantinova and T. V. Bavrina (1975) The callus model of plant flowering. Proc. R. Soc. Lond. B. 190; 333-340.

Chang, W.-C. and Y.-I. Hsing (1980) In vitro flowering of embryoids derived from mature root callus of ginseng (Panax ginseng). Nature 284; 341-342.

Chlyah, H. (1973a) Influence de la température sur la mise à fleur de plantes de Torenia fournieri (Lind.) issues de semis ou de boutures de feuilles, ou formées à partir de fragments d'organes cultivés in vitro. C. R. Acad. Sci., Paris, (Série D) 277; 1451-1454.

Chlyah, H. (1973b) Néoformation dirigée à partir de fragments d'organes de Torenia fournieri (Lind.) cultivés in vitro. Biol. Plant. 15; 80-87.

Chlyah, H. (1974a) Etude histologique de la néoformation

de méristèmes caulinaires et radiculaires à partir de segments d'entre-noeuds de Torenia fournieri cultivés in vitro. Can. J. Bot. 52; 473-476.

Chlyah, H. (1974b) Formation and propagation of cell division-centers in the epidermal layer of internodal segments of Torenia fournieri grown in vitro. Simultaneous surface observations of all the epidermal cells. Can. J. Bot. 52; 867-872.

Chlyah, H. (1974c) Inter-tissue correlations in organ fragments. Organogenetic capacity of tissues excised from stem segments of Torenia fournieri Lind. cultured separately in vitro. Plant Physiol. 54; 341-348.

Chlyah, H. (1978) Intercellular correlations: Relation between DNA synthesis and cell division in early stages of in vitro bud neoformation. Plant Physiol. 62; 482-485.

Chlyah, H. and K. M. Tran Thanh Van (1971) Analyse des capacités néoformatrices de méristèmes radiculaires et caulinaires (végétatifs et floraux) chez le Torenia fournieri (Lind.). C. R. Acad. Sci., Paris, (Série D) 273; 356-359.

Chlyah, H., K. M. Tran Thanh Van and Y. Demarly (1975) Distribution pattern of cell division centers on the epidermis of stem segments of Torenia fournieri during de novo bud formation. Plant Physiol. 56; 28-33.

Chouard, P. and D. Aghion (1961) Modalités de la formation de bourgeons floraux sur des cultures de segments de tige de tabac. C. R. Acad. Sci., Paris, 252; 3864-3867.

Cousson, A. and K. M. Tran Thanh Van (1981) In vitro control of de novo flower differentiation from tobacco thin cell layers cultured on a liquid medium. Physiol. Plant. 51; 77-84.

English, J., J. Bonner and J. Haagen-Smit (1939) The wound hormones of plants II. The isolation of a crystalline active substance. Proc. N. A. S. USA, 25; 323-329.

Evans, L. T. (1971) Flower induction and the florigen concept. Ann. Rev. Plant Physiol. 22; 365-394.

Feng, K. A. and A. J. Linck (1970) Effects of N-1-naphthylphthalmic acid on the growth and bud formation of tobacco callus grown in vitro. Pl. Cell Physiol. 11; 589-598.

Fridborg, G. and T. Eriksson (1975) Partial reversal by cytokinin and (2-chloroethyl)-trimethyl ammonium chloride of near ultraviolet inhibited growth and morphogenesis in callus cultures. Physiol. Plant. 34; 162-166.

Haberlandt, G. (1902) Kultiversuche mit isolierten Pflanzellen. Sber. Akad. Wiss. Wien 111; 69-92.

Harada, H. (1966) Effects of photoperiod on the formation of flower buds by flower stalk sections of Cichorium intybus in tissue culture. Bot. Mag. Tokyo 79; 119-123.

Hasegawa, P. M., T. Yasuda and T.-Y. Cheng (1979) Effect

of auxin and cytokinin on newly synthesized proteins of cultured Douglas fir cotyledons. Physiol. Plant. 46; 211-217.

Heide, O. M. (1968) Stimulation of adventitious bud formation in Begonia leaves by abscisic acid. Nature 219; 960-961.

Hillson, T. D. and C. E. LaMotte (1977) In vitro formation and development of floral buds on tobacco stem explants. Effects of kinetin and other factors. Plant Physiol. 60; 881-884.

Jayakar, M. (1970) In vitro flowering of Crepis capillaris. Phytomorphology 20; 410-412.

Kamada, H. and H. Harada (1979) Influence of several growth regulators and amino acids on in vitro organogenesis of Torenia fournieri Lind. J. Exp. Bot. 30; 27-36.

Kamate, K., A. Cousson, T. H. Trinh and K. M. Tran Thanh Van (1981) Influence des facteurs génétique et physiologique chez le Nicotiana sur la néoformation in vitro de fleurs à partir d'assises cellulaires épidermiques et sous-épidermiques. Can. J. Bot. 59; 775-781.

Kato, Y. (1978) Induction of adventitious buds on undetached leaves, excised leaves and leaf fragments of Heloniopsis orientalis. Physiol. Plant. 42; 39-44.

Kochhar, T. S., P. R. Bhalla and P. S. Sabharwal (1971a) Effect of tobacco smoke components on organogenesis in

plant tissues. Pl. Cell Physiol. 12; 603-608.

Kochhar, T. S., P. R. Bhalla and P. S. Sabharwal (1971b) In vitro induction of vegetative buds in tobacco callus by chelating agents. Can. J. Bot. 49; 391-394.

Kochhar, T. S. and P. S. Sabharwal (1977) Morphogenetic effects of indole-3-acetic acid and benz(a)anthracene on tobacco callus. Physiol. Plant. 40; 169-171.

Konar, R. N. and A. Konar (1966) Plantlet and flower formation in callus cultures from Phlox drummondii Hook. Phytomorphology 16; 379-382.

Konar, R. N. and A. Konar (1973) Interaction of CCC and gibberellic acid in morphogenesis of Rannunclus sceloratus tissue in vitro. Phytomorphology 23; 105-109.

Lee, T. T. and F. Skoog (1965) Effects of substituted phenols on bud formation and growth of tobacco tissue cultures. Physiol. Plant. 18; 396-402.

Lefebvre, R. (1972) Effets comparés d'un traitement anaérobie et de l'éthylène sur le bourgeonnement et la synthèse des pigments foliaires. C. R. Acad. Sci. Paris, (Série D) 275; 193-195.

Legrand, B. (1974) Influence des conditions d'éclairement sur la néoformation des bourgeons par les tissus de feuilles d'endive cultivés in vitro et sur l'activité peroxydasique de ces tissus. C. R. Acad. Sci. Paris, (Série D) 278; 2425-2428.

Margara, J. (1974) Sur les conditions de l'évolution, végétative ou inflorescentielle, des bourgeons néoformés à partir de fragments de hampe florale de Cichorium intybus L. C. R. Acad. Sci., Paris, (Série D) 278; 1195-1198.

Margara, J. (1977) Effets d'auxines et d'antiauxines sur la néoformation de bourgeons in vitro chez le chou-fleur (Brassica oleracea L., var botrytis). C. R. Acad. Sci., Paris, (Série D) 284; 1883-1885.

Margara, J. and A. Bouniols (1967) Comparaison in vitro de l'influence du milieu, liquide ou gélosé, sur l'initiation florale, chez Cichorium intybus L. C. R. Acad. Sci., Paris, (Série D) 264; 1166-1168.

Melchers, G. (1939) Die Blühhormone. Ber. Deutsch. Bot. Ges. 57; 29-48.

Miller, C. O., F. Skoog, F. S. Okumura, M. H. von Saltza and F. M. Strong (1956) Isolation, structure and synthesis of kinetin, a substance promoting cell division. J. Amer. Chem. Soc. 78; 1375-1380.

Murashige, T. (1961) Suppression of shoot formation in cultured tobacco cells by gibberellic acid. Science 134; 280.

Murashige, T. (1965) Effects of stem-elongation retardants and gibberellins on callus growth and organ formation in tobacco tissue. Physiol. Plant. 18; 665-673.

Murashige, T. (1974) Plant propagation through tissue

cultures. Ann. Rev. Plant Physiol. 25; 135-166.

Murashige, T. (1978) The impact of plant tissue culture on agriculture. In: Frontiers of plant tissue culture 1978. Edited by T. A. Thorpe, pp. 15-26.

Nitsch, C. and J. P. Nitsch (1967a) The induction of flowering in vitro in stem segments of Plumbago indica L. I. The production of vegetative buds. Planta 72; 355-370.

Nitsch, C. and J. P. Nitsch (1967b) The induction of flowering in vitro in stem segments of Plumbago indica L. II. The production of reproductive buds. Planta 72; 371-384.

Ogura, H. (1975) The effects of a morphactin, chlorflurenol, on organ differentiation from tobacco calluses cultured in vitro. Bot. Mag. Tokyo 75; 1-8.

Paulet, P. (1979) Sur la régulation de la néoformation florale in vitro. Physiol. Vég. 17; 631-641.

Paulet, P. and J. P. Nitsch (1964) La néoformation de fleurs sur sultures in vitro de racines de Cichorium intybus L.: étude physiologique. Ann. Physiol. Vég. 6; 333-345.

Peterson, J. B. and C. O. Miller (1976) Cytokinin in Vinca rosea L. crown gall tumor tissue as influenced by compounds containing reduced nitrogen. Plant Physiol. 57; 393-399.

Pierik, R. L. M. (1966) The induction and initiation of flower buds in vitro in tissues of Lunaria annua L. Naturwiss. 53; 45.

Rao, P. S., W. Handro and H. Harada (1973) Hormonal control

differentiation of shoots, roots and embryos in leaf and stem cultures of Petunia inflata and Petunia hybrida. Physiol. Plant. 28; 458-463.

Raste, A. P. and P. S. Ganapathy (1970) In vitro behaviour of inflorescence segments of Mazus pumilus. Phytomorphology 20; 367-374.

Ringe, F. and J. P. Nitsch (1968) Conditions leading to flower formation on excised Begonia fragments cultured in vitro. Pl. Cell Physiol. 9; 639-652.

Ross, M. K. and T. A. Thorpe (1973) Physiological gradients and shoot initiation in tobacco callus cultures. Pl. Cell Physiol. 14; 473-480.

Ross, M. K., T. A. Thorpe and J. W. Costerton (1973) Ultrastructural aspects of shoot initiation in tobacco callus cultures. Amer. J. Bot. 60; 788-795.

Rossini, M. E. and J. P. Nitsch (1966) Induction de la floraison in vitro chez une plante de jours courts, Streptocarpus nobilis. C. R. Acad. Sci., Paris, (Série D) 263; 1379-1382.

Rücker, W. and C. Paupardin (1969) Action de quelques acides-phénols sur la rizogenèse des tissus de tubercules de topinambour (variete violet de rennes) cultivés in vitro. C. R. Acad. Sci., Paris, (Série D) 268; 1279-1281.

Rücker, W. and B. J. Radola (1971) Isoelectric patterns of peroxidase isoenzymes from tobacco tissue cultures.

Planta 99; 129-198.

Sangwan, R. S., B. Norreel and H. Harada (1976) Effects of kinetin and gibberellin A_3 on callus growth and organ formation in Limnophila chinensis tissue culture. Biol. Plant. 18; 126-131.

Schott, H. and H. Schraudolf (1967) Die wirkung einiger derivate der 9-fluorenol-9-carbonsaire auf die regeneration von begonienblaltscheiben (Begonia rex). Z. Pflanzenphysiol. 56; 387-396.

Searle, N. E. (1965) Physiology of flowering. Ann. Rev. plant Physiol. 16; 97-118.

Sekiya, J. and Y. Yamada (1974) Cytokinin regulation of protein synthesis during the early stages of shoot formation from cultured tobacco cells. Bull. Inst. Chem. Res. Kyoto Univ. 52; 246-255.

Skoog, F. (1944) Growth and organ formation in tobacco tissue cultures. Amer. J. Bot. 31; 19-24.

Skoog, F. (1955) Growth factors, polarity and morphogenesis. Ann. Biol. 31; 1-11.

Skoog, F. and C. O. Miller (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Expt. Biol. 11; 118-131.

Skoog, F., R. Y. Schmitz, R. M. Bock and S. M. Hecht (1973) Cytokinin antagonists: Synthesis and physiological effects of 7-substituted 3-methylpyrazolo[4,3-d]-pyrimidines.

Phytochemistry 12; 25-37.

Skoog, F. and C. Tsui (1948) Chemical control of growth and bud formation in tobacco stem segments and callus cultured in vitro. Amer. J. Bot. 35; 782-787.

Skoog, F. and C. Tsui (1951) Growth substances and the formation of buds in plant tissues. In: Plant Growth Substances. Edited by F. Skoog, Univ. of Wisconsin Press. pp. 263-285.

Syōno, K. (1965) Physiological and biochemical changes of carrot root callus during successive cultures. Pl. Cell Physiol. 6; 371-392.

Tanimoto, S. and H. Harada (1980) Hormonal control of morphogenesis in leaf explants of Perilla frutescens Britton var. crispa Decaisne f. viridi-crispa Makino. Ann. Bot. 45; 321-327.

Tanimoto, S. and H. Harada (1982) Physiological and hormonal factors influencing organogenesis in Rudbeckia bicolor explants cultured in vitro. Pl. Cell Physiol. 23; 107-113.

Thorpe, T. A. (1978) Physiological and biochemical aspects of organogenesis in vitro. In: Frontiers of plant tissue culture 1978. Edited by T. A. Thorpe. Univ. of Calgary Press. pp.49- 58.

Thorpe, T. A. (1980) Organogenesis in vitro: Structural, physiological, and biochemical aspects. Int. Rev. Cytol.

11A; 71-111.

Thorpe, T. A. and T. Gaspar (1978) Changes in isoperoxidases during shoot formation in tobacco callus. In Vitro 14; 522-526.

Thorpe, T. A. and E. Laishley (1973) Glucose oxidation during shoot initiation in tobacco callus cultures. J. Exp. Bot. 24; 1082-1089.

Thorpe, T. A. and D. D. Meier (1972) Starch metabolism, respiration, and shoot formation in tobacco callus cultures. Physiol. Plant. 27; 365-369.

Thorpe, T. A. and D. D. Meier (1973) Effects of gibberellic acid and abscisic acid on shoot formation in tobacco callus cultures. Physiol. Plant. 29; 121-124.

Thorpe, T. A. and D. D. Meier (1974) Starch metabolism in shoot-forming tobacco callus. J. Exp. Bot. 25; 288-294.

Thorpe, T. A. and D. D. Meier (1975) Effect of gibberellic acid on starch metabolism in tobacco callus cultured under shoot-forming conditions. Phytomorphology 25; 238-245.

Thorpe, T. A. and T. Murashige (1968) Starch accumulation in shoot-forming tobacco callus cultures. Science 160; 421-422.

Thorpe, T. A. and T. Murashige (1970) Some histochemical changes underlying shoot initiation in tobacco callus cultures. Can. J. Bot. 48; 277-285.

Thorpe, T. A., K. M. Tran Thanh Van and T. Gaspar (1978)

Isoperoxidases in epidermal layers of tobacco and changes during organ formation in vitro. Physiol. Plant. 44; 388-394.

Tran Thanh Van, K. M. (1973a) Direct flower neoformation from superficial tissue of small explants of Nicotiana tabacum L. Planta 115; 87-92.

Tran Thanh Van, K. M. (1973b) In vitro control of de novo flower, bud, root, and callus differentiation from excised epidermal tissues. Nature 246; 44-45.

Tran Thanh Van, K. M. (1977) Regulation of morphogenesis. In: Plant Tissue Culture and its Bio-technological Application. Eduted by W. Barz, E. Reinhard and M. H. Zenk. Springer-Verlag, Berlin. pp. 367-385.

Tran Thanh Van, K. M. (1978) Morphogenesis in thin cell layers: Concept, methodology and results. In: Frontiers of plant tissue culture 1978. Edited by T. A. Thorpe, Univ. of Calgary Press. pp.37-48.

Tran Thanh Van, K. M. (1980) Control of morphogenesis by inherent and exogenously applied factors in thin cell layers. Int. Rev. Cytol. 11A; 175-194.

Tran Thanh Van, K. M., N. Thi Dien and A. Chlyah (1974) Regulation of organogenesis in small explants of superficial tissue of Nicotiana tabacum L. Planta 119; 149-159.

Trinh, T. H. (1978) Organogenèse induite in vitro sur des

fragments de racine de Nicotiana tabacum L. Can. J. Bot. 56; 2370-2374.

Trinh, T. H. and K. M. Tran Thanh Van (1981) Formation in vitro de fleurs à partir de couches cellulaires minces épidermiques et sous-épidermiques diploides et haploides chez le Nicotiana tabacum L. et chez le Nicotiana plumbaginifolia Viv. Z. Pflanzenphysiol. 101; 1-8.

Vasil, I. K. and V. Vasil (1980) Clonal propagation. Int. Rev. Cytol. 11A; 145-173.

Wardell W. L. (1975) Rapid initiation of thymidine incorporation into deoxyribonucleic acid in vegetative tobacco stem segments treated with indole-3-acetic acid. Plant Physiol. 56; 171-176.

Wardell, W. L. (1976) Floral activity in solutions of deoxyribonucleic acid extracted from tobacco stems. Plant Physiol. 57; 855-861.

Wardell, W. L. (1977) Floral induction of vegetative plants supplied a purified fraction of deoxyribonucleic acid from stems of flowering plants. Plant Physiol. 60; 885-891.

Wardell, W. L. and F. Skoog (1969a) Flower formation in excised tobacco stem segments; I. Methodology and effects of plant hormones. Plant Physiol. 44; 1402-1406.

Wardell, W. L. and F. Skoog (1969b) Flower formation in excised tobacco stem segments; II. Reversible removal

of IAA inhibition by RNA base analogues. Plant Physiol. 44; 1407-1412.

Wardell, W. L. and F. Skoog (1973) Flower formation in excised tobacco stem segments; III. Deoxyribonucleic acid content in sten tissue of vegetative and flowering tobacco plants. Plant Physiol. 52; 215-220.

White, P. R. (1939) Controlled differentiation in a plant tissue culture. Bull. Torrey Bot. Club 66; 507-515.

Winkler, H. (1903) Über regenerative Sprossbildung auf den Blättern von Torenia asiatica L. Ber. Deutsch. Bot. Ges. 21; 96-107.

Yamaguchi, T. and T. Nakajima (1974) Hormonal regulation of organ formation in cultured tissue derived from root tuber of sweet potato. In: Plant Growth Substances 1973. Hirokawa Publ., Tokyo pp. 1121-1127.

Yasuda, T., P. M. Hasegawa and T.-Y. Cheng (1980) Analysis of newly synthesized proteins during differentiation of cultured Douglas fir cotyledons. Physiol. Plant. 48; 83-87.

Zeevaart, J. A. D. (1976) Physiology of flower formation. Ann. Rev. Plant Physiol. 27; 321-348.

Zeevaart, J. A. D. (1978) Phytohormones and flower formation. In: Phytohormones and Related Compounds-- A Comprehensive Treatise. Vol. 2. Edited by D. S. Letham, P. B. Goodwin and T. J. V. Higgins. Elsevier/North-Holland

Biomedical Press, Amsterdam, pp. 291-327.

General Introduction

The elucidation of the mechanism of organ differentiation in higher plants is one of the most important and intriguing subjects in plant morphogenesis. Studies of various types on the differentiation have been conducted by a large number of research workers, but many questions still remain unsolved. It seems particularly important to choose appropriate experimental plant materials and methods for the investigation of organ differentiation in higher plants.

We have carried out a series of experiments on two important phases in organ differentiation, i.e. adventitious bud differentiation and flower formation. A simple but sensitive experimental system using in vitro culture of Torenia stem segments has been employed throughout the study. In this material, adventitious buds can easily be initiated from the epidermis, and floral buds can be differentiated on adventitious shoot apices.

In Part I, the effects of various chemical substances on bud initiation and biochemical changes taking place during the initial process of bud differentiation were investigated.

The favorable effects of cytokinin on adventitious bud

differentiation have already been reported with various plant materials. However, most of the reported instances were derived from the experiments, where cytokinin was applied throughout culture periods. Therefore, it was not clear to determine that cytokinin affects the inductive stage and/or developmental process of adventitious bud formation. In our study, the results of the investigation on cytokinin action during the inductive phase of adventitious buds are presented in Chapter 1.

In order to elucidate the mode of action of cytokinin on organogenesis, it is worthwhile to examine the effects of various cytokinins and related compounds having different structures and activities, as well as correlative effects of those chemicals. In this respect, a large number of substances with cytokinin-antagonistic or agonistic activities have been synthesized and their biological activities were reported by several research workers. However, only a few reports have dealt with their effects on organogenesis. In Chapter 2, individual and correlative effects of various cytokinins, anticytokinins and auxins on adventitious bud initiation are presented.

The treatment of explants with cytokinin must be responsible for certain physiological and biochemical changes occurring during the initial process of adventitious bud differentiation. Using callus tissues, several researchers

have investigated biochemical aspects of organogenesis. Bud formation from disorganized tissues like callus would not be ideal system for the biochemical study of organogenesis. In this regard, Torenia stem segments provide a significant advantage over other plant materials. For example, adventitious buds are directly induced from the epidermis, and the explants respond rapidly, showing high sensitivity to chemical treatments. Thus, we were able to investigate more specifically how cytokinin affects the process of protein synthesis during adventitious bud differentiation. Thus, the changes in endogenous amounts of protein-incorporated and free amino acids were examined (Chapter 3). Furthermore, quantitative and qualitative analysis on de novo synthesis of proteins were carried out (Chapter 4).

The studies on flower formation in Torenia stem segments constitute the Part II of the present work. Employing internodal segments of Torenia, we examined individual and correlative effects of chemical substances and the influences of environmental conditions and physiological state of explants on floral bud initiation and development.

First, a culture medium suitable for the studies on in vitro flowering was established, and the influence of several nutritional factors was investigated using stem segments excised from reproductive Torenia plants (Chapter 1).

As the process of *in vitro* flowering in *Torenia* stem segments comprises a series of events including adventitious bud formation, floral bud initiation and flower development, the effects of various factors should be examined in each developmental phase. First, histological examination of explants was carried out to clarify the process of floral bud formation. Secondly, the effects of several hormonal and nutritional treatments on flower initiation and development were investigated (Chapter 2).

The explants taken from the mother plants at the reproductive stage seemed to be potent to differentiate floral buds. Therefore, explants were excised from mother plants which had been kept at various physiological states, and their ability to form flower bud was examined. Furthermore, the effects of different environmental conditions on the expression of the flower-forming capacity were studied (Chapter 3).

The effects of various growth regulators on *in vitro* flowering have been investigated by several workers, but often their results seem to be inconsistent or difficult to explain. The inconsistency in the effects of growth regulators may be due to the differences in physiological state of explants. Therefore, the effects of some growth regulators on *in vitro* floral bud initiation and its development of *Torenia* stem segments in different physio-

logical states were also examined (Chapter 4).

PART I.

STUDIES ON THE INITIAL PROCESS OF ADVENTITIOUS BUD DIFFERENTIATION IN *TORENIA* STEM SEGMENTS CULTURED *IN VITRO*

Chapter 1

Effects of cytokinin on adventitious bud initiation

Summary

The effects of mineral nutrients and growth regulators added to a culture medium on the initial stage of adventitious bud differentiation were investigated using Torenia internodal segments cultured in vitro. Under favourable culture conditions, the formation of meristematic zones (MZ) occurred first in the epidermis of stem segments, then many of MZ developed to form adventitious buds. MZ and adventitious bud formation was rarely noted in the explants cultured on a nutrient medium without benzyladenine (BA). When explants were cultured on a medium composed of only BA and sucrose, MZ could be initiated but no adventitious bud formation was observed. However, if culture medium contained mineral nutrients and BA, both MZ and adventitious buds were abundantly formed. Applications of indole-3-acetic acid (IAA) and gibberellic acid (GA_3) stimulated neither MZ nor bud formation. When the explants were cultured on a medium contained only BA (1.0 mg/l) and sucrose during the first 6 days, then transferred to a nutrient medium without BA, adventitious buds were differen-

tiated at a high rate. Consequently, it seems that BA strongly promotes MZ formation (bud initiation) during the initial period of culture and mineral nutrients play major role in subsequent development of MZ to bud primordia.

Introduction

Hormonal factors controlling organogenetic processes in plant tissues and organs cultured *in vitro* have been studied by a large number of investigators. Skoog and Miller (1957) first demonstrated that a balance between auxin and cytokinin played an important role for the organogenesis in tobacco pith callus. A high ratio of cytokinin to auxin in a medium stimulated adventitious bud differentiation, while a reverse ratio of the two compounds promoted adventitious root formation. Stimulating effects of cytokinin on adventitious bud differentiation have been reported by a number of workers who used, as experimental materials, different tissues and organs of various plant species (review by Murashige, 1974). However, most of published results were based on experiments in which cytokinin was continuously applied throughout culture periods. Therefore, it has not been clear whether cytokinin stimulates only the induction of adventitious bud differentiation or its development or both of them. Chlyah (1974a) reported that organized cell divisions occurred prior to adventitious bud formation in epidermal cell layers of *Torenia* stem segments cultured *in vitro*. Kamada and Harada (1979) observed a promoting effect of cytokinin on bud formation in the same material. In this chapter, we report on some effects of cytokinin on the

initial stage of adventitious bud differentiation in *Torenia* stem segments cultured *in vitro*.

Materials and Methods

Plants of *Torenia fournieri* Lind. were grown in a growth room at a temperature of 25±2 °C under 16-hr light photoperiod. The second stem internodes (counting from the top) were taken from 6 to 8 week old vegetative plants and were surface-sterilized with an 8% sodium hypochlorite solution for 15 min after cut ends were sealed with molten paraffin. Stem segments, 5 mm long, were cut from the internodes using a sterile scalpel, and cultured on a 0.8% agar medium. The pH of medium was adjusted to 5.6 before adding the agar and autoclaving (120 °C for 15 min). The cultures were maintained in a growth room under the conditions described above. Media used in our experiments composed of mineral salts of Murashige and Skoog's medium (hereafter referred to as MS medium) or that of the 1/10-strength MS medium, and both contained 2% sucrose. Another medium containing only 2% sucrose and 0.8% agar (without mineral salts) was also used. Solutions of IAA and GA_3 were sterilized through a Millipore filter (pore size 0.45 μm).

In order to examine the initial process of adventitious bud differentiation, the epidermis was stripped off the stem segments cultured *in vitro* for 6 days. These epidermal strips were stained with acetocarmine (2% carmine in a 45% acetic acid solution), and observed immediately

under a microscope. As the stem segments of Torenia are more or less rectangular in section, three epidermal strips (excluding epidermis facing to the surface of agar medium) were excised from each of stem segments. Twenty-four replicates were used for each run and all experiments were repeated at least three times. The epidermal strips of 12 explants were used for microscopical observations and remaining 12 explants were examined macroscopically at the end of 6 week culture period. The results were expressed as an average number of meristematic zones per epidermal strip, an average percentage of cultures with adventitious buds, and also the degree of bud development.

Results

Control epidermal strips which were just peeled off the mother plants showed no cell division (Fig. 1A). Three days after the beginning of culture, meristematic zones (MZ) started to appear in the epidermis of stem segments cultured on a medium containing benzyladenine (BA) (Fig. 1B), and thereafter the number of MZ increased progressively attaining its maximum on the 6th day of culture (Fig. 1C). These MZ developed to form bud primordia at the end of 8 to 10-day culture period (Fig. 1D) and the buds became visible macroscopically 12 to 14 days after the start of culture (Fig. 1E). Figure 1F shows an aspect of shoots developed from neoformed buds at the end of 6-week culture.

The effects of mineral nutrients and three growth regulators on MZ and adventitious bud formation were summarized in Table 1. Among various media which were used without any addition of growth regulators, the 1/10-strength MS medium gave the best result in inducing MZ and bud formation. The mean number of MZ per epidermal strip was 4.9 and almost all of the explants formed adventitious buds. When explants were cultured either on the MS medium without BA or on a medium containing only sucrose, very limited number of MZ was formed, and the rate of the explants with adventitious buds was very low. Indole-3-acetic acid

(IAA) and GA_3 did not promote MZ and subsequent bud formation. When BA (0.1 or 1.0 mg/l) was added to the MS medium, a large number of MZ formed in the epidermis, and the cultures producing adventitious buds attained almost 100%. At a low concentration (0.01 mg/l), however, BA did not show stimulating effect, especially on the formation of MZ.

Some interesting results were obtained when a medium containing BA (1.0 mg/l) and sucrose but not mineral salts was used. Although the mean number of MZ per epidermal strip was relatively large (3.9), no adventitious bud was produced with this medium. This result suggested that MZ formation was stimulated by the application of BA, but the presence of mineral nutrients in a medium was required for MZ development leading to the formation of adventitious buds.

In order to investigate more precisely the effects of BA on bud differentiation, Torenia stem segments were cultured first on the media with BA (0.1 or 1.0 mg/l) but without mineral salts for different culture periods (2 to 14 days), and then transferred to the MS medium without BA. As shown in Fig. 2, the explants treated with 1.0 mg/l of BA for 6 days produced adventitious buds at a high rate (64%) after having been transferred to the MS medium. The degree of bud development was as high as

in those cultured on the MS medium with BA throughout the culture period. As seen in Fig. 2, when the explants were treated with BA at a concentration of 0.1 mg/l even for 14 days, the percentage of cultures with buds was not much higher than that in control explants which were cultured on the MS medium throughout the culture period.

Discussion

Adventitious bud differentiation in many plant species such as Nicotiana (Skoog and Miller, 1957), Petunia (Rao et al., 1973), Torenia (Kamada and Harada, 1979) and Perilla (Tanimoto and Harada, 1980) can be promoted by the application of cytokinin, but the mechanism of stimulatory action of cytokinin is still not clarified. For the elucidation of cytokinin action on adventitious bud differentiation, it is highly desirable to have plant materials which are histologically simple and sensitive to various treatments. Chlyah (1974a) reported that adventitious buds were easily formed in epidermal cells of Torenia stem segments. In Torenia, epidermal layer can be easily stripped off stem segments, and the process of cell division in the epidermis can be directly observed (Chlyah, 1974b). Using this material, he studied the distribution pattern of organized cell divisions in epidermal strips (Chlyah et al., 1975), as well as the relationship between cell division and DNA synthesis (Chlyah, 1978). However, detailed investigation on the effects of various constituents, including growth regulators, of culture medium on cell division and bud differentiation has not been conducted with this material.

The dilution of mineral salts comprising the MS medium promoted adventitious and floral bud formation in Torenia

stem segments, and similar promoting effects were also obtained when ammonium nitrate was completely eliminated from the MS medium (Part II, Chapter 1). The effects of ammonium nitrate on organogenesis is difficult to interpret. Mohanty and Fletcher (1976) reported that the presence of ammonium ion in a culture medium was closely related to the activity of nitrate reductase. The stimulating effects of the 1/10-strength MS medium on MZ and bud formation (Table 1) may be associated with such nitrogen metabolism.

Kamada and Harada (1979) reported that various cytokinins stimulated adventitious bud differentiation in Torenia stem segments. In their experiments, however, cytokinin was continuously present in the medium used. In Part II, we show that several growth substances differently affected in vitro flowering of Torenia stem segments. Their effects varied depending on the physiological state of plant materials used (Part II, Chapter 4), and on the time of application (Part II, Chapter 2). Our results presented here show that cytokinin seems to be required during the induction phase of adventitious buds rather than during bud development (Fig. 2). The presence of cytokinin (1.0 mg/l of BA) during the first 6 days of culture was sufficient in inducing adventitious bud differentiation, and subsequent development of bud primordia

required only mineral nutrients and sucrose. Thus, the process of adventitious bud differentiation in Torenia stem segments can be divided into the following 3 stages; the first stage (1st week) corresponding to adventitious bud induction (MZ formation), the second stage (2nd week) corresponding to bud initiation and the third stage (the 3rd week and thereafter) corresponding to bud development. A series of experiments is in progress to examine the physiological and biochemical changes occurring in the stem epidermis during the initial stage of adventitious bud differentiation.

Table 1. Effects of mineral nutrients and growth regulators on meristematic zone (MZ) and adventitious bud formation.

Mineral salts	Growth regulators (mg/l)	No. of MZ per strip of epidermis	Cultures with buds (%)	Degree of bud development
None	None	0.7	12	+
MS x 1/10	None	4.9	97	++
MS	None	0.5	11	+
None	BA 1.0	3.9	0	-
None	IAA 1.0	0.2	0	-
None	GA_3 1.0	0.8	8	+
MS	BA 0.01	0.9	22	+
MS	BA 0.1	4.1	92	++
MS	BA 1.0	16.5	98	+++
MS	IAA 1.0	0.4	13	+
MS	GA_3 1.0	1.0	12	+

All media contained 2% sucrose. Twenty-four segments were used for each treatment and all experiments were repeated 5 times. Data were scored 6 days (MZ) or 6 weeks (bud) after the start of culture. -, nil; +, low; ++, moderate; +++, high.

Fig. 1. Serial observations of *in vitro* adventitious bud differentiation in *Torenia* stem segments cultured on the MS medium containing BA (1.0 mg/l) and sucrose (2%). Bars represent 50 μm (A-D) or 1 cm (E, F).

A. Control (epidermis peeled off a mother plant). st: stomata, bhc: basal hair cell.

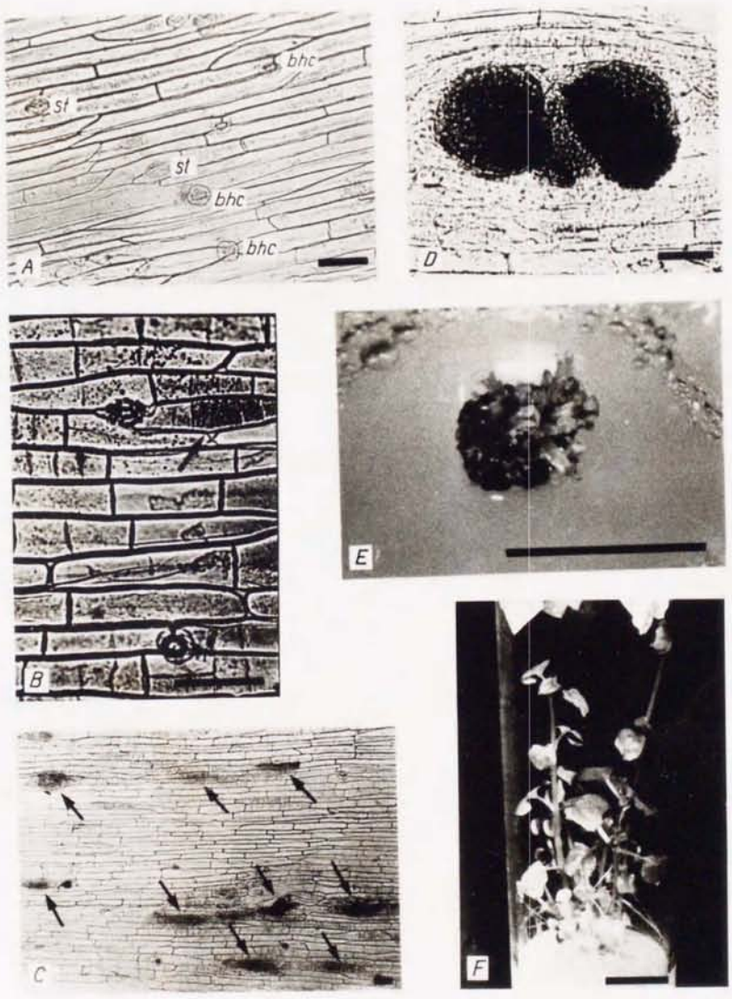
B. Initial aspects of MZ (arrow) formation 3 days after the start of culture.

C. Several MZ (arrows) formed 6 days after the beginning of culture.

D. Formation of bud primordia on the 8th day of culture.

E. Adventitious buds formed in a 2-week-old culture.

F. Development of vegetative shoots in a 6-week-old culture.


bhc
st
st
bhc
bhc
A
D
E
B
F
C

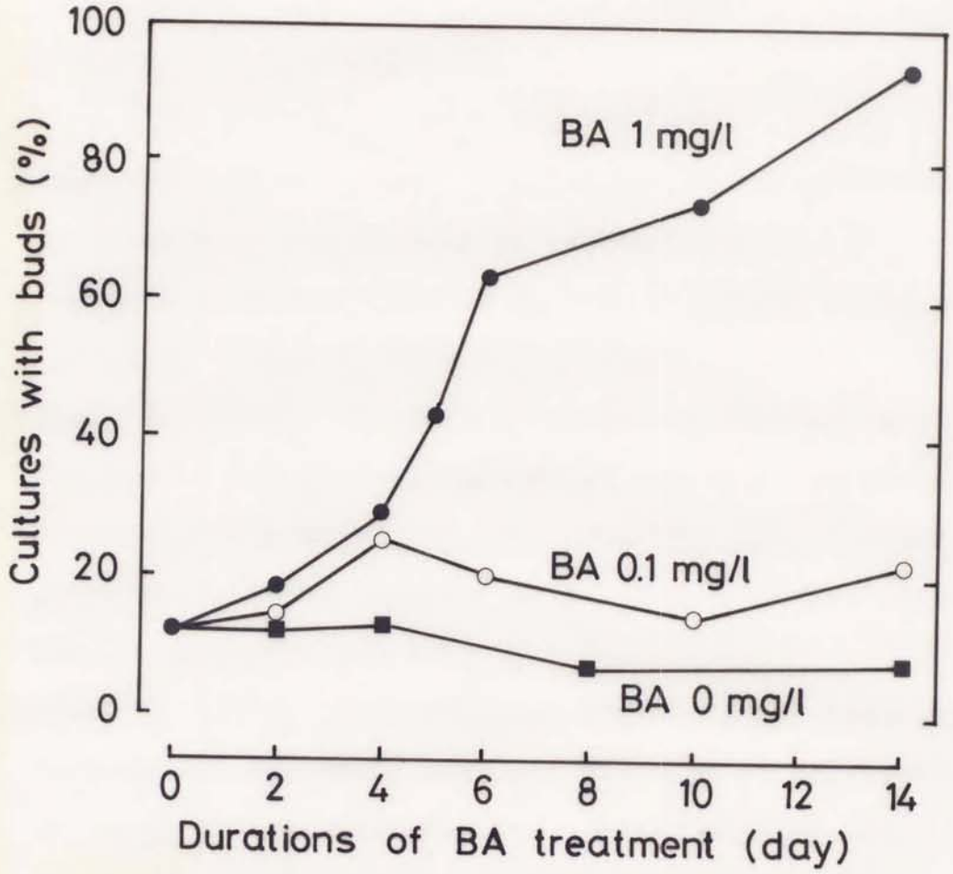


Fig. 2. Effects of various pre-treatments with BA on adventitious bud differentiation. Explants were cultured on a medium composed of BA (0, 0.1 or 1.0 mg/l), sucrose (2%) and agar (0.8%) without mineral salts during 2 to 14 days, and then transferred to the MS medium without BA. Data were scored 6 weeks after the start of culture. Twelve segments were used for each treatment and all experiments were repeated 4 times.

References

Chlyah, H. (1974a) Etude histologique de la néoformation de méristèmes caulinaires et radiculaires à partir de segments d'entre-noeuds de Torenia fournieri cultivés in vitro. Can. J. Bot. 52; 473-476.

Chlyah, H. (1974b) Formation and propagation of cell division-centers in the epidermal layers of internodal segments of Torenia fournieri grown in vitro. Simultaneous surface observations of all the epidermal cells. Can. J. Bot. 52; 867-872.

Chlyah, H. (1978) Intercellular correlations: Relation between DNA synthesis and cell division in early stages of in vitro bud neoformation. Plant Physiol. 62; 482-485.

Chlyah, H., K. M. Tran Thanh Van and Y. Demarly (1975) Distribution pattern of cell division centers on the epidermis of stem segments of Torenia fournieri during de novo bud formation. Plant Physiol. 56; 28-33.

Kamada, H. and H. Harada (1979) Influence of several growth regulators and amino acids on in vitro organogenesis of Torenia fournieri Lind. J. Exp. Bot. 30; 27-36.

Mohanty, B. and J. S. Fletcher (1976) Ammonium influence on the growth and nitrate reductase activity of Paul's

Scarlet rose suspension cultures. Plant Physiol. 58; 152-155.

Murashige, T. (1974) Plant propagation through tissue cultures. Ann. Rev. Plant Physiol. 25; 135-166.

Rao, P. S., W. Handro and H. Harada (1973) Hormonal control of differentiation of shoots, roots and embryos in leaf and stem cultures of Petunia inflata and Petunia hybrida. Physiol. Plant. 28; 458-463.

Skoog, F. and C. O. Miller (1957) Chemical regulation of growth and organ formation in plant tissue culture in vitro. Symp. Soc. Exp. Biol. 11; 118-131.

Tanimoto, S. and H. Harada (1980) Hormonal control of morphogenesis in leaf explants of Perilla frutescens Britton var.crispa Decaisne f. viridi-crispa Makino. Ann. Bot. 45; 321-327.

Chapter 2

Inhibition of cytokinin-promoted adventitious bud initiation by anticytokinin and auxin

Summary

In the epidermis of Torenia stem segments cultured in vitro, meristematic zones (MZ) were initiated prior to adventitious bud differentiation. Application of benzyladenine (BA) stimulated MZ and bud formation, and the average number of MZ per epidermal strip linearly increased with increasing concentrations of BA. The presence of naphthaleneacetic acid together with BA in a medium suppressed MZ formation. Some derivatives of 4-substituted 2-methylpyrrolo[2,3-d]pyrimidine which have anticytokinin activity inhibited BA-promoted MZ formation. Interactions of various cytokinins and anticytokinins in MZ and adventitious bud formation were also examined. The number of MZ formed by the treatment with 5 μM BA was reduced 50% by the simultaneous application of one of the anticytokinins at the same concentration.

Introduction

Hormonal control of organogenesis in in vitro cultured plant cells and tissues has been an important research subject and drawn much attetion of many plant physiologists. Subsequently a large number of experiments have been conducted in view of elucidating the mechanism of hormonal action on organ differentiation (review by Murashige, 1974; Thorpe, 1980). Early in 1957, Skoog and Miller reported that a high ratio of cytokinin to auxin in a medium stimulated adventitious bud differentiation in tobacco tissue cultures. In the cultures of organ fragments of Torenia (Kamada and Harada, 1979), Perilla (Tanimoto and Harada, 1980) and Rudbeckia (Tanimoto and Harada, 1982), however, the application of cytokinin alone was sufficient to induce bud differentiation. The mechanism of cytokinin action on growth and organ differentiation in intact plants, as well as in cultured tissues and cells, are still far to be clearly understood. To investigate effectively the action of cytokinin on organogenesis, we must have a sensitive and simple experimental system at hand. After an intensive survey of different plant materials, we found that Torenia stem segments provide a great advantage over other materials for this kind of study. Chlyah (1974) was first to report that meristematic cell divisions occur prior to bud differentiation in the epidermis of

Torenia stem segments. Using this plant material, we already showed that cytokinin was required only during the early period of adventitious bud differentiation (the first 6 days of culture) (Part I, Chapter 1). During this period, MZ were initiated in the epidermal layer then developed into adventitious buds even without further application of growth regulators.

As for the substances having anticytokinin activities, much effort has been devoted by several laboratories to synthesize such compounds, and a number of anticytokinins are now available. Biological activities of anticytokinins were often tested using a tobacco callus bioassay (Hecht et al., 1971, 1975; Iwamura et al., 1974, 1975; Skoog et al., 1975). For example, Iwamura et al. (1979) reported that some derivatives of 4-substituted-2-methylpyrrolo[2,3-d]pyrimidine exerted significant anticytokinin activity against simultaneously applied kinetin in the tobacco callus assay. However, only a few reports have dealt with the interactions between anticytokinins and cytokinins on organogenesis. Skoog et al. (1973) showed that one of the derivatives of 7-substituted-3-methylpyrazolo[4,3-d]pyrimidine inhibited bud formation in tobacco callus tissues.

In this chapter, we report some correlative effects of cytokinins, anticytokinins (derivatives of 4-substituted

2-methylpyrrolo[2,3-d]pyrimidine) and NAA on MZ and adventitious bud formation in the epidermis of Torenia stem segments cultured in vitro.

Materials and Methods

Plants of *Torenia fournieri* Lind. were raised in a growth room at a temperature of 25±2 °C under a 16-hr light photoperiod. The second stem internodes (counting from the top) of 6 to 8 week old plants, which most readily differentiated adventitious buds *in vitro* (Part II, Chapter 3), were excised and surface-sterilized. Several 5 mm long segments were cut out from each second internode and cultured under sterile conditions. The basal culture medium was comprised of the mineral salts of Murashige and Skoog's medium (Murashige and Skoog, 1962), sucrose (2%) and Difco Bacto agar (0.8%). In one series of experiments, BA, zeatin, kinetin, 4PU and 4PU-Cl were added individually to the basal medium. In another series of experiments, NAA or one of the derivatives of 4-substituted-2-methylpyrrolo[2,3-*d*]-pyrimidine including AA-P, BA-P, CB-P, CH-P, CP-P and HE-P was added in combination with one of the above cytokinins. Solutions of zeatin and the derivatives of 2-methylpyrrolo-[2,3-*d*]pyrimidine were sterilized through Millipore filter (0.45 μm).

After 7 days of culture, epidermal layers (ca. 5 x 2 mm) were stripped off the cultured stem segments, stained with aceto-carmine, and immediately observed under a microscope. The number of MZ were counted on at least 200 epidermal strips for each treatment, and the data were recorded as

the average number of MZ per epidermal strip. To evaluate the rate of bud formation, at least 75 explants were examined macroscopically at the end of a 6 week culture period; the results were expressed as the percentage of cultures with adventitious buds and also evaluated by the degree of bud development.

Results

The effects of BA on MZ and bud formation are summarized in Table 1. The addition of 0.5 μM BA to the basal medium was sufficient to induce bud differentiation in almost all the treated explants. The average number of MZ per epidermal strip was increased by raising the concentration of BA in the medium. Although some fluctuation was noted in the number of MZ produced among similarly treated epidermal strips (for instance, in the presence of 5 μM of BA, the number of MZ produced ranged from 8.1 to 17.1 MZ per epidermal strip), a linear correlation was found between the concentration of BA and the number of MZ produced in each experiments as indicated in Fig. 1.

Naphthaleneacetic acid counteracted stimulative effects of BA and the higher the concentration of NAA, the stronger the inhibitory effect (Table 2). A small number of adventitious buds were differentiated even in the presence of NAA but precise histological examination showed that these buds were originated from callus formed from the inner tissue and/or at the cut ends of segments.

Figure 2 shows interaction in MZ formation between a series of anticytokinins and BA (0.5 μM) applied simultaneously. At concentrations of 5 μM or higher, all the tested anticytokinins with the exception of HE-P, suppressed MZ formation to about the same extent. At a low concentration

(1.5 μM), however, the inhibition of MZ formation by the chemicals differed in degree as follows: CP-P caused the greatest inhibition (about 50% in terms of the number of MZ formed per epidermal strip); AA-P and CB-P caused moderate inhibition; BA-P and CH-P caused only a little; and HE-P was without inhibitory effect at any concentration tested (up to 50 μM).

In another series of experiments, BA and CB-P at various concentrations were incorporated into the basal medium. As shown in Fig. 3, epidermal strips of explants treated with 5 μM BA alone produced a large number of MZ (16.3 per epidermal strip). When 5 μM CB-P was added together with 5 μM BA, the mean number of MZ per epidermal strip fell to 8.5. When the medium contained 5 μM BA, 15 μM CB-P should be incorporated in order to obtain a comparable number of MZ which was produced by the treatment with 0.5 μM BA + 1.5 μM CB-P. Similar results were obtained with serial combinations of BA and all the other anticytokinins tested except HE-P.

The effects of various cytokinins and chemicals with cytokinin activity in promoting MZ and bud formation, as well as the counteracting action of CP-P against those stimulating substances, were also examined (Table 3). At 0.5 μM, kinetin and 4PU were not as effective in MZ induction as the other compounds; 4PU-Cl was most effective

in promoting MZ formation. When CP-P was added to the medium simultaneously with one of the cytokinins, more or less similar inhibitory effects on MZ formation were noted regardless of which substance was added with (Table 3). The strongest inhibitory effect of CP-P was shown against 4PU-Cl, decreasing the number of MZ to about 19%. Although CP-P clearly inhibited MZ formation, its suppressive effect on adventitious bud differentiation was less evident at the concentration used (15 μM).

Discussion

It is well known that many physiological phenomena in higher plants are regulated through the action of cytokinin. Various kinds of cytokinins and related compounds have been applied to different plant materials in attempts to elucidate their action mechanisms. So far, cytokinin activities have been tested in different bioassay systems including the growth of tobacco (Linsmaier and Skoog, 1965) and soybean (Miller, 1965) calluses, the proliferation of cells in tobacco pith discs (Bottomley et al., 1963), the inhibition of chlorophyll degradation in barley (Kende, 1965), and betacyanin synthesis in Amaranthus (Biddington and Thomas, 1973).

We reported here the effects of cytokinins and related compounds on MZ formation and adventitious bud differentiation in Torenia stem segments cultured in vitro. As we presented in Table 3, all of the cytokinins tested induced adventitious buds at high rates, but the number of MZ formed per epidermal strip was considerably different depending on the kind of cytokinin used. The number of MZ formed reflected better the degree of cytokinin activity of these chemicals in comparison with the percentage of cultures with buds. Since the number of MZ augmented linearly with the increasing concentration of BA (Fig.1), this experimental system seems to be suitable for a cytokinin

bioassay of bud formation.

Takahashi et al. (1978) reported that 4PU-Cl showed cytokinin activity 10 times higher than BA in the tobacco callus assay. Our results indicate that the number of MZ induced by treatment with 0.4 μM 4PU-Cl was greater than that produced by 5 μM BA (Table 3).

Although auxin (at a relatively low concentration) applied together with cytokinin generally stimulates adventitious bud differentiation (Murashige, 1974; Thorpe, 1980), such application somewhat inhibited bud formation in Torenia stem segments (Kamada and Harada, 1979). In the epidermis of Torenia stem segments, MZ formation was not stimulated by treatment with auxin alone (Part I, Chapter 1), and cytokinin-induced MZ formation was clearly suppressed by simultaneously applied NAA (Table 2). When auxin alone at a low concentration was added to the culture medium, adventitious bud formation was observed, but with a high concentration, it was suppressed (Table 2). A large proportion of the adventitious buds formed by the treatment with auxin did not originate from the MZ (i.e., not from the epidermis), but instead, from callus formed from the inner tissues and/or at the cut ends of segments.

Iwamura et al. (1979) examined the cytokinin-agonistic and -antagonistic activities of various 4-substituted-2-methylpyrrolo[2,3-d]pyrimidines using several bioassay

systems. Among them, CP-P and CB-P exhibited high anti-cytokinin activity against simultaneously applied kinetin (0.05 μM) in the tobacco callus assay, but the activity of BA-P, CH-P and AA-P was relatively low. HE-P did not show any anticytokinin activity in the tobacco callus assay. In the case of Torenia stem segments, these five compounds applied at concentrations of 5 μM or higher also suppressed BA-promoted MZ formation to about same extent. The concentrations of anticytokinins and cytokinins used in our experiments were much higher than those used in the callus assay. The physiological nature of interactions between cytokinin and anticytokinin at high concentrations could be different from that at low concentrations. In the assay system utilizing Amaranthus betacyanin synthesis, CH-P and CB-P produced little effect, but HE-P suppressed the action of 1 μM 6-(3-methyl-2-butenylamino)purine (Iwamura et al., 1979). However, HE-P did not show any anticytokinin activity in either tobacco callus assay (Iwamura et al., 1979) or in Torenia MZ formation (Fig. 2). These results indicate that BA-promoted MZ formation in the epidermis of Torenia stem segments can be suppressed by the same compounds which show anticytokinin activities in tobacco callus assay.

Table 1. Effects of BA on meristematic zone (MZ) and bud formation in the epidermis of Torenia stem segments cultured in vitro.

BA (μM)	No. of MZ per strip of epidermis[a]	Cultures with buds (%)[b]	Mean No. of buds per explants	Degree of bud development
0	0.5 (0.2-0.8)	7 ± 6.1	1.7	+
0.05	2.1 (1.2-3.1)	38 ± 16.5	2.3	++
0.5	6.5 (4.4-8.9)	95 ± 4.8	15.4	+++
1.5	9.9 (5.9-12.1)	96 ± 3.8	28.2	+++
5.0	12.5 (8.1-17.1)	98 ± 1.9	>40	++

[a]Values represent the average of 12 experiments, each of which consisted of at least 70 epidermal strips per treatment. Figures in parentheses indicate the minimum and maximum numbers of MZ per epidermal strip. Data were recorded 7 days after the beginning of culture.

[b]Values represent the average of 6 experiments, each of which had 25 replicates per treatment. Data were recorded 6 weeks after the beginning of culture.

+, low; ++, moderate; +++, high.

Table 2. Correlative effects of NAA and BA on meristematic zone (MZ) and bud formation in the epidermis of Torenia stem segments cultured in vitro.

BA (μM)	NAA (μM)	No. of MZ per strip of epidermis	Cultures with buds (%)	Degree of bud development
0	0	0.5	8	+
	0.5	0.1	47	+
	5.0	0.1	2	+
0.5	0	6.2	95	+++
	0.05	2.0	42	++
	0.5	0.8	27	+
	5.0	0.1	4	+
5.0	0	13.2	98	++
	0.5	1.3	62	+++
	5.0	0.3	34	+

+, low; ++, moderate; +++, high.

For each treatment, at least 200 epidermal strips and 75 explants were observed in order to calculate the average numbers of MZ and the percentages of cultures producing adventitious buds, respectively.

Table 3. Effects of 5 cytokinins and CP-P on meristematic zone (MZ) and bud formation in the epidermis of Torenia stem segments cultured in vitro.

Cytokinin	Conc. (μM)	No. of MZ per epidermis		Cultures with buds (%)	
		-CP-P	+CP-P (15 μM)	-CP-P	+CP-P (15 μM)
None		0.2 (100)	0.2 (100)	7	0
BA	0.5	7.9 (100)	3.0 (38)	95	69
	5.0	15.6 (100)	6.4 (41)	99	95
Kinetin	0.5	3.9 (100)	2.0 (51)	62	46
	5.0	4.8 (100)	2.3 (48)	74	56
Zeatin	0.5	8.6 (100)	4.9 (57)	100	79
4PU	0.5	4.9 (100)	3.0 (61)	83	82
4PU-Cl	0.4	17.5 (100)	3.3 (19)	100	97

For each treatment, at least 200 epidermal strips and 75 explants were observed in order to calculate the average numbers of MZ and the percentages of cultures producing adventitious buds, respectively. Numbers in parentheses indicate the percentage calculated on the basis of MZ numbers obtained with the application of respective cytokinins without CP-P.

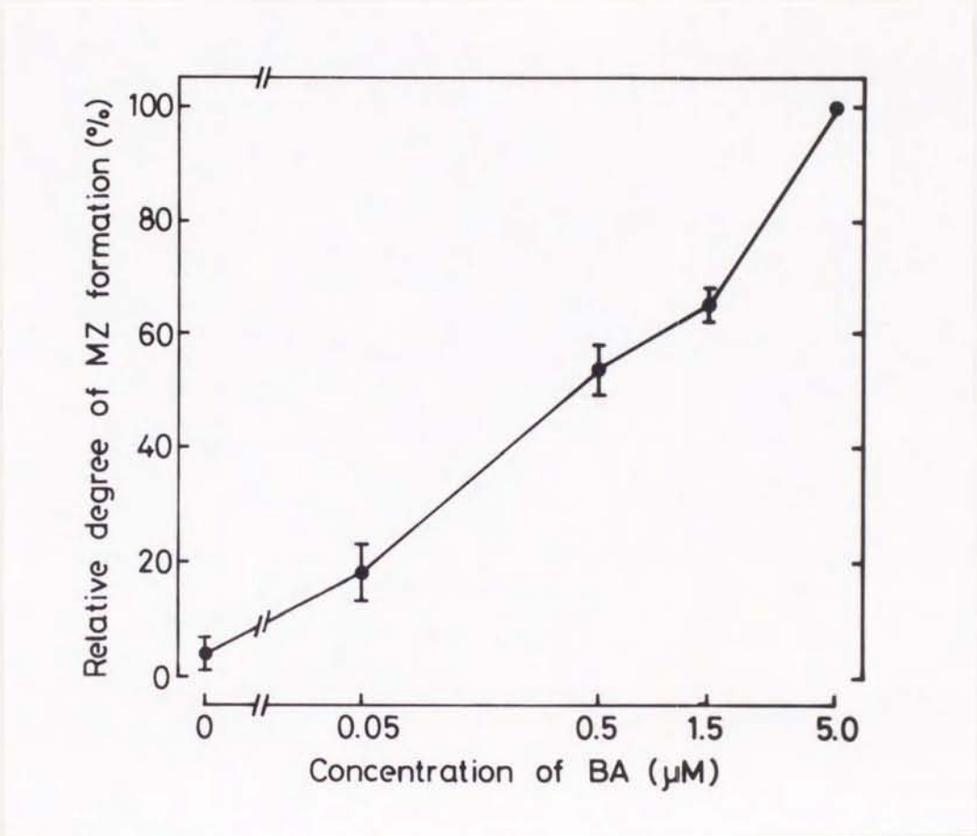


Fig. 1. Correlation between BA concentration and MZ formation in the epidermis of Torenia stem segments cultured in vitro. Abscissa: Concentration of BA in μM. Ordinate: Percent calculated basing on the MZ number obtained with 5 μM BA. Each point represents an average of 12 experiments, each of which had at least 70 epidermal strips per treatment. Vertical lines show standard errors.

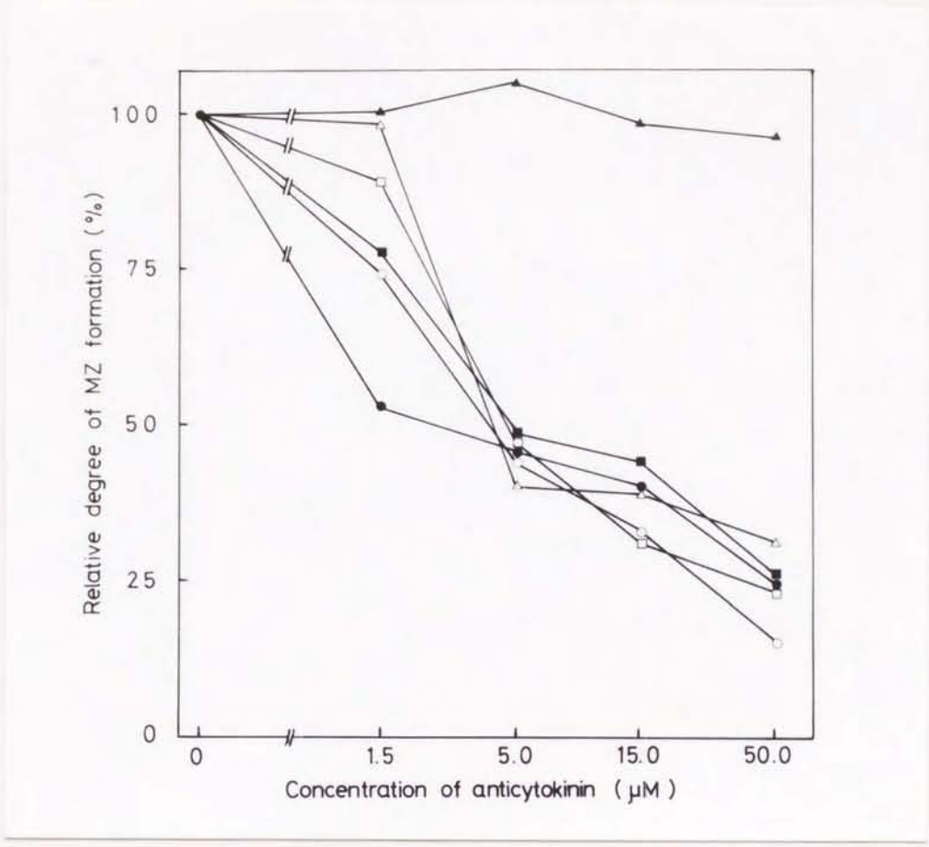


Fig. 2. Inhibitory effects of various anticytokinins on BA-promoted MZ formation in the epidermis of Torenia stem segments cultured in vitro. The control medium contained 0.5 μM BA without anticytokinin. The relative degree of MZ formation (ordinate) expresses the number of MZ formed per epidermal strip under BA plus anticytokinin treatment as a percentage of the number formed under treatment by 0.5 μM BA alone (which was 7.2 MZ per strip). —●—, CP-P; —■—, CB-P; —▲—, HE-P; —○—, AA-P; —□—, CH-P; —△—, BA-P.

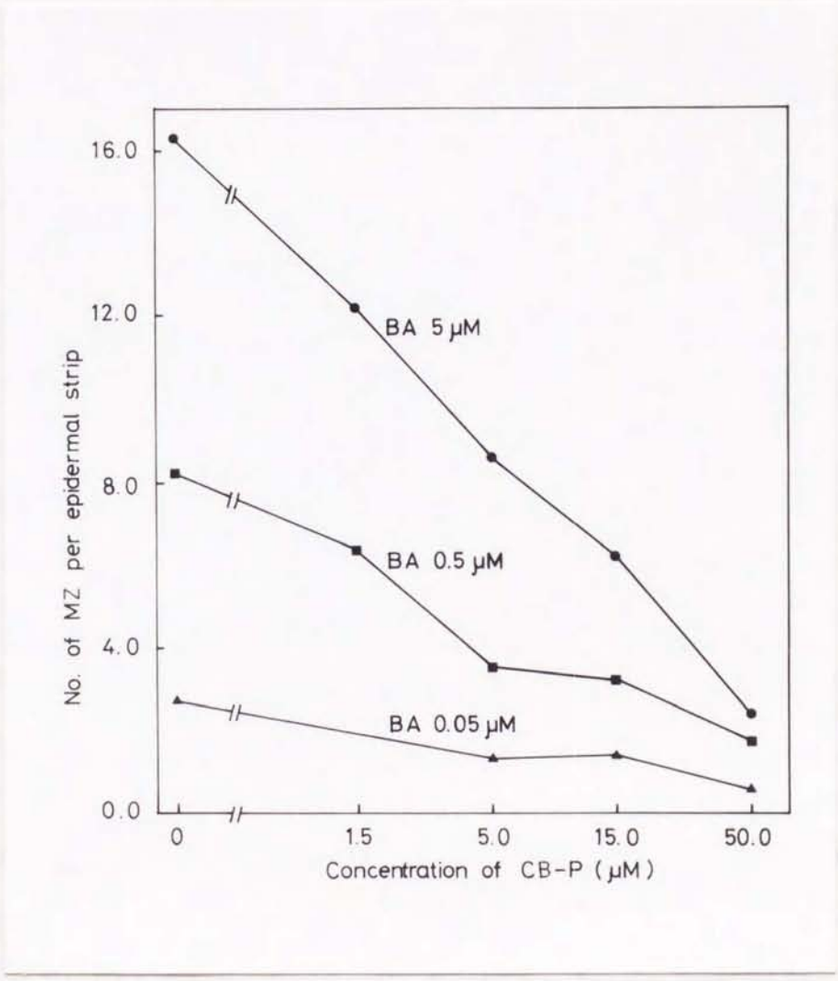


Fig. 3. Correlative effects of combined treatments with BA and CB-P in different concentrations on MZ formation in the epidermis of Torenia stem segments cultured in vitro. Each point represents an average of at least 200 epidermal strips.

References

Biddington, N. L. and T. H. Thomas (1973) A modified Amaranthus bioassay for the rapid determination of cytokinins in plant extracts. Planta 111; 183-186.

Bottomley, W., N. P. Kefford, J. A. Zwar and P. L. Goldacre (1963) Kinin activity from plant extracts. I. Biological assay and sources of activity. Aust. J. Biol. Sci. 16; 395-406.

Chlyah, H. (1974) Formation and propagation of cell division centers in the epidermal layers of internodal segments of Torenia fournieri grown in vitro. Simultaneous surface observations of all the epidermal cells. Can. J. Bot. 52; 867-872.

Hecht, S. M., R. M. Bock, R. Y. Schmitz, F. Skoog and N. J. Leonard (1971) Cytokinins: Development of a potent antagonists. Proc. Nat. Acad. Sci. USA 68; 2608-2610.

Hecht, S. M., R. B. Frye, D. Werner, S. D. Hawrelak, F. Skoog and R. Y. Schmitz (1975) On the activation of cytokinins. J. Biol. Chem. 250; 7343-7351.

Iwamura, H., T. Ito, Z. Kumazawa and Y. Ogawa (1974) Anti-cytokinin activity of 4-furfurylamino-7-(β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine. Biochem. Biophys. Res. Commun. 57; 412-416.

Iwamura, H., T. Ito, Z. Kumazawa and Y. Ogawa (1975)

Synthesis and anticytokinin activity of 4-substituted-7-(β-D-ribofuranosyl)-pyrrolo[2,3-d]pyrimidines. Phytochemistry 14; 2317-2331.

Iwamura, H., N. Masuda, K. Koshimizu and S. Matsubara (1974) Cytokinin-agonistic and antagonistic activities of 4-substituted-2-methylpyrrolo[2,3-d]pyrimidines, 7-deaza analogs of cytokinin-active adenine derivatives. Phytochemistry 18; 217-222.

Kamada, H. and H. Harada (1979) Influence of several growth regulators and amino acids on in vitro organogenesis of Torenia fournieri Lind. J. Exp. Bot. 30; 27-36.

Kende, H. (1965) Kinetinlike factors in the root exudate of sunflowers. Proc. Nat. Acad. Sci. USA 53; 1302-1307.

Linsmaier, E. M. and F. Skoog (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18; 100-127.

Miller, C. O. (1965) Evidence for the natural occurrence of zeatin and derivatives: Compounds from maize which promote cell division. Proc. Nat. Acad. Sci. USA 54; 1052-1058.

Murashige, T. (1974) Plant propagation through tissue cultures. Ann. Rev. Plant Physiol. 25; 135-166.

Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures.

Physiol. Plant. 15; 473-497.

Skoog, F. and C. O. Miller (1957) Chemical regulation of growth and organ formation in plant tissue culture in vitro. Symp. Soc. Exp. Biol. 11; 118-131.

Skoog, F., R. Y. Schmitz, R. M. Bock and S. M. Hecht (1973) Cytokinin antagonists: Synthesis and physiological effects of 7-substituted 3-methylpyrazolo[4,3-d]-pyrimidines. Phytochemistry 12; 25-37.

Skoog, F., R. Y. Schmitz, S. M. Hecht and R. B. Frye (1975) Anticytokinin activity of substituted pyrrolo-[2,3-d]pyrimidines. Proc. Nat. Acad. Sci. 72; 3508-3512.

Takahashi, S., K. Shudo, T. Okamoto, K. Yamada and Y. Isogai (1978) Cytokinin activity of N-phenyl-N'-(4-pyridyl)urea derivatives. Phytochemistry 17; 1201-1207.

Tanimoto, S. and H. Harada (1980) Hormonal control of morphogenesis in leaf explants of Perilla frutescens Britton var. criapa Decaisne f. viridi-crispa Makino. Ann. Bot. 45; 321-327.

Tanimoto, S. and H. Harada (1982) Physiological and hormonal factors influencing organogenesis in Rudbeckia bicolor explants cultured in vitro. Plant & Cell Physiol. 23; 107-113.

Thorpe, T. A. (1980) Organogenesis in vitro: Structural, physiological, and biochemical aspects. Int. Rev. Cytol. 11; 71-111.

Chapter 3

Changes in endogenous levels of amino acids during the initial stage of adventitious bud differentiation

Summary

Quantitative changes in protein-incorporated and free amino acids during adventitious bud differentiation in Torenia stem segments cultured on three different media were examined. The total amount of protein-incorporated amino acids significantly increased during the initial stage of culture when explants were cultured on a bud-forming medium. In the explants cultured on a medium containing only cytokinin (without mineral salts), a slight increase in the total amino acids in superficial layers were observed on the 3rd day of culture. On this medium, meristematic zones (MZ) were formed in the epidermis of explants, but bud initiation did not occur. Individual proportions of protein-incorporated amino acids remained almost the same in all the explants cultured on three different media. The total amount of free amino acids changed depending upon nutrient elements in a medium used. Its amount increased markedly in the explants cultured on nutrient-rich media. This increase was mainly due to

that in glutamine content, its rate being augmented from 7.4% at the start of culture to about 70% at the end of 3 day culture. The relative amounts of α-alanine and β-alanine gradually increased and that of aspartic acid, glutamic acid, proline, glycine, valine, leucine, lysine and arginine decreased with increasing time of culture.

Introduction

Kamada and Harada (1979) reported that in Torenia stem segments cultured in vitro, adventitious bud differentiation was strongly promoted by cytokinin added to nutrient media. We also showed (Part I, Chapter 1) that meristematic zones (MZ) in Torenia stem epidermis were induced prior to bud formation at the early stage of culture, and this induction was promoted if cytokinin was present in a medium (even without mineral nutrients). The presence of cytokinin for first 6 days of culture seemed to be sufficient to induce adventitious bud differentiation.

During this initial stage of adventitious bud differentiation, important physiological and biochemical changes must take place in stem epidermis. In this regard, Chlyah (1978) investigated the relationship between cell division and DNA synthesis using Torenia stem epidermis, and Hasegawa et al. (1979) and Yasuda et al. (1980) found that bud-forming cultures of Douglas fir cotyledons synthesized specific low molecular weight proteins during the first 2 to 4 days in culture. In bud-forming tobacco callus, a higher content of proteins was found in comparison with non-bud-forming callus (Thorpe and Meier, 1974). We attempted to examine the changes in the endogenous levels of protein-incorporated and free amino acids during an early process of adventitious bud differentiation in

Torenia stem segments cultured *in vitro*.

Materials and Methods

Plant materials and culture conditions used were the same as those described previously (Part I, Chapter 1). The following three media were used: a) "MS medium" composed of the mineral salts of Murashige and Skoog's medium and 2% sucrose; b) "MB medium" consisting of MS medium + 1.0 mg/l of benzyladenine; and c) "BA medium" containing only 1.0 mg/l of benzyladenine and 2% sucrose (without mineral salts).

Stem segments cultured on three media were separately collected on the 3rd, 6th and 12th day of culture (about 700 explants per treatment were used), and superficial tissues consisting of epidermis and a few cell layers of subepidermal parenchyma were stripped off the explants. These superficial tissues (about 2 g fresh weight per treatment) and the remaining tissues of internodal explants (about 4 g fresh weight per treatment) were separately homogenized for 5 min at 0°C with 10 ml of 0.1 M tetraborate buffer (pH 8.7) containing 0.5 M sodium chloride. The homogenates were centrifuged at 15,000 xg for 15 min at 4°C. Supernatants were mixed with an equal volume of 50% trichloroacetic acid (TCA) to precipitate proteins, allowed to stand at 4°C for 2 hr, and then centrifuged at 15,000 xg for 15 min. The supernatants were rinsed 4 times with ethyl ether and applied to a Dowex-50 column.

The TCA-precipitated protein fractions were sealed in vials and hydrolyzed with 6N HCl for 20 hr at 120°C. The hydrolisates were rinsed with ethyl ether and applied to a Dowex-50 column. Amino acids absorbed to the column were eluted with 4N HCl and evaporated in vacuo. The amount of various amino acids was measured in an amino acid analyzer (model JLE 6AS, JEOL Ltd. Japan).

Results

The effects of three culture media used on the formation of MZ and adventitious buds can be summarized as follows: (1) MZ and adventitious buds were rarely formed in the stem segments cultured on the MS medium; (2) the BA medium induced a large number of MZ but not bud differentiation; and (3) the MB medium strongly stimulated MZ and adventitious bud formation.

Changes in the total amount of amino acids in TCA-precipitated proteins during the initial stage of culture was shown in Fig. 1. In superficial tissues (Fig. 1A), the initial amount of protein-incorporated amino acids was 1.27 μmols/g fresh weight, and it significantly increased 6 days after the start of culture, reaching a level which was 7 times higher than the initial one on the 12th day of culture on the MB medium. With two other media, no clear change was noted as far as the quantity of protein-incorporated amino acids in superficial tissues was concerned, although it showed a slight increase on the 3rd day of culture with the BA medium. In the case of inner tissues (Fig. 1B), the initial amount of amino acids was low (0.19 μmoles/g fresh weight). In the explants cultured on the MB medium, the total amount of protein-incorporated amino acids rapidly increased showing a high rate of increase than that obtained with two other media used.

Table 1 shows the relative amounts of individual protein-incorporated amino acids as expressed in percentage of total amino acids in superficial tissues of the explants cultured on the MB medium. Major changes in the individual proportions of the amino acids during adventitious bud differentiation were noted in the case of glycine, leucine and proline. The relative amounts of glycine and leucine decreased, while that of proline increased with increasing time of culture. Although the changes in the individual proportions of the protein-incorporated amino acids showed a similar tendency with the three different media used, the changes observed with glutamic acid in the explants cultured on the MS medium was different from that in the explants cultured on two other media. The relative proportion of glutamic acid remained nearly constant throughout the culture period, at least with the MB and BA medium (13 to 16%). When explants were cultured on the MS medium, however, its proportion increased attaining 24% on both the 6th and 12th days of culture. The changes in the relative amounts of individual protein-incorporated amino acids in inner tissues were similar to that of superficial tissues regardless of the media used.

The changes in the total amount of free amino acids during adventitious bud initiation are shown in Fig. 2.

Both superficial tissues (Fig. 2A) and inner tissues (Fig. 2B) exhibited comparable tendencies in all of the three media used. The explants cultured on the MS or MB medium showed considerable increase in the quantity of free amino acids, but those cultured on the BA medium showed only a slight increase. On the 6th and 12th days of culture, a larger amount of free amino acids was accumulated in the explants cultured on the MS medium than in those cultured on the MB medium.

Individual changes in the relative amounts of free amino acids were summarized in Table 2. The data presented concern only with the superficial tissues of the explants cultured on the MB medium because the data obtained with inner tissues were nearly the same as those shown in Table 2. During 3 days of culture, the proportion of glutamine increased from 7.4% to 70.8%. The relative amounts of α-alanine and β-alanine gradually increased and that of aspartic acid, glutamic acid, proline, glycine, valine and arginine decreased with increasing time of culture.

The individual proportions of some free amino acids in the explants cultured on the three different media were shown in Table 3. In the explants cultured on the MS medium, the proportion of threonine-serine decreased rapidly during the first 3 days of culture (5.8%), but its decrease was gradual when the BA medium was used.

With the MB medium, the level of threonine-serine remained relatively high throughout the culture period. The relative proportions of certain amino acids such as glutamic acid, arginine and α-alanine varied depending upon the medium used.

Discussion

When Torenia stem segments were cultured on the MB medium, the total amount of protein-incorporated amino acids in superficial tissues seemed to be constant during the first 3 days of culture but, thereafter, started to increase considerably. As reported earlier, the mean number of MZ per epidermal strip, the rate of bud formation and the degree of bud development were very high with the MB medium. Consequently, it is conceivable that the increase in amino acid level mentioned above may be related to the formation of adventitious buds. It was previously shown (Part I, Chapter 1) that the formation of MZ can be observed in epidermis of Torenia stem segments 3 days after the start of culture. Figure 1A shows a small peak on the 3rd day of culture with the BA medium, and this slight increase might have some significance related to MZ formation. Yasuda et al. (1980) reported that the presence of specific proteins was detected 2 days after the start of culture and they attained the maximum level on the 4th day of culture in the case of bud-differentiating cultures obtained from Douglas fir cotyledons. It is necessary to examine whether or not certain specific proteins directly concerned with adventitious bud differentiation are also involved in the case of Torenia stem segments.

As it was presumed, a large amount of free amino acids

was found in the explants cultured on the MS or MB medium (contained mineral salts) than in those cultured on the BA medium (without mineral salts), owing mainly to an accumulation of glutamine. Glutamine is thought to have a function of nitrogen redistribution to other amino acids through an action of glutamate synthetase (Miflin and Lea, 1976). The accumulation of glutamine was noted during the maturation and germination of Gossipium embryos (Capedevila and Dure III, 1977) and also during the somatic embryogenesis in carrot cell culture (Kamada, 1979). Between the 3rd and the 6th days of culture, the total amount of protein-incorporated amino acids in superficial and inner tissues cultured on the MB medium increased considerably. This increase seems to be related in some way to the total amount of free amino acids which decreased or remained in a constant level during the same period in the explants cultured on the same medium.

Sangwan (1978) reported that the quantity of free threonine-serine increased markedly during pollen embryogenesis in Datura metal anther culture, but our results showed a decreasing tendency in the relative proportions of threonine-serine during adventitious bud formation in Torenia.

Kamada and Harada (1979) reported various effects of exogenously applied amino acids on Torenia stem segments

cultured *in vitro*. For example, they showed that glutamic acid, asparagine and alanine stimulated adventitious bud formation when added to a BA-containing nutrient medium. In a medium without BA, glutamic acid and aspartic acid also promoted bud formation. Several other amino acids were more or less inhibitory for bud formation. In our experiments (Table 3), the relative amount of glutamic acid in explants remained at comparably high levels when the explants were cultured on a medium containing BA, but decreased when cultured on a medium without BA (MS medium).

So far, only limited studies have been made on the changes in endogenous levels of amino acids in relation to *in vitro* organogenesis. Our results presented here are mostly in accordance with those obtained in relation to the formation of somatic embryos in *in vitro* culture of *Datura* (Sangwan, 1978) and carrot (Kamada, 1979). Histological, physiological and biochemical similarities and differences between adventitious bud differentiation and somatic embryogenesis are being investigated using *Torenia* stem segments with a view of explaining those two important phenomena in terms of the changes in amino acid metabolism.

Table 1. Relative amounts of protein-incorporated amino acids as expressed in percentage of the total amino acids in superficial tissues of the explants cultured on the MB medium.

Amino acids	Days of culture			
	0	3	6	12
Aspartic acid	9.7	10.3	10.1	11.8
Threonine	5.8	6.6	5.7	6.3
Serine	7.4	8.0	7.3	7.4
Glutamic acid	13.8	15.9	12.5	14.0
Proline	-	-	6.5	7.0
Glycine	18.6	12.6	10.7	10.2
Alanine	4.8	6.5	6.4	6.8
Cysteine-Valine	13.6	15.7	16.0	17.5
Methionine	-	-	-	0.9
Isoleucine	3.9	4.8	5.2	4.0
Leucine	8.3	6.9	6.3	4.0
Histidine	0.5	0.6	0.3	0.1
Lysine	9.4	8.2	7.1	7.5
Arginine	4.2	3.9	4.0	2.7

-, trace. Each figure represents the results obtained with about 700 explants.

Table 2. Relative amounts of free amino acids as expressed in percentage of the total amino acids in superficial tissues of the explants cultured on the MB medium.

Amino acids	Days of culture			
	0	3	6	12
Aspartic acid	4.5	0.3	0.3	-
Threonine-Serine	17.1	9.3	14.2	13.6
Glutamic acid	18.2	8.6	8.1	8.7
Glutamine	7.4	70.8	56.7	54.6
Proline	4.5	0.3	0.5	0.5
Glycine	14.6	1.1	1.3	1.4
α-Alanine	5.0	4.2	9.0	8.7
Valine	3.8	0.5	0.8	0.9
Isoleucine	1.0	-	-	0.2
Leucine	2.9	-	-	0.3
β-Alanine	-	3.0	6.2	4.8
γ-ABA[a]	4.9	1.0	3.1	3.7
Ornithine	1.3	0.4	0.2	0.3
Lysine	6.0	0.1	0.2	0.4
Histidine	2.6	0.2	0.5	1.2
3-methyl-histidine	-	0.3	0.8	1.3
Arginine	5.0	-	-	1.2

[a] γ-ABA, γ-amino-n-butyric acid; -, trace. Each figure represents the result obtained with about 700 explants.

Table 3. Relative amounts of some free amino acids as expressed in percentage of the total amino acids in superficial tissues of the explants cultured on three different media.

Amino acids	Media	Days of culture			
		0	3	6	12
Threonine-Serine	MS	17.1	5.8	5.2	5.8
	BA	17.1	14.3	6.5	6.0
	MB	17.1	9.3	14.2	13.6
Glutamic acid	MS	18.2	7.5	3.4	3.9
	BA	18.2	13.5	16.3	10.3
	MB	18.2	8.6	8.1	8.7
Glutamine	MS	7.4	71.0	68.5	61.8
	BA	7.4	36.8	60.1	50.5
	MB	7.4	70.8	56.7	54.6
Proline	MS	4.5	6.8	0.4	0.7
	BA	4.5	3.1	-	-
	MB	4.5	0.3	0.5	0.5
α-Alanine	MS	5.0	2.6	7.5	7.1
	BA	5.0	5.5	2.4	2.7
	MB	5.0	4.2	9.0	8.7
β-Alanine	MS	-	1.2	8.8	6.1
	BA	-	2.4	2.7	6.2
	MB	-	3.0	6.2	4.8
Arginine	MS	5.0	0.3	-	0.3
	BA	5.0	1.5	0.7	9.1
	MB	5.0	-	-	1.2

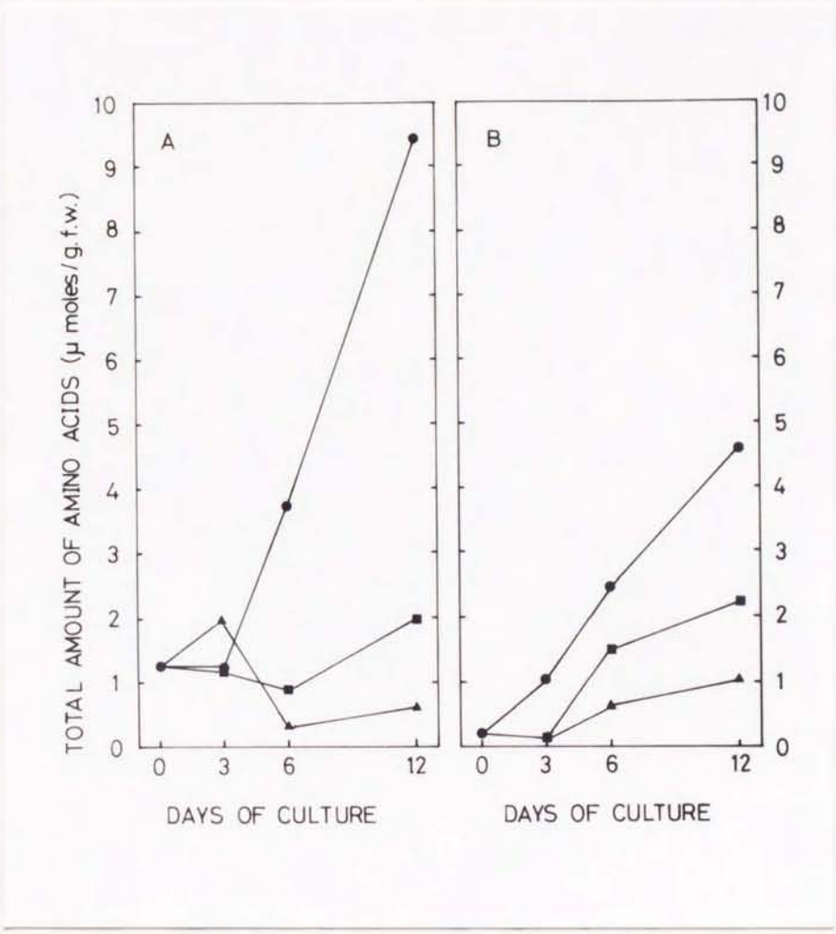


Fig. 1. Changes in the total amount of protein-incorporated amino acids in superficial (A) and remaining inner (B) tissues of the explants cultured in three different media.
MB (—●—), MS (—■—) and BA (—▲—) medium.

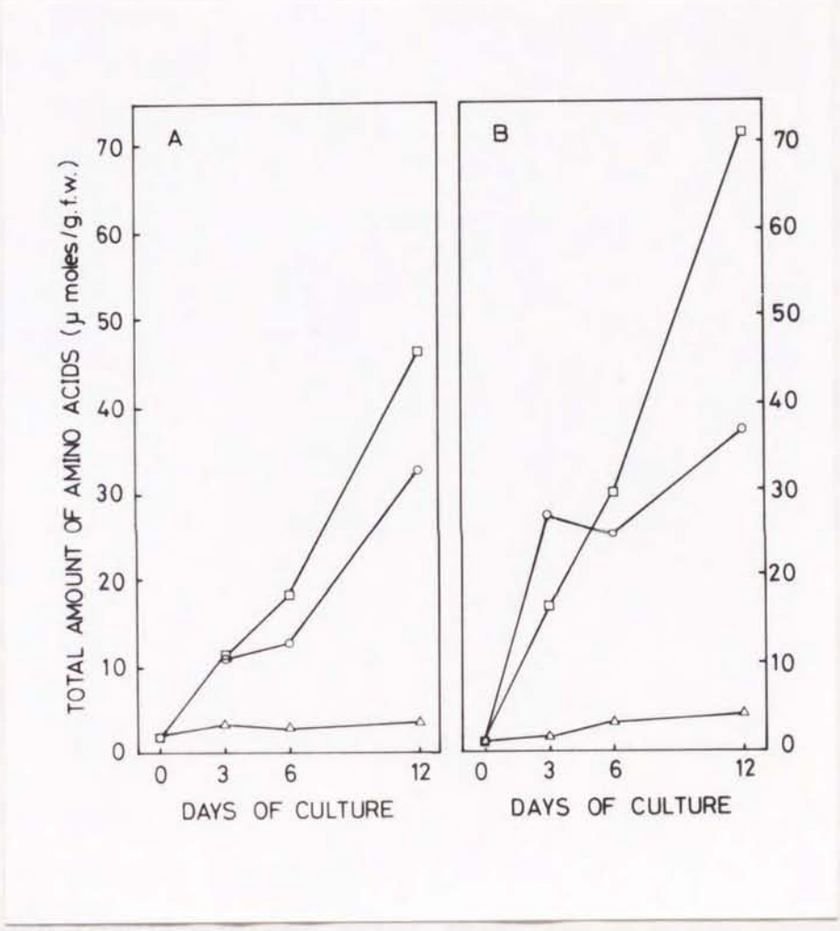


Fig. 2. Changes in the total amount of free amino acids in superficial (A) and remaining inner (B) tissues of the explants cultured in three different media.
MB (—○—), MS (—□—) and BA (—△—) medium.

References

Capdevila, A. M. and L. Dure III (1977) Developmental biochemistry of cotton seed embryogenesis and germination VIII. Plant Physiol. 59; 268-273.

Chlyah, H. (1978) Intercellular correlation: Relation between DNA synthesis and cell division in early stages of in vitro bud neoformation. Plant Physiol. 62; 482-485.

Hasegawa, P. M., T. Yasuda and T. Y. Cheng (1979) Effect of auxin and cytokinin on newly synthesized proteins of cultured Douglas fir cotyledons. Physiol. Plant. 46; 211-217.

Kamada, H. (1979) Effects of exogenously applied growth regulators and amino acids on the organogenesis in higher plant tissue cultures. Ph. D. Thesis. The University of Tsukuba, Ibaraki, Japan.

Kamada, H. and H. Harada (1979) Influence of several growth regulators and amino acids on in vitro organogenesis of Torenia fournieri Lind. J. Exp. Bot. 30; 27-36.

Miflin, B. J. and P. J. Lea (1976) The pathway of nitrogen assimilation in plants. Phytochemistry 15; 873-885.

Sangwan, R. S. (1978) Amino acids metabolism in cultured anthers of Datura metal. Biochem. Physiol. Pflanzen. 173; 355-364.

Thorpe, T. A. and D. D. Meier (1974) Starch metabolism in shoot-forming tobacco callus. J. Exp. Bot. 25; 288-294.

Yasuda, T., P. M. Hasegawa and T. Y. Cheng (1980) Analysis of newly synthesized proteins during differentiation of cultured Douglas fir cotyledons. Physiol. Plant. 48; 83-87.

Chapter 4

Protein synthesis during adventitious bud initiation in superficial layers of *Torenia* stem segments

Summary

In *Torenia* stem segments cultured *in vitro*, adventitious bud initiation takes place in epidermal cells. The initiation was promoted by a cytokinin and inhibited by some inhibitors of RNA and protein synthesis such as actinomycin-D, cordycepin, 5-fluorouracil and cycloheximide. The inhibitory effect was evident when the inhibitors were applied during the first 2-3 days of culture. During this period, extensive incorporation of radioactive leucine to protein and rapid increase in protein contents were observed in superficial layers of the stem segments cultured on the medium containing benzyladenine (BA). The rapid increase in protein contents may be attributed to the inhibition of protein degradation by BA. The turnover rates of proteins in superficial layers of the explants cultured with or without BA for 3 days were 2.2 and 4.7 % per hr, respectively. The SDS-polyacrylamide gel electrophoretic profile of labelled proteins in superficial layers of the explants cultured on BA-containing medium

resulted in some differences to the one obtained from the hormone-free medium.

Introduction

There have been several reported instances demonstrating the biochemical regulation of protein synthesis associated with the organogenesis of several plant species. In this respect, Thorpe and Meier (1974) pointed out higher rate of protein content in bud-forming tobacco callus than in non-bud-forming callus. The presence of specific proteins related to adventitious bud differentiation in carrot callus was suggested by Syōno (1965). Similar observation was also made in the organogenesis from tobacco callus by Sekiya and Yamada (1974). Recently, Hasegawa et al. (1979) and Yasuda et al. (1980) showed that bud-forming cultures of Douglas fir cotyledons synthesized specific proteins of low molecular weight.

Previously we reported that the total amount of amino acids in superficial layer of Torenia stem segments increased significantly when the explants were cultured on the medium containing BA (Part I, Chapter 3). The process of bud differentiation in this system can be characterized by the appearance of MZ in the epidermis during the early stage of culture (around the 6th day). However, the development of buds from the MZ does not require exogenouly supplied growth regulators (Part I, Chapter 1). Therefore, it would be reasonable to conclude that important biochemical events responsible for the organogenesis of Torenia stem

segments may take place in the process of MZ formation.

In this chapter, the outcome of the experiments on the de novo synthesis of proteins during the early stage of adventitious bud differentiation in Torenia stem segments will be presented.

Materials and Methods

Experimental materials and culture conditions

Plants of Torenia fournieri Lind. were grown under a long-day condition (16 hr light/8 hr dark) for 6 to 8 weeks. Stem segments (5 mm in length) were taken from the upper part of the stem and cultured on defined media. Other culture conditions were the same as those described earlier (Part I, Chapter 1). Basal culture medium (MS medium) was comprised of the mineral salts of Murashige and Skoog (1962) formulation, sucrose (2%), and Difco Bacto agar (0.8%). In the case where extensive adventitious bud differentiation was desirable, 0.5 μM of BA was added to the MS medium (denote to MB medium). Inhibitors of RNA and protein synthesis such as Act.D, cordycepin, 5-fluorouracil and CHI were added to the media when necessary. The solutions of these inhibitors were sterilized through a Millipore filter (0.45 μm). To count the number of MZ, the epidermis of stem segments (ca. 5 x 2 mm) were peeled off after 7 days of culture, and stained with aceto-carmine, which were in turn provided for the macroscopic observation.

Measurements of protein contents in the superficial layers

The epidermis with a few underlying cell layers of the cultured stem segments (denote to superficial layers) were

stripped off, and the fresh weight of the strips of superficial layer was determined. The amount of water-soluble protein in the superficial layer was estimated by Coomassie Brilliant Blue G-250 binding assay (Bradford, 1976). About 500 strips of superficial layer were used for each treatment, and the results were shown in mg fresh weight and μg protein per 100 strips of superficial layer.

Preparation and analysis of radioactively labelled proteins

Stem segments cultured for various periods were transferred to a medium containing either L-[4,5-^{3}H(N)]-leucine (specific activity: 60 Ci/mmol) or L-[^{14}C(U)]-leucine (specific activity: 343 mCi/mmol) at 0.5 μCi/ml. Compositions of incubation media were the same as those used for the culture of the explants except that agar was omitted and that non-radioactive leucine was added at a final concentration of 20 μM. After 4 hr, the superficial layer was peeled from each stem segments and homogenized with a denaturation buffer containing 100 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.02% bromophenol blue at 4°C. The homogenate was boiled at 100°C for 5 min to denature proteins, followed by the centrifugation at 15,000 xg for 10 min. The supernatant was dialysed overnight against the same buffer. To measure the total incorporation of radioactivity to proteins,

50 µl each of dialyzed protein samples was mixed with 2 ml of 10% TCA. The precipitate was collected on a Whatman GF/C glass filter paper, and subjected to 5 times of successive wash with 5 ml of 5% TCA and 75% ethanol. Then, the filter paper was dryed and transferred into a vial containing 5 ml of a scintillator (1 l of toluene, 4 g of 2,5-diphenyloxasol and 0.1 g of 1,4-bis-2-(5-phenyloxasolyl)benzene). The radioactivity was recorded by a Beckman liquid scintillation spectrometer (Model LS-250). For pulse-chase experiments, the stem segments were first labelled with radioactive leucine for 2 hr, then incubated for 1, 2 and 4 hr with the MS or MB medium without radioactive leucine but containing 1 mg/l CHI.

SDS-polyacrylamide gel electrophoresis

To analyze protein samples by SDS-polyacrylamide gel electrophoresis, stem segments which had been cultured either on the MS or on the MB medium for 3 days, were transferred to the same medium containing ^{3}H- or ^{14}C-leucine, respectively. After 4 hr of labelling, superficial layers of the stem segments of both media were combined together, and provided for the co-extraction of water-soluble proteins. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli's method (1970) with a slight modification; 0.1% tetramethylethylenediamine

was used for polymerization of acrylamide. Separation gel (190 x 100 x 1.2 mm) was made with a gradient of acrylamide (12-20%). About 50 µl of a protein sample was applied on a gel. After electrophoresis at a constant current of 45 mA for 4 hr, the gel was immersed in a solution containing 10% TCA, 10% acetic acid and 30% ethanol for 2 hr; and washed overnight in the same solution. Each gel sliced (2 mm thickness) was placed in separate vials containing 1 ml 30% hydrogen peroxide at 60°C overnight. To each vial, 5 ml scintilltor (1 l of toluene, 500 ml of Triton X-100, 6 g of 2,5-diphenyloxasol and 0.3 g of 1,4-bis-2-(5-phenyloxasolyl)benzene) was added, then the radioactivity was counted. Calibration proteins used for the determination of molecular weight were bovine serum albumin (66,000 daltons), egg albumin (45,000 daltons), ovalbumin (24,000 daltons), β-lactoglobulin (18,400 daltons) and lysozyme (14,300 daltons).

Results

Effects of the inhibitors of RNA and protein synthesis

As described in the Materials and Methods, the basal medium containing BA (MB medium) significantly promoted both MZ and adventitious bud formation at the approximate rates of 12-16 hold. The BA-promoted MZ and bud formation was inhibited when one of the inhibitors of RNA or protein synthesis was incorporated in the MB medium (Table 1). The concentrations of inhibitors which substantially suppressed bud formation were 3 mg/l for cordycepin, 30 mg/l for 5-fluorouracil, 3 mg/l for Act. D and 0.3 mg/l for CHI.

In the next experiment, the relation between the time of the incorporation of Act.D or CHI in the MB medium and adventitious bud differentiation was examined (Fig. 1). When the explants were first cultured on the MB medium for 3 days, then transferred to the medium with Act.D (3 mg/l), 24% of cultures were able to differentiate adventitious buds. As to the treatment with CHI (1 mg/l), 4 days of the preculture without CHI were required to initiate bud formation.

Changes in fresh weight and protein content

Figure 2A summarizes the time-course of fresh weight changes of the superficial layers of excised stem segments

cultured for a given period. The fresh weight was decreased immediately after the onset of culture, but gradually increased after the 2nd day of culture in both the MS and MB medium. On the 8th day, there was about 1.4 hold increase of fresh weight with both the MS and MB medium, whilst addition of CHI (1 mg/l) to the MB medium was inhibitory.

The changes in protein contents in the superficial layers were shown in Fig. 2B. The superficial layers initially contained about 40 μg of proteins per 100 strips. The protein contents were decreased slightly during the first 2 days, regardless of the medium used, and started to increase on the 3rd day of culture with both the MS and MB medium. The increment of protein was much greater in the MB medium than the MS medium. Cycloheximide added in the MB medium substantially lowered the protein level.

Protein synthesis

The rate of ^{14}C-labelled leucine incorporation to proteins in the superficial layers was examined with the explants cultured for 8 days. Three kinds of media; MS, MB, and MB plus 1 mg/l CHI, were used in this experiment. The results are summarized in Fig. 3. When the MB medium plus CHI was used, relatively small amount of radioactive leucine was incorporated in protein throughout culture

periods. The highest rate of radioactively labelled proteins was observed on the 2nd day, where the MB medium resulted in two hold gain of radioactivity over the MS medium.

To obtain more detailed information on protein synthesis associated with MZ formation in Torenia superficial layers, stem segments were labelled with radioactive leucine for 2 hr, and the radioactivity remained in protein was counted at 1, 2 and 4 hr chase periods. As shown in Fig. 4, the radioactivity of the protein sample prepared from the explants cultured on the MB medium did not significantly decrease with the chase time. On the other hand, after the 4 hr chase period, the radioactivity of protein samples prepared from the explants cultured for 0, 1, 2 and 3 days on the MS medium decreased to 49, 53, 59 and 71 %, respectively, of the initial value. After 4 or 5 days, the changes were insignificant with the explants kept on the same medium.

Qualitative changes in newly synthesized proteins

Qualitative changes in newly synthesized proteins during the initial stage of bud formation were examined by SDS-polyacrylamide gel electrophoresis of doubly-labelled samples. Figure 5 shows a typical profile of gel electrophoresis of the samples prepared from the explants cultured

for 3 days on the MS and MB media. There were four major peaks of newly synthesized proteins which corresponded to molecular weight (in daltons) ranging from 50,000 to 60,000 (I), 35,000 to 40,000 (II), 24,000 to 30,000 (III), and 11,000 to 14,000 (IV), respectively. As to the distribution of radioactive proteins prepared from the explants cultured on the MS or MB medium, some differences were noted. For example, the profile obtained from the explants cultured on the MS medium showed larger quantity of radioactive proteins in the peak IV than that from those cultured on the MB medium. The reverse trend was observed in the peak I and III.

Discussion

As we presented above, some inhibitors of RNA and protein synthesis suppressed adventitious bud differentiation in Torenia stem segments. The critical period during which the inhibitors can exert their action seems to be the first 2-3 days of culture. This is coincided with the observation in bud-forming cultures of Douglas fir cotyledons, where specific proteins appeared during the first 2 to 4 days in culture (Yasuda et al., 1980).

It is a well known fact that cytokinin stimulates the incoporation of labelled amino acids to protein, as reported with Lemna (Fankhauser and Erisman, 1969), radish (Paranjothy and Wareing, 1971), and tobacco (Richmond et al., 1971; Maaß and Klambt, 1977; Grierson et al., 1977). Extensive incorporation of radioactive leucine to protein fraction was also observed in Torenia explants cultured for 2 days on the medium containing BA, and protein content started to increase continuously on the 3rd day of culture. Thus, the protein synthesis in the early period of explant culture seems essential for adventitious bud initiation in Torenia stem segments.

It would be worthwhile to point out that, in the presence of BA, the degradation of newly synthesized proteins did not occur during the 4 hr chase period, while, in the absence of BA, nearly one-third of those proteins in

superficial layers of the initial explants and ones cultured for 1, 2 or 3 days, degraded within 4 hr.

The favorable effects of cytokinin on the prevention of protein degradation was supported also by calculating the protein turnover rate according to the formula of Holleman and Key (1967). The rate of protein turnover in initial explants was less than 1% per hr (Table 2). In the case of the explants cultured on the MS medium, the rate increased to 4.7% per hr after 3 days of culture, then decreased to 1.0% per hr at the end of 6 days culture. When the MB medium was used, the rate was only 2.2% per hr on the 3rd day of culture, which was in the range of other reported instances such as 2.1% per hr in soybean hypocotyls (Holleman and Key, 1967) and 0.5% per hr in tobacco mesophyll protoplasts (Sakai and Takebe, 1970).

In the early work of biochemical analysis of organogenesis, Syōno (1965) suggested the possible involvement of specific proteins in the adventitious bud differentiation from carrot root callus. Working with early stage of organogenesis of Douglas fir cotyledons, Hasegawa et al. (1979) reported the presence of major proteins in the following ranges of molecular weight (in daltons); 76,000 to 84,000, 52,000 to 58,000, 37,000 to 42,000, 24,000 to 27,000 and 16,000 to 20,000. The three peaks of proteins found in superficial layers of Torenia stem segments (Fig. 5)

have similar molecular weights as those reported in Douglas fir cotyledons. The low molecular weight proteins (16,000 to 20,000 daltons) which seemed to be related to bud formation in Douglas fir explants (Hasegawa et al., 1979; Yasuda et al., 1980), were not detected in our material. The proteins located in the peak I (50,000 to 60,000 daltons) and peak III (24,000 to 30,000 daltons) were found in large quantities in Torenia explants cultured on the MB medium than in those cultured on the MS medium. These proteins seems to play some role for adventitious bud induction.

Efforts are being made in order to secure more informations regarding the regulation of gene expression during the process of organogenesis in Torenia.

Table 1. Effects of the inhibitors of RNA and protein synthesis on meristematic zone (MZ) and bud formation in the epidermis of Torenia stem segments cultured in vitro.

Inhibitors	mg/l	No. of MZ per epidermal strip		Cultures with buds (%)
Control		6.9	(100)	94
Cordycepin	0.1	4.0	(59)	36
	1.0	0.6	(9)	25
	3.0	0.0	(0)	0
Actinomycin-D	0.1	4.5	(65)	66
	1.0	1.1	(16)	31
	3.0	0.1	(1)	0
5-Fluorouracil	0.3	3.9	(57)	91
	1.0	2.6	(38)	78
	3.0	2.0	(29)	65
	10.0	0.1	(1)	13
	30.0	0.0	(0)	0
Cycloheximide	0.01	3.2	(47)	98
	0.1	0.8	(12)	7
	0.3	1.0	(14)	0
	1.0	0.6	(9)	0
	3.0	0.6	(9)	0

MS medium containing 0.5 μM BA (MB medium) was used in this experiment. For each treatment, at least 200 epidermal strips and 75 stem segments were observed to calculate the average numbers of MZ and the percentages of cultures with buds, respectively. Numbers in parentheses indicate the percentage calculated on the basis of MZ numbers obtained with the control.

Table 2. The rate of protein turnover in superficial layers of Torenia stem segments cultured on the MS medium with or without BA for various periods. The protein turnover rate was calculated according to Holleman and Key (1967). Each figure represents an average value per 100 segments of superficial layers.

Culture period (days)	Culture media	Leucine incorporation to protein[a] (n moles/hr)	Leucine content in protein[b] (μ moles)	Turnover rate (%/hr)
0	(MS)[c]	0.129	0.027	0.5
	(MS+BA)[c]	0.225	0.027	0.8
3	MS	0.564	0.012	4.7
	MS+BA	0.818	0.037	2.2
6	MS	0.364	0.038	1.0
	MS+BA	0.482	0.141	0.3

[a]Calculated from the results shown in Fig. 3.

[b]Calculated from the results of amino acids analysis (Part I, Chapter 3).

[c]Initial segments sampled at day 0 were directly incubated with the respective medium (MS or MS+BA) containing ^{14}C- or ^{3}H-labelled leucine.

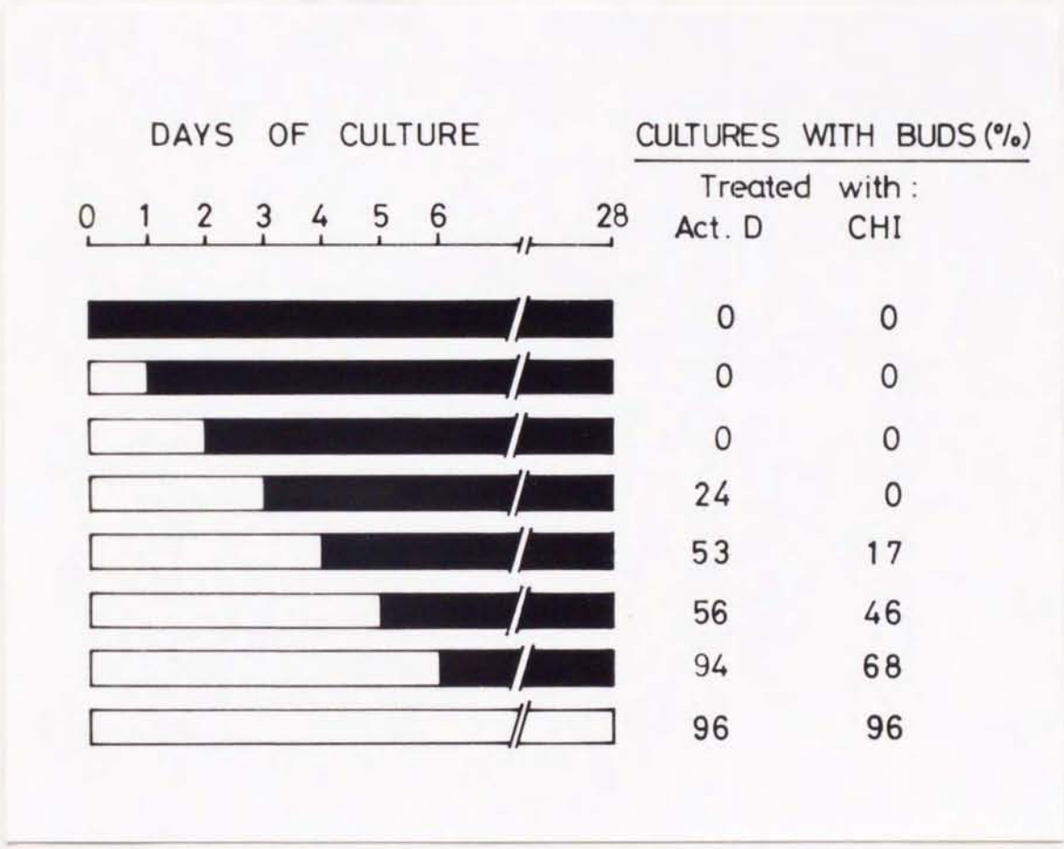


Fig. 1. Effects of Act.D and CHI, applied for different periods during culture, on bud formation in the epidermis of Torenia stem segments. The explants were initially cultured on the MB medium for different periods (□), and then transferred to the MB medium with Act.D (3 mg/l) or CHI (1 mg/l) for remaining periods (■). At least 75 explants were used for each treatment, and the data were recorded 28 days after the beginning of culture.

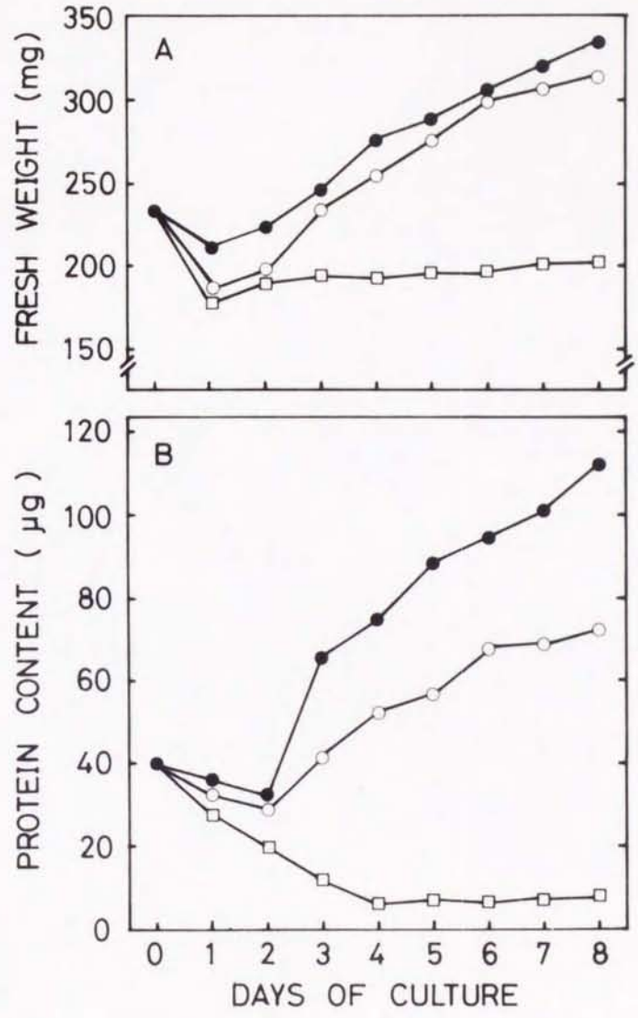


Fig. 2. Changes in fresh weight (A) and protein content (B) of superficial layers of Torenia stem segments during the early stage of culture. At least 500 segments of superficial layers were used for each treatment. The data were expressed in mg fresh weight or µg protein per 100 segments of superficial layers. The explants were cultured on the MS (○), MB (●) or MB medium with 1 mg/l of CHI (□).

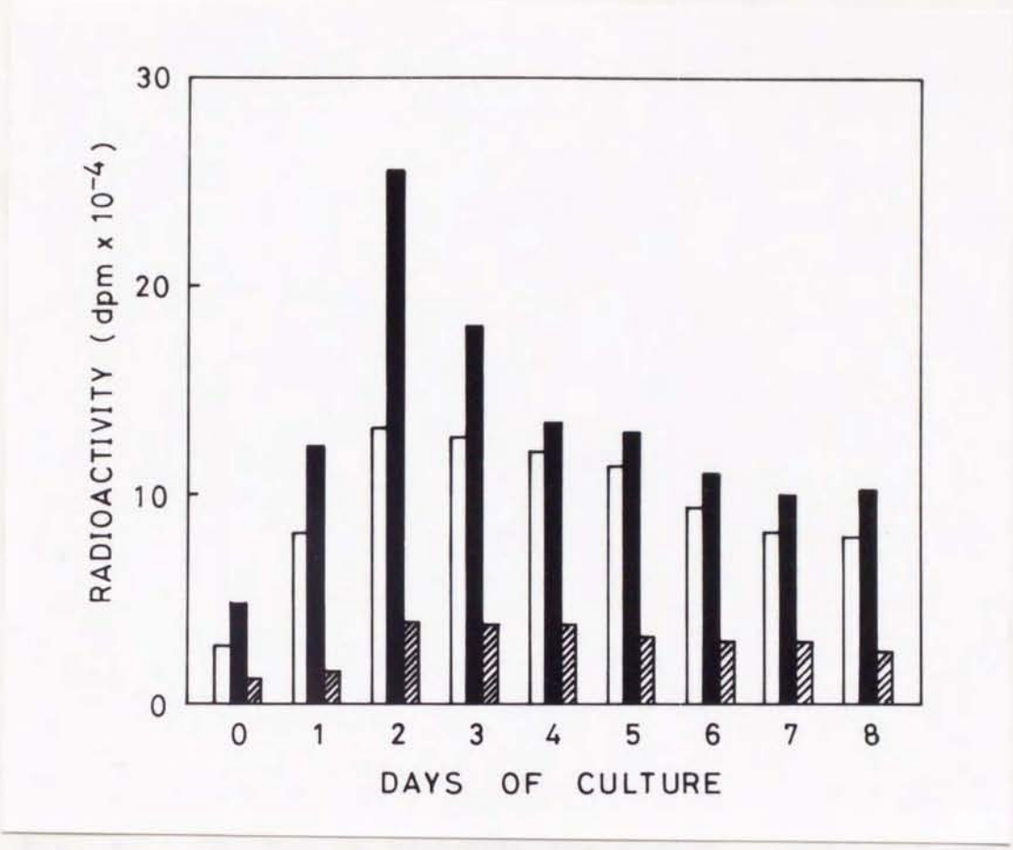


Fig. 3. The total incorporation of radioactivity to proteins in superficial layers of Torenia stem segments. The explants were cultured on the MS (□), MB (■) or MB+CHI (1 mg/l) (▨) medium for given periods, and then incubated for 4 hr with the same medium containing ^{3}H- or ^{14}C-labelled leucine. At least 500 segments of superficial layers were used for each treatment. The results were expressed in radioactivity (dpm) per 100 segments of superficial layers.

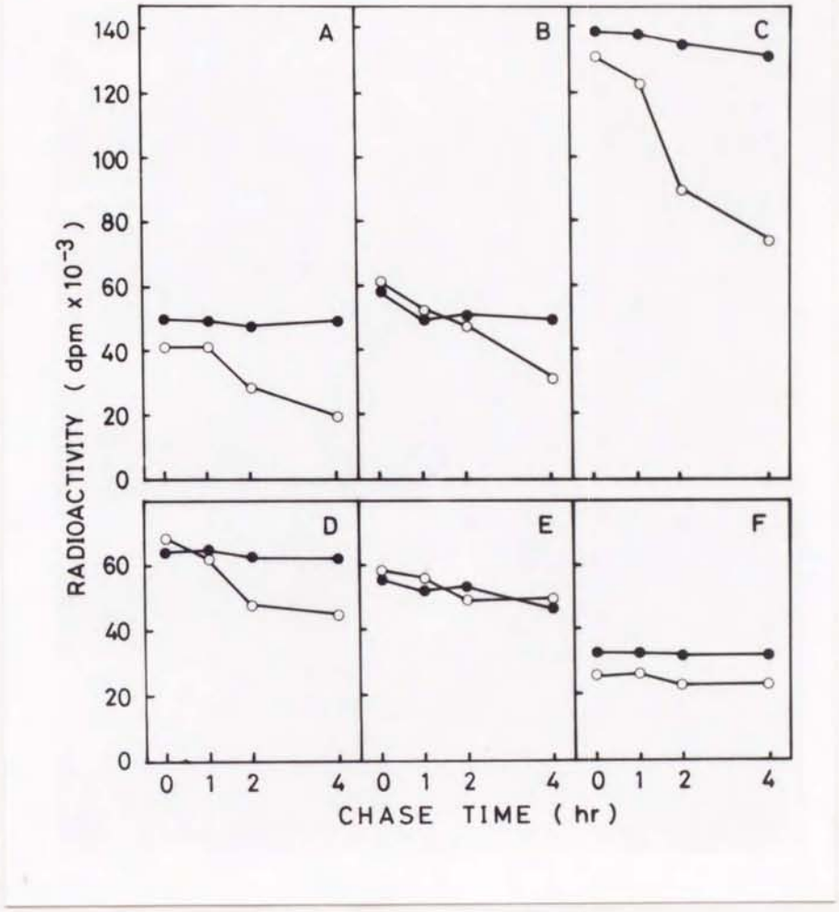


Fig. 4. Changes in the radioactivities in proteins during the chase period. The explants were first cultured on the MS (○) or MB (●) medium for 0 (A), 1 (B), 2 (C), 3 (D), 4 (E) and 5 (F) days, and then transferred to the same medium containing radioactive leucine. Following 2 hr pulse labelling, the chase was done with the respective media lacking radioactive leucine. At least 500 segments of superficial layers were used for each treatment. The results were expressed in radioactivity (dpm) per 100 segments of superficial layers.

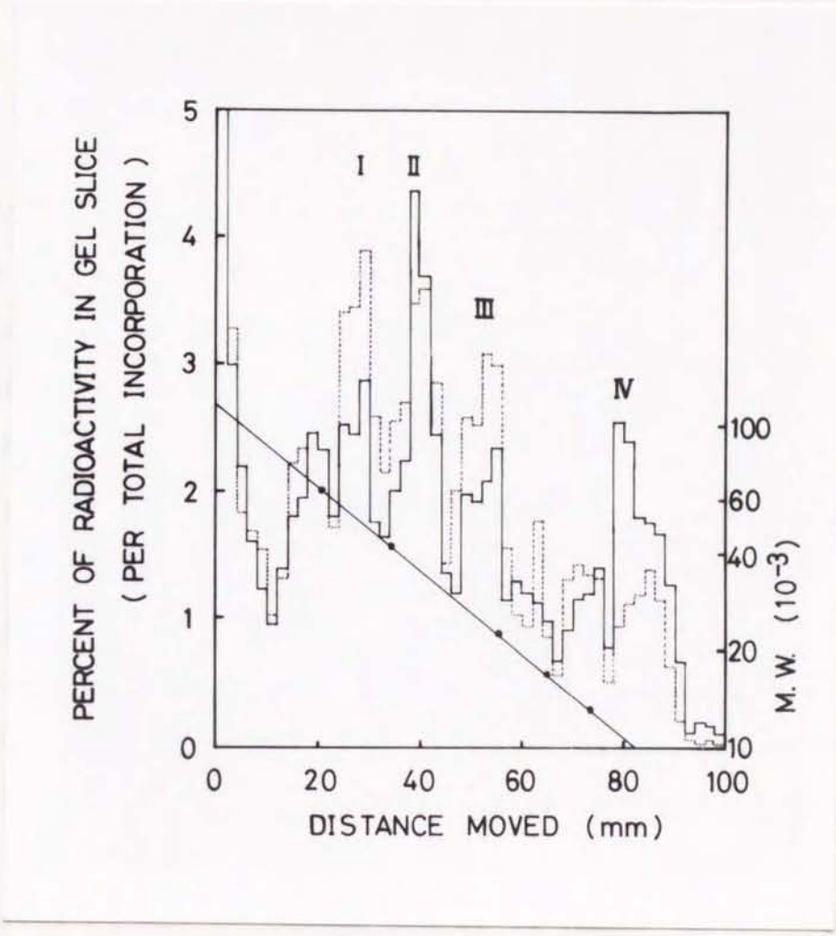


Fig. 5. A histogram showing the distribution of radioactivities after the electrophoresis of doubly labelled proteins prepared from superficial layers of Torenia stem segments. A protein sample prepared from the explants cultured on the MS medium (^{3}H, ——) was co-electrophoresed with that prepared from the explants cultured on the MB medium (^{14}C, ·····). The point on the straight line indicate the position of the following calibration proteins: bovine serum albumin (mol.wt. 66.000), egg albumin (mol.wt. 45,000), ovalbumin (mol. wt. 24,000), β-lactoglobulin (mol. wt. 18,400), and lysozyme (mol. wt. 14,300).

References

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72; 248-254.

Frankhauser, M. und K. H. Erismann (1969) Über die Wirkung kurzfristinger Kinetingaben auf Protein, Aminosäuren und RNS bei Lemna minor L. Planta 88; 332-343.

Grierson, D., S. E. Chambers and L. P. Penniket (1977) Nucleic acid and protein synthesis in discs cut from mature leaves of Nicotiana tabacum L. and cultured on nutrient agar with and without kinetin. Planta 134; 29-34.

Hasegawa, P. M., T. Yasuda and T. Y. Cheng (1979) Effect of auxin and cytokinin on newly synthesized proteins of cultured Douglas fir cotyledons. Physiol. Plant. 46; 211-217.

Holleman, J. M. and J. L. Key (1967) Inactive and protein precursor pools of amino acids in the soybean hypocotyl. Plant Physiol. 42; 29-36.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227; 680-685.

Maaß, H. and D. Klambt (1977) Cytokinin effect on protein

synthesis in vivo in higher plants. Planta 133; 117-120.

Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures Physiol. Plant. 15; 473-497.

Paranjothy, K. and P. F. Wareing (1971) The effects of abscisic acid, kinetin and 5-fluorouracil on ribonucleic acid and protein synthesis in senescing radish disks. Planta 99; 112-119.

Richmond, A. E., B. Sachs and D. J. Osborne (1971) Chloroplasts, kinetin and protein synthesis. Physiol. Plant. 24; 176-180.

Sakai, F. and I. Takebe (1970) RNA and protein synthesis in protoplasts isolated from tobacco leaves. Biochem. Biophys. Acta 224; 531-540.

Sekiya, J. and Y. Yamada (1974) Cytokinin regulation of protein synthesis during the early stages of shoot formation from cultured tobacco cells. Bull. Inst. Chem. Res. Kyoto Univ. 52; 246-255.

Syōno, K. (1965) Physiological and biochemical changes of carrot root callus during successive cultures. Plant & Cell Physiol. 6; 371-392.

Thorpe, T. A. and D. D. Meier (1974) Starch metabolism in shoot-forming tobacco callus. J. Exp. Bot. 25; 288-294.

Yasuda, T., P. M. Hasegawa and T. Y. Cheng (1980) Analysis of newly synthesized proteins during differentiation of cultured Douglas fir cotyledons. Physiol. Plant. 48; 83-87.

PART II.

STUDIES ON FLORAL BUD INITIATION AND DEVELOPMENT IN *TORENIA* STEM SEGMENTS CULTURED *IN VITRO*

Chapter 1

Nutritional factors controlling floral bud differentiation

Summary

Internodal segments of Torenia fournieri Lind. were cultured on various media to investigate chemical factors influencing in vitro flowering. The elimination or dilution of ammonium nitrate from Murashige and Skoog's medium increased the formation of adventitious buds which subsequently differentiated floral buds. The dilution of mineral salts in Murashige and Skoog's medium enhanced adventitious bud formation, but did not influence the ratio of cultures with floral buds to those with adventitious buds. Among various media tested, in vitro floral bud formation and development in Torenia stem segments was best on a medium having the 1/5 of the mineral salts of Murashige and Skoog's medium and no ammonium nitrate. Eighty-seven percent of the cultures produced floral buds on this medium. Using this medium, the effects of various sugars were also examined. Increasing the concentration of sucrose in the medium (up to 6%) increased the rate of cultures with floral buds, and stimulated the development of floral buds led to anthesis.

Introduction

Since Skoog (1955) observed floral bud formation in the course of tobacco tissue culture, many investigators have attempted *in vitro* flower induction using the stem segments of tobacco plants (Chouard and Aghion, 1961; Aghion-Prat, 1965; Wardell and Skoog, 1969; Hillson and LaMotte, 1977) and of other plants (Harada, 1966; Konar and Konar, 1966; Ringe and Nitsch, 1968). Some of them demonstrated the presence of a gradient of flower forming capacity along the stem; it being greater in higher portions of stems than in the lower portions. *In vitro* induction of floral buds from strictly vegetative materials has also been observed in *Plumbago* (Nitsch and Nitsch, 1965, 1967a, b), *Lunaria* (Pierik,1966a), *Cichorium* (Pierik, 1966b), and *Streptocarpus* (Rossini and Nitsch, 1966). Nitsch and Nitsch (1967a, b) described in detail some experimental conditions favourable for flower initiation in vegetative stem segments of *Plumbago indica*.

Detailed observations of these results revealed that adventive floral buds were induced from intermediate callus formed on explants. Adventive floral bud induction *via* callus formation tends to complicate physiological studies of flowering process. Other appropriate experimental systems should also be sought for the investigation of *in vitro* floral bud formation. Recently it has been

reported that floral buds can be differentiated in in vitro directly from the epidermal tissue of stem segments of Nicotiana (Tran Thanh Van, 1973) as well as from the shoots regenerated from the epidermal tissue of Torenia (Chlyah, 1973a, b).

There have been reports indicating the effects of the physiological condition of explants on floral bud formation in tobacco stem segments (Aghion-Prat, 1965; Wardell and Skoog, 1969; Tran Thanh Van, 1973; Tran Thanh Van et al., 1974) and Perilla leaf discs (Tanimoto and Harada, 1980). Some of them mentioned also the effects of chemical substances added to media on in vitro flowering. It is apparent that a close relationship exists between the physiological state of explants and the amount and/or kinds of endogenous growth substances present in the tissue of explants.

In order to study the relation between the effects of physiological state of explants and that of chemical compounds added to media, it is useful to start with a simple culture medium. In many investigations on in vitro flowering, auxin and/or cytokinin were generally added to a culture medium. Working with Torenia fournieri, Bajaj (1972) induced floral buds on excised leaves using the MS medium containing IAA and kinetin. The influence of different sugars on in vitro floral bud formation has been described in Plumbago (Nitsch and Nitsch, 1967b) and Nicotiana (Tran

Thanh Van et al., 1974; Tran Thanh Van, 1977). In both cases, some growth regulators were added to culture media used. In cultures of organ fragments of Torenia, Chlyah (1973a) reported that floral buds differentiated on a more simplified medium which was composed only of inorganic salts and sucrose. However, detailed investigation of the effects of inorganic salts and sugars on in vitro flowering in the absence of growth regulators in a culture medium has not so far been reported.

The present series of studies will report on various chemical factors controlling floral bud formation of Torenia stem segments cultured in vitro. This chapter will consider the effects of nutritional factors such as the amount and kinds of inorganic salts and sugars added to a medium.

Materials and Methods

Plants of Torenia fournieri Lind., a quantitative short-day plant, were cultivated in a growth room at a temperature of 25±2 °C with a daily 16-hr light period (Mitsubishi day-light type fluorescent lamps, ca. 5,000 lux at plant level) during the first 6 weeks and then transferred to an 8-hr short-day condition. The second stem internodes (counting from the top) were excised from 12-week-old mother plants (reproductive stage) and were surface-sterilized with an 8% sodium hypochlorite solution for 15 min after the cut ends were sealed with molten paraffin. Stem segments, 5 mm length, were cut from the internodes using a sterile scalpel, and cultured on 0.8% semi-solidified agar medium prepared in 3 x 12 cm glass culture tubes. The pH of medium was adjusted to 5.6 with 1 N HCl and 1 N KOH before adding the agar and autoclaving (120 °C for 15 min). The cultures were maintained in a growth room at 25±2 °C and were exposed to short-day photoperiod of 8-hr light/16-hr dark at the intensity of ca. 5,000 lux.

In order to find out the most appropriate medium for floral bud formation, we examined the effects of individual elements comprising MS medium as well as different dilutions of the medium. The basal medium used in our experiments contained only mineral salts and sugar, because the addition

of various organic elements stimulated callus proliferation and vegetative bud initiation, but inhibited shoot development and floral bud differentiation. The effects of various concentrations of different sugars such as sucrose, glucose, fructose, maltose and galactose added to the medium were also tested.

Twelve replicates were used in all experiments which were repeated at least three times. Data were recorded at the end of 12-week culture period with macroscopic observation of the cultured stem segments. The results were expressed as an average percentage of cultures producing adventitious buds, floral buds and flowers fully bloomed, and also occasionally, as an average number of floral buds per explant.

Results

Effects of MS medium with or without growth regulators

Table 1 shows some results of preliminary experiments concerning the effects of MS medium with or without NAA and BA on adventitious and floral bud formation. Generally, an addition of BA to the medium gave a high rate of adventitious bud formation. Thirty-three percent of cultures formed floral buds and a few of them reached full anthesis when the medium contained 0.1 mg/l of NAA and 1 mg/l of BA. It is interesting to note that a few cultures produced floral buds on MS medium in the absence of both NAA and BA. This prompted us to investigate the each effect of five major salts comprising in the MS medium.

Individual effects of five major salts comprising MS medium

Table 2 shows the results obtained with explants cultured on 5 modified MS media, each of which was lacking one of the 5 major salts (NH_4NO_3, KNO_3, KH_2PO_4, $CaCl_2$, $MgSO_4$). The elimination of NH_4NO_3 increased adventitious buds which subsequently differentiated floral buds in 58% of the cultures. The elimination of the other salts did not show the promoting effect on adventitious bud formation and lowered the ratio of cultures with floral buds to those with adventitious buds. In other words, the presence of NH_4NO_3 in a medium clearly inhibited adventitious and floral bud formation. The elimination of micro elements from MS

medium affected neither the formation nor development of adventitious and floral buds.

Effects of different concentrations of NH_4NO_3 in the MS medium were also investigated (Table 3). The standard MS medium contains 1650 mg/l of NH_4NO_3. As the concentration of NH_4NO_3 decreased, the percentages of cultures with adventitious buds and with floral buds increased, though the ratio of cultures with floral buds to those with adventitious buds did not vary significantly. When the amount of NH_4NO_3 was 1/20 of that of normal MS medium, 83% of cultures formed floral buds.

Effects of various dilutions of MS medium

Effects of different concentrations of inorganic salts on floral bud formation were also investigated. As shown in Table 4, dilutions of whole mineral salts of the MS medium caused significant stimulation of adventitious bud formation, but had much less effect on the ratio of cultures with floral buds to those with adventitious buds. A little more than 40% of cultures differentiated floral buds when 1/5- or 1/10-strength of MS medium was used. In a medium containing only sucrose (without mineral salts), the rate of adventitious bud formation was low and floral bud was not observed.

The influence of various dilutions of the MS medium without NH_4NO_3 was also investigated. The results (Table 5)

indicate that the most appropriate medium for floral bud formation in Torenia stem segments was 1/5-strength MS medium without NH_4NO_3, which gave also a high rate of in vitro flowering. Therefore, in the following experiments, thus modified MS medium was employed.

Effects of different sugars on floral bud formation

In previous experiments, the sucrose concentration used was 20 g/l in all media. Effects of various concentrations of sucrose in the 1/5-strength MS medium lacking NH_4NO_3 were tested (Table 6). High rates of floral bud formation were obtained with concentrations of sucrose ranging from 20 g/l to 80 g/l. No bud formation was observed with the explants cultured on the medium without sucrose. With the medium containing 100 g/l of sucrose, adventitious bud formation was suppressed and explants rapidly turned brown or red. The percentage of cultures with fully developed flowers was very high (76%) with 60 g/l of sucrose. The mean number of floral buds per explant increased also with the increasing concentrations of sucrose.

The effects of different sugars on adventitious and floral bud formation are summarized in Table 7. In the case of glucose, the effects were more or less similar to those obtained with sucrose within concentrations ranging from 5 to 40 g/l. The maximum rates of floral bud formation were obtained at the concentrations of 20 g/l and 40 g/l,

and the percentage of cultures with fully developed flowers was the highest at the concentration of 40 g/l of glucose. At concentrations higher than 60 g/l, adventitious bud formation was strongly inhibited, and explants showed necrosis. In the case of fructose, strong inhibitory effects were observed even at the concentration of 40 g/l. The stimulatory effects of maltose on floral bud formation and its development were rather weak as compared with those of sucrose and glucose.

Discussion

Although the effects of growth regulators on *in vitro* flowering have been extensively investigated, the studies on those of mineral nutrients are rather limited. In *Nicotiana*, which has been frequently used by a number of investigators, most of the studies have been done using MS medium with auxin and cytokinin (Wardell and Skoog, 1969; Tran Thanh Van *et al.*, 1974; Hillson LaMotte, 1977). However, Chouard and Aghion (1961) used Knop's solution without growth regulators. In the case of other plant materials, the MS medium has rarely been employed. *In vitro* floral bud formation was induced in organ fragments of *Lunaria* (Pierik, 1966a), *Cichorium* (Pierik, 1966b) and *Begonia* (Ringe and Nitsch, 1968) when cultured on a medium composed of Knop's mineral salts. *In vitro* flowering in organ fragments of *Streptocarpus* (Rossini and Nitsch, 1966) and *Plumbago* (Nitsch and Nitsch, 1967b) were obtained by using Nitsch and Nitsch's medium (Nitsch and Nitsch, 1967a). In *Torenia fournieri*, Chlyah (1973a) reported that a high rate of floral bud formation was obtained in leaf and stem fragments using a modified Hoagland's medium without auxin and cytokinin. Finally, as we presented, 87% of *Torenia* stem segments produced floral buds when cultured with 1/5-strength MS medium devoid of NH_4NO_3 and growth regulators.

The four culture media mentioned above, namely, Knop's,

Nitsch and Nitsch's, modified Hoagland's and the MS medium modified by us have two common characteristics, i.e., relatively low concentrations of mineral salts and the absence of ammonium nitrate. With the exception of tobacco, in vitro floral bud formation in many plant species can be stimulated with a low mineral nutrient medium and inhibited when ammonium nitrate is present in a medium.

The effects of low concentrations of mineral nutrients on flowering of intact plants is also known for many plant species. Some reports indicated a certain degree of promotion of flowering by a low nutrient level, but reverse cases have also been presented. Diomaiuto-Bonnard (1974) described Nicotiana glutinosa which flowered under a long-day condition if abundant nutrient was given, but it responded as a quantitative short-day plant with low nutrient supplies.

There are a few reports concerning the effects of ammonium nitrate on in vitro floral bud formation. In Lemna plants, flowering of both long-day (Kandeler, 1969) and short-day species (Tanaka and Takimoto, 1975) were completely inhibited by the addition of ammonium ions to a medium. The stimulative effects of reduced nitrogenous compounds have been reported in somatic embryogenesis in carrot (Halperin and Wetherell, 1965; Reinert et al., 1967; Wetherell and Dougall, 1976). Kamada and Harada (1979) indicated that reduced nitrogen was required for the development of carrot somatic

embryos, but not for initiation of these embryos. Mohanty and Fletcher (1976) reported that the presence of ammonium ions in a medium increased the activity of nitrate reductase and promoted growth of suspension cultures of Paul's Scarlet rose. It is difficult to explain the effects of ammonium nitrate on in vitro flowering from the results presented here. However, it seems that the absence of ammonium nitrate in a medium may stimulate flowering through a decrease of nitrate reductase activity and subsequent modification of the plant's nitrogen metabolism.

Nitsch and Nitsch (1967b) demonstrated the effects of various sugars on in vitro flowering of Plumbago. Sucrose and maltose at a concentration of 9×10^{-2} M increased flowering, but they were ineffective at a low concentrations. Lactose, cellobiose and mannitol were totally ineffective. Flower formation from epidermal strips of Nicotiana was promoted by each addition to a medium of glucose (1/6 M), sucrose (1/12 M) and fructose (1/12 M) (Tran Thanh Van, 1977). Harada (1966) also used a high sucrose concentration (50 g/l) for in vitro studies of flowering of Cichorium intybus. These results, together with our results obtained with Torenia stem segments, agree with the classical theory that a high carbon/nitrogen ratio is stimulatory for flowering.

Table 1. Effects of NAA and BA added to the MS medium on *in vitro* adventitious and floral bud formation of *Torenia* stem segments.

BA (mg/l)	NAA	Percentage of cultures with		
		Adventitious buds	Floral buds	Flowers
0	0	16	11 (69)	0 (0)
0	0.1	68	23 (34)	0 (0)
0	1.0	0	-	-
0.1	0	95	10 (11)	0 (0)
1.0	0	100	0 (0)	-
1.0	0.1	94	33 (35)	4 (12)
1.0	1.0	67	5 (7)	0 (0)

Twelve segments were used for each treatment and repeated 5 times. Data was recorded 12 weeks after the beginning of culture. Figures in parentheses indicate the percentage of cultures with floral buds, calculated on the basis of the number of cultures with adventitious buds (middle column), and the percentage of cultures with fully developed flowers, calculated on the basis of the number of cultures with floral buds (right column).

Table 2. Effects of the individual elimination of 5 macro elements of the MS medium on *in vitro* adventitious and floral bud formation of *Torenia* stem segments.

Media	Percentage of cultures with		
	Adventitious buds	Floral buds	Flowers
MS	17	11 (69)	0 (0)
MS - NH_4NO_3	83	58 (70)	15 (26)
MS - KNO_3	20	5 (25)	0 (0)
MS - KH_2PO_4	38	13 (34)	5 (38)
MS - $CaCl_2$	17	2 (12)	0 (0)
MS - $MgSO_4$	12	5 (42)	5 (100)

Twelve segments were used for each treatment and repeated 3 times. Data were recorded 12 weeks after the beginning of culture. For figures in parentheses, see the footnote of Table 1.

Table 3. Effects of NH_4NO_3 on *in vitro* adventitious and floral bud formation of *Torenia* stem segments.

NH_4NO_3 (mg/l)	Percentage of cultures with				
	Adventitious buds	Floral buds		Flowers	
1650	16	12	(75)	0	(0)
1320	38	15	(39)	8	(53)
990	23	19	(83)	12	(63)
660	38	27	(71)	8	(30)
330	56	44	(79)	16	(36)
165	84	76	(90)	35	(46)
83	92	83	(90)	21	(25)
0	84	63	(75)	28	(44)

Twelve segments were used for each treatment and repeated 3 times. Data were recorded 12 weeks after the beginning of culture. For figures in parentheses, see the footnote of Table 1.

Table 4. Effects of various dilutions of the MS medium on *in vitro* adventitious and floral bud formation of *Torenia* stem segments.

Media	Percentage of cultures with				
	Adventitious buds	Floral buds		Flowers	
MS x 1.0	17	13	(76)	0	(0)
MS x 0.8	7	2	(29)	0	(0)
MS x 0.6	18	14	(78)	0	(0)
MS x 0.4	47	25	(53)	2	(8)
MS x 0.2	81	44	(54)	2	(5)
MS x 0.1	87	48	(55)	8	(17)
MS x 0.05	95	33	(35)	0	(0)
MS x 0.01	100	33	(33)	0	(0)
MS x 0	50	0	(0)	-	

Twelve segments were used for each treatment and repeated 3 times. Data were recorded 12 weeks after the beginning of culture. For figures in parentheses, see the footnote of Table 1.

Table 5. Effects of various dilutions of the MS medium lacking NH_4NO_3 on *in vitro* adventitious and floral bud formation of *Torenia* stem segments.

MS minus NH_4NO_3	Percentage of cultures with				
	Adventitious buds	Floral buds		Flowers	
x 1.0	83	58	(70)	23	(40)
x 0.8	74	69	(93)	23	(33)
x 0.6	89	69	(78)	23	(33)
x 0.4	88	68	(77)	27	(40)
x 0.2	98	87	(89)	39	(45)
x 0.1	90	59	(67)	28	(47)

Twelve segments were used for each treatment and repeated 5 times. Data were recorded 12 weeks after the beginning of culture. For figures in parentheses, see the footnote of Table 1.

Table 6. Effects of sucrose at various concentrations on *in vitro* adventitious and floral bud formation of *Torenia* stem segments.

Sucrose concentration (g/l)	Percentage of cultures with			Mean No. of floral buds per explant
	Adventitious buds	Floral buds	Flowers	
0	0	-	-	-
5	73	37 (51)	3 (8)	3.0
10	84	39 (46)	16 (41)	2.7
20	96	81 (84)	24 (30)	2.4
40	85	74 (87)	46 (62)	3.3
60	89	81 (91)	76 (94)	7.4
80	85	64 (75)	54 (84)	8.8
100	55	15 (27)	8 (53)	2.0

The 1/5-strength MS medium lacking NH_4NO_3 was used as basal medium. Twelve segments were used for each treatment and repeated 4 times. Data were recorded 12 weeks after the beginning of culture. For figures in parentheses, see the footnote of Table 1.

Table 7. Effects of various sugars on *in vitro* adventitious and floral bud formation of *Torenia* stem segments.

Sugars	Conc. (g/l)	Percentage of cultures with		
		Adventitious buds	Floral buds	Flowers
None		0	-	-
Glucose	5	100	25 (25)	0 (0)
	10	94	52 (55)	24 (46)
	20	94	77 (82)	49 (64)
	40	82	71 (87)	63 (89)
	60	8	0 (0)	-
	80	0	-	-
	100	0	-	-
Fructose	10	75	50 (67)	25 (50)
	20	58	42 (72)	26 (60)
	40	9	4 (44)	0 (0)
Maltose	10	95	43 (45)	10 (23)
	20	88	56 (64)	20 (38)
	40	62	38 (61)	23 (61)
Galactose	10	88	46 (52)	17 (37)
	20	80	68 (85)	36 (53)
	40	84	72 (86)	30 (42)

The 1/5-strength MS medium lacking NH_4NO_3 was used as basal medium. Twelve segments were used for each treatment and repeated 3 times. Data were recorded 12 weeks after the beginning of culture. For figures in parentheses, see the footnote of Table 1.

References

Aghion-Prat, D. (1965) Néoformation de fleurs in vitro chez Nicotiana tabacum L. Physiol. Vég. 3; 229-303.

Bajaj, Y. P. S. (1972) Effects of some growth regulators on bud formation by excised leaves of Torenia fournieri. Z. Pflanzenphysiol. 66; 284-287.

Chlyah, H. (1973a) Néoformation dirigée à partir de fragments d'organes de Torenia fournieri (Lind.) cultivés in vitro. Biol. Plant. 15; 80-87.

Chlyah, H. (1973b) Influence de la température sur la mise à fleur de plantes de Torenia fournieri (Lind.) issues de semis ou de boutures de feuilles, ou formées à partir de fragments d'organes cultivés in vitro. C. R. Acad. Sci. (Paris) Série D, 277; 1451-1454.

Chouard, P. and D. Aghion (1961) Modalités de la formation de bourgeons floraux sur des cultures de segments de tige de tabac. C. R. Acad. Sci. (Paris) Série D, 252; 3864-3867.

Diomaiuto-Bonnard, J. (1974) Variations du comportement photopériodique du Nicotiana glutinosa L. en fonction de la fréquence de l'apport en substances minerales au cours des arrosages. C. R. Acad. Sci. (Paris) Série D, 278; 49-52.

Halperin, W. and D. F. Wetherell (1965) Ammonium requirement for embryogenesis in vitro. Nature 205; 519-520.

Harada, H. (1966) Effects of photoperiod on the formation of flower buds by flower stalk sections of Cichorium intybus in tissue culture. Bot. Mag. Tokyo 79; 119-123.

Hillson, T. D. and C. E. LaMotte (1977) In vitro formation and development of floral buds on tobacco stem explants. Plant Physiol. 60; 881-884.

Kamada, H. and H. Harada (1979) Studies on the organogenesis in carrot tissue cultures. II. Effects of amino acids and inorganic nitrogenous compounds on somatic embryogenesis. Z. Pflanzenphyiol. 91; 453-463.

Kandeler, R. (1969) Hemmung der Blütenbildung von Lemna gibba durch Ammonium. Planta 84; 279-291.

Konar, R. N. and A. Konar (1966) Plantlet and flower formation in callus cultures from Phlox drummondii Hook. Phytomorphology 16; 379-382.

Mohanty, B. and J. S. Fletcher (1976) Ammonium influence on the growth and nitrate reductase activity of Paul's Scarlet rose suspension cultures. Plant Physiol. 58; 152-155.

Nitsch, J. P. and C. Nitsch (1965) Néoformation de fleurs in vitro chez une espece de jours courts; Plumbago indica L. Ann. Physiol. végét. 7; 251-256.

Nitsch, C. and J. P. Nitsch (1967a) The induction of flowering in vitro in stem segments of Plumbago indica L. I. The production of vegetative buds. Planta 72; 355-370.

Nitsch, C. and J. P. Nitsch (1967b) The induction of flowering *in vitro* in stem segments of *Plumbago indica* L. II. The production of reproductive buds. Planta 72; 371-384.

Pierik, R. L. M. (1966a) The induction and initiation of flower buds *in vitro* in tissues of *Lunaria annua* L. Naturwiss. 53; 45.

Pierik, R. L. M. (1966b) The induction and initiation of flower buds *in vitro* in root tissues of *Cichorium intybus* L. Naturwiss. 53; 387.

Reinert, J., M. Tazawa and S. Semenoff (1967) Nitrogen compounds as factors of embryogenesis *in vitro*. Nature 216; 1215-1216.

Ringe, F. and J. P. Nitsch (1968) Conditions leading to flower formation on excised *Begonia* fragments cultured *in vitro*. Plant & Cell Physiol. 9; 639-952.

Rossini, L. M. E. and J. P. Nitsch (1966) Induction de la floraison *in vitro* chez plante de jours courts, *Streptocarpus nobilis*. C. R. Acad. Sci. (Paris) Série D. 263; 1379-1382.

Skoog, F. (1955) Growth factors, polarity and morphogenesis. Ann. Biol. 31; 1-11.

Tanaka, O. and A. Takimoto (1975) Suppression of long-day flowering by nitrogenous compounds in *Lemna perpusilla* 6746. Plant & Cell Physiol. 16; 603-610.

Tanimoto, S. and H. Harada (1980) Hormonal control of morphogenesis in leaf explants of Perilla frutescens Britton var. crispa Decaisne f. viridi-crispa Makino. Ann. Bot. 45; 321-327.

Tran Thanh Van, K. M. (1973) Direct flower neoformation from superficial tissue of small explants of Nicotiana tabacum L. Planta 115; 87-92.

Tran Thanh Van, K. M. (1977) Regulation of morphogenesis. In Plant Tissue Culture and its Bio-technological Application. Edited by W. Barz, E. Reinhard and M. H. Zenk. pp. 367-385. Springer-Verlag, Berlin.

Tran Thanh Van, K. M., N. Thi Dien and A. Chlyah (1974) Regulation of organogenesis in small explants of superficial tissue of Nicotiana tabacum L. Planta 119; 149-159.

Wardell, W. L. and F. Skoog (1969) Flower formation in excised tobacco stem segments. I. Methodology and effects of plant hormones. Plant Physiol. 44; 1402-1406.

Wetherell, D. F. and D. K. Dougall (1976) Sources of nitrogen supporting growth and embryogenesis in cultured wild carrot tissue. Physiol. Plant. 37; 97-103.

Chapter 2

Effects of IAA, zeatin, ammonium nitrate and sucrose on the initiation and development of floral buds

Summary

In Torenia stem segments cultured on a defined medium from which ammonium nitrate and growth regulators were omitted, adventitious buds were readily formed from the epidermal tissue, with subsequent differentiation of floral buds. Using this plant materials, the correlation between the time of application of various chemicals and the time-course of floral bud differentiation was investigated. Histological examination showed that adventitious buds were vegetative during the first two weeks of the culture, and floral primordia appeared after about three to four weeks of culture. We divided the flowering process in Torenia stem segments into the following 3 phases: the first phase (first 2 weeks) during which adventitious buds are formed, the second phase (3rd and 4th weeks) during which floral buds are initiated and the third phase (5th to 12th weeks) during which floral buds develop. Then we added to IAA, zeatin, ammonium nitrate or a high concentration of sucrose to the medium during one, two or

three of these phases. Ammonium nitrate added during the third phase suppressed floral bud development, but the high concentration of sucrose given during this phase stimulated it. These two chemicals influenced only the development of floral buds previously initiated. The application of IAA during the first phase promoted both the initiation and development of floral buds. However, its application after 2 weeks of culture failed to promote floral bud formation. Zeatin inhibited floral bud formation in a manner similar to ammonium nitrate, but if it was added to the medium only during the first phase, it slightly promoted the initiation and development of floral buds.

Introduction

Chemical factors influencing *in vitro* flowering have previously been examined by many investigators. Floral bud differentiation was induced in organ segments of *Lunaria* (Pierik, 1966a), *Streptocarpus* (Rossini and Nitsch, 1966), *Cichorium* (Pierik, 1966b), *Plumbago* (Nitsch and Nitsch, 1967) and *Begonia* (Ringe and Nitsch, 1968) when they were cultured on a medium from which ammonium nitrate was omitted. We reported previously (Part II, Chapter 1) that the elimination of ammonium nitrate from a basal medium promoted the formation of floral buds on stem segments of *Torenia*. A high sucrose concentration promoted *in vitro* flowering of *Cichorium* (Harada, 1966), *Plumbago* (Nitsch and Nitsch, 1967), *Nicotiana* (Tran Thanh Van, 1977) and *Torenia* (Part II, Chapter 1). The effects of growth regulators on floral bud differentiation varied depending on the physiological state of the plant material used. In *Torenia* stem segments, IAA stimulated, and zeatin suppressed, *in vitro* flowering if explants were excised from mother plants which were at the reproductive stage (Part II, Chapter 4). In these experiments, the chemicals in question were applied throughout the whole culture period. As the process of *in vitro* flowering comprises a series of physiological, biochemical and morphological events, including adventitious bud formation, floral bud initiation and flower bud development leading

to anthesis, it is necessary to examine the effects of these various substances on each phase of the developmental process. In this chapter, we first conducted some histological examinations to clarify the time of floral bud initiation. Secondly, we examined the effects on flowering of ammonium nitrate, sucrose, IAA and zeatin, in relation to their application time.

Materials and Methods

Plants of Torenia fournieri Lind. were grown under a long-day regime (16 hr) for 6 weeks after sowing and then transferred to a short-day regime (8 hr). The second stem internodes (counting from the top) of the 12- to 14-week-old plants (reproductive stage) were excised and surface-sterilized. Stem segments, 5 mm length, were cut from the internodes and cultured under sterile conditions. The basal medium used was composed of sucrose (2%) and mineral salts of Murashige and Skoog's medium at 1/5-strength minus the ammonium nitrate.

For histological examination, the apices of adventitious buds were excised at different times, fixed with formalin-acetic acid-alcohol, dehydrated through a series of n-butanol solutions and then embedded in paraffin. Serial sections (10 μm) were cut longitudinally and stained with safranin and fast green.

In order to examine the effects of ammonium nitrate, sucrose, IAA and zeatin at each stage of flower formation, sets of explants were each cultured on medium containing one of these substances during a particular developmental stage, being cultured on the basal medium during the remaining period. The experimental scheme is shown in Fig. 3. Control cultures were also transferred to a fresh basal medium after 2 and 4 weeks of culture like the experimental

explants to eliminate any differences which might be caused by the transfer itself. Data were recorded at the end of a 12-week culture period, based on macroscopic observations of cultured stem segments. Twelve replicates were used for each treatment, and all experiments were repeated at least three times. Other techniques used were as described previously (Part II, Chapter 1).

Results

The formation of adventitious vegetative buds and of floral buds were observed macroscopically after 2 and 5 weeks, respectively, of *in vitro* culture of stem segments (Fig. 1). Microscopic observations of the apices of adventitious buds showed that during the first 2 weeks the apices seemed to remain in a vegetative state (Fig. 2A, 2B). Apical meristems became dome-shaped after 3 weeks of culture (Fig. 2C), and developed into floral primordia at the end of 4 weeks of culture (Fig. 2D). Figure 2E shows a typical example of two differentiated floral primordia arising from an apex on the 33th day of culture. It seems, therefore, the floral bud initiation occurs between the third and fourth weeks after initiation of culture. Based on these histological observations, the process of *in vitro* flowering in *Torenia* stem segments was divided into the following 3 phases: the first phase (first 2 weeks) corresponding to adventitious bud formation, the second phase (3rd and 4th weeks) corresponding to floral bud initiation and the third phase (5th to 12th weeks) corresponding to floral bud development (Fig. 3).

The effects of IAA, zeatin, ammonium nitrate and highly concentrated sucrose added to a basal medium throughout the culture period are summarized in Table 1. The application of ammonium nitrate (330 mg/l) or zeatin (0.1 mg/l)

partially suppressed the initiation and development of floral buds, producing a relatively large number of nodes (4.2 or 4.8 respectively) before floral bud initiation, as compared to the control (2.9). The application of IAA (0.1 mg/l), or a high concentration of sucrose (6%), did not change the number of nodes produced before floral bud initiation, but greatly increased the percentage of cultures with fully developed flowers.

As shown in Table 2, ammonium nitrate given throughout the culture period suppressed the initiation and development of floral buds, while given just during the 3rd phase it only inhibited the development of floral buds.

The effects of the highly concentrated sucrose given during each of the 3 phases were also examined. As shown in Table 3, 6% sucrose given during the 3rd phase stimulated both the initiation and development of floral buds. Most of the differentiated floral buds reached anthesis when 6% sucrose was added to the medium both in the second and the third phases.

The effects of IAA and zeatin given during only the 2nd or the 3rd phase were not consistent. Therefore, we applied these growth regulators either during the first phase or throughout the second and the third phases, and obtained the results shown in Table 4. The application of IAA during the first 2 weeks of culture stimulated the initia-

tion and development of floral buds. About 90 percent of the cultures produced floral buds, and the majority of them (80% or more) reached anthesis. However, IAA given after 2 weeks of culture did not promote floral bud formation. Zeatin inhibited floral bud differentiation if it was added to the medium after 2 weeks of culture, but was less inhibitory if added throughout the culture period. When zeatin was added to the medium only during the first 2 weeks, however, it slightly promoted the initiation and development of floral buds.

Discussion

The four chemicals affected the *in vitro* flowering of the stem segments differently depending on the period of their application. Ammonium nitrate inhibited floral bud development when applied in the third phase (Table 2), but it inhibited flower bud initiation only when applied continually throughout the three phases. Floral bud initiation in *Lemna* was also reported to be inhibited by the addition of ammonium ions to its medium (Kandeler, 1969; Tanaka and Takimoto, 1975), but it is not known whether it would inhibit floral bud development when applied after the floral bud initiation.

High sucrose concentration in the medium acted in a manner opposite to that of ammonium nitrate. It promoted flower development when applied during the third phase, but did not stimulate floral bud initiation when applied during the first and second phases (Table 3). Nitsch and Nitsch (1967) reported that high concentration of various sugars promoted *in vitro* flowering of *Plumbago*, and similar results were obtained in *Cichorium* (Harada, 1966), *Nicotiana* (Trah Thanh Van, 1977) and *Torenia* (Part II, Chapter 1). The results presented here show that high sugar concentration in a medium promotes the development but not the initiation of floral buds.

The inhibitory effects of zeatin on the initiation and

development of floral buds were evident when it was applied after 2 weeks of culture. In *in vitro* cultured organ segments of *Torenia* (Chlyah, 1973) and tobacco (Tran Thanh Van *et al.*, 1974), cytokinin also inhibited floral bud formation. However, if zeatin was applied only during the first phase of culture, the initiation and development of floral buds were promoted, perhaps due to its stimulation of shoot formation from vegetative bud primordia.

The period during which IAA was effective differed from that of the 3 other chemicals. Stimulation of floral bud initiation and development was observed when it was applied in the first phase of the culture. In this period, apices of adventitious buds are still in the vegetative state (Fig. 2A), suggesting that the early process of floral bud initiation (evocation) can be influenced by IAA treatment. As regards the effects on *in vitro* flowering of auxin added to the culture medium, rather contradictory results have been reported by several investigators. For example, auxin was inhibitory for flower formation in *in vitro* cultured *Plumbago* (Nitsch and Nitsch, 1967) and *Torenia* (Chlyah, 1973) explants, but was required in organ segments of tobacco (Wardell and Skoog, 1969; Tran Thanh Van *et al.*, 1974) and *Perilla* (Tanimoto and Harada, 1980). In addition, IAA promoted *in vitro* flowering of *Streptocarpus* (Rossini and Nitsch, 1966) and *Begonia* (Ringe and Nitsch, 1968).

The effects of various chemicals on floral bud formation seem to be highly influenced by the physiological state of the specimen, and that the physiological state of the explants depends largely on their original position on the mother plants and on the physiological state of the mother plants themselves. It will be necessary to examine in detail among such factors as the chemical substances utilized, the conditions of the mother plants and explants cultured, and the developmental stages of the plant specimens in order to make this kind of study as complete as possible.

Table 1. Effects of various chemical substances on *in vitro* floral bud initiation and development of *Torenia* stem segments.

1/5 MS medium with (+) or without (-) NH_4NO_3 (330 mg/l)	Sucrose (%)	Growth regulators (0.1 mg/l)	Percentage of cultures with adventitious buds	Percentage of cultures with floral buds	Percentage of cultures with flowers	Position of 1st floral bud: average node number
- (Control)	2	None	97.4±2.3	87.3±3.2 (89)[a]	39.4±2.0 (45)[b]	2.9
+	2	None	91.3±7.2	44.5±2.5 (48)	2.6±1.5 (5)	4.2
-	6	None	89.3±6.8	81.3±3.4 (91)	76.4±4.5 (94)	2.8
-	2	IAA	96.2±3.2	94.2±4.3 (97)	84.3±5.2 (89)	2.5
-	2	Zeatin	96.4±3.4	42.1±2.1 (43)	16.3±3.1 (38)	4.8

Twelve segments were used for each treatment, and experiments were repeated 8 times. Data was recorded 12 weeks after the beginning of culture. Figures in parentheses indicate [a]the percentage of cultures with floral buds, calculated on the basis of the number of cultures with adventitious buds, and [b]the percentage of cultures with fully developed flowers, calculated on the basis of the number of cultures with floral buds.

Table 2. Effects of ammonium nitrate applied at 3 different culture stages on *in vitro* floral bud initiation and development of *Torenia* stem segments.

Ammonium nitrate (330 mg/l)			Percentage of cultures with		
1-2w[a]	3-4w	5-12w	adventitious buds	floral buds	flowers
-	-	-	94.8±5.0	52.1±6.2 (55)	25.0±11.8 (48)
-	-	+	88.1±11.5	54.2±3.5 (63)	12.5±9.9 (22)
-	+	-	90.7±5.8	61.2±4.1 (67)	29.6±9.8 (49)
-	+	+	96.4±3.6	60.0±2.9 (62)	14.5±9.5 (24)
+	+	+	88.0±7.5	38.0±3.7 (43)	6.0±4.5 (16)
+	+	-	90.7±7.8	66.6±8.6 (73)	25.9±6.2 (39)
+	-	+	92.3±7.4	55.7±9.2 (60)	11.5±1.4 (21)
+	-	-	90.2±2.3	68.6±11.4 (76)	35.3±16.1 (51)

Twelve stem segments were used for each treatment, and experiments were repeated 4 times. Data were recorded 12 weeks after the beginning of culture. For figures in parentheses, see the footnote of Table 1.

[a]Ammonium nitrate present (+) or absent (-) during the 1st and 2nd weeks (1-2w), etc.

Table 3. Effects of sucrose applied at different concentrations and at different culture stages on *in vitro* floral bud initiation and development of *Torenia* stem segments.

Sucrose concentrations			Percentage of cultures with				
			adventitious	floral		flowers	
1-2w[a]	3-4w	5-12w	buds	buds			
+	+	+	86.5±3.6	46.1±1.2	(53)	23.1±7.5	(50)
+	+	+++	76.9±3.2	71.2±3.6	(93)	63.5±6.6	(89)
+	+++	+	85.0±6.9	51.7±3.9	(61)	16.7±0.7	(32)
+	+++	+++	85.0±1.3	75.0±4.3	(88)	71.7±5.9	(96)
+++	+++	+++	67.8±11.1	57.6±13.8	(85)	52.5±7.8	(91)
+++	+++	+	83.1±2.2	47.5±0.8	(57)	13.6±5.3	(29)
+++	+	+++	73.7±11.6	61.4±5.8	(83)	36.8±9.3	(60)
+++	+	+	80.0±10.5	38.3±1.8	(48)	15.0±2.6	(39)

Twelve stem segments were used for each treatment, and repeated 5 times. Data was recorded 12 weeks after the beginning of culture. For figures in parentheses, see the footnote of Table 1.

[a]Sucrose present at 2% (+) or 6% (+++) concentration during the 1st and 2nd weeks (1-2w), etc.

Table 4. Effects of IAA and zeatin applied at different culture stages on *in vitro* floral bud initiation and development of *Torenia* stem segments.

Growth regulators (0.1 mg/l)		Percentage of cultures with					Position of 1st floral bud: average node number
1-2w[a]	3-12w	adventitious buds	floral buds		flowers		
None	None	92.5±0.9	42.5±9.4	(46)	15.0±9.4	(35)	4.0
None	IAA	96.2±3.3	53.8±3.3	(56)	26.9±9.8	(50)	4.0
IAA	None	100	85.7±9.2	(86)	71.4±2.6	(83)	2.7
IAA	IAA	100	96.3±3.6	(96)	76.9±3.6	(80)	2.8
None	Zeatin	100	8.0±6.3	(8)	0	(0)	5.3
Zeatin	None	100	70.6±6.3	(71)	47.1±5.5	(66)	4.2
Zeatin	Zeatin	100	19.9±8.1	(20)	6.7±4.8	(34)	4.8

Twelve stem segments were used for each treatment, and experiments were repeated 3 times. Data was recorded 12 weeks after the beginning of culture. For figures in parentheses, see the footnote of Table 1.

[a]IAA or zeatin, or neither (none), present in the medium during the 1st and 2nd weeks (1-2w), etc.

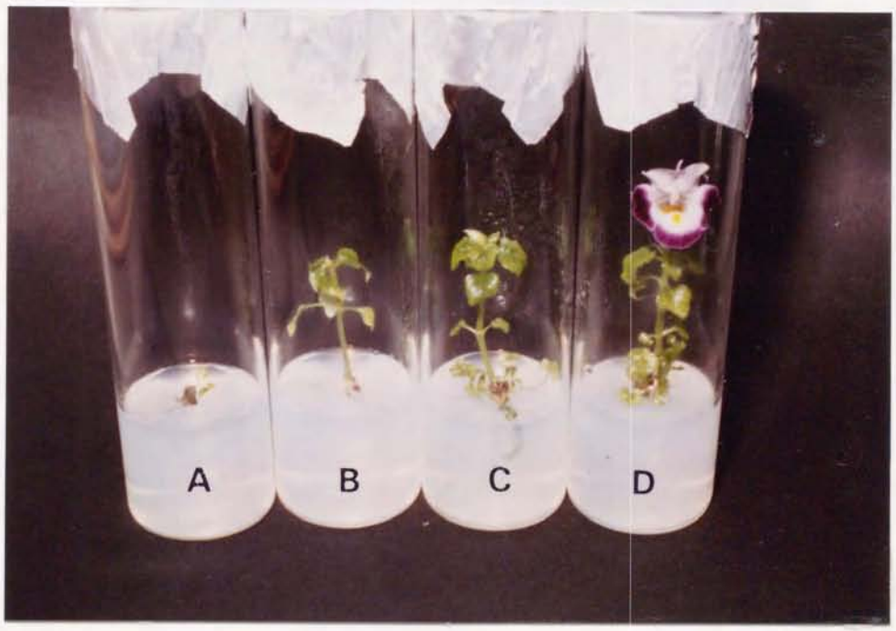


Fig. 1. Serial observations of in vitro floral bud initiation and development in Torenia stem segments. A. Adventitious vegetative bud formation after 2 weeks of culture. B. Development of vegetative shoot in a 4-week-old culture. C. Floral bud formation after 6 weeks of culture. D. Fully bloomed flower in a 8-week-old culture.

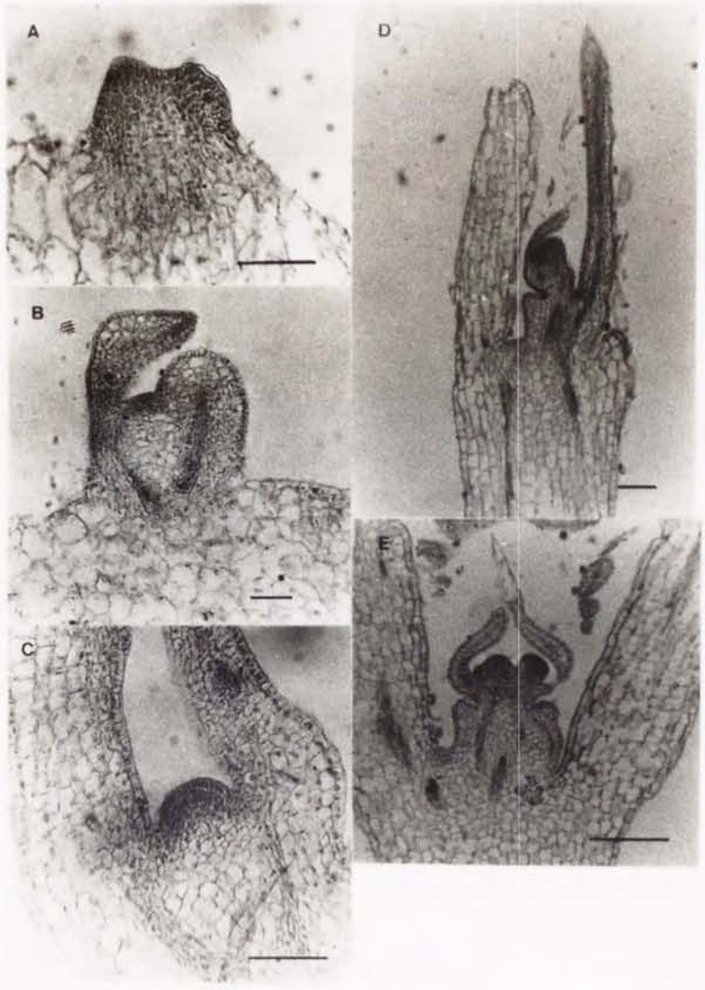


Fig. 2. Histological observations of vegetative and floral bud initiation in in vitro cultured stem segments of Torenia. (Bars represent 0.1 mm) A. Longitudinal section of an adventitious bud differentiated after 15 days of culture. B. An adventitious bud with the first 2 leaves after 18 days of culture. C. Dome-shaped structure in apical meristem after 23 days of culture. D. Well developed dome-shaped structure in apical meristem after 28 days of culture. E. Two flower primordia differentiated after 33 days of culture.

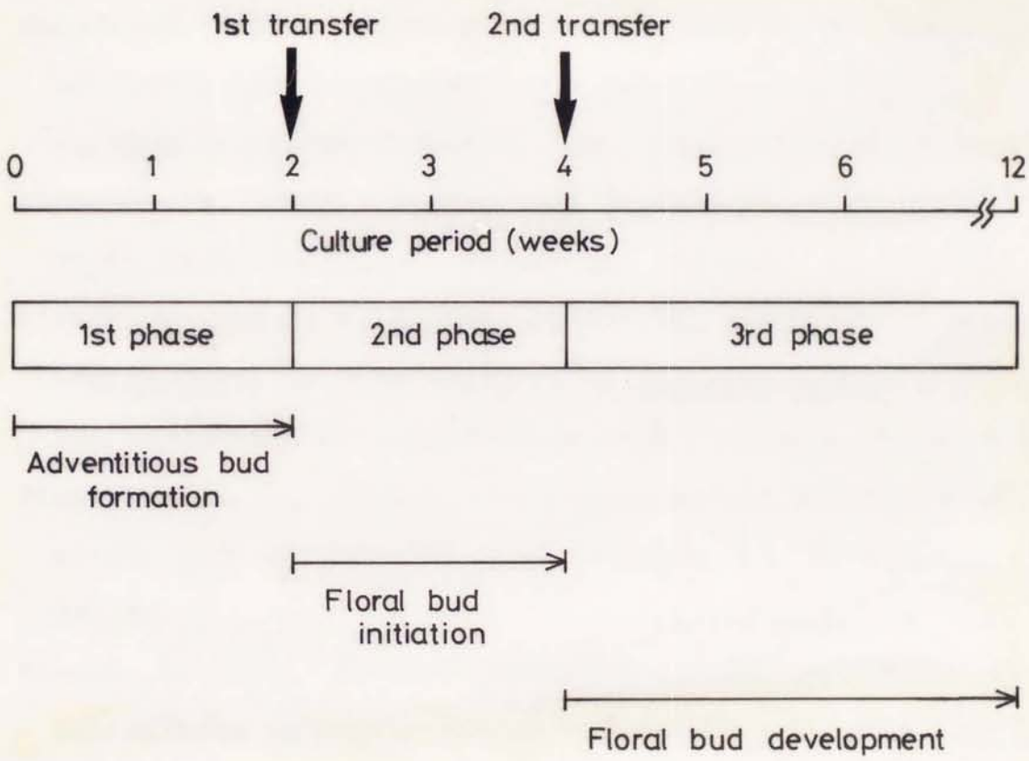


Fig. 3. A scheme showing the process of floral bud differentiation in Torenia stem segments cultured in vitro and the transfer time of explants from one medium to another.

References

Chlyah, H. (1973) Néoformation dirigée à partir de fragments d'organes de Torenia fournieri (Lind.) cultivés in vitro. Biol. Plant. 15; 80-87.

Harada, H. (1966) Effects of photoperiod on the formation of flower buds by flower stalk sections of Cichorium intybus in tissue culture. Bot. Mag. Tokyo 79; 119-123.

Kandeler, R. (1969) Hemmung der Blutenbildung von Lemna gibba durch Ammonium. Planta 84; 279-291.

Nitsch, C. and J. P. Nitsch (1967) The induction of flowering in vitro in stem segments of Plumbago indica L. II. The production of reproductive buds. Planta 72; 371-384.

Pierik, R. L. M. (1966a) The induction and initiation of flower buds in vitro of Lunaria annua L. Naturwiss. 53; 45.

Pierik, R. L. M. (1966b) The induction and initiation of flower buds in vitro in root tissues of Cichorium intybus L. Naturwiss. 53; 387.

Ringe, F. and J. P. Nitsch (1968) Conditions leading to flower formation on excised Begonia fragments cultured in vitro. Plant & Cell Physiol. 9; 639-652.

Rossini, L. M. E. and J. P. Nitsch (1966) Induction de la floraison in vitro chez une plante de jours courts, Streptocarpus nobilis. C. R. Acad. Sci. (Paris) Série D 263; 1379-1382.

Tanaka, O. and A. Takimoto (1975) Suppression of long-day flowering by nitrogenous compounds in Lemna perpusila 6746. Plant & Cell Physiol. 16; 603-610.

Tanimoto, S. and H. Harada (1980) Hormonal control of morphogenesis in leaf explants of Perilla frutescens Britton var. crispa Decaisne f. viridi-crispa Makino. Ann. Bot. 45; 321-327.

Tran Thanh Van, K. M. (1977) Regulation of Morphogenesis. In Plant Tissue Culture and its Bio-technological Application. Edited by W. Barz, E. Reinhard and M. H. Zenk. pp. 367-385. Springer-Verlag, Berlin.

Tran Thanh Van, K. M., N. Thi Dien and A. Chlyah (1974) Regulation of organogenesis in small explants of superficial tissue of Nicotiana tabacum L. Planta 119; 149-159.

Wardell, W. L. and F. Skoog (1969) Flower formation in excised tobacco stem segments. I. Methodology and plant hormones. Plant Physiol. 44; 1402-1406.

Chapter 3

Influences of environmental and physiological conditions on floral bud differentiation

Summary

Internodal segments of Torenia differentiated floral buds when they were cultured on a defined nutrient medium lacking ammonium nitrate. Two environmental factors, temperature and light, strongly affected the floral bud formation. A temperature of 25±2 °C, 8 hr short day photoperiod, and a light intensity of 5,000 lux gave the best flowering response. Physiological factors such as the size of explants, the age of mother plants and the stem portions from which the explants were taken, also influenced floral bud differentiation. The highest percentage of floral bud formation was obtained with 4 mm long explants prepared from the 2nd stem internodes (counting from the top) of 12 week old mother plants (reproductive stage), of which 91% of cultures produced floral buds.

Introduction

In studies of flowering, many investigators have attempted *in vitro* flower induction using organ fragments of various plant species. Floral bud initiation in cultured explants was largely influenced by some chemical, environmental and physiological factors. In *Nicotiana tabacum*, the degree of *in vitro* differentiation of floral buds from epidermal cells depended on the physiological state of mother plants (Tran Thanh Van *et al.*, 1974) and also on the part of stems of mother plants from which explants were excised (Tran Thanh Van, 1973). This means that the flower forming potentiality of cells must partly depend on physiological state of tissues used. In *Torenia fournieri*, floral buds can be differentiated from excised leaves cultured on the Murashige and Skoog's medium containing indoleacetic acid and kinetin (Bajaj, 1972), and from leaf discs and stem segments cultured on modified Hoagland's medium (Chlyah, 1973). However, little investigation has been carried out on this material concerning precise environmental and physiological conditions required for *in vitro* floral bud differentiation. In this chapter, we report some effects of environmental and physiological conditions on flower induction in internodal segments of *Torenia* and discuss the potentiality of cells for floral bud differentiation.

Materials and Methods

Plants of Torenia fournieri Lind. were grown under a short day condition (8 hr) at a temperature of 25±2 °C. Unless otherwise stated, 4 mm long segments were taken from the second stem internodes (counting from the top) of mother plants (reproductive stage) and cultured under sterile conditions.

These cultures were maintained in a growth room and exposed to various conditions. To test the effects of temperature, experiments were conducted under 5 different temperatures (15, 20, 25, 30 and 35 °C) with a single light condition of 8 hr light (3,000 lux). When the influences of light were investigated, temperature was always set at 25±2 °C. Other techniques used are the same as those described previously (Part II, Chapter 1).

All experiments consisted of 12 replicates and were repeated at least three times. Data were recorded at different intervals throughout culture periods in parallel with the macroscopical observation. The results were expressed as an average percentage of cultures producing vegetative and floral buds, an average number of nodes formed before flower initiation and the culture time required for the appearance of the first visible floral bud in each lot.

Results

Effects of environmental conditions on the floral bud differentiation

The effects of two environmental factors, temperature and light, were investigated. Four mm long segments were excised from the 2nd internodes of 12-14 week old flowering plants. All cultures were maintained under short day photoperiod (8 hr light, ca. 3,000 lux) but treated with five different temperatures namely, 15, 20, 25, 30 and 35 °C. The results obtained are presented in Table 1. At a temperature of 15 °C, occasional callus formation was observed, and no bud initiation was noted. As the temperature raised, bud formation was induced and its rate attained 100% at 30 °C. However, when the cultures were maintained at 30 °C, regenerated shoots were slender (Fig. 1A), and barely 14% of cultures produced floral buds after forming 6 nodes, and the culture time required for the appearance of the first visible floral bud was twice as long as the one required at 25 °C. The highest percentage of floral bud differentiation was obtained at 25 °C and 75% of cultures produced floral buds after forming 3-4 nodes.

The effects of light conditions, daylength and light intensity, on floral bud differentiation were studied at a constant temperature of 25±2 °C. The results (Table 2)

showed that the percentages of explants forming floral buds were higher with the explants grown under short days than the ones grown under long days or continuous illumination.

High rates of floral bud differentiation were observed when cultured under a light intensity of 5,000 lux, and a high light intensity of 12,000 lux completely inhibited floral bud formation under both daylengths. Subsequently, in the following experiments, cultures were maintained at 25±2 °C under 8 hr short day photoperiod with a light intensity of 5,000 lux.

Effects of physiological state of explants on floral bud differentiation

Different physiological factors such as the size (length) of explants, the age of mother plants, and the portion of stems from which explants were taken, also affected floral bud differentiation. First, the correlation between the length of explants and the rate of floral bud differentiation was examined. Explants were excised from the 2nd internodes of 14 week old, fully bloomed plants. As shown in Table 3, 1 mm long segments were unable to differentiate adventitious bud, but the segments longer than 2 mm initiated buds at high rates. Two to 8 mm long segments formed floral buds, and the average number of nodes formed before flowers and the culture period required for floral bud

initiation did not differ much. Ten to 12 mm long segments produced no floral buds, though all explants initiated vegetative buds which developed well.

The effects of the age of mother plants were also investigated. Developmental stages of mother plants used were as follows: 2 week old plants consisted of a hypocotyl and a pair of cotyledons; the 1st leaves unfolded at the 4th week; 8 week old plants had 4 pairs of unfolded leaves; floral buds became visible during 12th week: flowered at the 14th week and flower abscission occurred during the 18th week after germination. Explants were excised from hypocotyl of 2 week old plants and the 2nd internodes (longer than 1 cm) of 4 to 18 week old plants. In all cases (Table 4), explants initiated floral buds, but the culture periods required for floral bud formation significantly differed depending on the age of mother plants. In the case of segments taken from 2 week old plant, floral buds became visible only after 18 weeks of culture, while only 5 weeks were necessary for the explants taken from 12 week old plants. The average number of nodes formed before flowers was 7 with the segments excised from 2 week old plants compare to 3.1 with the explants prepared from 12 week old plants (Fig. 1B). Explants excised from 12 to 16 week old plants exhibited high rates of floral bud differentiation.

As shown in Table 5, a gradient of floral bud forming capacity along the stem was noted. The internodal segments taken from the lst and the 2nd stem internodes exhibited high rates of floral bud formation than ones taken from the 3rd to 7th internodes. The highest rate was obtained with the explants prepared from the 2nd internodes.

Discussion

The following conditions induced the highest rate of floral bud differentiation, namely, explants of 4 mm long excised from the 2nd stem internodes of 12 week old flower-forming plants and cultured at 25±2 °C under 8 hr short day photoperiod with a light intensity of 5,000 lux. When these conditions were met, the explants initiated shoot buds in the end of two week culture (Fig. 1C) and the shoots rapidly developed attaining a height of 5 cm in five weeks. Floral buds were observed macroscopically on the shoot apices after 5-6 weeks of culture (Fig. 1D) and flowered normally 8-9 weeks after the beginning of culture (Fig. 1E).

Nitsch and Nitsch (1967a,b) distinguished two aspects of the flower formation *in vitro*; 1. the expression of physiologically induced state already present in plant tissues, and 2. the flower induction and development in the explants taken from completely vegetative materials and maintained under non-inductive conditions. The case of *Torenia* presented here, falls in the first category. Tran Thanh Van *et al.*(1974) reported that stem explants excised from tobacco plants of which flowers were in the process of fructification, formed floral buds most readily. When the flowers of a mother plant were in full bloom, explants did not form floral buds. In our experiments,

the highest rate of floral bud differentiation was obtained with the explants excised from mother plants which were just initiating flower buds. The physiological state of mother plants seems to be one of important factors for in vitro floral bud formation in many species. The difference in the rate of floral bud formation observed with explants taken from various portions of a stem revealed the presence of a gradient along a stem of floral bud forming capacity. This kind of gradient was already reported by several investigators in Nicotiana (Aghion-Prat, 1965; Wardell and Skoog, 1969; Tran Thanh Van, 1973; Hillson and LaMotte, 1977). In this chapter, we showed that flowering Torenia stems also maintain and exhibit a similar gradient.

Explants of 2 to 8 mm long were capable of differentiating floral buds. It may be presumed that 2 mm long explants are large enough to retain a necessary amount of unknown flower initiating substance(s) or a particular balance of different substances favorable for flower initiation. If it is so, why 10 and 12 mm long segments produced no floral buds? The ratio of the area of cut-ends to the volume of an explant may explain this situation. In the case of short explants, substance(s) inhibitory for floral bud formation might be more readily released compare to long ones, through the cut-ends of explants into culture medium, making the balance of flower inducing and suppressing

substances more favourable for flowering.

Aghion-Prat (1965) found that floral buds can be formed on stem segments of Nicotiana cultured in vitro without an addition of auxin in medium, while Tran Thanh Van et al. (1974) showed that both auxin and cytokinin were necessary for floral bud formation from the epidermal strips of the same species. This difference may be explained by a possibility that the stem segments contain relatively large amounts of endogenous growth regulators compare to the epidermal strips. In our experiments, internodal segments of Torenia produced floral buds in the absence of growth regulators in culture medium. It seems that a sufficient amount of substance(s) stimulatory for floral bud differentiation may be accumulated in the tissues of Torenia stems used under our growing conditions of mother plants.

Table 1. Effects of temperature on floral bud formation.

Temperature (°C)	Percentage of cultures with		Position of 1st floral bud: average node number	No. of weeks required for the 1st visible floral bud formation
	adventitious buds	floral buds		
15	0	-	-	-
20	40	20	3.2	8
25	83	75	3.6	6
30	100	14	6.0	12
35	100	0	-	-

Four mm long explants were excised from the 2nd internodes of 12-14 week old plants at reproductive stage. Cultures were maintained under 8 hr light (3,000 lux)/16 hr dark.

Table 2. Effects of daylength and light intensity on floral bud initiation.

Day length (light/dark)	Light intensity (lux)	Percentage of cultures with		Position of 1st floral bud: average node number
		adventitious buds	floral buds	
8 hr/16 hr	1,500	73	21	3.0
	5,000	84	71	3.4
	8,000	82	56	3.3
	12,000	100	0	-
16 hr/8 hr	1,500	70	8	3.5
	5,000	87	24	4.0
	8,000	87	15	4.3
	12,000	100	0	-
24 hr/0 hr	1,500	82	3	5.0
	5,000	88	16	5.2
	8,000	100	3	4.8
	12,000	100	0	-

Four mm long explants were excised from the 2nd internodes of 12-14 week old plants at reproductive stage. Cultures were maintained at 25 ± 2 °C.

Table 3. Correlation between the length of explants and the capacity of floral bud initiation.

Length of explants (mm)	Percentage of cultures with adventitious buds	Percentage of cultures with floral buds	Position of 1st floral bud: average node number
1	0	-	-
2	82	56	4.8
3	75	53	3.9
4	95	60	4.0
6	100	45	4.3
8	100	33	4.0
10	100	0	-
12	100	0	-

Cultures were maintained under 8 hr light (5,000 lux)/16 hr dark at 25±2 °C. Explants were excised from the 2nd internodes of 14 week old plants at reproductive stage.

Table 4. Effects of the age of mother plants on floral bud initiation.

Age of mother plants (weeks)	Percentage of cultures with		Position of 1st floral bud: average node number	No. of weeks required for the 1st visible floral bud formation
	adventitious buds	floral buds		
2	93	3	7.0	18
4	89	13	4.6	12
6	100	28	5.9	10
8	100	48	4.7	6
10	92	46	4.1	6
12	96	91	3.1	5
14	94	68	3.6	6
16	100	72	4.0	9
18	95	35	3.9	9

Cultures were maintained under 8 hr light (5,000 lux)/16 hr dark at 25 ± 2 °C. Four mm long explants were excised from the 2nd internodes of mother plants of different ages.

Table 5. Effects of different stem portions of mother plants on floral bud initiation.

Origin of explants	Percentage of cultures with		Position of 1st floral bud: average node number	No. of weeks required for the 1st visible floral bud formation
	adventitious buds	floral buds		
1st internode	100	50	3.5	10
2nd internode	84	67	3.2	5
3rd internode	91	31	4.3	9
4th internode	79	24	4.0	9
5th internode	69	29	4.0	9
6th internode	86	32	5.0	9
7th internode	64	15	5.4	9

Cultures were maintained under 8 hr light (5,000 lux)/16 hr dark at 25±2 °C. Four mm long explants were excised from different parts of stems of 14-16 week old plants at reproductive stage.

Fig. 1. Morphogenesis in stem segments of Torenia fournieri cultured in vitro. Four mm long explants were excised from the 2nd internodes of 12-14 week old plants (A, C, D and E). Cultures were maintained under 8 hr (A, B, C and D) with a light intensity of 3,000 lux (A) or 5,000 lux (B, C, D and E) at 30 °C (A) or 25 °C (B, C, D and E).

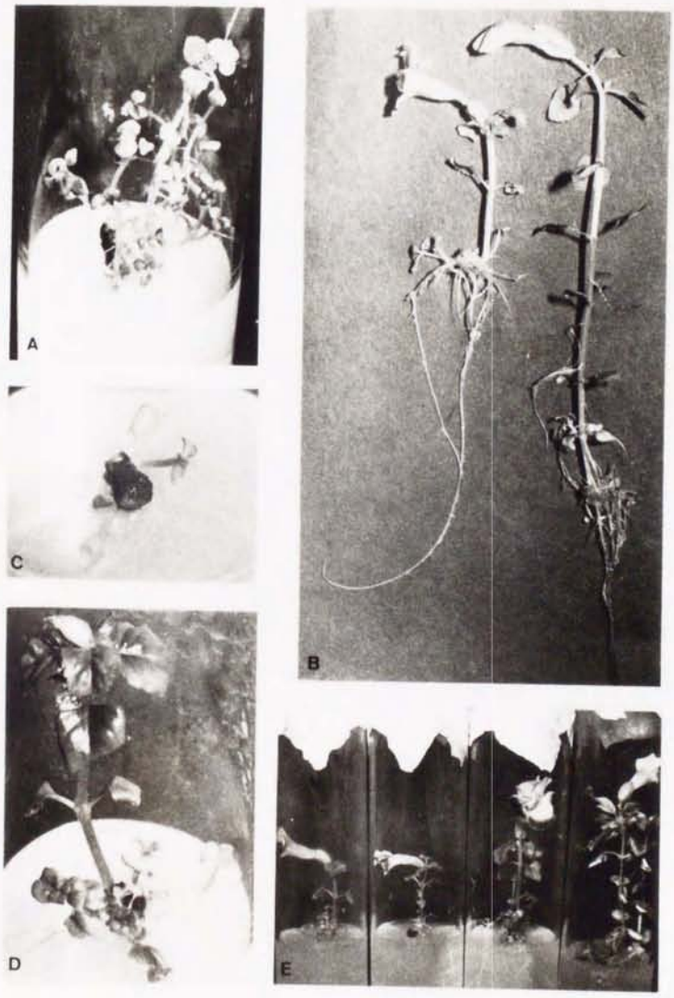
A. Slender shoots developed at a temperature of 30 °C. (x 1).

B. Difference in number of nodes formed before flowers. (x 1).
Left: a flowering shoot developed from an explants prepared from a 12 week old mother plant.
Right: a flowering shoot developed from an explants prepared from a 2 week old mother plant.

C. Shoot buds initiated at the end of 2 week culture. (x 2.5).

D. Floral bud formation after 6 weeks of culture. (x 2).

E. Flowering after 9 weeks of culture. Two cultures in the left were maintained under 8 hr light and two in the right were maintained under 16 hr light. (x 0.6).


A
B
C
D
E

References

Aghion-Prat, D. (1965) Néoformation de fleurs in vitro chez Nicotiana tabacum L. Physiol. Vég. 3; 229-303.

Bajaj, Y. P. S. (1972) Effects of some growth regulators on bud formation by excised leaves of Torenia fournieri. Z. Pflanzenphysiol. 66; 284-287.

Chlyah, H. (1973) Néoformation dirigée à partir de fragments d'organes de Torenia fournieri (Lind.) cultivés in vitro. Biol. Plant. 15; 80-87.

Hillson, T. D. and C. E. LaMotte (1977) In vitro formation and development of floral buds on tobacco stem explants. Plant Physiol. 60; 881-884.

Nitsch, C. and J. P. Nitsch (1967a) The induction of flowering in vitro in stem segments of Plumbago indica L. I. The production of vegetative buds. Planta 72; 335-370.

Nitsch, C. and J. P. Nitsch (1967b) The induction of flowering in vitro in stem segments of Plumbago indica L. II. The production of reproductive buds. Planta 72; 371-384.

Tran Thanh Van, K. M. (1973) Direct flower neoformation from superficial tissue of small explants of Nicotiana tabacum L. Planta 115; 87-92.

Tran Thanh Van, K. M., N. Thi Dien and A. Chlyah (1974) Regulation of organogenesis in small explants of superficial tissue of Nicotiana tabacum L. Planta 119; 149-159.

Wardell, W. L. and F. Skoog (1969) Flower formation in excised tobacco stem segments I. Methodology and effects of plant hormones. Plant Physiol. 44; 1402-1406.

Chapter 4

Correlative effects of phytohormones and physiological factors on floral bud differentiation

Summary

Correlative effects between growth regulators added to a medium and different physiological states of explants on adventitious bud formation and flowering were investigated using Torenia stem segments cultured in vitro. Indoleacetic acid stimulated floral bud formation and its development in explants taken from reproductive plants. These stimulative effects were clearly seen in explants taken from plants in which flower abscission was taking place, but insignificant when explants were prepared from younger materials. Abscisic acid acted in a reverse way to auxin, greatly promoting floral bud initiation and flowering of originally vegetative explants. Zeatin at a concentration of 1 mg/l inhibited floral bud formation, and at a low concentrations it was generally ineffective. However, floral bud initiation and flowering of explants taken either from basal parts of stems or from 18- to 20-week-old plants were promoted by zeatin treatment. The action of gibberellic acid seemed rather indirect: at a

concentration of 0.01 mg/l, it generally stimulated floral but initiation but at a concentration of 1 mg/l, it was often inhibitory.

Introduction

In studies on *in vitro* flowering, many workers have investigated the effects of various growth regulators added to a culture medium, and sometimes their results seemed to be inconsistent or difficult to explain. For example, floral buds were induced on stem segments of tobacco cultured *in vitro* without growth regulators (Aghion-Prat, 1965), while floral bud formation from epidermal strips of the same species required both auxin and cytokinin (Tran Thanh Van *et al.*, 1974). These differences in the requirement of growth regulators seem to be due, at least partially, to the difference in the physiological state of explants. Therefore, we planned a series of experiments in which *Torenia* stem segments of different physiological states were used as test materials. We reported previously some physiological factors closely related to the *in vitro* floral bud formation of *Torenia* (Part II, Chapter 3). A gradient of floral bud forming capacity along the stem was noted and different developmental stages of mother plants also influenced floral bud differentiation. It seems worth-while to examine the effects of growth regulators using the same plant materials in different developmental stages.

Another problem to be considered in this kind of study is related to the composition of culture medium. In order

to induce floral buds on explants, adventitious vegetative buds should first be differentiated except a few cases, and this process generally requires the presence in a medium, of growth regulators such as auxin and cytokinin. Addition of these chemicals to a culture medium tends to complicate investigation of the physiological process of *in vitro* flowering. In the Chapter 1 of the 2nd Part, we reported that *Torenia* stem segments produced floral buds *in vitro* when cultured on the medium composed of only mineral salts and sugar. In this chapter we mainly report the effects of growth regulators such as auxin, cytokinin, gibberellic acid and abscisic acid on *in vitro* floral bud initiation and development of *Torenia* stem segments which were in different physiological states. A simple culture medium was used to make the interpretation of results obtained easier.

Materials and Methods

Plants of Torenia fournieri Lind. were grown under a long-day condition (16 hr) for the first 6 weeks and then transferred to a short-day condition of 8 hr light period. Developmental stages of mother plants grown under these conditions were as follows: 10-week-old plants were still in vegetative state, floral buds became visible during the 12th week, flowered at the 14th week and flower abscission occurred during the 19th week after germination. Internodal stem segments were excised from apical parts of stems of mother plants in each stage and cultured in a sterile condition (in the case of 15- to 16-week-old plants, both apical and basal parts of stems were used).

Aqueous solutions of IAA, Z, GA_3 and ABA were sterilized through a Millipore filter (0.45 μm) and added to a basal medium composed of 1/5-strength mineral salts of Murashige and Skoog's medium without ammonium nitrate. Twelve replicates were used in all experiments which were repeated at least three times. Other techniques used are the same as those described in the preceding chapter (Part II, Chapter 1).

Results

Effects of growth regulators on floral bud initiation and their development were examined using explants obtained from the apical portions of stems of 5- to 6-week-old vegetative plants (Table 1). The percentage of cultures producing floral buds from these explants was the lowest among the series tested. With no additives, barely 22% of the cultures formed floral buds and only 4% developed full flowers. Addition of 1 mg/l of Z to the basal medium strongly inhibited for floral bud formation, whereas IAA (0.1-1.0 mg/l) and GA_3 (0.01-0.1 mg/l) promoted floral bud formation. A significant effect was obtained by the treatment with ABA: the percentage of cultures with floral buds was about three times as high as the control, in addition, 0.1 mg/l of ABA also stimulated development of flowers.

Similar treatments were applied to explants taken from 12- to 14-week-old plants (Table 2). In this case, more than 80% of control cultures produced floral buds, the highest rate among the different developmental stages examined. Zeatin, at a concentration of 1 mg/l, inhibited floral bud formation. ABA, at a concentration of 1 mg/l, suppressed adventitious bud formation. Treatment with IAA (0.1 mg/l) and GA_3 (0.01 mg/l) increased the rate of explants with fully developed flowers.

Correlation between the effects of growth regulators and the gradient of flowering capacity along the stem was also investigated. Explants were excised from both apical and basal parts of stems of 15- to 16-week-old flowering plants. The results for apical stem segments (Table 3) show responses more or less comparable to those obtained with the 12- to 14-week-old materials (Table 2), except for ABA and IAA at 1 mg/l where adventitious bud formation was completely (ABA) or strongly (IAA) inhibited. When explants were excised from basal parts of stems (Table 4), all growth regulators used at a concentration of 1 mg/l exhibited similar inhibitory action on floral bud formation. Effects of Z on floral bud formation were interesting, promoting floral bud formation at 0.01 mg/l (81%) and at 0.1 mg/l (65%) or completely suppressing it at 1 mg/l.

A further experiment was carried out using 18- to 20-week-old plants in which flower abscission was taking place. Results are summarized in Table 5. IAA, Z, and GA_3 at concentrations of 0.01 and 0.1 mg/l promoted floral bud formation and its subsequent development, but 1 mg/l of each compound had little effect. ABA (0.1 and 1 mg/l) suppressed both adventitious and floral bud formation.

Discussion

The physiological stages of our experimetal materials, Torenia stem segments, can be divided into the following three ones: 1. vegetative stage, at which the segments show a low floral bud forming capacity on the basal medium without growth regulators; 2. reproductive stage, at which the segments show a high flower differentiating capacity; and 3. aged stage (including the stem segments taken from the basal part of reproductive materials), at which the segments have lost their flower differentiating capacity. The effects of growth regulators on floral bud formation vary depending on these physiological stages. For example, ABA application enhanced the rate of cultures bearing floral buds and flowers only with vegetative explants, and stimulative effects of Z (0.01 and 0.1 mg/l) were exhibited only with aged materials. Reasons for differences in the response of explants in various physiological states to growth regulators are not well understood and more studies in this field are expected.

We will discuss our results in the light of various data published by other authors.

Auxin--Inhibition of flower formation by an application of auxin has been reported with a number of plant species using intact plants (review by Zeevaart, 1978). On excised plant organs cultured in vitro, auxin also inhibited flower

formation in Plumbago (Nitsch and Nitsch, 1967) and Torenia (Chlyah, 1973). On the other hand, floral bud formation on tobacco stem segments (Wardell and Skoog, 1969) and epidermal strips (Tran Thanh Van et al., 1974) required the presence of IAA (1 μM) in a medium, but higher levels of IAA were inhibitory. IAA greatly promoted in vitro floral bud formation in Streptocarpus (Rossini and Nitsch, 1966) and Begonia (Ringe and Nitsch, 1968). It should be noted that in the case of Plumbago and Torenia, explants were taken from mother plants in the vegetative stage, while floral stalks were used as explants in Begonia and tobacco. It seems, therefore, that the treatment with IAA suppresses in vitro flowering, if explants in vegetative state are used, and stimulates it, if explants are in reproductive state. In our experiments, the promotive effects of IAA on floral bud formation and its subsequent development were observed in most of the explants examined, but the effects was prominent in the explants at an aged stage (Table 5). Wardell and Skoog (1969) reported that IAA was required for normal development of flower buds differentiated on tobacco stem segments cultured in vitro.

Cytokinin--Using organ fragments taken from 45-day-old vegetative Torenia plants, Chlyah (1973) reported that BA prevented floral bud formation as its concentration in a medium increased. A similar response to Z was

observed with our 5- to 6-week-old materials. A high cytokinin concentration in a medium was inhibitory but its presence in a medium at a low concentration was favourable for in vitro flowering of explants excised from floral stalks of tobacco (Tran Thanh Van et al., 1974) and from aged Torenia stems (Table 5). Cytokinin requirement was also shown in the case of Plumbago (Nitsch and Nitsch, 1967), Streptocarpus (Rossini and Nitsch, 1966) and Perilla (Tanimoto and Harada, 1980). In cultures of floral stalk fragments of Begonia (Ringe and Nitsch, 1968), floral buds were produced with the addition of BA and adenine. The exact role of cytokinin on in vitro flowering is not clear, but our results show that the effects of cytokinin varied depending on the physiological state of explants.

Gibberellic acid--GA_3 is known to be capable of inducing floral buds in cold-requiring plants such as Chrysanthemun morifolium cv. Shuokan (Harada and Nitsch, 1959) and in long-day plants such as Rudbeckia (Harada, 1963) under non-inductive conditions. In short-day plants, GA_3 usually can not induce flower formation under non-inductive conditions. In cultures of excised organ fragments, inhibitory effects of GA_3 have been observed in Plumbago (Nitsch and Nitsch, 1967) and Streptocarpus (Rossini and Nitsch, 1966). In the case of Torenia which is a quantitative

short-day plant, GA_3 treatment at high concentrations tends to be inhibitory and at low concentrations stimulatory, the degree depending on the physiological state of explants.

Abscisic acid--Except a few cases, the effects of ABA on flowering have not been yet fully investigated. Harada et al. (1971) described that an ABA application could not induce flower initiation of Pharbitis under a strictly non-inductive, long-day condition, but it was stimulatory when the plants were slightly induced. In vitro flowering of Plumbago (Nitsch and Nitsch, 1967) was promoted by ABA treatment under an inductive photoperiod. Stimulation of floral bud formation in Torenia stem segments by ABA treatment was observed only in young vegetative materials. These promotive effects of ABA seem to take place as a result of the suppression of vegetative growth.

Conclusion--Different effects of the growth regulators used on floral bud initiation and its development are in a close relation with the physiological state of Torenia stem segments used as explants. It is necessary to carry out more detailed investigation of hormonal action on flowering, keeping in mind the importance of the physiological state of experimental materials used.

Table 1. Effects of growth regulators on *in vitro* adventitious and floral bud formation in apical stem segments of vegetative *Torenia* plants.

Compounds	Conc. (mg/l)	Percentage of cultures with adventitious buds	floral buds		flowers	
None		98	22	(22)	4	(18)
IAA	0.01	81	26	(32)	0	(0)
	0.1	100	43	(43)	3	(7)
	1.0	52	26	(50)	0	(0)
Z	0.01	100	28	(28)	0	(0)
	0.1	100	19	(19)	3	(16)
	1.0	91	3	(3)	0	(0)
GA_3	0.01	93	57	(61)	14	(25)
	0.1	93	40	(43)	3	(8)
	1.0	90	14	(16)	0	(0)
ABA	0.01	100	65	(65)	3	(5)
	0.1	97	62	(64)	21	(34)
	1.0	65	62	(95)	15	(24)

Explants were taken from 5-6 weeks old plants. Twelve segments were used for each treatment and repeated 4 times. Figures in parentheses indicate the percentage of cultures with floral buds, calculated on the basis of the number of cultures with adventitious buds (middle column), and the percentage of cultures with fully developed flowers, calculated on the basis of the number of cultures with floral buds (right column).

Table 2. Effects of growth regulators on in vitro adventitious and floral bud formation in apical stem segments of reproductive Torenia plants.

Compounds	Conc. (mg/l)	Percentage of cultures with adventitious buds	floral buds		flowers	
None		98	83	(85)	40	(48)
IAA	0.01	92	79	(86)	53	(58)
	0.1	97	94	(97)	84	(89)
	1.0	76	57	(75)	52	(91)
Z	0.01	100	91	(91)	41	(45)
	0.1	97	65	(67)	24	(37)
	1.0	96	4	(4)	4	(100)
GA_3	0.01	84	81	(96)	68	(84)
	0.1	85	65	(76)	29	(34)
	1.0	88	58	(66)	13	(22)
ABA	0.01	97	83	(86)	50	(60)
	0.1	89	71	(80)	40	(56)
	1.0	53	42	(79)	26	(62)

Explants were taken from 12-14 weeks old plants with floral buds. Twelve segments were used for each treatment and repeated 5 times. For figures in parentheses, see the footnote of Table 1.

Table 3. Effects of growth regulators on *in vitro* adventitious and floral bud formation in apical stem segments of reproductive *Torenia* plants.

Compounds	Conc. (mg/l)	Percentage of cultures with adventitious buds	floral buds		flowers	
None		83	65	(78)	40	(62)
IAA	0.01	85	76	(89)	54	(71)
	0.1	92	78	(85)	70	(90)
	1.0	26	17	(65)	13	(76)
Z	0.01	95	73	(77)	27	(37)
	0.1	98	88	(90)	48	(55)
	1.0	83	13	(16)	4	(31)
GA_3	0.01	82	64	(78)	46	(72)
	0.1	66	46	(70)	34	(74)
	1.0	87	39	(45)	9	(23)
ABA	0.01	80	68	(85)	44	(65)
	0.1	63	55	(87)	35	(64)
	1.0	0	-		-	

Explants were taken from apical parts of stems in 15-16 weeks old plants which were in full bloom. Twelve segments were used for each treatment and repeated 5 times. For figures in parentheses, see the footnote of Table 1.

Table 4. Effects of growth regulators on *in vitro* adventitious and floral bud formation in basal stem segments of reproductive *Torenia* plants.

Compounds	Conc. (mg/l)	Percentage of cultures with adventitious buds	floral buds		flowers	
None		65	34	(52)	15	(44)
IAA	0.01	47	33	(70)	20	(61)
	0.1	50	44	(88)	19	(43)
	1.0	20	10	(50)	0	(0)
Z	0.01	100	81	(81)	63	(78)
	0.1	90	65	(72)	30	(46)
	1.0	45	0	(0)	-	
GA_3	0.01	56	33	(59)	22	(67)
	0.1	55	35	(64)	15	(43)
	1.0	73	7	(10)	7	(100)
ABA	0.01	38	38	(100)	15	(39)
	0.1	0	-		-	
	1.0	0	-		-	

Explants were taken from basal parts of stems in 15-16 weeks old plants which were in full bloom. Twelve segments were used for each treatment and repeated 3 times. For figures in parentheses, see the footnote of Table 1.

Table 5. Effects of growth regulators on *in vitro* adventitious and floral bud formation in apical stem segments of aged *Torenia* plants.

Compounds	Conc. (mg/l)	Percentage of cultures with adventitious buds	floral buds		flowers	
None		75	34	(45)	3	(9)
IAA	0.01	91	81	(89)	56	(69)
	0.1	69	61	(88)	53	(87)
	1.0	30	18	(60)	10	(56)
Z	0.01	95	61	(64)	21	(34)
	0.1	93	50	(54)	25	(50)
	1.0	67	36	(54)	17	(47)
GA_3	0.01	72	59	(82)	38	(64)
	0.1	68	47	(69)	34	(72)
	1.0	91	32	(35)	29	(91)
ABA	0.01	73	46	(63)	8	(17)
	0.1	41	14	(34)	0	(0)
	1.0	11	0	(0)	-	

Explants were taken from 18-20 weeks old plants, in which flower abscission was taking place. Twelve segments were used for each treatment and repeated 3 times. For figures in parentheses, see the footnote of Table 1.

References

Aghion-Prat, D. (1965) Néoformation de fleurs *in vitro* chez *Nicotiana tabacum* L. Physiol. Vég. 3; 229-303.

Chlyah, H. (1973) Néoformation dirigée à partir de fragments d'organes de *Torenia fournieri* (Lind.) cultivés *in vitro*. Biol. Plant. 15; 80-87.

Harada, H. (1962) Etudes des substances naturelles de croissance en relation avec la floraison--Isolement d'une substance de montaison. Rev. Gén. Bot. 69; 201-297.

Harada, H., T. K. Bose and J. Cheruel (1971) Effects of four growth regulating chemicals on flowering of *Pharbitis nil*. Z. Pflanzenphysiol. 64; 267-269.

Harada, H. and J. P. Nitsch (1959) Flower induction in Japanese chrysanthemums with gibberellic acid. Science 129; 777-778.

Nitsch, C. and J. P. Nitsch (1967) The induction of flowering *in vitro* in stem segments of *Plumbago indica* L. II. The production of reproductive buds. Planta 72; 371-384.

Ringe, F. and J. P. Nitsch (1968) Conditions leading to flower formation on excised *Begonia* fragments cultured *in vitro*. Plant & Cell Physiol. 9; 639-652.

Rossini, L. M. E. and J. P. Nitsch (1966) Induction de la floraison *in vitro* chez une plante de jours courts,

Streptocarpus nobilis. C. R. Acad. Sci. (Paris) Série D 263; 1379-1382.

Tanimoto, S. and H. Harada (1980) Hormonal control of morphogenesis in leaf explants of Perilla frutescens Britton var, crispa Decaisne f. viridi-crispa Makino. Ann. Bot. 45; 321-327.

Tran Thanh Van, K. M., N. Thi Dien and A. Chlyah (1974) Regulation of organogenesis in small explants of superficial tissue of Nicotiana tabacum L. Planta 119; 149-159.

Wardell, W. L. and F. Skoog (1969) Flower formation in excised tobacco stem segments. I. Methodology and effects of plant hormones. Plant Physiol. 44; 1402-1406.

Zeevaart, J. A. D. (1978) Phytohormones and flower formation. In Phytohormones and Related Compounds--A Comprehensive Treatise. Vol. 2. Edited by D. S. Letham, P. B. Goodwin and T. J. V. Higgins pp. 291-327. Elsevier/North-Holland Biomedical Press, Amsterdam.

General Conclusion

As we discussed in each of 8 foregoing chapters, the following conclusion can be drawn from the present studies on adventitious bud differentiation and flower formation in Torenia stem segments cultured in vitro.

I. Effects of cytokinin on the formation of MZ and adventitious buds

The treatment of excised stem segments of Torenia with cytokinin significantly promoted adventitious bud differentiation. The extensive studies were made on the cytokinin actions on the formation of meristematic zone (MZ) and adventitious buds. The followings summarize the outcome of the experiments.

1. The formation of MZ in the epidermis can be induced when explants are treated with cytokinin at the early stage of culture.
2. Auxin and cytokinin-antagonists counteract the stimulative effects of cytokinin on adventitious bud induction when applied simultaneously.
3. Actinomycin-D and cycloheximide can completely suppress the cytokinin-promoted adventitious bud induction.

The critical period for the action of inhibitors is the first 2-3 days of culture.

4. During the initial period, individual proportions of protein-incorporated amino acids remain almost same. However, some qualitative differences in newly synthesized proteins can be found in the cytokinin-treated explants.

5. Protein contents rapidly increase in the superficial layer of the explants cultured on the cytokinin-containing medium. This may be attributed to the protective effect of cytokinin to protein degradation.

6. The turnover rate of proteins in the superficial layers of the explants cultured with and without cytokinin for 3 days are about 2.2 and 4.7 % per hr, respectively.

7. Early events in the organogenesis of Torenia stem segments may be associated with an alteration of specific proteins.

II. Effects of chemical, physical and biological factors on flowering

In vitro floral bud differentiation in Torenia stem segments was influenced by some chemical, environmental and physiological factors. The examination of individual and correlative effects of those factors on the initiation

and development of floral buds resulted in the following conclusion.

1. The appropriate culture medium for inducing floral bud in Torenia stem segments was determined. The medium must contain mineral nutrients at low level and 2-6 % sucrose, but phytohormones and ammonium nitrate must be excluded.
2. The best flowering response can be achieved when explants are cultured at 25°C under 8-hr short-day photoperiod with a light intensity of ca. 5,000 lux.
3. The development of floral buds can be suppressed by the application of ammonium nitrate, and promoted by increasing the concentration of sucrose in culture medium. These results are in agreement with the classical theory suggesting that a high C/N ratio is stimulatory to flowering.
4. Addition of phytohormone like indoleacetic acid to culture medium also stimulate the initiation of floral buds.
5. The highest rate of floral bud formation can be obtained when explants are excised from mother plants at reproductive stage. Thus, these explants seem to possess themselves the potentiality to initiate flower buds. Reverse phenomenon can be observed with the explants taken from vegetative or aged plants.

6. Abscisic acid stimulate floral bud formation in originally vegetative explants but inhibit in aged explants. Zeatin acts in a reverse way to abscisic acid.

7. Possible existence of a gradient of floral bud forming capacity was found to occur in the longitudinal section of the stems. The rate of floral bud formation in the explants taken from basal parts of stems is low, but it can be increased by the addition of zeatin to culture medium.

8. The action of phytohormones on floral bud formation is closely related to the physiological state of explants. This may be due to the endogenous level of phytohormones contained in explants.

As a conclusion, it is hoped that the informations secured in this investigation will serve for the understanding of the fundamental mechanism responsible for the in vitro organogenesis in higher plants.

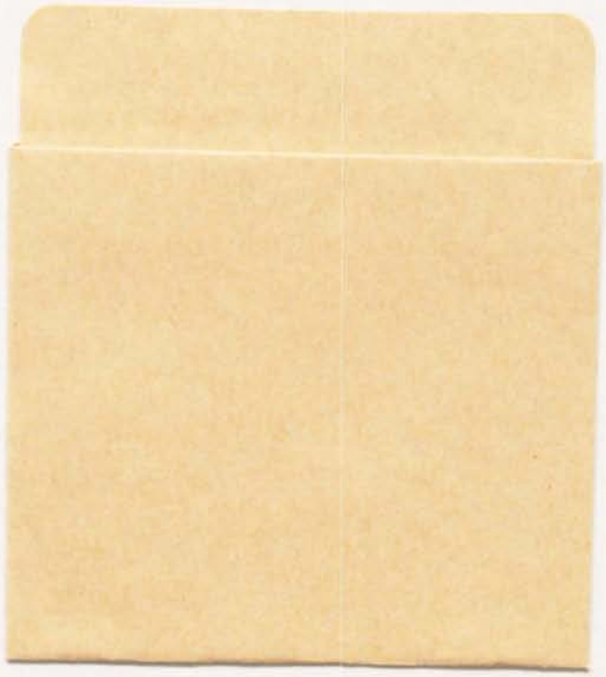
Acknowledgements

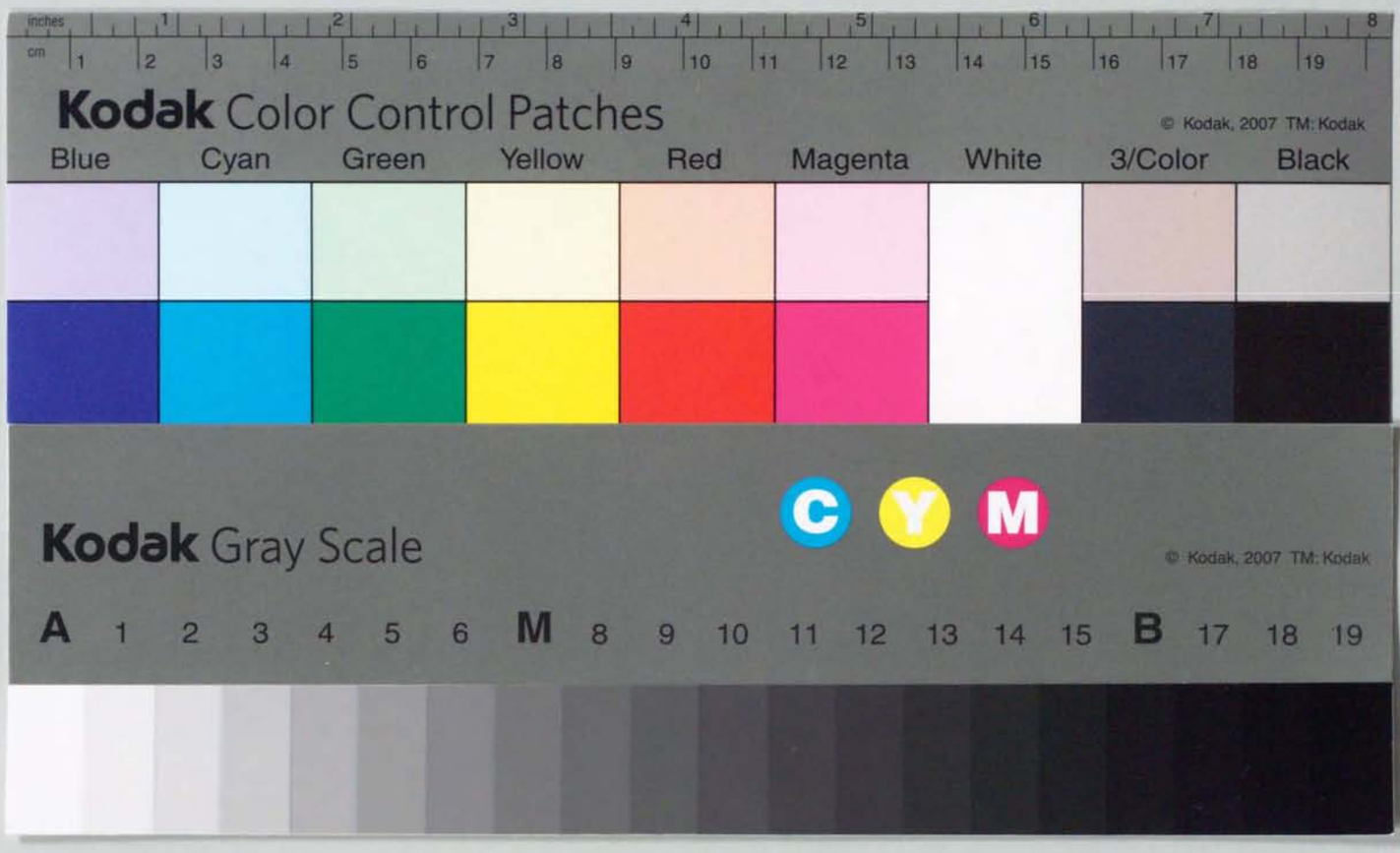
I wish to express my sincere appreciation to Professor Hiroshi Harada of the University of Tsukuba, for his invaluable suggestions and constructive criticisms throughout the course of my research and the preparation of the manuscript.

I am also indebted to Associate Professor Tadashi Fujii and Assistant Professor Hirofumi Uchimiya for their invaluable advices and discussions with respect to certain aspects of this research. My sincere thanks are also due to Drs. H. Kamada and J. Imamura for their valuable suggestions and criticisms. Appreciations are expressed to Mrs. A. Noda and Mr. S. Satoh for their technical assistances and suggestions. Special thanks must be accorded to all members of the laboratory for useful discussions on the results.

I am also grateful to Dr. K. Shudo, Tokyo University, for kindly supplying me with 4PU and 4PU-Cl, and Dr. H. Iwamura, Kyoto University, for kindly supplying me with some derivatives of 2-methylpyrrolo[2,3-d]pyrimidine.

My sincere thanks and appreciations go to my family for their continued supports throughout the duration of my research.




inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19