Acta Bot. Neerl. 22(4), August 1973, p. 390–398.

THE FORMATION OF ADVENTITIOUS ORGANS. I. CYTOKININ-INDUCED FORMATION OF LEAVES AND SHOOTS IN CALLUS CULTURES OF POPULUS NIGRA L. 'ITALICA'*

CATHARINA J. VENVERLOO

Botanisch Laboratorium, Leiden

SUMMARY

One- to four-year-old callus cultures of the Lombardy poplar (*Populus nigra* L. '*Italica*') are able to form roots and leaves or shoots. There may be single leaves, small buds, elongating shoots, or a combination of these organs. Of the four cytokinins used to stimulate organ formation, zeatin was the most active, stimulating shoot formation at a concentration as low as 10^{-8} M. The highest zeatin and BA concentrations (10^{-5} M) stimulated leaf formation, but fewer elongated shoots were formed than at a lower cytokinin concentration. 2,4-D was inhibitory to organ formation, even in low concentrations that stimulated callus growth (0.05–0.1 mg/l). This 2,4-D-inhibition is discussed in relation to the suppression of tissue differentiation.

1. INTRODUCTION

The study of the formation of adventitious organs may contribute to a better understanding of organ formation in general. In many species adventitious organs are formed in the normal course of development, or can be induced, for instance in cuttings of root, stem, or leaf. Some tissues, such as the epidermis and pericycle of stem and root, seem to be preferential sites of organ formation, whereas other tissues are not easily stimulated to form organs. In the light of modern views on the totipotency of mature plant cells, it is important to know which differentiated cells are still able to form organs under suitable conditions. This raises two questions: 1. Which cells or tissues can be induced to form adventitious organs? and 2. Which factors play a role in the initiation and during the ontogenesis of organs? Hormones, nutrients, and physical factors are known to influence organ formation. If organ formation is seen as a complex process comprising several successive steps, it is clear that optimal conditions for the separate steps may be different. When the process takes place in a fully differentiated tissue, successive stages – recognized from morphological characteristics – can be distinguished: cytological changes preceding the first mitoses; the first mitoses and cytokineses; further divisions and formation of a group of meristematic cells; pattern formation leading to a primordium; and functioning

* Dedicated to Professor Karstens on the occasion of his retirement.

of the apical meristem of this primordium. Furthermore, we should like to know at what point the determination to root or shoot takes place. Is this as early as the initiation of the first divisions or much later, when a group of meristematic cells is present? Perhaps a stage of determination to "organ" precedes the determination to "root" or "shoot".

It seems important to choose an experimental sytem with possibilities for root and shoot formation in the same tissue; for example small explants (TRAN THANH VAN & DRIRA 1971; KATO & KAWAHARA 1972), callus cultures (THORPE & MURASHIGE 1970), or organ cultures (BONNETT & TORREY 1965, 1966). By choosing a simple system we hope to exclude, at least partially, the normal internal regulation connected with differentiation patterns and directions of transport. For the present study, callus cultures of the Lombardy poplar were choosen.

In callus cultures of poplar species roots are often found under conditions stimulating callus growth (JACQUIOT 1964; MATHES 1964). Shoot formation usually requires a cytokinin (MATHES 1964; WINTON 1968, 1970; WOLTER 1968; BAWA & STETTLER 1972), but sometimes takes place when the tissue is transferred to a medium with a very low auxin concentration (JACQUIOT 1964). Budding has also been observed on young roots under the influence of cytokinins (BRAND & VENVERLOO 1973). Cuttings of older parts of the root readily form "rootsuckers", i.e. adventitious shoots (ELIASSON 1961), and stem cuttings kept in a moist atmosphere may form callus with shoots. This organ formation by cuttings takes place without an external supply of growth substances. Endogenous levels of growth regulators, which change with the seasons, have a strong influence on organ formation (ELIASSON 1971). Recently, several cytokinins, among which zeatin and its riboside seems to be the most important, were isolated from buds of *Populus robusta* (HEWETT & WAREING 1973).

The experiments reported here may be seen as a study on the optimal conditions for the process of shoot formation as a whole. The continuation of this work is expected to result in a description of the successive stages of shoot formation in tissue cultures.

2. MATERIAL AND METHODS

Tissue culture strains derived from internodes of 1- or 2-year-old branches of trees of the Lombardy poplar (*Populus nigra* L. '*Italica*') were grown on a medium composed of MURASHIGE & SKOOG's (1962) mineral salts and some organic additions. Iron was given by adding 2.78 ml/l of a solution prepared with 6.8 g KOH, 10.4 g EDTA, and 10.0 g $FeSO_4.7H_2O$ per litre. Organic constituents per litre medium were 1 mg pyridoxine hydrochloride, 1 mg nicotinic acid, 1 mg thiamine hydrochloride, 100 mg meso-inositol, 20 g sucrose, and 8 g Difco agar Noble.

For standard procedures, one auxin was added as the only growth substance. Most isolations were made with 2,4-D 0.5 mg/l, but isolations with lower 2,4-D concentrations or with NAA were also successful. Five strains were subcultured,

and of these strains substrains were made at different times. When used for experiments, these substrains had grown for at least a year on the same medium.

The cytokinins used were 6-benzylaminopurine (Fluka), 6-(γ,γ-dimethylallylamino)purine (Sigma), kinetin (NBC), and zeatin (Sigma). Auxins and 6-benzylaminopurine (BA) were autoclaved with the medium unless otherwise mentioned. For separate sterilization of cytokinins, Sartorius membrane filters were used.

The standard procedure for the induction of shoot formation was to transfer rather large pieces of callus tissue from a culture grown on an auxin medium to an auxin-free medium containing BA (0.2 mg/l).

The tissues were grown in glass tubes provided with 25 ml agar medium, at 23 °C in alternating periods of 12 hours of darkness and 12 hours of fluorescent light (Philips TL 55), and were checked weekly under a dissecting microscope for the appearance of leaves, shoots, and roots.

3. RESULTS

3.1. Callus growth

Generally, good callus growth requires the presence of an auxin. 2,4-dichlorophenoxyacetic acid (2,4-D) in a concentration of 0.5 mg/l gives strong proliferation, and with 0.1 mg/l 2,4-D or 1 mg/l α-naphtalene acetic acid (NAA), callus growth is less abundant. The tissue can be cultured with lower concentrations of 2,4-D (0.05 mg/l) or NAA (0.2 mg/l), but at these levels there is less growth and results are variable. The poplar strains under study could not be grown on a medium with IAA instead of 2,4-D or NAA, probably due to the high indoleacetic acid oxydase activity of the tissue.

3.2. Roots

Root formation occurs in callus cultured with NAA or low concentrations of 2,4-D (0.01–0.05 mg/l), but not in cultures with a high 2,4-D concentration (0.5 mg/l). Stimulation of root formation can be achieved by lowering the auxin concentration of the medium. Numerous roots may form when callus is transferred to a medium without auxin or with a low cytokinin concentration. Higher concentrations of cytokinins (10^{-5} M) inhibit both root formation and root growth. An interesting phenomenon is the appearance of a few small roots with limited growth during a short period after transfer to a cytokinin medium.

3.3. Leaves and shoots

Cytokinins are usually obligatory for bud induction, but the spontaneous formation of small leaf-like organs was found in a slow-growing isolation on an NAA medium. The same type of small abnormal organ is sometimes found when tissue is transferred to a medium low in or lacking auxins.

In the presence of cytokinins, more and better leaves and shoots are obtained. BA is just as effective for bud induction in Lombardy poplar cultures as it is n culture s of *P. tremuloides* (WINTON 1968) and *P. trichocarpa* (BAWA & STETT-

LER 1972). When rather large pieces of callus were transferred from an auxin medium to a cytokinin medium with no auxins, leaf-like organs appeared in all or most of the cultures after 10–20 days. This organ formation is almost always preceded by callus formation, the new callus being compact and having a high percentage of dry matter.

The organs are usually formed on the periphery of the callus close to the surface of the agar, but sometimes occur in the agar or on the top of the callus. At first we see small shining green protuberances; later, leaf-like organs with a smooth surface are recognizable. The organs may appear singly or in small or large groups (*figs. 3, 4*). Often they do not seem to belong to a bud, or they belong to a bud with an abnormal number of leaves. Initially, shoots are lacking; but in a later stage most cultures have not only leaves but also some elongated shoots (*fig. 1*). The first shoots appear after about four weeks, and may reach a length of some centimetres. The leaves on these shoots are narrow and sometimes fasciated or teratomic (*fig. 2*). Some shoots with a normal appearance were cultivated to whole plants, for which purpose they were isolated and kept in the dark to induce root formation. The resulting plants showed a normal appearance and good growth.

3.4. Capacity for organ formation of poplar strains

The organ-forming capacity of strains of different ages was tested. From five strains isolated one to four years previously, substrains were started at various intervals and with different auxin concentrations. These substrains had been kept for at least one year on the same medium before being transferred to the cytokinin medium (*table 1*). In most of the substrains BA (0.2 mg/l) induced leaf-like organs in a high percentage of the cultures, but organ formation in substrains transferred from the highest 2,4-D concentration was strikingly low and retarded by two to three weeks. Tissue of the same substrains showed a high percentage of organ formation when grown for 70 days on a lower 2,4-D concentration before transfer to the cytokinin medium, which means that no lasting change in the capacities of the strain had taken place under the influence of the high 2,4-D concentration. The inhibition of organ formation by 2,4-D seems to be due either to accumulated 2,4-D still present in the tissue or to the differentiation pattern induced by the high 2,4-D concentration.

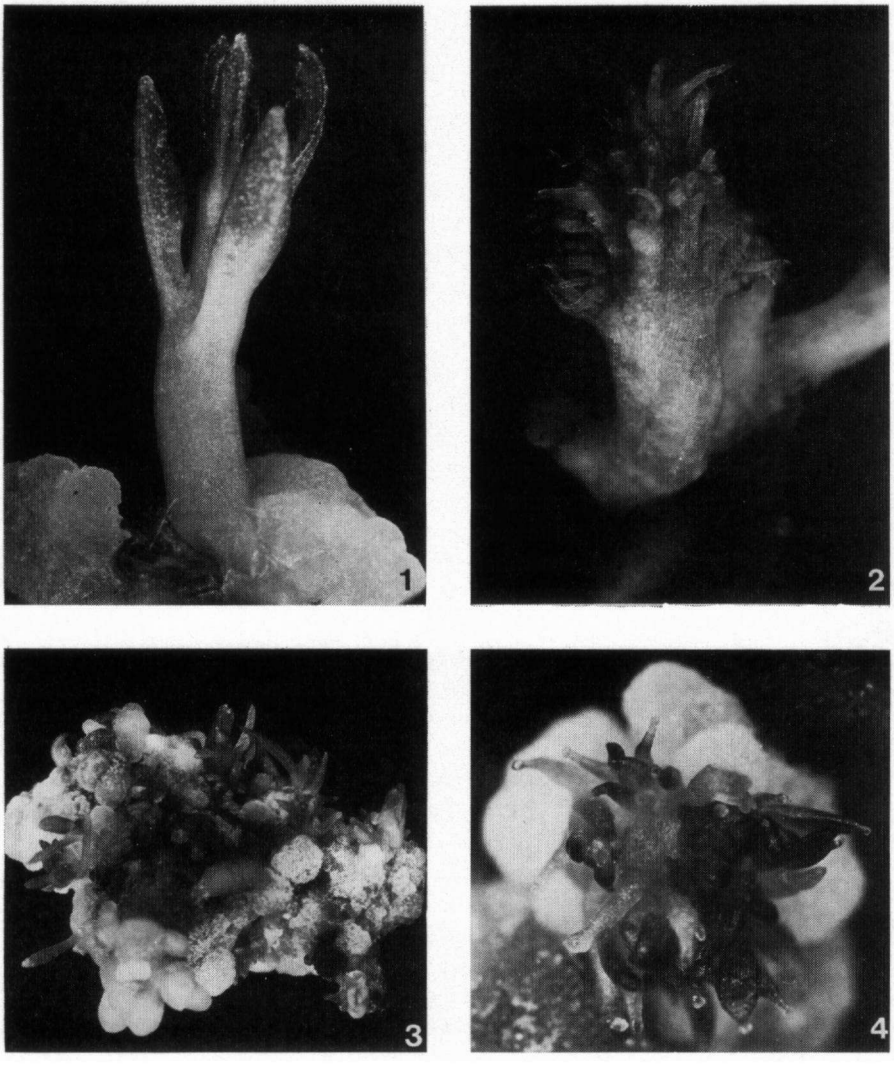
True elongated shoots were not found in all substrains, but were lacking in some substrains of strain A. In another substrain, however, the capacity to form shoots was still present $3\frac{1}{2}$ years after isolation. This substrain had been grown initially for 28 months on a medium with 0.5 mg/l 2,4-D and then on one containing 0.05 mg/l. Elongated shoots were often absent in tissue that had been transferred directly from the high 2,4-D concentration to BA.

All strains showed root formation at some time.

3.5. Inhibition of organ formation by 2,4-D

Tissue transferred from a medium with a high concentration of 2,4-D (0.5 mg/l) shows feeble and late organ formation under the influence of cytokinin. The

histological differentiation of these strains appears to be lower than that of other strains. There are very few or no nodules, and few tracheids and phloem elements. The latter can be recognized by the presence of sieve plates and callose.



Figs. 1–4. Organ formation in 5-week-old BA cultures. Fig. 1. Young elongated stem with normal narrow leaves. × 7. Fig. 2. Elongated thick stem with abnormal leaves. × 7. Fig. 3. Culture with leaves and young buds; on the left a single leaf. × 2. Fig. 4. Group of young leaves, most of them more or less flattened but some conical. A small terminal knob is often present. × 11.

Table 1. Organ formation under the influence of BA (0.2 mg/l).

	medium of substrain (mg/l)	time after isolation (months)	% cult.[1] with leaves	cult. with shoots
strain A, isol. 1968	NAA 1.0	43	93	+
	2,4-D 0.05	44	100	+
	2,4-D 0.1	45	100	–
	2,4-D 0.5	46	58	–
	2,4-D[2]	46	100	–
strain B, isol. 1969	2,4-D 0.05	27	97	++
	2,4-D 0.5	30	75	–
	2,4-D[2]	30	100	++
strain C, isol. 1970	2,4-D 0.5	23	83	–
	2,4-D[2]	23	100	++
strain D, isol. 1970	2,4-D 0.1	20	100	+
strain E, isol. 1970	2,4-D 0.1	14	100	+
	2,4-D 0.5	15	42	+
	2,4-D[2]	15	100	+

[1] At least 12 cultures of each substrain were used.
[2] Tissue from the substrain on 2,4-D 0.5 mg/l after two periods of 35 days on a medium with a lower 2,4-D concentration (0.1 mg/l).

After a short period of growth on a medium with a lower 2,4-D concentration, more tissue differentiation can be observed. BA also induces strong differentiation of the callus tissue: nodules are numerous and there are superficial zones with mitotic activity at places where organ differentiation has started.

The inhibition of bud formation was further investigated with media containing BA (5.10^{-7} or 10^{-6} M; filter sterilized) and various concentrations of 2,4-D. Good organ formation occurred with media containing BA plus 2,4-D in a concentration of 0.01 mg/l, but with the higher 2,4-D concentrations bud formation was strongly inhibited (0.05 and 0.1 mg/l) or ceased (0.2 –1.0 mg/l).

The lowest concentrations of 2,4-D that were inhibitory for bud formation lay in the same range as the lowest concentrations used for continuous culture of poplar strains. The strong inhibition of BA-induced bud formation was not found with other auxins. Bud formation under the influence of BA was not inhibited by the presence of NAA (0.1 or 1.0 mg/l) or IAA (1.0 or 10 mg/l) in the medium. Due to rapid oxidation, IAA is probably not available to the cultures for any appreciable length of time. Bud formation induced by zeatin (10^{-6} M; filter sterilized) was totally inhibited by the presence of 2,4-D in concentrations of 0.1 or 0.5 mg/l.

Root formation is also inhibited by 2,4-D. The inhibition is total at a concentration of 0.5 mg/l, and in most strains also at 0.1 mg/l. With lower 2,4-D concentrations, roots are sometimes formed. The best rooting is observed, however, on media lacking auxins. These results suggest that 2,4-D suppresses meristematization or pattern formation, and thus prevents the formation of primordia.

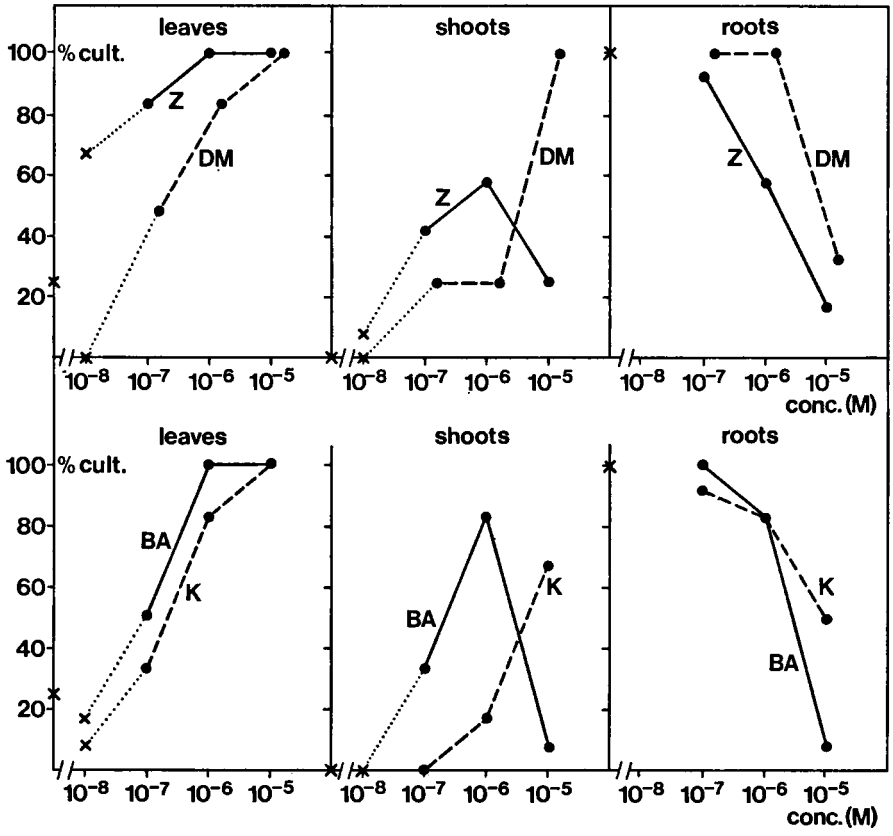


Fig. 5. Percentage of cultures showing organs after 8 weeks of growth on a cytokinin medium The data represent two experiments with the same substrain (●●● and × × ×). In controls without growth substances all of the cultures showed roots and 25% had leaves; shoots were lacking. BA = 6-benzylaminopurine, DM = 6-(γ,γ-dimethylallylamino)purine, K = kinetin, Z = zeatin, shoots = normal organs with a stem length of at least 2 mm.

3.6. Comparison of the activity of some cytokinins

The influence of some cytokinins was studied in callus from a B-substrain grown on a medium with a low auxin concentration (0.05 mg/l). In these experiments the cytokinins were filter-sterilized separately and then added to the agar medium. In the control lacking growth substances, a low number of the cultures (25%) showed some small leaves or buds, but no elongated shoots were formed. Kinetin, 6-(γ,γ-dimethylallylamino) purine (DM), and zeatin (Z) were effective inducers of bud formation (*fig. 5*). The appearance of the organs is comparable with that of cultures grown on BA. There are, however, differences in callus growth and number of elongated shoots formed. Whereas zeatin induces bud formation at a concentration as low as 10^{-8} M, the other cytokinins must be used in higher concentrations. Kinetin is the least active; bud induction is still

low with 10^{-7} M. At the highest concentration (10^{-5} M) of zeatin and BA, fewer cultures with normal shoots having a stem length of more than 2 mm are found than at 10^{-6} M. At the highest zeatin concentration the cultures have numerous leaf-like organs, but normal elongated shoots are scarce. This inhibition of the formation or elongation of shoots is not found at the highest concentration of DM ($1.64.10^{-5}$ M) or kinetin (10^{-5} M). At the highest DM concentration most cultures have several (up to 8) well-developed shoots.

Media inducing leaf and shoot formation often give some callus growth. Profuse callusing was observed with the intermediate DM concentration ($1.64.10^{-6}$ M). In general, the cytokinin-grown cultures are compact, having a relatively high percentage of dry matter. The newly formed callus consists of rather small cells with nodules of xylem and phloem or with small bundles of differentiated tissue.

4. DISCUSSION

Although an external supply of cytokinins was not always needed for the initiation of leaves, many more were formed when cytokinins were added to the medium. The formation of elongated shoots was even entirely dependent on an external cytokinin supply. Zeatin and DM, both of which are naturally occurring cytokinins, are very active inducers of organ formation. Shoot formation is enhanced by a very low zeatin concentration (10^{-8} M). The first organs induced by cytokinins vary from a single leaf to smaller or larger groups of leaves. Often, small groups of leaves are arranged as though belonging to one bud. Few of these organs or organ complexes grow into normal elongated shoots. Where the leaves are not arranged in a regular pattern it is difficult to tell whether a shoot apex is present. It is conceivable that the formation of a shoot apex is preceded by the development of some leaves. In studies on the induction of *normal* shoots, conditions leading to a high quantity of teratomes and inhibiting the formation of elongated shoots must be excluded. The further study of shoot formation requires culture methods giving leaves of normal shape as well as large numbers of shoots in relation to other "green organs". For anatomical investigations, numerous shoots per culture and a predictable development will be favourable.

Since we are working with tissue cultures, i.e. a population of dividing cells, attention must be paid to the growth phase at the moment of transfer to a cytokinin medium. The best results seem to be reached with cultures in the phase of rapid growth. The induction of activity leading to organ formation starts at a different level in tissue cultures than in the mature tissues of explants. In the latter "dedifferentiation" plays an important role, whereas in callus cultures dividing cells are present and even small meristems forming part of nodules are often available. Tissue differentiation in *Populus* callus appears to be correlated with the auxin content of the medium. The parenchymatic tissue grown under the influence of the high 2,4-D concentration – which almost always lacked differentiated cells and meristems – was not easily induced to form organs.

Although 2,4-D-inhibition of organ formation has often been reported (e.g.

ELIASSON 1961), rather high concentrations of 2,4-D were usually needed to stop the process. In *Taraxacum* callus cultures, however, organ formation was prevented by the relatively low 2,4-D concentration of 0.5 mg/l (BOWES 1970). Of importance in this respect are the observations of HALPERIN & WETHERELL (1965), who found that during the embryoid formation in *Daucus* callus cultures, polarized growth and histological differentiation are inhibited when proembryos are exposed to a concentration of 2,4-D higher than 0.1 mg/l.

ACKNOWLEDGEMENT

The author is greatly indebted to Professor W. K. H. Karstens for his interest, encouragement, and suggestions. She wishes also to thank Miss E. K. P. Cool for her skilled technical assistance, and Messrs. G. P. G. Hock and H. Verkijk for the preparation of the illustrations.

REFERENCES

BAWA, K. S. & R. F. STETTLER (1972): Organ culture with black cottonwood: morphogenetic response of female catkin primordia. *Can. J. Bot.* **50**: 1627–1631.

BONNETT, H. T. & J. G. TORREY (1965): Chemical control of organ formation in root segments of Convolvulus cultivated in vitro. *Plant Physiol.* **40**: 1228–1236.

— & — (1966): Comparative anatomy of endogenous bud and lateral root formation in Convolvulus arvensis roots cultivated in vitro. *Amer. J. Bot.* **53**: 496–507.

BOWES, B. G. (1970): Preliminary observations on organogenesis in Taraxacum officinale tissue cultures. *Protoplasma* **71**: 197–202.

BRAND, R. & C. J. VENVERLOO (1973): The formation of adventitious organs. II. The origin of buds formed at young adventitious roots of Populus nigra L. 'Italica'. *Acta Bot. Neerl.* **22**: 399–406.

ELIASSON, L. (1961): The influence of growth substances on the formation of shoots from aspen roots. *Physiol. Plant.* **14**: 150–156.

— (1971): Growth regulators in Populus tremula. IV. Apical dominance and suckering in young plants. *Physiol. Plant.* **25**: 263–267.

HALPERIN, W. & D. F. WETHERELL (1965): Ontogeny of adventive embryos of wild carrot. *Science* **147**: 756–758.

HEWETT, E. & P. G. WAREING (1973): Cytokinins in Populus × robusta: changes during chilling and bud burst. *Physiol. Plant.* **28**: 393–399.

JACQUIOT, C. (1964): Structure des racines excisées ou des organes néoformés par le tissu cambial d'arbres cultivés in vitro. *Rev. Cytol. Biol. végét.* **27**: 319–322.

KATO, Y. & S. KAWAHARA (1972): Bud formation in leaves, leaf fragments and midrib pieces of Helioniopsis orientalis (Liliaceae). *Planta* **107**: 111–120.

MATHES, M. C. (1964): The in vitro formation of plantlets from isolated aspen tissue. *Phyton* **21**: 137–141.

MURASHIGE, T. & F. SKOOG (1962): A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**: 473–497.

THORPE, T. A. & T. MURASHIGE (1970): Some histochemical changes underlying shoot initiation in tobacco callus cultures. *Can. J. Bot.* **48**: 277–285.

TRAN THANH VAN & A. DRIRA (1971): Definition of a simple experimental system of directed organogenesis de nuevo: organ neoformation from epidermal tissue of Nautilocalyx Lynchei. *Coll. Intern. C.N.R.S.* **193**, Les cultures de plantes: 170–176.

WINTON, L. L. (1968): Plantlets from aspen tissue cultures. *Science* **160**: 1234–1235.

— (1970): Shoot and tree production from aspen tissue cultures. *Amer. J. Bot.* **57**: 904–909.

WOLTER, K. E. (1968): Root and shoot initiation in aspen callus cultures. *Nature* **219**: 509–510.