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MANIPULATION OF ORGAN INITIATION IN PLANT TISSUE CULTURES

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Abstract

Some of the parameters that deserve systematic evaluation in developing tissue culture methods of plant propagation have been presented. Propagule multiplication ***in vitro*** can occur by one of three ways: (1) asexual embryogenesis, (2) adventitions shoot formation, and (3) enhancement of axillary shoot development. Major factors that need consideration are: (1) characteristics of explant, (2) nutrient medium composition and form, (3) light provisions, and (4) temperature requirements. The plants obtained through tissue culture should be examined for recovery of genotype and freedom from pathogens when applicable.

Introduction

A major economic application of plant tissue cultures has been in the area of clonal multiplication, and benefits have already been demonstrated. The tissue culture procedure has been utilized effectively to enhance substantially the multiplication of plants that can be propagated asexually only slowly through cuttings, division, or similar materials where rooting is involved, e. g., Orchidaceae (Marston and Voraurai, 1967). Even with plants the asexual multiplication of which through cuttings is already achievable at a substantial rate, e. g., *Chrysanthemum* (Ben-Jaacov and Langhans, 1972; Earle and Langhans, 1974), the *in vitro* procedure can provide significant enhancement; an increase in the rate of multiplication of a million-fold per year over conventional methods is not unrealistic. Plant tissue cultures also remain as one of the more effective aids in the exploration of the physiology which underlies root and shoot initiation, and it is highly probable that ultimately all plants, including trees, will be propagatable asexually via tissue cultures or through information derived from tissue culture investigations.

The successful employment of tissue cultures in asexual plant propagation requires the manipulation of certain organ-forming processes, namely shoot initiation, root initiation and asexual embryogenesis. The available information

indicates that these processes can be regulated in some plants, although not all. The woody tree genera as a group, in contrast to many herbaceous plants, have not been propagatable through tissue culture methods, even though cultures of callus and other tissues have been establishable. With few exceptions, e.g., *Populus* (Winton, 1970) and *Citrus* (Grinblat, 1972), organized development remains relatively unattainable in tree tissue cultures.

This report is based largely on investigations carried out in the author's laboratory. Whereas successful experiments have been restricted to the herbaceous genera, more widespread applicability of the information is not discounted. The information could serve at least as the basis of experiments with woody plants and other difficult-to-propagate genera.

There are currently three principal methods by which clonal multiplication can be attained *in vitro*. These are illustrated in Fig. 1. With some plants, multiplication can be achieved by means of asexual embryo formation in callus and other tissues. In others it has been possible to induce adventitious shoot initiation; these shoots can then be treated as stem cuttings and rooted to give rise to whole plants. A third method involves the enhancement of axillary shoots, followed by rooting of shoot cuttings. Perhaps the most rapid multiplication is via adventive embryogenesis, but its applicability has been confined to plants of a few families. In spite of suggestions to the contrary, it is not now possible to induce embryogenesis *in vitro*, although it has been possible to achieve an enhancement or repression in normally embryogenic tissues. Propagation via adventitious shoots can be a very rapid process with some plants. But this method, too, is not always applicable and is frequently associated with a high incidence of genetically aberrant plants. More general applicability is found in the technique of hastening development of axillary shoots. This procedure may be the slowest of the three; nevertheless, it is employable with virtually any plant and is least likely to produce genetically deviant plants. In developing a tissue culture procedure of plant propagation, it may be helpful to decide first on the morphogenic process by which the plant increase is to be accomplished.

The propagation of plants through tissue cultures often entails a sequence of steps *in vitro*, each step necessitating a specific set of conditions. The extreme case is one in which there are three distinct steps: (1) initial establishment of aseptic culture, (2) multiplication of propagule, and (3) preparation of propagules for transfer to soil. This report will be concerned with a consideration of some of the key parameters associated with propagule multiplication.

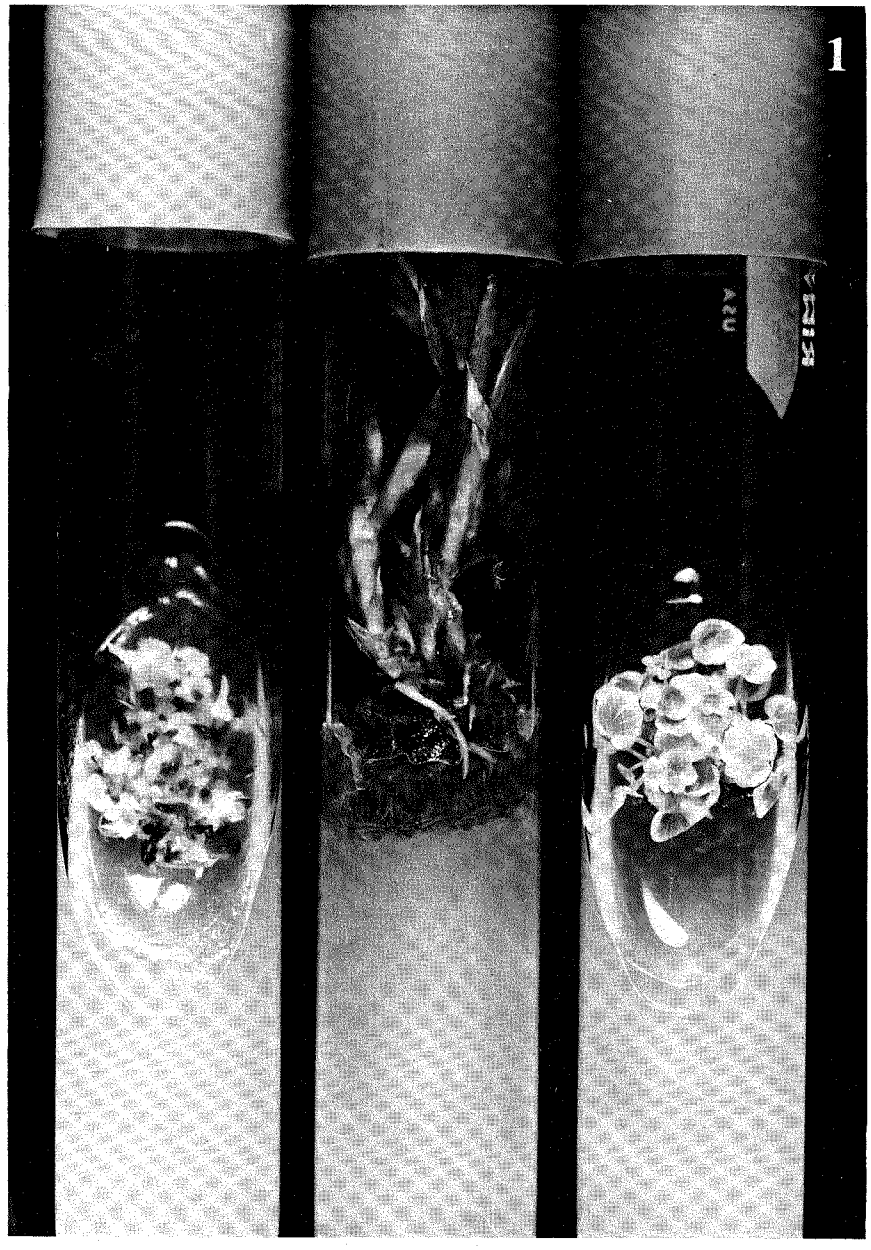


Fig. 1. Principal methods of propagule multiplication in vitro. Left to right: asexual embryogenesis (*Ophiopogon*), adventitious shoot formation (*Dracaena*) and axillary shoot increase (*Saxifraga*).

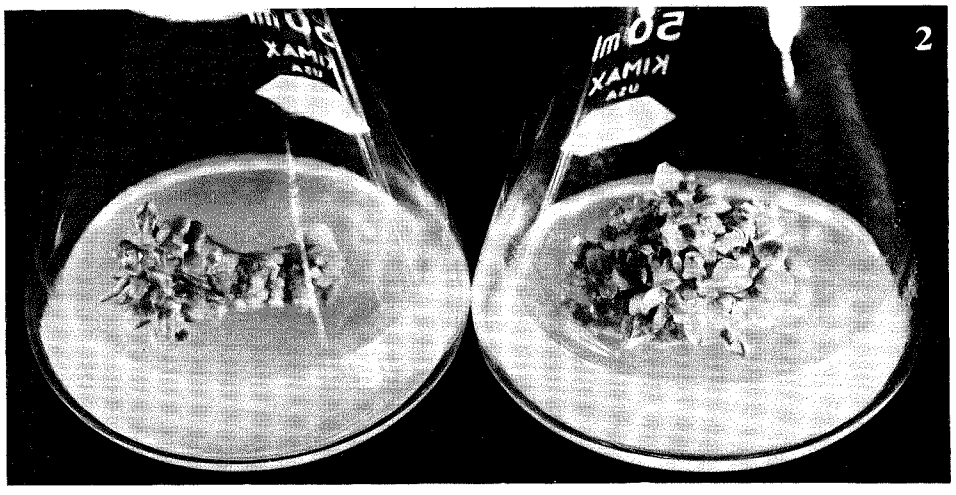


Fig. 2. Comparison of regenerative behavior of *Petunia* internode (left) and shoot tip explants.

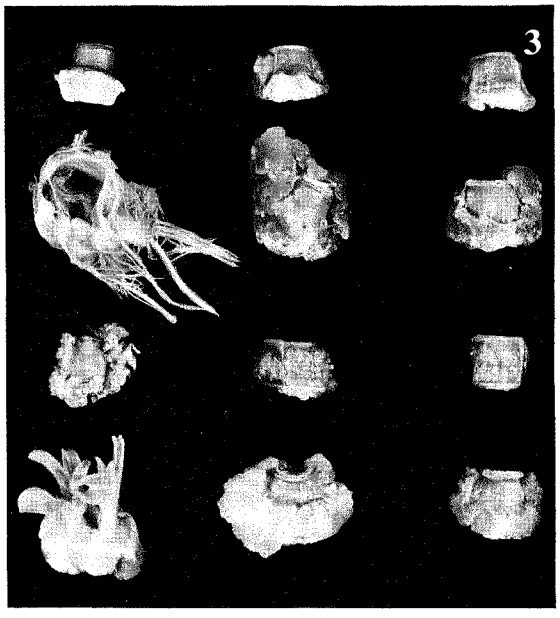


Fig. 3. Relationship between ontogenetic age and organogenetic behavior of *Nicotiana* stem section explants. Column on left: sections from near apex, middle: sections from midway down stem, and right: sections from base of plant.

Selection of a suitable explant

The manipulatability of organ formation in tissue cultures is often determined by the choice of the explant. While it is generally accepted that totipotentiality is characteristic of all plant cells, the manifestation of this potential appears to be restricted to certain cells. It is therefore desirable that for each plant in question a systematic search be made of explants that contain suitable cells. The investigation should consider (a) the organ that is to serve as the source of explant, (b) the age of the organ, (c) the size of the explant, (d) the season in which the explant is being obtained, and (e) the overall quality of the source plant. Virtually every plant organ or tissue has been used successfully. Shoot tips, sometimes erroneously referred to as meristems, have been used extensively in the propagation of numerous genera. Sections of root, stem, leaf, inflorescence, cotyledon, and other parts have been satisfactory with still other genera. The nucellus has been cultured to produce asexual embryos in the *Rutaceae*. The sometimes critical role of the explant is readily observed in tissue cultures of *Nicotiana glauca*. Internodal sections and callus of this species rarely yield roots or shoots in response to auxin/cytokinin treatments. However, their excised stem tips produce adventitious organs profusely under the same conditions. In other plants the difference among diverse kinds of explants can be seen in the degree to which the organogenetic potential is expressed. Fig. 2 is an example of this difference in behavior between stem segment and shoot tip of *Petunia*. Explants of *Petunia* shoot tips produce adventitious shoots apparently without restriction, whereas those of stem sections show some repression. In the case of *N. glauca* or *Petunia*, then, the choice is clearly one of employing shoot tip, not stem section, as explant.

The variation in regenerative behavior among explants is sometimes attributable to the age of the tissue or organ and the extent to which constituent cells are differentiated. In *N. tabacum*, for example, the preponderance of root and shoot initiation occurs in internode explants taken from near the apical region, or the youngest portion, of the stem. Organogenesis is absent in explants obtained from more mature regions (Fig. 3). It has been known in horticultural practice that cuttings of younger plant parts frequently root better than those of mature parts. Moreover, the regeneration of roots is possible even in difficult-to-root tree genera when cuttings are taken from stems that are in the juvenile stage of development. Indeed, with tissue cultures of some plants, it has been possible to obtain organized growth by employing explants obtained from embryos, germinating seeds, and other structures of the early stages of plant ontogeny. Sometimes the kind of organ

regenerated *in vitro* may be dependent on the age of the organ that is used as source of explant. Raju and Mann (1970) reported that sections of young leaves of *Echeveria elegans* regenerated only roots, whereas those of old leaves formed shoots; leaves of more intermediate age gave explants that produced roots as well as shoots.

The regeneratability may be polarized further within a given organ. Robb (1957) as well as Hackett (1969) noted that the initiation of new bulbs in cultures of bulb scales of *Lilium* occurred principally in the basal region of each scale. Hackett further indicated that the tendency to produce adventitious bulbs was highest among the older, outer scales of a bulb and decreased progressively towards the younger, inner scales.

The probability of isolating tissues that are free from microbial contamination, including viruses, is usually related inversely to the size of the explant. The smaller the explant, the more likelihood of a clean tissue. But the frequency of surviving cultures and the rate of their development into reculturable structures are often in direct correlation with initial size. Thus, for example, whereas cultures of the true shoot apical meristem are more likely to result in exclusion of viruses and other microscopic inhabitants of tissues and cells, they are ordinarily impractical, particularly so in rapid asexual multiplication, because the survival rate of the extremely small explant is very low and the speed of development *in vitro* among those that survive is distinctly poorer than that of the larger shoot tip explants.

The season in which explants are obtained may influence the regenerative behavior *in vitro* of plants that are adapted to certain climates. Bulb scale explants of *Lilium speciosum*, a temperate climate perennial, have been reported to regenerate adventive bulbs more freely when obtained from spring and fall sources, but not from those of summer or winter (Robb, 1957). Robb suggested the seasonal variation to be correlated with phases in the development of the lily plant, the regeneration being presumably associated with periods of vegetative growth.

The behavior of cultured tissues is sometimes a reflection of the overall quality of the plant that is used as the source of explants. Explants obtained soon after the source plant had been provided fertilizer may respond to the nutrient medium differently from those taken substantially later or from an unfertilized plant. Also, healthy plants may provide explants the developmental behavior of which may be different from those of diseased plants.

Manipulating organogenesis via the nutrient medium

The formation of roots and shoots in plant tissue cultures appears to be regulated basically by balances in the availability of two hormonal substances,

auxin and cytokinin, as proposed by Skoog and Miller (1957). Other substances seem primarily to modify the effectiveness of either or both growth regulators. The concept that auxin/cytokinin interactions underlie the control of organogenesis apparently applies to all plants, and an inability to extend it to some plants indicates simply a limitation in accessory factors, e. g., unsuitable choice of cells, inadequate light or temperature, deficiency or excess of certain metabolites, etc. Briefly, Skoog and Miller's concept states that both auxin and cytokinin are fundamental in the regulation of growth and development of plant tissue cultures, and that the pattern or direction of development of tissue cultures is determined by balances in the supply of the two substances. A relatively high level of auxin combined with one of low cytokinin promotes the initiation of roots, whereas the reverse relationship leads to shoot formation.

Our investigations with herbaceous plants have enabled us to identify some of the key ingredients of nutrient media of tissue cultures that are intended for shoot or root multiplication. These are found in Table 1. For purposes of simplification, the ingredients have been placed under three categories: inorganic salts, organic constituents, and natural complexes. In all investigations in our laboratory it has been found that the salt requirement can be satisfied

Table 1. *Ingredients of culture media intended for shoot or root multiplication*

Inorganic Salts

Murashige & Skoog, 1962

(Additional $NaH_2PO_4 \cdot H_2O$, 170 mg/l; $\pm NH_4NO_3$)

Organic Constituents

Carbohydrate: 3% sucrose

Vitamins: thiamine•HCl, 0.4 mg/l

Amino acid: L-tyrosine, 100 mg/l

Purine: adenine sulfate•$2H_2O$, 80 mg/l

Hormonal Substances:(1)

Auxin (0.1-10 mg/l): IAA, IBA, NAA, CPA, 2, 4-D

Cytokinin (0.03-30 mg/l): 2iP, BA, kinetin

Others: inositol, 100 mg/l; ascorbic acid, 100 mg/l; citric acid, 150 mg/l

Natural Complexes

Endosperm fluid: coconut water

Extracts of malt or yeast

Fruit material: orange juice, tomato juice, banana pulp

Animal extracts: fish emulsion

Protein hydrolysates: casein hydrolysate, lactalbumin hydrolysate, peptones

(1) IAA=indole-3-acetic acid, IBA=indole-3-butyric acid, NAA=naphthaleneacetic acid, CPA=4-chlorophenoxyacetic acid, 2, 4-D=2, 4-dichlorophenoxyacetic acid, 2iP=N_6-isopentenyladenine, BA=N_6-benzyladenine

by the Murashige and Skoog formulation. Enrichment of the inorganic phosphate in this formula by supplements of $NaH_2PO_4 \cdot H_2O$ has been beneficial to some plants. The applicability of other salt formulations to certain plants is not precluded. In fact, it is not unrealistic to encounter adverse effects on some plants by the Murashige and Skoog formulation, especially in view of its relatively high concentrations of all salts and its content of the NH_4^+ ion. The natural complexes are used primarily when a chemically definable medium containing presumably optimum concentrations of all known morphogenetically active substances is still ineffective. Sometimes their inclusion may not be critical, but benefits can be obtained from certain natural complexes through additional increments of tissue culture growth.

With most plants the carbohydrate requirement is readily satisfied by sucrose in a concentration near 3%. Other carbohydrates may be used occasionally, but none has shown consistent superiority over sucrose. Numerous vitamins have been employed in plant tissue cultures; however, we have found only one vitamin to be critical. Thiamine·HCl appears to be essential in the culture of most plants, and severe nutrient medium deficiencies of this vitamin have been observed to reduce the number and vigor of new organs and to induce severe foliar chlorosis. Extremely low concentrations, 0.1–1.0 mg/l, of thiamine·HCl have been adequate. Inositol has not been essential; nevertheless, clearly beneficial effects have been observed by incorporating this substance at a rate of 100 mg/l in the culture medium of numerous plants. Ascorbic acid and citric acid are also used when necessary. These two substances appear to be helpful in retarding the browning of freshly excised tissues, a phenomenon associated with high activities of polyphenol oxidase. Some amino acids or their amides have been helpful in the culture of certain tissues as enhancers of organ initiation. The key ones have been arginine, aspartic acid or asparagine, glutamic acid or glutamine, and tyrosine. Skoog and Miller (1957) observed that the same stimulation of initiation of both shoots and roots in tobacco callus cultures by casein hydrolysate could be obtained by the sole addition of L-tyrosine. Only the L forms of amino acids are apparently effective, the D form often negating the effectiveness of the other if provided in a racemic mixture. Inclusion of amino acids as nutrient constituents should recognize possible antagonisms among them. Unfavorable antagonisms could result in repression of tissue culture development. The inclusion of adenine may be desirable, although not critical, for the initiation of shoots in tissue cultures of some plants. The beneficial effects of adenine on shoot initiation was first observed by Skoog and Tusi (1948). Subsequently, Miller and Skoog (1953) discovered that adenine was able to counteract the

inhibition of shoot initiation by auxin; the antagonism between adenine sulfate and IAA was quantitative and almost stoichiómetric.

Indeed, the most critical constituents of nutrient media intended for the multiplication of roots or shoots have been auxin and cytokinin. Both classes of hormonal substances should be examined in kind and concentration. The numerous auxins differ significantly in stability, effectiveness, and influence on organogenesis. In our laboratory, the first choice among the auxins has been IAA. This substance shows a minimum of adverse effects on organ formation, although it is perhaps the weakest auxin and is inactivated readily in tissue cultures of some plants. The auxin 2, 4-D appears to be the most potent and, whereas it stimulates favorably callus cultures of most plants, it often antagonizes organized development very severely. In Table 1, the auxins have been listed in virtually increasing order of physiological activity and adverse morphogenetic effects. The Table also lists three cytokinins—2iP, BA and kinetin—which are currently available for routine and large-scale use. The cytokinin 2iP has been the most active of the three and therefore preferred. The concentration of cytokinin required in tissue culture media, especially for the stimulation of shoot initiation, is usually in the neighborhood of 3 mg/l. But a level 30 mg/l and even higher may not be unrealistic with some plants. It should be remembered that cytokinins, while enhancing shoot initiation, tend to suppress rooting; similarly, auxins stimulate rooting, but they also are likely to repress shoot formation (see Figs. 4 & 5).

While we have not found many other substances to be significant, their importance has been reported in some tissue cultures. Lee and Skoog (1965) described the enhancement of shoot formation in tobacco callus cultures by a number of phenolic substances, and Rucker and Paupardin (1969) noted that still other phenolics enhanced rooting. Whether a phenol stimulated root or shoot initiation appears to be correlated with its effect on auxin inactivation; one that promotes inactivation increases shoot formation, while another that represses IAA destruction tends to enhance rhizogenesis. Feng and Linck (1970) reported that the auxin antagonist N-1-napthylphthalamic acid promoted shoot formation in tobacco callus cultures. Similarly, 2, 4, 6-trichlorophenoxyacetic acid has been described to enhance asexual embryogenesis in *Daucus carota*, presumably by negating an inhibitory effect of excessive auxin (Newcomb and Wetherell, 1970). Gibberellins, while stimulating growth of organs, generally suppress organ initiation processes (Murashige, 1963). There are exceptions, inasmuch as Gautheret (1969) has observed that gibberellins could substitute for light in stimulating root initiation in *Helianthus tuberosus* tuber sections. Root formation in these sections is inhibited by gibberellin in the presence of light; nevertheless, in darkness, especially in combination with an auxin, it is

reported to stimulate rooting markedly. Chelating agents have been claimed to promote shoot initiation in cultures of haploid tobacco callus (Kochar, Bhalla and Sabharwal, 1971). The extent to which it applies to diploid cells or to other genera remains unestablished. NH_4^+ has been known to increase asexual embryogenesis in callus cultures of certain species. This effect is probably not specific to NH_4^+, since other nitrogen compounds are also effective (Reinert, Tazawa and Semenoff, 1967).

The physical characteristics of the culture medium may play as significant a role as its chemical composition in determining success or failure with a given plant in vitro. Many bromeliads studied in this laboratory have been culturable only when provided with a liquid nutrient; this has also been true of *Cattleya*, but not *Cymbidium* orchid. In contrast, shoot tip cultures of *Asparagus* or *Gerbera* have required the use of an agar gel medium. In most instances of plant tissue culture the choice between liquid and gel media has been made rather arbitrarily, the decision being dependent on available facilities and accustomed practice of the tissue culturist. This can be an unwise and sometimes hazardous procedure. As early as in 1939, White (1939) observed that *Nicotiana* callus cultures differentiated shoots better in a liquid than in an agar nutrient medium. This observation has been confirmed by Skoog (1944). Both investigators suggested that oxygen gradients were involved in the regulation of plant organ initiation. More recently, Kessel and Carr (1972) reported that asexual embryogenesis and adventitious shoot formation in carrot callus cultures were enhanced by a reduced level of oxygen, and root initiation was favored by increased oxygen. Thus, relatively anaerobic conditions favor embryogenesis and shoot initiation, while a more aerobic situation favors rhizogenesis.

In employing a gel medium it is significant to consider both the gel concentration and the quality of the gelling agent. Romberger and Tabor (1971) noted that the best growth of excised *Picea abies* shoot apical meristems occurred in a nutrient medium solidified with Difco "Purified" agar and, surprisingly, the poorest growth was obtained with Difco "Noble" agar. Difco "Bacto" agar gave more intermediate results. High concentrations of agar, resulting in an excessively hard gel, can inhibit growth of excised plant tissues. The required concentration of agar should be established systematically by considering specific needs of each case.

Liquid nutrient formulations can be provided in various ways. Successful cultures have been attained sometimes with the tissue submerged in a nutrient solution that is maintained in the stationary state. At other times a filter-paper bridge, glass-wood, or similar support has been used with stationary liquid media. Batch cultures of cells and tissues have also been carried out

in special apparati called phytostats. Usually, when the nutrient medium has to be provided in the liquid form, especially as it is the case of some freshly excised tissues, gentle agitation of the medium may be beneficial. A continuously revolving apparatus has been found effective for many plants, and the optimum rate of agitation has been about 1 rpm.

There are other physical characteristics of the nutrient medium that may require consideration. These include the pH and the quantity of medium utilized. The usual practice has been to set the initial pH of media at some value within the range 5.0–6.0. Unfortunately, drifts in pH occur during the course of tissue culture and little has been established with respect to the influence of these drifts on the cultures. With very small explants, the incidence of surviving explants as well as the rate of growth of tissue cultures is often dependent on the provided quantity of medium. Cell population density requirements compel the use of medium quantities that are proportionate with the explant size; the smaller the explant, the smaller should be the nutrient medium volume. Nevertheless, with established tissue cultures, the rate of growth of the cultures may be in direct relation to the quantity of medium provided. Indeed, this has been the case with tobacco callus cultures (Murashige and Skoog, 1962).

Influence of light on organogenesis in vitro

One of the significant observations made in our laboratory has been that, in spite of presumably optimum concentrations of auxin and cytokinin in the nutrient medium, tobacco callus cultures failed to initiate shoot buds when maintained under constant darkness. However, similar cultures exposed to low intensities of artificial illumination responded as expected to the high cytokinin-low auxin medium by forming numerous shoots. This observation as well as some others led us to explore the significance of light in tissue cultures, and particularly in relation to organogenesis. The findings indicate that all three characteristics of illumination—intensity, length of the daily exposure period and spectral quality—deserve consideration. In Fig. 6 can be seen cultures of *Gerbera* obtained under various light intensities. The light was provided 16 hr daily, employing Sylvania Gro Lux lamps. As evidenced in the Figure, the optimum intensity of illumination for the initiation of shoots in *Gerbera* cultures was in the neighborhood of 1000 lux; an intensity of 300 lux was inadequate, and still fewer shoots were produced under complete darkness. The explants used in this case were shoot tips excised from axillary buds of the mature plant. Light intensities higher than 1000 lux—3000 and 10,000 lux—were increasingly and severely repressive of shoot differentiation. We have found this relationship between light intensity and shoot differentiation

to be consistent among a large number of herbaceous genera. Using the Gro Lux lamp and a 16 hr exposure period, a 1000 lux intensity has been optimum for most plants. Nebel and Naylor (1968) reported that bud formation in the moss *Physcomitrium* was dependent on the extent of illumination. They found that the time required for buds to form was inversely related to the light intensity, at least in the range up to 7000 lux. Adventitious root initiation in *Helianthus tuberosus* tuber sections has been observed by Gautheret (1969) to be stimulated by extremely low light intensities. An intensity of 600 lux resulted in 80% of the sections producing roots; the optimum was 5000 lux. An intensity exceeding 7000 lux resulted in lowering of the incidence of root-forming cultures to 50%. Although the favorable intensity of illumination in root and shoot formation has been in a relatively low range, the possible need of substantially higher light intensities in other organogenetic processes should not be discounted. For example, in the differentiation of cladophylls in asparagus tissue cultures, a phenomenon which appears critical in preparing asparagus plants for their transfer from the laboratory to soil, it has been necessary to raise the light intensity from 1000 lux, the optimum for spear and root initiation, to 10,000 lux (Hasegawa, Murashige and Takatori, 1973).

Continuous illumination does not allow maximum organogenesis to occur. With all plants studied in this laboratory, the optimum daily exposure period to illumination has been 16 hr. The experiments employed a light intensity of 1000 lux, supplied by Sylvania Gro Lux lamps. Cultures of tobacco callus showing shoot forming responses to the daily illuminated period can be seen in Fig. 7. No shoots resulted under continuous darkness or an 8-hr exposure to light. The optimum photoperiod was 16 hr. Continuous illumination was distinctly unfavorable, and shoot initiation was repressed significantly. More quantitative data can be seen in Fig. 8, where the responses with respect to both root and shoot formation in tobacco callus can be seen. The optimum daily exposure period to illumination for rhizogenesis was also 16 hr. These observations do not necessarily signify a truly photoperiodic phenomenon. It appears that the best light exposure period for a given tissue culture is dependent upon the intensity of the illumination employed, and probably other factors. With tissue cultures of *Plumbago* Nitsch and Nitsch (1967) observed that a 9-hr daily exposure period. using a 7000 lux light intensity, gave the maximum yield of shoots. Similarly, Margara (1969) found a 9-hr exposure to be optimum when a 4000 lux light intensity was used for *Brassica* cultures. These findings suggest that shorter exposure periods are sufficient when relatively high intensities of illumination are used and longer daily exposure periods may be necessary with very low intensities. It is possible, however, that plants that are normally sensitive to photoperiod *in vivo* will have

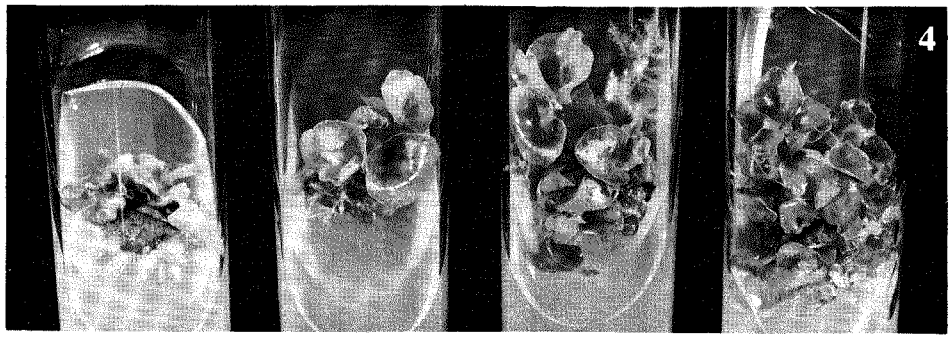


Fig. 4. Effects of increasing the nutrient medium concentration of kinetin on shoot and root formation in *Begonia* shoot-tip cultures. Left to right: 0, 3, 10, and 30 mg/l.

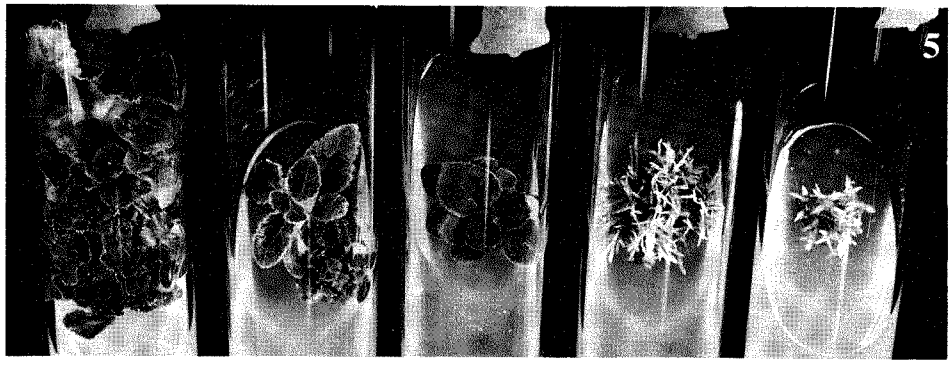


Fig. 5. Effects of increasing the nutrient medium concentration of NAA on root and shoot formation in *Gloxinia* shoot-tip cultures. Left to right: 0, 0.1, 0.3, 1, and 3 mg/l.

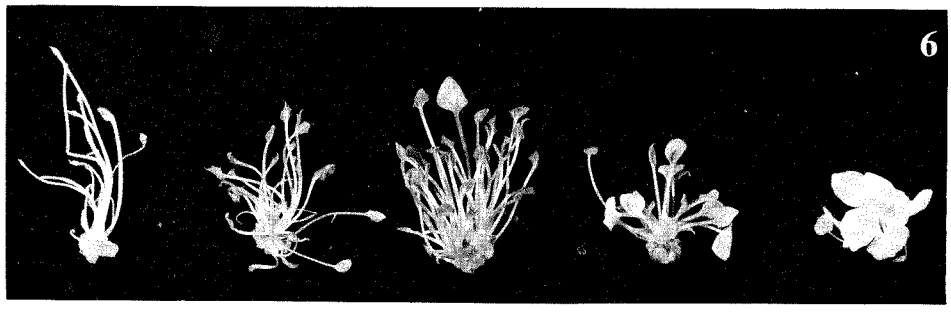


Fig. 6. Influence of light intensity on shoot formation in *Gerbera* tissue cultures. Left to right: 0, 300, 1000, 3000, and 10,000 lux. Sylvania Gro Lux lamps used, 16 hr/da.

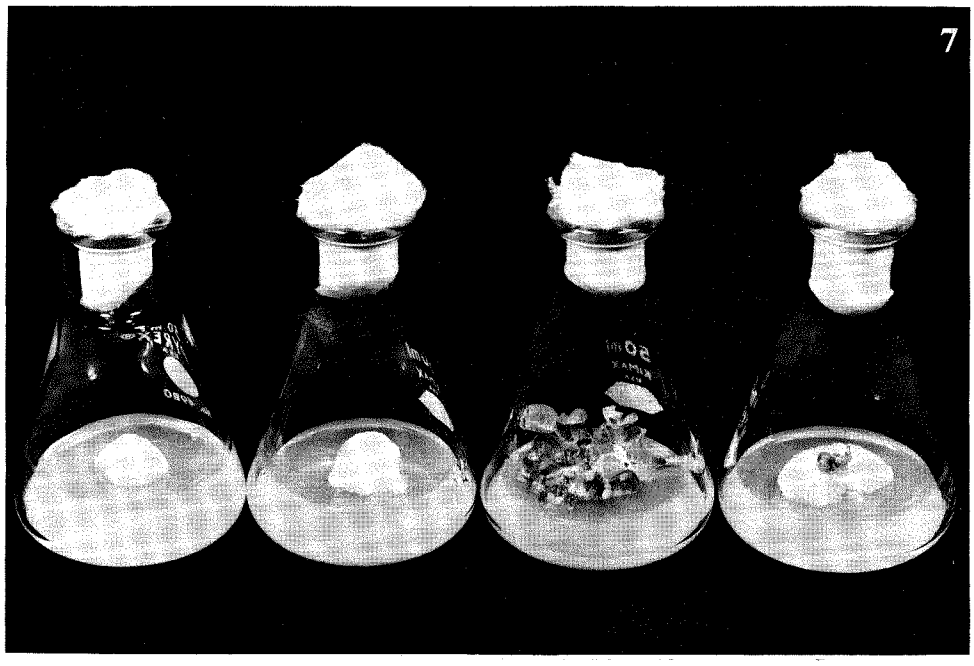


Fig. 7. Shoot-forming response of *Nicotiana* callus to variations in period of daily exposure to illumination. Left to right: 0, 8, 16, and 24 hr. Sylvania Gro Lux lamps, 100 lux intensity.

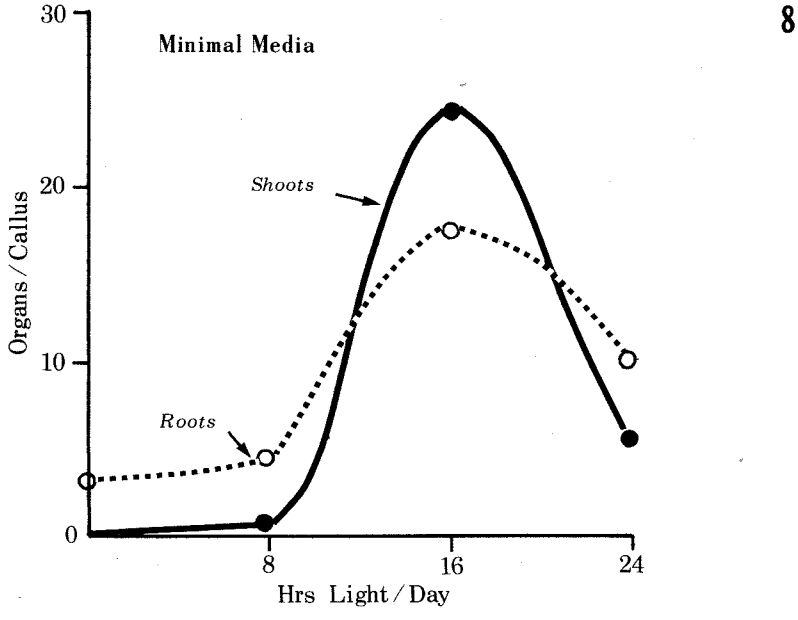


Fig. 8. Root and shoot formation in *Nicotiana* callus in response to length of daily illuminated period. Sylvania Gro Lux lamps, 1000 lux intensity.

photoperiodic requirements that must be met *in vitro*. The experiments of Alleweldt and Radler (1961) with grape varieties indicated that the behavior *in vitro* of stem sections reflected the natural photoperiodic requirements of a given variety. Root initiation in tissue cultures of at least one variety was dependent on satisfaction of its short-day requirement.

The spectral quality of the lamps utilized has significant effects on root and shoot initiation in tissue cultures. Furuya and Torrey (1964) found that lateral root initiation was inhibited by red light and, moreover, this inhibition could be negated by far red light. The phenomenon was phytochrome mediated and reversible, the morphogenetic response being determined by the last used light. The stimulation of adventitious root initiation in plant tissue cultues, however, appears to be promoted by red light. Letouze and Beauchesne (1969) found that the effective region of the light spectrum for initiation of roots in *Helianthus tuberosus* tuber sections was near 660 nm. Thus the formation of lateral and adventitious roots appars to have different light requirements. The critical portion of the light spectrum for shoot induction has been shown to be in the blue region; red light is evidently without effect (Weis and Jaffe, 1969). More precise studies by Seibert (1973) showed that the most effective monochromatic radiation was at 467 nm. For use with tissue cultures intended for plant multiplication, the lamp should contain adequate doses of blue and red light, since both root initiation and shoot formation will be desired at one time or another. The moss *Pohlia nutens* (Mitra, Misra and Prabha, 1965) requires a balanced exposure to both red and blue light for bud formation alone, although a few shoot buds develop when exposed solely to red light. The role of other spectral regions in still other physiological processes of plant tissue cultures should not be ignored in selecting the lamp.

Temperature and organogenesis in vitro

The general practice among plant tissue culturists has been to provide an environment in which the temperature is held constant, usually around 25°C. This practice fails to recognize the fluctuations, diurnally and seasonally, to which many plants are normally exposed and possibly adapted. Constant temperature may be adequate for tissue cultures of annuals and of tropical species, whose life cycles are completed in a climate of relatively uniform temperature conditions. But it may be appropriate to explore periodically varied temperatures with tissue cultures of temperate and some desert climate plants. Kefford and Caso (1972) reported a maximum yield of adventitious shoots in *Chondrilla juncea* root cultures in a diurnally fluctuated environment of 21–27°C day temperature and 16–22°C at night. Other diurnal temperature combinations resulted in fewer shoots. According to Gautheret (1969) the

optimum environment *in vitro* for the rooting of *Helianthus tuberosus* tuber sections included alternating temperatures of 26°C during the day and 15°C at night. Gautheret suggested that the higher day temperature was essential for the formation of cambia and the lower night temperature enabled the differentiation of cambia into root primordia.

When constant conditions are satisfactory, it may still be necessary to establish optimum temperatures for specific cases. For *Nicotiana tabacum*, Skoog (1944) showed that shoot initiation in callus cultures was best at 18°C; a temperature of 33°C was too high and 12°C too low. Hasegawa, Murashige and Takatori (1973) found a constant 27°C temperature to be satisfactory for the increase of both spears and roots in asparagus cultures.

Plants of the temperate climate may not lend themselves to the tissue culture method of plant propagation unless their seasonally associated temperature requirements have been satisfied. Work in this laboratory with *Lilium* tissue cultures indicates that the attainment of bulbs from bulb scale sections of certain varieties is possible only after the bulbs have been exposed to a low temperature, circa 5°C, for a prescribed period (Fig. 9). Furthermore, bulbs and plants which develop in tissue cultures become dormant upon their transfer to soil, unless they have first been exposed to the low temperature. Hildebrandt (1971) described the same phenomenon in *Gladiolus hortulans*. *Gladiolus* corms and plants obtained from tissue culture could not be established in soil unless they had been treated with a temperature of 2°C for a period of 4-6 weeks, just prior to the transfer to soil.

The plants obtained through tissue culture

Clonal propagation signifies that all plants derived from a plant are genetically identical. However, reproduction of genotype has not always been the case, and aberrant plants have been a significant occurrence with some species when propagated through tissue cultures, e. g., *Asparagus officinalis* (Malnassy and Ellison, 1970), *Brassica oleracea* (Horak, Landa and Lustinec, 1971), *Chrysanthemum morifolium* (Ben-Jaacov and Langhans, 1972), *Crepis capillaris* (Sacristan, 1971), *Dianthus caryophyllus* (Hackett & Anderson, 1967), *Nicotiana tabacum* (Murashige & Nakano, 1967), *Oryza sativa* (Nishi, Yamada and Takahashi, 1968) and *Saccharum* sp. (Heinz and Mee, 1969). Usually the abnormality has been in the form of polyploid plants arising from diploids. Other genetic alterations including changes in flower pigmentation and branching patterns have been reported in some plants, e. g., *C. morifolium*, *D. caryophyllus* and *O. sativa*. A sport obtained from tissue culture of the bromeliad, *Aechmea fasciata*, is seen in Fig. 10. Tissue cultures should be used cautiously to propagate chimera varieties. Their components may separate very easily,

resulting in loss of the desired traits of the cultivar. This has happened in the White Sim carnation (Hackett and Anderson, 1967), the Indianapolis White chrysanthemum (Ben-Jaacov and Langhans, 1972), and in some variegated plants that we have studied, e. g., *Cryptanthus* 'It' and the variegated pineapple. The frequency of deviant plants is expected to be higher in instances where organogenesis has occurred by an adventitious process. In contrast, plants multiplied by way of enhanced axillary shoot formation, e. g., shoot tip cultures of *Asparagus officinalis* (Hasegawa, Murashige and Takatori, 1973) and *Gerbera jamesonii*, appear to produce significantly fewer mutants.

Continuous and repeated reculturing of plant tissues *in vitro* is often associated with a progressively declining organ-forming tendency. Such declines have been observed in *Allium cepa* (Fridborg, 1971), *Daucus carota* (Syono, 1965), *Linaria vulgaris* (Charlton, 1965), *Nicotiana tabacum* (Murashige and Nakano, 1965), *Pisum sativum* (Torrey, 1967) and *Saccharum* sp. (Heinz, Mee and Nickell, 1969). In each case the decline appears attributable to the attainment of predominance by polyploid cells, especially aneuploids. Plants derived from such cells are indeed polyploids. The karyotypic change occurs quickly in tissue cultures of some plants. For instance, in callus cultures of *Citrus limon* (Murashige, Nakano and Tucker, 1968), the frequency of diploid cells has been observed to decline from 100 to 71% within the first passage, and to 33% by the end of the third passage. This decrease of diploid cells in successive subcultures has been compensated for by rises in tetraploid, octaploid, and ultimately aneuploid cells.

The pathogen-free state of plants obtained through tissue culture methods must be ascertained through appropriate indicator tests, and any claim of freedom from disease should specify the pathogens for which tests have been negative. Simply following *in vitro* procedures is not sufficient basis to claim disease-free plants. Absence of obvious colony development does not invariably signal absence of infection. Even parasites much larger than viruses sometimes miss detection. For example, the fungus *Physoderma citrii*, although not externally evident in callus cultures of *Citrus*, has been observed in histological sections of the tissue. An example of such a tissue can be seen in Fig. 11. The organisms in this instance appear as spherical bodies with hyphal extensions.

Some fundamental considerations

While sufficient data have been accumulated and guidelines can be offered for the development of specific procedures for the propagation of many crops, the available information remains inadequate for application to all plants. Hopefully, there will be major breakthroughs in investigations with the woody

genera. The woody plants constitute a large group of economically important plants, many of which cannot be propagated rapidly enough by any conventional asexual method.

The tissue culture method of plant propagation owes its success largely to plant physiologists who have been studying the processes of organized development. The discovery by Skoog and Miller (1957) of the relationship of auxin and cytokinin to root and shoot formation has been most important. The participation of further substances, and of light, temperature, and other factors, has now been disclosed. Nevertheless, there remains much to be learned before tissue cultures can produce widespread benefits.

It is significant that, in spite of the totipotentiality of all plant cells, the regeneration of organized structures has been observed in some cells only. The manifestation of organized development seems limited to the truly undifferentiated cells, or meristemoids as Torrey (1966) has called them in tissue cultures. The fundamental misconception has been that tissue cultures, callus cultures clearly included, are composed of undifferentiated cells. This is rarely the case. In most cultured tissues the cells are distinctly vacuolated, highly varied in shape and size, lacking in conspicuous nuclei or cytoplasm, and characterized by cell walls with varying degrees of secondary depositions, In contrast, meristemoids when present appear as nests or clusters of tightly held definitely smaller cells with dense cytoplasm and prominent nuclei; they show little evidence of vacuolation. An example of such a nest found in a callus culture of tobacco can be seen in Fig. 12. According to Torrey (1966), it is the meristemoid that is capable of responding to organogenetic stimuli, and, depending upon the direction of stimulation, of organizing into either root, shoot, or embryo. Observations of adventitious bud and lateral root initiation in *Convolvulus arvensis* root cultures have indicated that the pattern of organization among meristemoids is undetermined (Bonnet and Torrey, 1966). The experiments with *Nasturtium officinale* nodal explants by Ballade (1972) confirm this interpretation; primordial cells usually present in nodal segments of *Nasturtium* or in roots of *Convolvulus* can be stimulated to organize as either root or shoot, depending on the relative supplies of auxin and cytokinin. Champagnat (1971) has similarly observed that the apical meristem in root tips of the orchid *Neottia* can be morphogenically redirected to differentiate protocorms.

The absence of organized development in callus cultures of many species might be attributed to a lack of meristemoids or of cells that can be transformed into meristemoids. Conversely, it is possible to suggest that successes with shoot tip explants of many plants are due to the natural meristemoidal cluster, the apical meristem, that the explants contain.

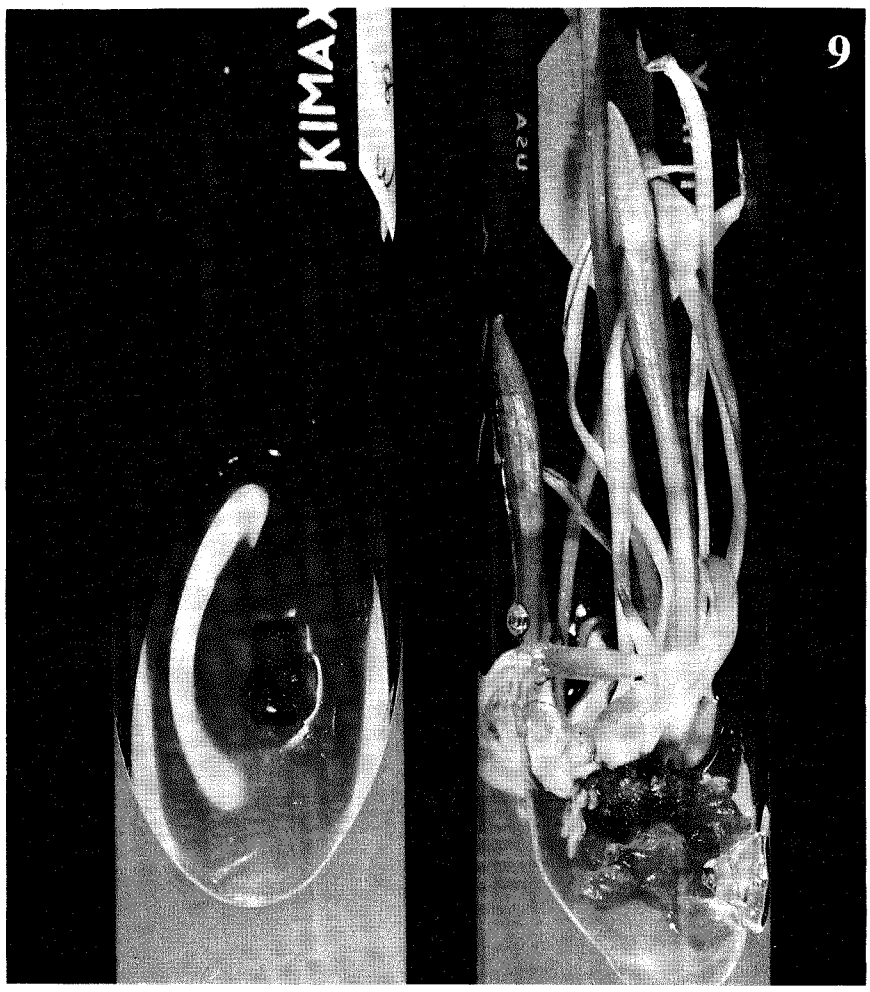


Fig. 9. Chilling requirement of *Lilium* bulb scale sections. Left: explant from unchilled bulb; right: explant from bulb exposed to 5°C, 8 wk.

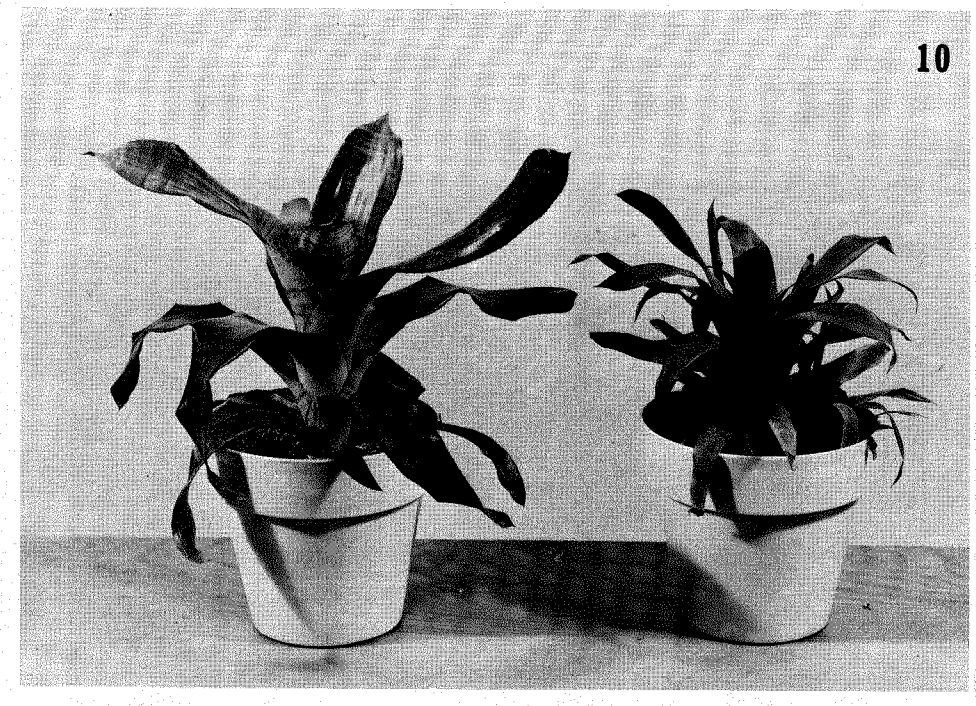
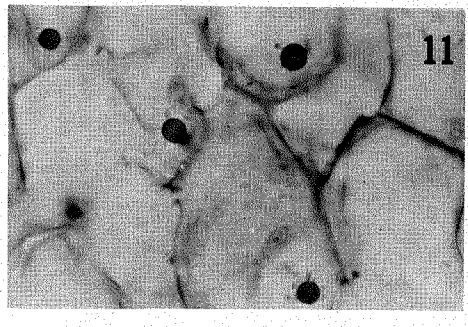


Fig. 10. Mutant (right) derived through tissue culture propagation of *Aechmea*.



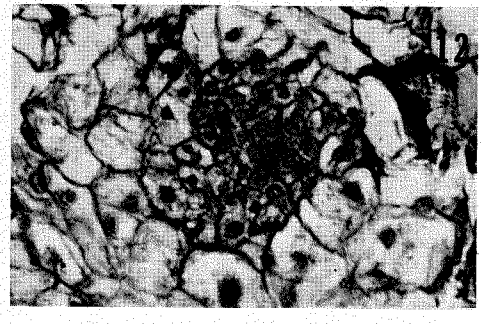


Fig. 11. Histological section of *Citrus* callus showing internal development of *Physoderma* fungus (spheres with short hyphae). Callus looked normal and no colony formation was observed externally.

Fig. 12. Meristemoid cluster in tobacco callus. Note small size, dense cytoplasm and prominent nuclei of cells in the nest.

The challenge is to determine how meristemoids might arise from differentiated cells. Thorpe and Murashige (1970) have observed that one event that precedes the formation of meristemoids in tobacco callus culture has been the accumulation of substantial amounts of starch. This observation has been confirmed by Brossard (1970). Further studies by Thorpe and Meier (1972) have disclosed that several sugars would cause significant concentration of starch in cells which ultimately became meristemoids. It is interesting that gibberellin prevented starch accumulation and suppressed organized development in tobacco callus. As differentiation of the meristemoids into organ primordia occurred and the primordia evolved into recognizable shoots, the starch diminished, indicating its utilization in the organogenetic process.

The significance of nucleic acids in organogenesis has also been studied. Vasseur (1972) has been able to associate shoot initiation in *Cichorium intybus* leaf sections with alterations in the pattern of synthesis of RNA and certain nucleotides. An initial period of callus formation in explants has been associated with a significantly high rate of RNA synthesis, and a subsequent one of shoot initiation has been related to a rise in the UMP/CMP ratio. Kovacs (1971) has noted that shoot formation in *Nicotiana* tissue cultures follows increases in the RNA/DNA and histone/DNA ratios. The development of asexually arising embryos in *D. carota* callus has been correlated with DNA synthesis (Wochok, 1973); DNA synthesis increased in readily identifiable patterns as embryo development progressed from the proembryonic mass to the torpedo stage.

The phenomenon of asexual embryogenesis in tissue cultures has been explored extensively and discussed at length (Halperin, 1969). A study of asexual embryogenesis in the *Rutaceae* just completed by Esan (1973) has disclosed some new and very significant information and deserved special consideration. Esan's study has revealed that the nucellus of all members of the *Rutaceae* possess the capacity to develop into embryos. However, the extent to which this capacity is manifested varies from one cultivar or species to another. These variations are relatable to differences in concentrations within the necelli of a substance with potent anti-embryogenic properties. This substance has been transmissible through grafts between nucelli. It has also been observed to diffuse through nutrient agar. More significant, it causes an irreversible repression of embryo initiation among affected cells, signifying probable action at the level of gene expression. The applicability of Esan's observation to other plants remains to be explored. A preliminary study has shown that the *Rutaceae* factor also suppresses embryogenesis in callus cultures of the wild carrot, *Daucus carota* "Queen Anne's Lace". Should these observations be confirmed in tests with a suitable range of species and tissues, it

would indicate that the manipulation of organized development in plants, as well as animals, would probably require an ability to manipulate and achieve genetic derepression. The inability of cells to express their organogenetic potential is clearly a reflection of repressed genes. Indeed, without repression, development of any multicellular organism would be wholly chaotic. In tissue cultures, however, the intent is to achieve resumption of organogenetic activity among normally repressed cells and the goal is to be able to unmask and enable transcription of the appropriate genes.

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植物組織培養中器官初生之誘導

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本文討論植物組織培養技術用於植物無性繁殖各項方法，及影響器官形成的諸因子。