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ORGANOGENESIS AND PLANT REGENERATION OF *CYCLAMEN PERSICUM* MILL. IN VITRO

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Christopher J. Catanzaro

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ABSTRACT

Thirteen week old F_1 hybrid cyclamen seedlings which had been germinated axenically were divided into cotyledon, cotyledon petiole, and tuber explants. Explants were cultured on modifications of Murashige and Skoog (MS) medium supplemented with an auxin (IAA, NAA or 2,4-D) and a cytokinin (BA or kinetin) in order to establish callus cultures. Petiole tissue produced large masses of callus when the growth regulator combination of 0.5 mg l^{-1} NAA and 2.5 mg l^{-1} BA was included in the medium, whereas prolific callus growth occurred on tuber tissue cultured in the presence of 2.5 mg l^{-1} NAA and 0.5 mg l^{-1} kinetin. Shoots were produced sporadically on explants cultured in the presence of NAA (1.0 mg l^{-1}) and BA (1.0 mg l^{-1}), BA alone (1.0 mg l^{-1}), or kinetin (0.5 mg l^{-1}), but were produced more consistently and in higher numbers on explants cultured on Wicart medium supplemented with NAA (0.5 mg l^{-1}) and BA (2.5 mg l^{-1}). Root formation was promoted on explants cultured in the presence of NAA (0.5 or 2.5 mg l^{-1}). Plantlets were successfully regenerated both through callus tissue and directly from parent tissue. A small number of propagules were established in vitro, and were subsequently established as freely growing in vivo plants.

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CHAPTER I

INTRODUCTION

The genus Cyclamen belongs to the Primulaceae, the largest family of the order Primulales. The members of the genus are closely related to primulas, primroses and polyanthus. However, the fact that cyclamen grow from tubers clearly sets them apart from the other members of the Primulaceae. The taxonomy of the genus remains partially unresolved even today, due mostly to genetical heterogeneity within the genus (Widmer, 1980). Estimates of the number of species range from 13 to 24. Several species are indigenous throughout the Mediterranean region, while others are native only to specific areas thereof. Cyclamen persicum, the wild species from which modern cultivars were derived, is found in southern Turkey, Crete, Cyprus, Syria, Palestine and Tunisia, but is not native to Persia (modern day Iran), as implied by its species name.

The breeding of fancy florists' strains of Cyclamen persicum (hereinafter referred to as cyclamen) was begun in the mid-nineteenth century when the plant started to achieve economic significance. Early forms had small flowers with pink hues, but soon new forms with more intense petal colors and broader, flatter petals were developed. The first large-flowered persicum forms, much like present day cultivars, became available under a number of names, 'Giganteum' among them. The 'Giganteum' phenotype has

since become a popular floricultural pot plant crop. The popularity of modern cultivars is due to the combination of their intricately marbled or silvered foliage and their unique flower petals in shades of scarlet, lilac, carmine, red, rose or white.

Commercial propagation of cyclamen is primarily from seed. In the early days of cyclamen production, 15 months or more elapsed from seed sowing to flowering of the finished plant, making costs high for the grower and ultimately for the buyer as well. Today the trend is toward fast-crop cyclamen, using selected, early-flowering strains of genetically uniform F_1 hybrids. However, the production of hybrid seed is an expensive and laborious process in itself. Other production problems include slow and erratic germination of seed, and low percentage yield of seedlings, especially under poor germination conditions.

The disadvantages of cyclamen production from seed have provided the impetus to explore alternate, asexual methods of propagation. The regenerative capabilities of notched tubers have been proven, but the technique required is too laborious to be a feasible method for the vegetative cloning of cultivars. Tissue culture has been explored as an alternate technique for vegetative cloning. Callus cultures have the potential to serve as a source for large numbers of propagules, as well as a source for individual plantlets which have undergone mutations such as resistance to various stress factors. Mutated plants would be ideal for vegetative cloning themselves, or for incorporation into breeding

programs. Although tissue culture techniques at present do not allow for the mass propagation of cyclamen, in vitro callus cultures, as well as cultures of anther, leaf, petiole and tuber tissue have all proven the ability to regenerate organs. Tuber tissue has shown the highest morphogenetic potential when compared with the other differentiated tissues which have been utilized as initial explants (Geier, 1977).

The tuber of cyclamen is formed during germination. A recently developed procedure utilizes F_1 hybrid seedlings as a source for tuber explants (Wainwright and Harwood, 1985), thus taking advantage of the plant's precocious tuberization. Juvenile seedling tuber tissue has been shown to posess high regenerative potential in vitro, as have cotyledon, petiole and root explants. Thus, in this study, sterile, uniform explants obtained from axenically germinated seedlings of hybrid cyclamen cultivars have been utilized in order to:

(1) Explore further the regenerative potential of juvenile tubers, petioles and cotyledons, and pieces thereof.

(2) Determine whether explants are more responsive to a culture medium containing a low or high concentration of macronutrients.

(3) Attempt to regulate organogenesis by varying the concentration and type of auxin and cytokinin in the medium.

(4) Acclimatize plantlets originated in vitro to conditions for autotrophic growth.

CHAPTER II

LITERATURE REVIEW

A. SEED DISINFESTATION

Anderson and Widmer (1975) explored the reasons for slow and inconsistent germination of cyclamen, and reported that seed and seedling vigor was a more likely cause than seed dormancy. The surface disinfestation of fully imbibed seed in 5% sodium hypochlorite (active ingredient basis) was recommended.

Lyons and Widmer (1979) found that soaking cyclamen seeds in water for 12 h hastened germination by up to one week. However, the total crop production time for plants from dry and soaked seeds was similar. Germination of some of the cultivars which were tested was slower when seeds were pretreated with sodium hypolchlorite, in contrast to the findings of Anderson and Widmer (1975).

Sweet and Bolton (1979) tested a variety of sterilizing agents to develop a standard procedure for the surface decontamination of seeds of many species. The use of 5% calcium hypochlorite in a 0.5% phosphate buffer (pH 6) for 10 min, followed by three sterile water rinses, was one of the most effective and least injurious sterilization procedures.

Jeon (1983) compared the in vitro germination of cyclamen seeds that had been sterilized with the germination of seeds sown

in a greenhouse bed. The in vitro germination entailed soaking the seeds in a solution containing one part Clorox bleach and nine parts Alconox (0.75% w v^{-1}) for two min, and then placing them on Knudson's medium (without sucrose). Employing the in vitro method resulted in a higher percentage of germinated seedlings and a shorter period of time required for germination to occur.

B. EXPLANT CULTURE

In 1920, Hill demonstrated the power of regeneration of the hypocotyl of the cyclamen seedling. After he removed the upper portion of the tuber, adventitious leaves, which were shown to be in the "embryonic" condition, arose from the tuber just below the cut surface, and in time a normal plant formed from the decapitated seedling tuber.

Since that time, a number of workers have demonstrated cyclamen's ability to regenerate callus, shoot buds and roots from tuber explants obtained from greenhouse-grown plants. Mayer (1956) controlled organogenesis by the addition of various concentrations of naphthalene acetic acid (NAA) and adenine. Adenine inhibited root and callus formation but induced shoot formation. Both Mayer (1956) and Stichel (1959) obtained limited whole plant regeneration from tuber explants. Okumoto and Takabayashi (1969) obtained buds or roots by supplementing the culture medium with NAA either with or without adenine sulfate.

Loewenberg (1969) used tuber pieces to establish callus cultures on a defined medium, and found that the callus retained the capacity to form roots and shoots after more than six years of subculture.

A generally high degree of internal microbial contamination of mature tuber tissue made whole plant regeneration from such tissue difficult to obtain. Stichel (1959) reported that the addition of the antibiotic Achromycin to the culture medium suppressed bacterial growth without affecting the growth of the explants, but Geier (1977) found that culturing the tissue for long periods in the presence of Achromycin decreased the viability of explants. He developed a method in which he precultivated tuber explants in the antibiotic, and later transferred them to a medium without it. Okumoto and Takabayashi (1969) studied the possibility of reducing microbial infection by drying explants for 24 h and then placing them onto the culture medium with only minimum contact.

Morel (1975) used leaf petiole callus to produce meristematic nodules which grew into plantlets as somatic embryos would. He called for further refinements of the technique regarding culture media.

Geier (1977) and Geier et al. (1979) obtained callus, shoot buds, and roots using tuber, leaf or somatic anther tissue. Geier found that combinations of indoleacetic acid (IAA) and kinetin at

appropriate concentrations caused the simultaneous induction of shoots and roots from tuber explants, whereas combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin did not. Geier et al. (1979) confirmed Geier's earlier findings, that the concentration and type of cytokinin and auxin (especially the type of auxin) which were added to cultures played an important role in the control of morphogenesis, and that only cultures of tuber tissue produced buds and roots simultaneously.

Nakayama (1980) examined the regenerative capabilities of notched tubers isolated from greenhouse-grown cyclamen. The technique proved to be laborious and thus was not feasible for propagation on a large scale.

Fersing et al. (1982) reported that a modified Murashige and Skoog medium had been used to regenerate plants from cultured organ fragments. Adult leaves formed callus which gave rise to shoot buds and tuber-like structures, and juvenile seedling tissue also underwent organogenesis.

Ando and Murasaki (1983) reported that the regenerative potential of etiolated petiole explants was superior to that of non-etiolated petioles. The explants were cultured in darkness on a one-third strength Murashige and Skoog medium.

Jeon (1983) found that explanted tuber pieces obtained from tubers which were produced in vitro regenerated shoots and roots on Knudson's medium and Murashige shoot-tip rooting medium, both

supplied with exogenous auxin and kinetin. Tubers retained high regenerative capacity when they were divided into halves or thirds, but not when divided into fourths.

Wicart et al. (1984) specified the nature of regenerated organs as being roots, shoot buds, tuber-like structures and embryo-like structures. Using morphological and histological examinations, the structures were compared with cyclamen seed embryo and young seedling tuber, and evidence was presented that all regenerated structures arose from a single somatic embryogenetic process by means of a "pre-embryogenic state".

Wainwright and Harwood (1985) used seedling tissue of Cyclamen persicum cv. 'Rosamunde' to obtain adventitious shoots and roots. Cotyledon, petiole, tuber and root explants were cultured on media containing benzyladenine (BA) and/or NAA. Shoot formation required BA (1.0 - 2.5 mg l^{-1}), whereas root formation increased with increasing NAA (up to 5.0 mg l^{-1}). However, only a small number of in vivo plants were successfully regenerated from explants.

CHAPTER III

MATERIALS AND METHODS

A. GENERAL PROCEDURES

All manipulations of plant material, sterile media and sterile culture vessels were performed in a laminar flow hood under aseptic conditions. At all other times, cultures were incubated in a growth chamber at 20° C.

B. SEED SOURCE

Cyclamen seeds of the cultivars 'Bright Red', 'Cattleya', 'Lilac', 'Pure White', and a mixture of miniature 'Kaori' shades were donated by T. Sakata & Company of Yokohama, Japan. The seeds were stored under refrigeration until used.

C. SEED DISINFESTATION AND GERMINATION

Seeds, in lots of 100 or 200, were surface disinfested by immersion and constant agitation for 10 min in a solution of 0.5% (w v^{-1}) reagent grade calcium hypochlorite, or 50, 75, or 100% Clorox bleach (5.25% sodium hypochlorite at commercial strength), to which a few drops of wetting agent (1% Tween 80) had been added. The seeds were subsequently rinsed three times in sterile, deionized, distilled water, and then placed in a single layer on moist, sterile filter paper in a petri dish. After 48 hr, the

fully imbibed seeds were once again disinfested in order to ensure a more complete surface sterilization.

The germination medium was prepared using Murashige and Skoog (MS) macronutrients (see page 12) at full, one-quarter or zero strength. Following adjustment of the pH of the medium to 6.0 and the addition of agar (10 gm l^{-1}), the medium was heated to boiling, distributed in 50 ml aliquots to GA7 culture vessels (Magenta Corp., Chicago, IL), and then autoclaved at 121° C and 1.03 bar for 15 min.

Fully imbibed and twice disinfested seeds were placed on the solidified medium, 10 seeds per vessel. The vessels were incubated in darkness for three weeks, after which they were supplied with a day/night regime of 16/8 h.

D. MEDIA SELECTION AND PREPARATION

Modifications of Murashige and Skoog (MS) (1962) medium (see pages 12 and 13) were used to induce callus on explants derived from seedling tissues. The media were supplied with macronutrients at full or one-quarter strength, and with an auxin (IAA, NAA or 2,4-D) and a cytokinin (BA or kinetin) in various combinations and at various concentrations (see page 14). A special modification of MS medium, hereinafter referred to as Wicart (1984) medium, contained macronutrients (one-quarter strength), glycine (2.0 mg l^{-1}), nicotinic acid (0.5 mg l^{-1}), pyridoxine (0.5 mg l^{-1}), thiamine HCl (5.0 mg l^{-1}), NAA (0.5 mg l^{-1}),

BA (2.5 mg l^{-1}), and sucrose (6%), with the remaining constituents included as listed in Tables 1 and 2.

The following combinations of medium constituents were used for the induction of organs in callus cultures, (S.1 - S.6) for shoot-induction and (R.1 - R.2) for root-induction:

(S.1) MS macronutrients (full strength), adenine sulfate (80 mg l^{-1}), NaH_2PO_4 (170 mg l^{-1}), L-tyrosine (100 mg l^{-1}), IAA (2.5 mg l^{-1}), and kinetin (0.5 mg l^{-1}) (a further modification of MS medium for tobacco shoot induction on callus, which had been modified previously by Murashige).

(S.2) MS macronutrients (one-quarter strength in this medium and in all succeeding media), adenine sulfate (80 mg l^{-1}), NaH_2PO_4 (170 mg l^{-1}), L-tyrosine (100 mg l^{-1}), NAA (2.0 mg l^{-1}), and kinetin (0.5 mg l^{-1}).

(S.3) MS macronutrients, NAA (1.0 mg l^{-1}), and BA (1.0 mg l^{-1}).

(S.4) MS macronutrients and BA (1.0 mg l^{-1}).

(S.5) MS macronutrients and kinetin (0.5 mg l^{-1}).

(S.6) MS macronutrients alone.

(R.1) MS macronutrients and NAA (2.5 mg l^{-1}).

(R.2) MS macronutrients and NAA (0.5 mg l^{-1}).

All remaining constituents of the media listed above were included as listed in Tables 1 and 2.

TABLE 1

COMPOSITION OF INORGANIC NUTRIENTS (SALTS)
OF MURASHIGE AND SKOOG (1962) MEDIUM

Inorganic nutrients	mg l^{-1}
Macronutrients[a]	
NH_4NO_3	1650.000
KNO_3	1900.000
$CaCl_2 \cdot 2H_2O$	440.000
$MgSO_4 \cdot 7H_2O$	370.000
KH_2PO_4	170.000
Micronutrients	
H_3BO_3	6.200
$MnSO_4 \cdot 4H_2O$	22.300
$ZnSO_4 \cdot 7H_2O$	8.600
KI	0.830
$Na_2MoO_4 \cdot 2H_2O$	0.250
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
$Na_2EDTA \cdot 2H_2O$[b]	37.300
$FeSO_4 \cdot 7H_2O$	27.800

[a]Stock solutions of macro- and micronutrients were prepared and then stored under refrigeration.

[b]A stock solution of $Na_2EDTA \cdot 2H_2O$ and $FeSO_4 \cdot 7H_2O$ (iron) was prepared separately.

TABLE 2

COMPOSITION OF ORGANIC NUTRIENTS OF MURASHIGE AND SKOOG (1962) MEDIUM

Organic nutrients	mg l^{-1}
Vitamins and amino acids[a]	
Glycine	2.000
Nicotinic acid	0.500
Pyridoxine HCl	0.500
Thiamine HCl	0.100
Myo-inositol	100.000
Other organic constituents[b]	percent
Sucrose	3.00%
Agar	1.00%

[a]Glycine, nicotinic acid and pyridoxine HCl were excluded from the medium and the concentration of thiamine HCl was increased to 0.4 mg l^{-1} in accordance with Linsmaier and Skoog (1962) medium.

[b]Other constituents include indoleacetic acid and kinetin at a range of concentrations.

TABLE 3

COMPOSITION OF GROWTH REGULATORS OF CALLUS-INDUCTION MEDIA

Auxin	mg l^{-1}	cytokinin	mg l^{-1}
IAA	2.5	kinetin	0.5
NAA	2.0	kinetin	0.5
NAA	2.5	kinetin	0.5
2,4-D	2.5	kinetin	0.5
NAA	0.5	BA	2.5
NAA	2.5	BA	2.5

The media were prepared using deionized, distilled water. The macronutrients were mixed with water, after which the micronutrients, vitamins and amino acids, growth regulators, and sucrose were added as needed. Following adjustment of the pH to 5.7 and the addition of the agar, the solution was brought up to final volume, autoclaved at 121° C and 1.03 bar for 15 min, and then distributed in 25 ml aliquots to sterile, 100 x 15 mm petri dishes. All media were stored under refrigeration until used.

E. CALLUS PRODUCTION

Approximately 13 weeks after the seeds had been placed on the germination medium, the seedlings were divided into cotyledon, cotyledon petiole, and tuber explants. The explants were sorted by type, trimmed in order to maximize the uniformity within each explant type, and then placed on a solid callus-induction medium in 15 x 100 mm petri plates. The cultures were subsequently incubated in darkness.

F. SHOOT AND ROOT PRODUCTION

Callus tissue produced by the initial explant tissues was isolated and transferred onto a fresh nutrient medium once it had reached a size sufficient for it to be divided into several callus fragments ranging from 5 - 10 mm in diameter and 20 - 100 mg in weight (Street, 1969). The tissue fragments were initially placed on a shoot-induction medium, and were supplied with a day/night regime of 16/8 h. The rate of tissue growth and the incidence of organ formation on the initial shoot-induction medium played a decisive role in the selection of the medium or media utilized for subsequent transfers, as well as the frequency with which the transfers were made. In most cases, MS macronutrients alone (the constituents of medium S.6) were used for the establishment of freely growing in vitro plantlets.

G. HISTOLOGICAL PROCEDURES

Histological procedures were performed on callus, organs regenerated from callus, and organs regenerated directly from parent tissue. Tissues were placed in a solution of formalin, acetic acid and ethanol (50% FAA) (Johansen, 1940, Jensen, 1962), aspirated thoroughly, and allowed to stand at least 24 h. The fixed tissue samples were dehydrated by passing them through an ethanol-t-butanol (TBA) series (Johansen, 1940, Sass, 1958), and embedded in paraffin. The paraffin blocks were sectioned on a rotary microtome and the sections (10 µm thick) were mounted on slides, following which the slides were stained directly through the paraffin in 0.1% aqueous toluidine blue for 60 min, rinsed, dried overnight, and passed through clearing fluid. In the final step, resin was used to affix cover glasses to the slides.

Tissues representative of a given treatment were preserved in polyethylene glycol 400 (PEG 400) (Graham et al., in press) so that they could be stored at a particular stage of development for photographic purposes at a later date. The tissues were fixed in 50% FAA, allowed to stand in three successive changes of 50% ethanol, immersed in a 1:1 mixture of 50% ethanol and PEG 400 for 24 h, and then placed in a 60° C oven. Once the ethanol and some of the water initially present were driven off, a final fluid change was made into 80% PEG.

CHAPTER IV

RESULTS

A. SEED DISINFESTATION AND GERMINATION

Preliminary germination tests in which seeds were disinfested with calcium hypochlorite or sodium hypochlorite revealed little difference between treatments in residual contamination of the seeds. Similarly, no differences were noted in the number of seeds which underwent germination, or in their subsequent development. Sodium hypochlorite, available in a 75% solution of Clorox bleach (5.25% sodium hypochlorite at commercial strength) was chosen as the standard solution for disinfestation after it was proven that sodium hypochlorite at high concentrations was in no way detrimental to seed germination. Commercial bleach was selected as the source of sodium hypochlorite due to its low cost and ready availability.

Subsequent tests were performed in order to compare the germination of seeds on a medium supplied with macronutrients at full, one-quarter or zero strength. The percentage of seeds which had germinated on the one-quarter and zero strength media were comparable one month after sowing, but the percentage of seeds which had germinated on the full strength medium was considerably lower. However, those seedlings which germinated on the medium supplied with macronutrients at full or one-quarter strength

developed earlier than did those on the zero strength medium, as indicated by the greater expansion of their cotyledons. Seed which was exposed to the standard disinfestation procedure and then sown on the medium supplied with macronutrients at one-quarter strength exhibited an overall average germination rate of roughly 60%, and those seedlings which developed (Plate 1, Fig. 1) tended to have larger cotyledons and a more extensive root system than comparable seedlings with no exogenous nutrient supply.

B. CALLUS PRODUCTION

In the first phase of callus-induction experiments, the medium which was employed contained macronutrients at either full or one-quarter strength, and was supplemented with the growth regulator combination of IAA (2.5 mg l^{-1}) and kinetin (0.5 mg l^{-1}). Each type of explant tissue produced at least small clumps of callus on the medium supplied with either level of macronutrients. Cotyledon tissue supplied with full strength nutrients demonstrated the least propensity to produce callus. Petiole and tuber tissue on the one-quarter strength macronutrients produced considerably more callus than cotyledon tissue on the one-quarter strength macronutrients or petiole, tuber and cotyledon tissue on the full strength macronutrients.

Those tissues which were noted for their superior ability to produce callus on the twice-diluted macronutrients were also noted for their ability to undergo organogenesis. Tuber tissue tended

Plate 1. Seedling and callus-bearing explants cultured on modifications of Murashige and Skoog medium.

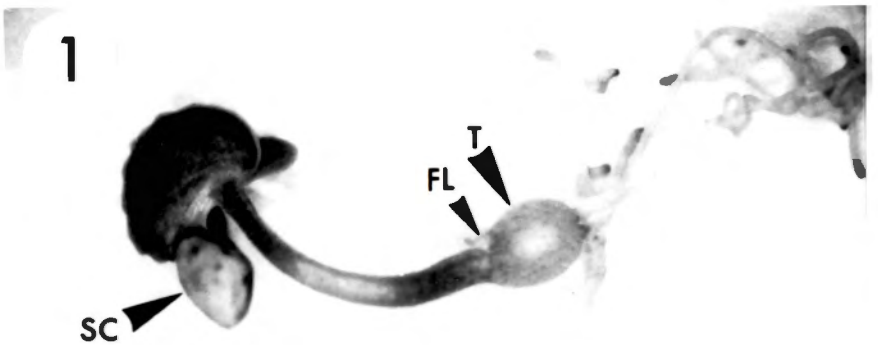
Fig. 1. Eleven week old 'Lilac' seedling. Cultured on one-quarter strength MS macronutrients. X 4

Fig. 2. Callus-bearing petiole explant. Cultured on MS medium supplemented with NAA and BA, fixed in 50% FAA, and immersed in PEG 400. X 4

Fig. 3. Callus-bearing tuber explant. Cultured on MS medium supplemented with NAA and BA, fixed in 50% FAA, and immersed in PEG 400. X 7

Fig. 4. Tuber-derived callus tissue. Cultured on MS medium supplemented with NAA and BA. X 7

c = callus, fl = first leaf, pt = parent tissue, sc = seed coat, t = tuber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FL
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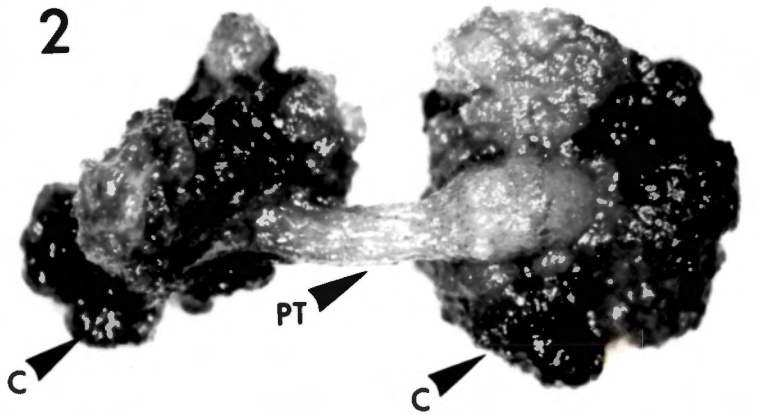
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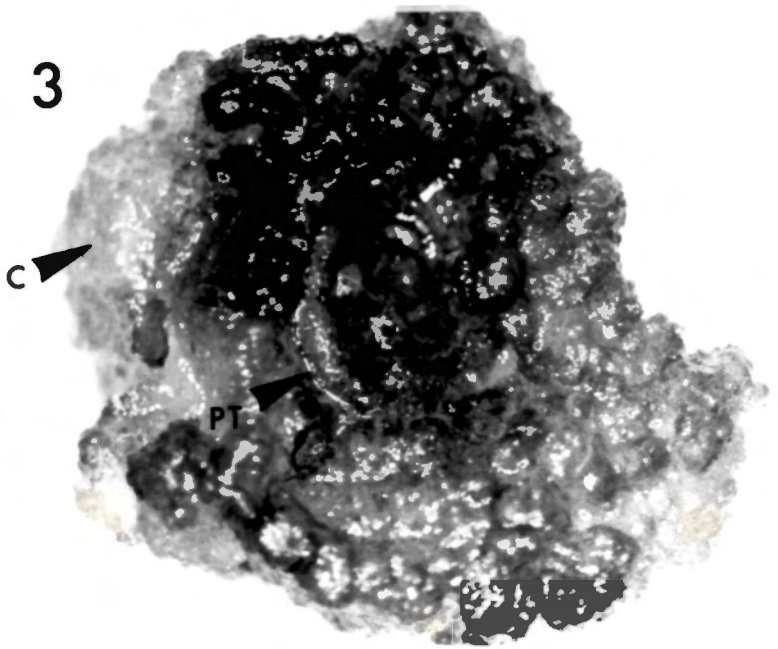
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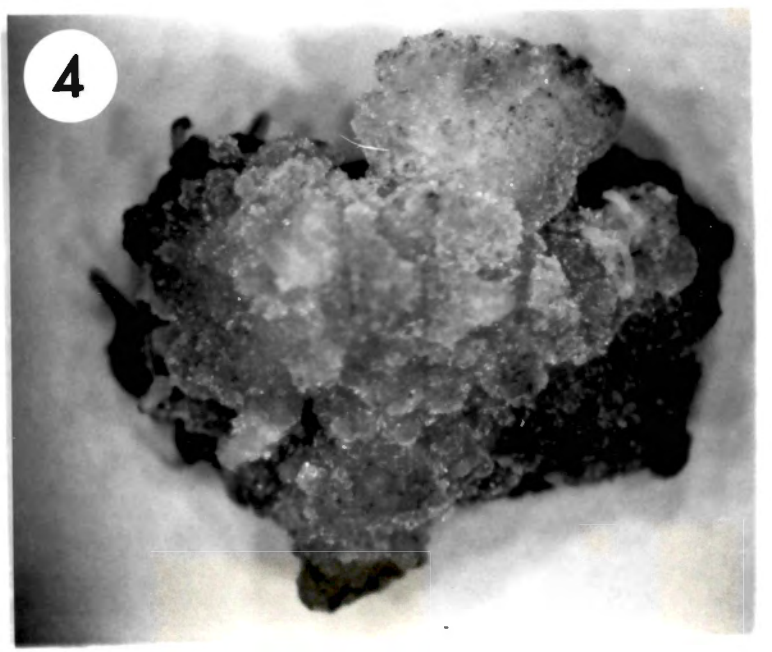
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to form roots, but also formed shoots sporadically. Petiole tissue showed slightly less tendency to undergo organ formation than tuber tissue.

In later experiments, IAA was replaced by NAA or 2,4-D as the auxin source of the callus-induction media. Explants which were cultured in the presence of 2,4-D (2.5 mg l^{-1}) produced considerable callus, but all tissues which were exposed to 2,4-D gradually became very dark in color, indicating that they were adversely affected by the growth regulator at the given concentration. Thus, the use of 2,4-D was discontinued in favor of NAA (0.5 - 2.5 mg l^{-1}), which, in the presence of kinetin or BA, stimulated considerable callus production within seven weeks of the initial date of explant culture. Petiole and tuber tissue proved superior to cotyledon tissue in their ability to produce callus (Plate 1, Figs. 2, 3 and 4), which had been demonstrated in the first phase of experiments as well. Large masses of callus were produced on petiole tissue when the growth regulator combination of 0.5 mg l^{-1} NAA and 2.5 mg l^{-1} BA was included in the medium, and the combination of 2.5 mg l^{-1} NAA and 0.5 mg l^{-1} kinetin induced similar callus masses on tuber tissue. In addition to the large amounts of callus it was capable of producing, tuber tissue tended to produce roots, again in agreement with earlier findings.

The production of callus by explants cultured on the MS and Wicart media, both supplemented with 0.5 mg l^{-1} NAA and

2.5 mg l^{-1} BA, was similar. Callus production on petiole tissue was very high on both media, although it was produced at a slightly accelerated rate on the Wicart medium. However, MS proved to be the superior medium for the promotion of callus on tuber tissue.

Explant tissue which was cultured on the Wicart medium underwent organogenesis (Plate 2) at a much higher rate than it did on any of the other modifications of MS medium. In one experiment, 26 of 98 (26%) cotyledon expants cultured on the Wicart medium underwent shoot induction, as did 26 of 46 (56%) petiole explants. On some explants shoots were produced singly, while on others up to four viable shoots were formed.

C. SHOOT AND ROOT PRODUCTION

Shoot-induction media 1 and 2 (S.1 and S.2) were unsuccessful in the induction of shoots from callus tissue. Only sporadic shoot formation occurred when callus tissue was subcultured on media S.3 and S.4. Callus tissue which was subcultured on medium S.5 underwent shoot formation either sporadically or not at all.

Results from previous shoot-induction experiments led to the development of an experiment in which callus tissue from petiole and tuber explants was divided into four homogeneous groups, with each group being subcultured on a modified MS medium.

Plate 2. Shoot-bearing explants cultured on Wicart medium.

Fig. 5. Shoot-bearing leaf explant. X 4

Fig. 6. Shoot-bearing leaf explant. X 4

Fig. 7. Shoot-bearing petiole explant. X 4

Fig. 8. Shoot-bearing tuber explant. Transferred whole to MS medium supplemented with NAA. X 4

s = shoot, sm = shoot meristem

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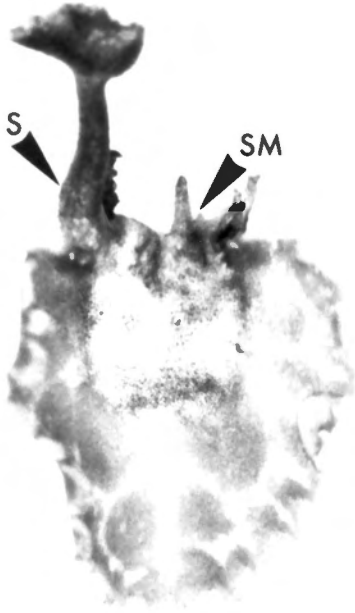

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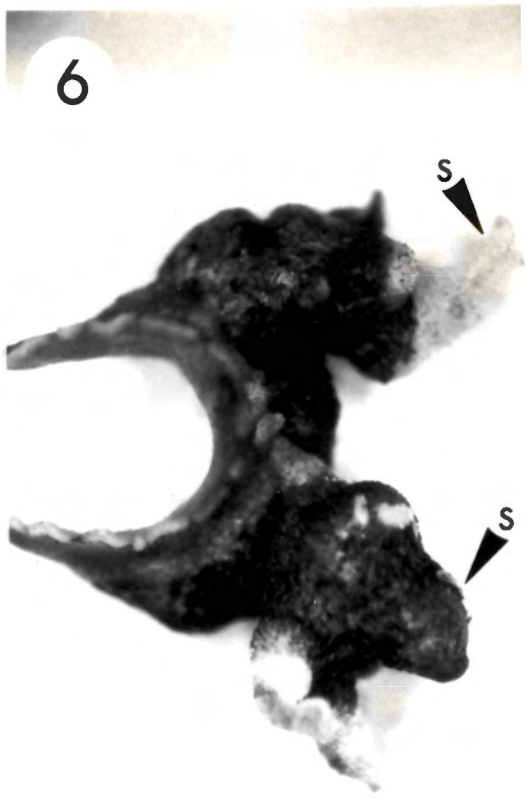

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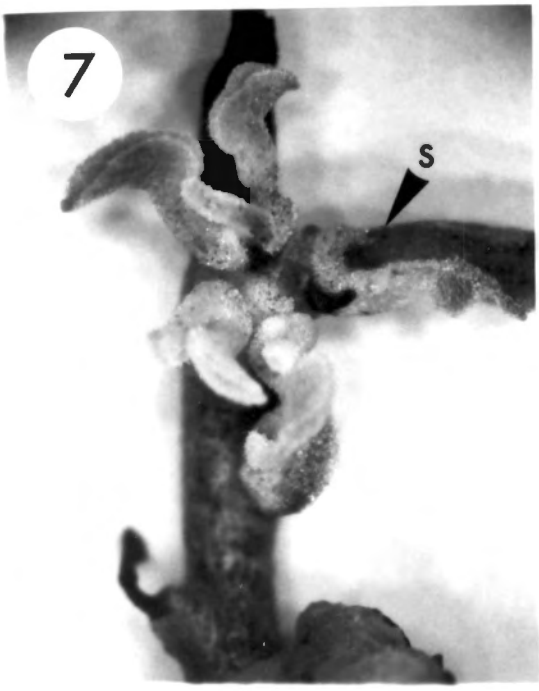

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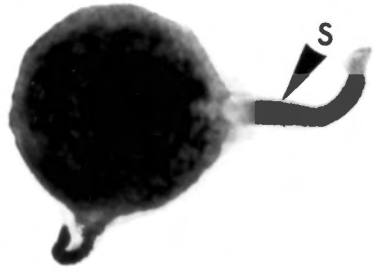


Plate 2
Figs. 5 - 8
24
Catanzaro
1986

Each of the media was supplemented with one of the following:

(1) NAA (0.5 mg l^{-1}), BA (2.5 mg l^{-1}) and adenine sulfate (80 mg l^{-1}).

(2) NAA (0.5 mg l^{-1}) and BA (2.5 mg l^{-1}).

(3) Adenine sulfate (80 mg l^{-1}) alone.

(4) None of the above constituents.

A few shoots were formed on tissues on the media supplemented with adenine sulfate (Plate 3, Fig. 9), but none were initiated on the tissues without it. It was also noted that after five weeks in culture, 27 of 60 (45%) tuber-derived tissue fragments which were cultured on the media without NAA and BA had formed roots, whereas only 3 of 60 (5%) callus fragments cultured in the presence of the growth regulators had done so.

Although medium S.6 did not prove useful in the induction of shoots, it was successful in supporting newly isolated, regenerated shoots and plantlets as they established themselves as freely growing in vitro plants (Plate 3, Fig. 10). In addition to medium S.6, the root-induction media (R.1 and R.2, supplemented with 2.5 and 0.5 mg l^{-1} NAA respectively), were successful in the promotion of rooting and the establishment of plantlets (Plate 3, Fig. 11). Of the two concentrations of NAA used, 2.5 mg l^{-1} was found to be slightly superior in the promotion of rooting. Tuber-derived shoots which were removed from parent tissue during their transfer to medium R.1 underwent a relatively high incidence of root formation (6 of 16 shoots isolated) (Plate 3, Fig. 12),

Plate 3. Regenerated shoots and whole plantlets cultured on modifications of Murashige and Skoog medium.

Fig. 9. Shoot-bearing callus tissue. Cultured in the presence of adenine sulfate, and derived from tuber tissue. X 4

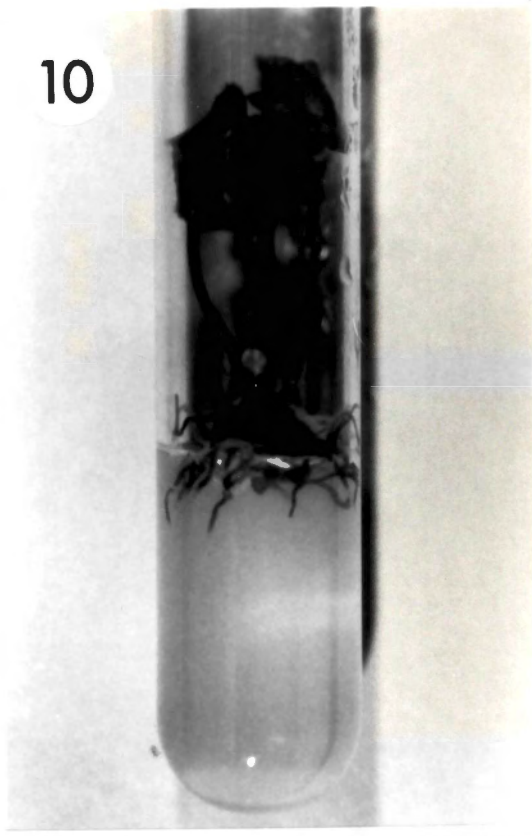
Fig. 10. Regenerated plantlet derived from tuber tissue. Tissue originally cultured on MS medium supplemented with NAA, later transferred to basal MS medium (medium S.6). X 1.1

Fig. 11. Regenerated plantlets derived from cotyledon tissue. Tissue originally cultured on MS medium supplemented with IAA and kinetin. Plantlets cultured in the presence of NAA. X 1.2

Fig. 12. Isolated plantlet derived from tuber tissue. Tissue cultured on Wicart medium, later rooted on MS medium supplemented with NAA. X 4

r = roots, s = shoot, sm = shoot meristem


9
SM
S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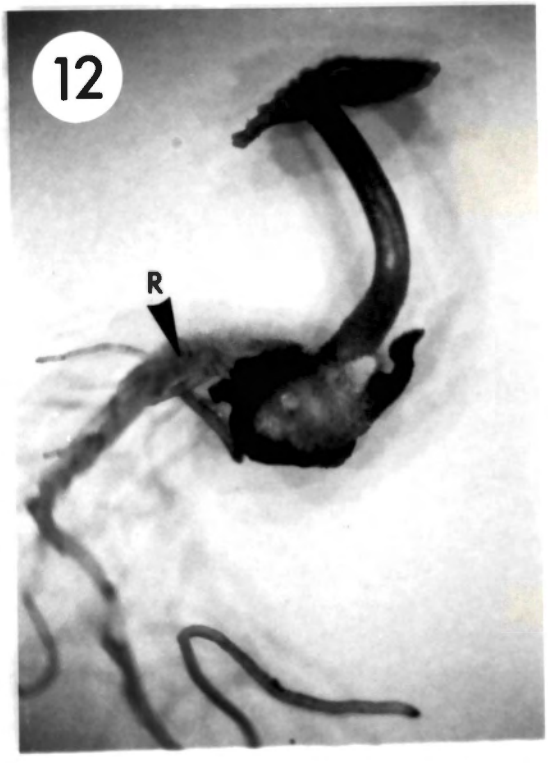
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whereas most of the shoots which were not isolated at the time of passage gradually became necrotic.

Regenerated plantlets proved somewhat difficult to establish in vivo. Casualties were high, as only 7 of 21 (33%) propagules survived the hardening off process. The propagules which underwent this process were removed from the tissue from which they had developed, planted in Pro-Mix soilless medium, and placed under shade and mist for one week. Following their removal from the mist, the propagules were treated with a Benlate-Truban mixture and returned to a 20° C incubator with a day/night regime of 16/8 h.

D. HISTOLOGICAL PROCEDURES

Shoots and plantlets were examined histologically in order to obtain a better understanding of the distinctions between organs which were regenerated through callus and those which were formed directly from parent tissue. Callus tissue has a dramatically different appearance from the regenerated shoots which arose from it, which is evident in both transverse sections and longitudinal sections (Plate 4, Figs. 13 and 14), since cellular organization is lacking in the callus tissue. At the boundary between callus tissue and differentiated tissue lies a band of small, thin-walled, meristematic cells which clearly demarcate the two types of tissue. Transverse sections (Plate 4, Fig. 15) and longitudinal sections (Plate 4, Fig. 16) of shoots which were

Plate 4. Light micrographs of sections (10 µm thick) of shoot-bearing explants cultured on Wicart medium.

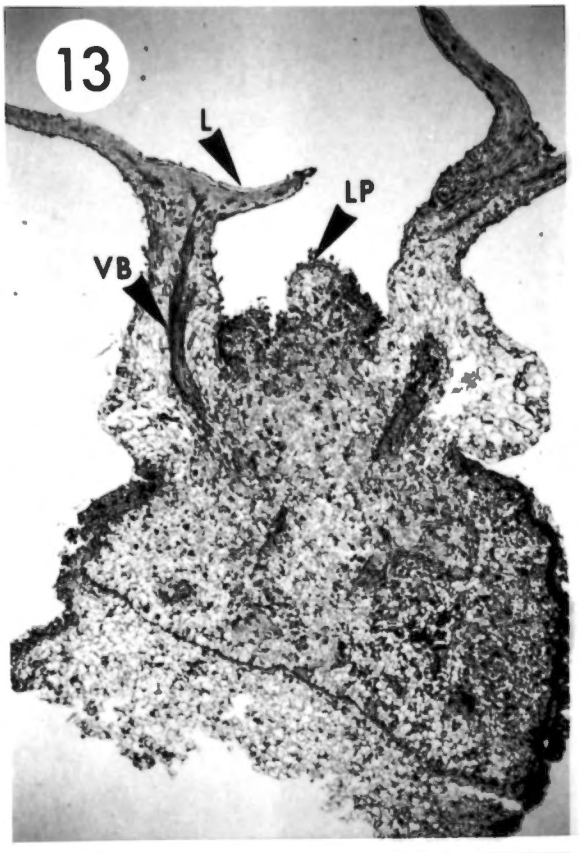
Fig. 13. Longitudinal section of shoot-bearing callus tissue. Tissue derived from petiole explant. X 25

Fig. 14. Longitudinal section of shoot-bearing callus tissue. Tissue derived from petiole explant. X 22

Fig. 15. Transverse section of shoot-bearing petiole tissue. Shoot bud derived from parent tissue. X 22

Fig. 16. Longitudinal section of shoot-bearing tuber tissue. Shoot derived from parent tissue. X 25

c = callus, l = leaf, lp = leaf primordium, s = shoot, sb = shoot bud, sm = shoot meristem, vb = vascular bundles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L
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VB


14
SM
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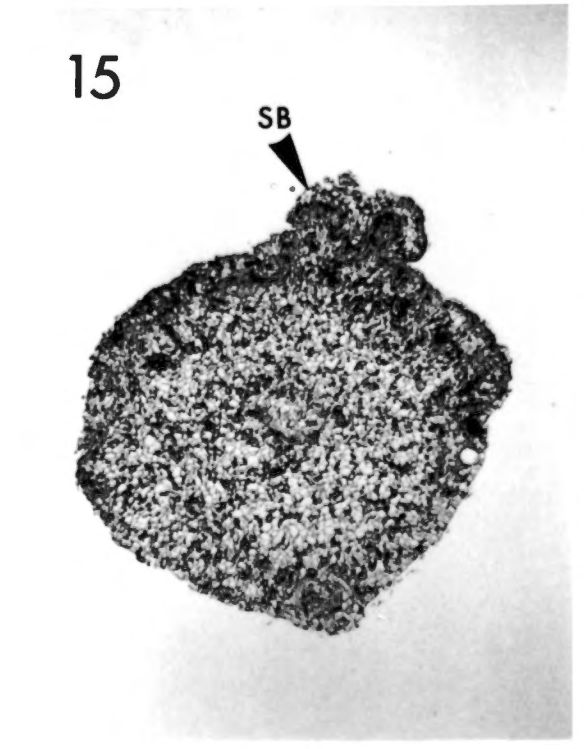
C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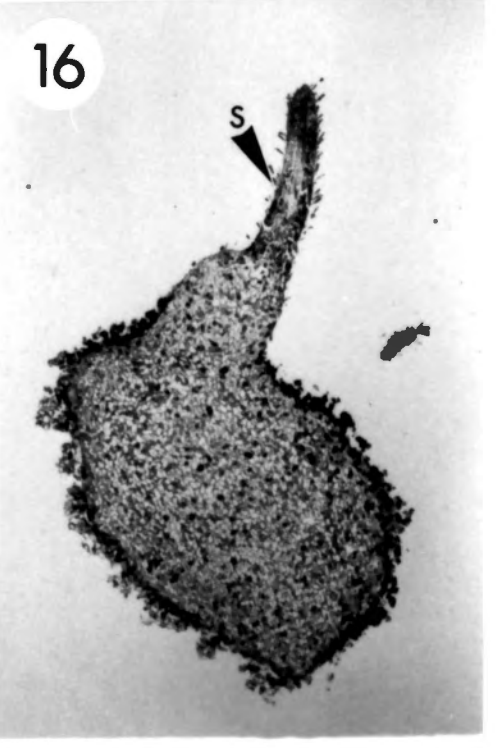
SB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S

regenerated from parent tissue revealed that the shoots had arisen from the parenchymatous cortex only a few cellular layers below the epidermis.

CHAPTER V

DISCUSSION

A. SEED DISINFESTATION AND GERMINATION

Seed which was surface disinfested in a 100% solution of Clorox bleach underwent germination and subsequent development no differently than seed disinfested in a 30% solution of bleach. One possible reason for the lack of detrimental effects of sodium hypochlorite, even at high concentrations, concerns the extraordinarily thick walls which are characteristic of the endosperm cells of cyclamen seeds (Jeon, 1983). Hypochlorite might be unable to penetrate the endosperm, due to the thickness of its cell walls. However, seed was not completely disinfested even when treated with a full strength solution of commercial bleach to which a few drops of 1% Tween 80 had been added. A higher concentration of wetting agent might have been necessary to break the surface tension at the outer surface of the furrowed seed coat, enabling the sodium hypochlorite to more effectively remove the microorganisms which were present.

A difference in the germination rates of seeds supplied with MS macronutrients at one-quarter or zero strength was not expected, due to the presence of endosperm in cyclamen seed, and indeed no such difference between the two treatments was found. One-quarter strength macronutrients proved sufficient to meet the

nutritional requirments of seedlings for the period during which they were cultured on the germination medium.

B. CALLUS PRODUCTION

All three types of explant tissue (cotyledon, cotyledon petiole, and tuber) were capable of producing callus tissue, although the amount produced was dependent on factors such as the type of explant tissue utilized, the size of explant pieces utilized, and the constituents included in the callus-induction medium, especially the concentration and type of auxin and cytokinin. Petiole and tuber tissue on the one-quarter strength macronutrients supplemented with 2.5 mg l^{-1} IAA and 0.5 mg l^{-1} kinetin demonstrated an above average ability to produce callus, which was realized more fully when IAA was replaced by NAA (0.5 - 2.5 mg l^{-1}) as the auxin source. NAA in combination with kinetin or BA stimulated considerable callus production within seven weeks of the initial date of explant culture. Petiole explants often had the characteristic appearance of a dog's bone after being in culture for some time, since callus tissue was formed most prolifically at the cut ends of the explant tissue. Similar results were recorded by Wainwright and Harwood (1985) with root explants.

Those tissues which were noted for their superior ability to produce callus on the MS media were also noted for their ability to undergo organogenesis. Considering its role in root production

in the intact plant, tuber tissue predictably formed roots in vitro as well; however, it was also noted for the sporadic formation of shoots. Explant tissue which was cultured on the Wicart medium initiated shoot buds at a higher rate than it did on any of the other modifications of MS medium. This increased incidence of shoot formation is apparently the result of the interaction of explant tissue with the additional amino acids and/or the doubled concentration of sucrose with which the medium is supplemented.

C. SHOOT AND ROOT PRODUCTION

None of the media which were intended to be used for the induction of shoots from callus tissue were as successful in the promotion of shoot formation as the Wicart medium, which had been utilized originally as a callus-induction medium. The control of organogenesis in vitro depends on several factors including cell to cell interactions, cell and tissue interactions, factors inherent in the explants, and all external factors (Tran Thanh Van, 1980). The lack of organogenesis on small callus fragments might thus be explained by the lack of certain cell to cell interactions necessary for organogenesis to occur, or by the lack of the necessary external factors, or even by a combinative lack of factors. Any of these factors might also help to explain the difficulties encountered during attempts to alter the course of organogenetic pathways, i.e., during attempts to induce formation of the other organ type once shoots or roots have been initiated.

D. HISTOLOGICAL PROCEDURES

Although the PEG method developed by Graham et al. was developed primarily as an alternate procedure for sectioning tough herbaceous stems, the method was also effective in enabling in vitro grown tissue pieces or plant parts to be preserved and subsequently photographed in a likelike manner. In addition to acting as a preservative, PEG 400 prevents the dehydration of infiltrated specimens by clinging to them after they are removed from the fluid in order to be photographed. Specimens can be easily preserved at any point of interest in their development, and the task of photographing them can be delayed until a considerable number have been accumulated, making the method efficient as well as convenient. Specimens which are returned to PEG after being photographed can be preserved indefinitely.

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