

AZALEA TISSUE CULTURE
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ABSTRACT

A procedure for in vitro proliferation of callus tissue from Azalea variety Mollis was developed.

Hypocotyl sections were shown to be acceptable tissue source and were used as the primary explant source. Rapidly growing stem tissue and anther-pollen tissue from varieties Pluto, Roadrunner, and Hinodegiri were also investigated concerning their utility as explant sources.

The influence of the auxin-cytokinin ratio (using 2,4-D, IAA, and BA) on callus initiation and growth was investigated. Additionally, the influence of the major and minor inorganic media components was studied. Factors such as reduced salt concentrations were investigated.

A reliable procedure for the establishment of callus was developed.

AZALEA TISSUE CULTURE

INTRODUCTION

Plant tissue culture has provided many research opportunities for the exploration of applied and fundamental questions pertaining to tissue and cell development. Tissue culture embraces a wide spectrum of experimental systems and as a result, the following introduction is presented in two parts, first considering the primary somatic tissue explants with the hypocotyl section as the primary explant type and then considering anther-pollen culture. Citations include recent selected papers which summarize progress in these two areas, in addition to listing the plants that have been successfully propagated in vitro.

The objective of this study is to provide information for the development of procedures for the in vitro propagation of Azalea plants. This includes delineating factors influencing the procurement of suitable explants, relative growth of explants on various media types, the effects of growth regulators and hormones on the production of callus.

Plant tissue culture is a valuable technique by which cells and tissues can be controlled experimentally, although it is recognized that tissues grown in vitro are not necessarily entirely comparable to the parent tissues from which they were derived. In general, the first steps in establishment of a culture involve the

recrudescence of division and in some cases, the selective differentiation of some cells (Reinert et al. 1977). It is evident that a complex explant theoretically may give rise to a callus consisting of a very heterogenous mixture of cell types (Bornman 1974), but in general, parenchyma cells proliferate. Relevant literature concerning the establishment of tissue cultures permits no broad generalizations because of wide differences in the origin of explants, physiological states, endogenous hormone levels, and general culture conditions (i.e., mineral salts, carbohydrates, light, and temperature, Kohlembach 1977).

Various growth regulators have been investigated in a wide variety of plant systems. Organogenesis and embryogenesis in culture are complex events involving a number of interacting chemical and physical factors. One hypothesis proposes that small quantitative changes in the ratio of media components are critical while another hypothesis proposes that the synthesis of organ specific substances is involved in shoot, leaf and root organogenesis (Reinert et al. 1977). Certain general observations can be noted: (1) It has been shown that low concentrations of auxin stimulate vessel member formation (Aloni 1979) and auxin-sugar combinations can modify cellular development, e.g., 1.0 ppm auxin and high sucrose results in phloem development in Nicotiana tabacum callus (Bornman 1974, Bornman et al. 1977). (2) A high ratio of auxin relative to cytokinin favors root formation, whereas the opposite relationship of increased cytokinin to auxin favors shoot buds in Lycopersicon (Kohlenback 1977) and Beta vulgaris (Welanders

1974). (3) In triggering embryogenesis the ratio of nitrogen to auxin has been shown to be important, i.e., the presence of reduced nitrogen favors embryogenesis (Kamada and Harada 1979b). (4) A requirement for cell division in the determination of subsequent cellular development has not been clearly established (Reinert et al. 1977). Evidence from one study of Avena sativa L. coleoptiles, indicates that protein synthesis is required for initiation of auxin induced growth (Bates et al. 1979). (5) Reports have not delineated the role of cytokinin (kinetin, K) in auxin (indoleacetic acid, IAA) induced cell elongation. Low concentrations of K enhance IAA-induced elongation in cucumber (Katsumi and Kazama 1978) but K inhibits auxin promoted elongation in soybean (Vanderhoef et al. 1973). Auxin stimulates cell enlargement and also promotes the synthesis of m-RNA and t-RNA. An auxin-induced cell wall loosening may influence wall plasticity through the action of a protein membrane factor which interacts with RNA polymerase directly or may transport the hormone (IAA) into the nucleus, thus altering the transcription pattern as reported using soybean hypocotyl callus tissue (Hardin et al. 1972).

Numerous studies cited by Reinert et al. (1977) state that a certain ratio of auxin to cytokinin in the nutrient medium is necessary for successful morphogenesis in selected plants. Comparison between reports dealing with a variety of plants reveals considerable variability in the response of isolated tissues. In Nicotiana tabacum L. c.v. Wiscon 38, cytokinins characteristically induce thylakoids and grana formation in plastids (Szweykouska 1974)

and in general influence development in a pattern similar to the effects of light on watermelon cotyledons (Longo et al. 1979). Cytokinins have been shown to act complementarily with auxin, enhancing the synthesis rates of RNA and DNA in pea parenchyma (Shinger 1978) while cytokinin prevents m-RNA destruction (Reinert et al. 1977). At certain concentrations and ratios, the simultaneous application of cytokinin (benzyladenine, BA) and various auxins stimulates the proliferation of isolated carrot callus explants (Kamada and Harada 1979a). Skoog and Miller (1957) reported that K and IAA are requisites for tissue growth and differentiation of N. tabacum tissue. This relationship in tobacco tissue has been confirmed in additional studies (Linsmaier and Skoog 1965).

Extensive reports have examined the nutritional requirements and the physical parameters which influence the growth and development of isolated tissues. Brando and Salema (1977) reported that darkness facilitated Sedum telephuna L. callus formation by promoting the division of isolated cells. Photosynthesis is not a limiting activity since a carbohydrate source is incorporated into the medium. Light, however, regulates certain in vitro morphogenetic processes as indicated using isolated Nicotiana (Murashige 1974) and cucumber tissues (Kazama and Karsumi 1978). Numerous tissues have been grown using standard media containing Murashige and Skoog 1962 mineral salts (MS), a carbohydrate source and agar. This medium was selected as a standard in our studies because of favorable preliminary results with Azalea anthers (MS

delaying necrosis) and the desirability of using standardized media for comparative purposes as suggested by Gamborg et al. (1976). This basal culture medium is often modified by the addition of various types and concentrations of growth regulators.

According to Kamada and Harada (1979b) the presence of reduced nitrogen promotes somatic embryogenesis in carrot tissue cultures (MS is rich in reduced nitrogen, Murashige and Skoog 1962). Kohleback (1977) further emphasized the role of this form of nitrogen in embryo formation and root formation. Coconut milk (CM), an undefined media additive, has long been recognized as a growth promoting additive in plant tissue culture systems. Steward (1976) noted that despite extensive analytical studies, whole CM is more stimulative than solutions designed to accurately duplicate the content of natural CM. Bajaj and Mader (1974) found that 10% CM, when combined with various levels of auxin and cytokinin, greatly increased the amount of root and shoot differentiation in Anagallis arvensis cultures. Reduced nitrogen, as found in CM or in the form of amino acids, is frequently added to basal media. Studies using carrot (Kamada and Harada 1979b) or Vigna sinensis (Murthyreddy 1974) indicated the beneficial influences of quantitative and qualitative changes in exogenous amino acids. There is, however, little unifying information concerning the influence of exogenous amino acids in plant tissue culture (Sandsted and Skoog 1960).

A wide variety of tissues and organs (leaves, roots, stems, seeds and seedlings) have been used as explant sources. In this study the suitability of azalea seedling was initially explored in

order to determine an acceptable explant source. Azalea seeds were germinated and the seedlings were placed in culture (intact or divided into epicotyl, hypocotyl and root sections) on selected media. The development of callus tissue on these preliminary treatments indicated the acceptability of azalea hypocotyl sections in studies dealing with the factors which influence cell proliferation and development. An additional factor is the relative ease of obtaining a large number of uniform, sterile hypocotyl explants. Various studies have employed hypocotyl explants (Gossypium arboreum--Smith et al. 1977; Indigofera enneaphylla--Bharal and Rashid 1979; Brassica alboglabra--Zee and Hui 1977; Sandalwood--Rao and Bapot 1978). Studies of auxin-cytokinin interactions and plantlet regeneration from callus of Chinese kale (Zee and Hui 1977), indicated that, in culture, root primordia were initiated at the cut surface of the hypocotyl segment (in 4 to 6 days) with shoot primordia formed at the opposite end after approximately two additional days (Zee et al. 1978). In a study using Xanthium hypocotyls (Suthers 1978), callus formation was stimulated on MS medium which had been modified by the addition of casein hydrolysate, kinetin and the auxin, naphthalenaecetic acid (NAA). Using cotton hypocotyls, a 2.0 ppm IAA and 1.0 ppm kinetin balance was required for callus initiation while a medium containing 2.0 ppm NAA and 0.5 to 1.0 ppm BA was found necessary for the vigorous growth of subcultured callus (SMith et al. 1977). For differentiation of shoots and buds on callus isolated from the legume, Indigofera enneaphylla, IAA and BA were required (Bharal and

Rashid 1979). Investigations using carrot hypocotyl tissue showed that the addition of cytokinins, in the absence of exogenous auxin, did not induce organogenesis. In this system low concentrations of auxins (IAA, NAA or IBA at 0.1 to 1.0 ppm) induced roots while somatic embryos were produced at higher auxin concentrations. Simultaneous application of BA, with various auxins, stimulated, in differing degrees, the formation of callus composed of parenchymatous cells while inhibiting the stimulatory action of auxin in root and somatic embryo formation (Kamada and Harada 1979a). Friedman et al. (1979) reported that auxin increased the number of roots formed in cultures of bean hypocotyls. This wide range of fragmented information, although often difficult to link together in a coherent manner, illustrates that the hypocotyl is a viable explant for the study of growth and differentiation of excised plant cells.

Pollen grains represent an additional source of isolated cells which can be used in studies of developmental parameters. The first haploid cultures were obtained from the grains of Ginko biloba as a result of divisions of the vegetative cells in the absence of organogenesis. Anther cultures of the angiosperm Datura innoxia produced haploid embryo-like structures which had their origin from pollen grains (Vasil and Nitsch 1975). Since then, either by anther or isolated pollen cultures, many additional angiosperm pollens have been switched from their normal sexual role to vegetative plant formation either directly via embryogenesis or indirectly via callus (Sunderland and Dunwell 1977). The stages of pollen development

vary so it is important to recognize the critical points where angiosperm pollen can be switched from the normal pattern of development (Nitsch and Nitsch 1969, Sunderland and Wicks 1969). Sunderland and Dunwell (1977) identified six anther stages based on cytological events. In Datura innoxia and Nicotiana tabacum, for successful induction of androgenetic events, anthers should be cultivated just before, during, or immediately following microspore mitosis (Sunderland and Dunwell 1977). Studies using Nicotiana established that the most successful results with anther culture were obtained at the time of microspore mitosis leading to the formation of the generative and the vegetative cells. In these studies, mitosis produced two equal daughter cells with similar staining characteristics, instead of the typically large vegetative cell and small generative cell. Where detailed ontogenetic studies have been made, the haploid callus or the embryoids arise from the activity of the vegetative cell alone (Vasil and Nitsch 1975). Sunderland and Wicks (1971) noted that embryogenetic grains of N. tabacum lack starch and the vegetative nucleus can be seen clearly against the lightly-stained (using acetocarmine) background of the cytoplasm. The nonembryogenic grains develop starch and the cytoplasm stains densely using acetocarmine. In other species the changes in staining properties of the vegetative cell are much less obvious (Sunderland and Dunwell 1977). Dale (1975) established that the distribution of pollen callus in cultured anthers of barley follows very closely the distribution of nonstaining grains of in vivo anthers. Generally only 0.5 to 5.0 percent of the pollen

grains undergo androgenetic development (Vasil and Nitsch 1975).

Embryoids, which are formed from pollen grains in vitro, generally follow the typical pattern of development as exemplified by sexually produced zygotic embryos. The globular, heart-shaped, torpedo-shaped and cotyledonary stages of embryos develop in vitro. When a callus is initiated (Oryza sativa), Brassica oleracea, Lycopersicon esculentum), embryoids or plantlets can be obtained by transferring the callus tissues to media containing appropriate concentrations of plant growth substances (e.g., auxins, cytokinins and/or other complex factors such as coconut milk; Vasil and Nitsch 1975, Reinert and Bajaj 1976).

The nutrient medium for inducing androgenesis in anthers of Datura and Nicotiana can contain only sucrose and mineral salts in the absence of organic substances including vitamins and plant growth substances (Nitsch and Nitsch 1969; Nitsch 1974). Iron has been reported to be required for the growth of embryoids beyond the globular stage of development (Reinert and Bajaj 1976), while embryoid stages are not produced in the absence of sugars. The addition of auxin or cytokinin leads to the formation of callus instead of embryoids and also stimulates the proliferation of the diploid somatic anther wall or filament tissue (Vasil and Nitsch 1975). Based on an analysis of extracts of embryogenic and nonembryogenic anthers of Nicotiana, Nitsch (1974) found glutamine, serine and myo-inositol should be added to the nutrient media for the culture of isolated microspores of Datura and tobacco. These specialized nutritive requirements have been shown to vary from

species to species (Sunderland and Dunwell 1977).

The most critical stage in the induction of androgenetic development has been shown to be the period beginning just before microspore mitosis and ending soon after the first mitotic division. Vasil and Nitsch (1975) link r-RNA and selective t-RNA production in the vegetative cell with the proposed shift of the microspores to a sporophytic pattern of development. They also propose that low temperature treatment of excised flower buds, prior to anther culture, may promote the accumulation of a larger percentage of pollen grains in the critical stages of development. The influence of cold treatment is also supported by studies of N. tabacum (Duncan and Heberle 1976) but biochemical effects of cold treatment remain unclear. Nitsch (1974) notes that treatments such as cold shock (Tradescantia, Datura and Nicotiana), gravity stresses (Swangwan-Norreel 1977, investigation of Datura) modify mitosis, triggering isolated microspores to act as somatic cells which may give rise to plantlets.

Haploid plants provide potentially useful material for the study of various fundamental and applied problems. In the haploid condition all genes present express themselves in the phenotype so various recessive characters are not hidden and mutations are therefore easily identifiable (Reinert and Bajaj 1976).

Although the literature for the successful culture of anthers and pollen increases rapidly, this increase is not matched by a corresponding insight about the fundamental processes underlying the induction process (Sunderland and Dunwell 1977).

MATERIAL AND METHODS

Basal medium. The basal standardized nutrient medium used throughout this investigation consisted of major and minor salts according to Murashige and Skoog (1962; abbreviated as MS), 3% sucrose, 1% Difco-Bacto agar, 100 ppm myo-Inositol, and 0.1 ppm thiamine-HCl. Various concentrations of growth regulators including Indoleacetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), benzylaminopurine (BA), with or without coconut milk (CM; prepared by heating coconut milk to 60 C, cooling and coarsely filtering to remove sediment) were added. The levels of salts, sucrose, CM and the growth regulators were also altered as various parameters were investigated in the study of the initiation and growth of Azalea callus tissue.

Anther agar nutrient media. Three media, Z (Zenkeler 1972), NA (Nitsch and Nitsch 1969) and G (Gresshoff and Doy 1972) were used in preliminary experiments in which whole anthers were placed in culture. All media were autoclaved for 15 minutes at 121 C under 15 psi.

To determine the influence of the addition of exogenous amino acids on the initiation of anther derived callus or plantlet development, agar solidified MS was modified by the addition of the following amino acid supplements: (1) 800 ppm glutamine and 100 ppm

serine (MS-NA), (2) 2.62 ppm hydroxy-proline, 3.60 ppm threonine, 6.30 ppm serine, 11.50 ppm proline, 3.10 ppm histidine, 3.50 ppm arginine (azalea amino acid supplement; MS-Aaa), (3) MS modified by the addition of 2 ppm glycine (MS-gly). The amino acid supplements were filter sterilized at 0.2 micron and added to autoclaved basal MS. As recommended by Nitsch (1974) only minimal organics were added, omitting cytokinins, auxins and the vitamin, nicotinic acid.

Anther and pollen culture liquid nutrient media. Liquid media, NP (Nitsch 1974), developed for the culture of tobacco pollen culture was used and modified by the addition of the azalea amino acid supplement (NP-Aaa). Additionally, liquid MS with minimal organics was also utilized for liquid culture of entire anthers and isolated microspores.

Stem and hypocotyl segments nutrient media. In addition to MS, media were utilized in which the salt concentrations of MS were reduced along with other modifications. Ma and Wang (1977) medium (MW) used 1/2 MS salts, 2% sucrose, 0.7% agar, 20% CM and in ppm, 100 myo-Inositol, 0.4 thiamine-HCl, 5 kinetin, 500 malt extract. Preil and Engelhardt (1977) medium (PE) contained 1/10 MS salts, 2% sucrose, 0.6% agar and 5 ppm BA. The pH of the media was adjusted to 5.6 prior to autoclaving. The media MS-Aaa, MS-gly and NP-Aaa, formulated for anther and pollen culture, were also used in preliminary stem cultures.

All cultures were incubated in growth cabinets at 25 C, either in darkness or on a 12 hr photoperiod of low intensity light in agar or liquid shake cultures (30 rotations per minute).

Observations and fresh weight were recorded at appropriate intervals.

Experimental material. Azalea plants were obtained from Le Mac Nurseries Inc., Hampton, Virginia, in March and November and again in February. Bushes in 8 inch pots were collected and placed in cold storage (38 -40 C) on a 12 hr photoperiod of low intensity light. Two varieties, Hinodegiri and Roadrunner, were used to provide sources of anthers.

To study callus initiation in Azalea, two actively growing systems were chosen, stems and seedlings, with explants obtained from both sources. Stems from three Azalea varieties, Roadrunner, Hinodegiri and Pluto, were obtained from the fields and greenhouses of Le Mac Nurseries Inc. Actively elongating shoots were chosen, leaves and terminal buds were removed and the stems were surface sterilized for various time periods in 95% ETOH, followed by 5.25% sodium hypochlorate (commercial bleach) solution with one drop Tween-20 (wetting agent) and rinsed at least three times with sterile distilled water. Aseptic manipulations were performed at a laminar flow hood. Internode sections were cut with a razor blade into 10mm lengths, placed on the appropriate medium and incubated either in the dark or on a 12 hr photoperiod at low intensity light at 25 C.

Anthers. After excising the flower buds from the twigs, the buds were surface sterilized by immersion in commercial bleach (5.25% sodium hypochlorate) with a drop of Tween-20 (wetting agent) for five to ten minutes. This was immediately followed with at least three rinses with sterile distilled water. The outer bud

scales and flower petals were removed with sterile instruments and the anthers were carefully excised by gently cutting the base of the filament. The anthers were transferred aseptically to an appropriate nutrient medium.

A number of culture procedures were employed using explants from a variety of sources. Whole anthers were placed in liquid media in sterile 5 cm Petri plates containing a filter paper disk (folded to form a wick) ensuring uniform moisture distribution. In the second series of liquid cultures a simplified technique, as described by Sunderland and Roberts (1977), involved anthers which were simply floated on the liquid medium. The plates were sealed with para-film and incubated in darkness or on a 12 hr photoperiod under low intensity light.

Pollen. Whole anthers, excised aseptically, were precultured in liquid medium for 3 to 5 days before preparing the suspension as recommended by Nitsch (1974). With a glass rod the anthers were gently pressed against the sides of a glass breaker, releasing the pollen cells from the anther. The suspensions were filtered through silk bolting cloth which retained the nonpollen debris. After filtration the suspension was transferred to centrifuge tubes and spun down gently in a hand centrifuge, forming a pellet of pollen cells. The supernatant was removed, the pollen washed with medium, resuspended, centrifuged again. After discarding the supernatant, the final pellet was resuspended in the same medium as used in the precultures. Three ml aliquots were transferred to 5 cm Petri plate, sealed with para-film and incubated in either darkness or on

a 12 hr photoperiod of low intensity light.

Hypocotyls. Azalea seeds, variety Glacier, an open pollinated white evergreen azalea, were obtained from Le Mac Nurseries Inc., and the variety Mollis was obtained from Herbst Brothers Seedmen Inc., Brewster, New York. The seeds were surface sterilized for 30 minutes in commercial bleach (5.25% sodium hypochlorate) with one drop Tween-20, followed immediately by at least three rinses with sterile distilled water. After sterilization, the seeds were placed on basal medium and incubated in the dark at 25 C in Petri plates which were sealed with para-film. After 28 to 38 days the seedlings were aseptically dissected in a laminar flow hood. Ten mm hypocotyl segments were selected as the primary experimental material. The excised segments were placed in Erlenmyer flasks or in Petri plates on the appropriate media containing selected supplements and incubated under appropriate conditions.

RESULTS AND DISCUSSION

Anther and pollen cultures. Anthers were cultured in attempts to obtain haploid Azalea plants. The initial studies (summary table 3) used three media (tables 1 and 2) which provided three different concentrations of nutrients. These studies used whole anthers from the Azalea variety Hinodegiri and tested a range of inorganic and organic components. Medium NA (Nitsch and Nitsch 1969) is characterized by low levels of inorganic salts and high concentration of vitamins. Medium G (Gresshoff and Doy 1972) contains the highest inorganic salt levels and moderate levels of vitamins. All three contain indoleacetic acid. These media, developed for anther or pollen cultures from plants other than azalea, have been used for haploid plantlet production. A sequential range of anthers at different progressive developmental stages, from microspore mother cell in immature anthers to fully developed pollen, were placed in culture. Petal length and color were noted in order to morphologically characterize each phase of the developmental sequence. The morphological stages have been clearly delineated in tobacco studies but the stages in azalea are not clearly defined. Despite differences in nutrient levels in the media, there did not appear to be differences in anther response as noted in table 3. Neither did there appear a difference between

Table 1. Inorganic salt Composition (mg/l) of selected Media for Growth of Azalea Tissues

<u>Components</u>	<u>media (a)</u>				
	Z	G	NA	NP	MS
MgSO ₄ ·7H ₂ O	370	1250	185	740	370
KNO ₃	1900	5000	950	3800	1900
KCl		1500			
NH ₄ NO ₃	1650		720	2900	1650
CaCl ₂			166	664	
KH ₂ PO ₄	170		68	272	170
MnSO ₄ ·4H ₂ O	22.3		25		8.6
H ₃ BO ₃	6.2	3	10		6.2
KI	0.83	3.75			0.83
ZnSO ₄ ·7H ₂ O	8.6	3	10		
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.25	0.25		0.25
CaCl ₂ ·2H ₂ O	440				440
CaCl ₂ ·H ₂ O		750			
CoCl ₂					0.025
CoCl ₂ ·6H ₂ O	0.025	0.25			
CuSO ₄		0.25			0.025
CuSO ₄ ·6H ₂ O	0.025				
CuSO ₄ ·5H ₂ O			0.025		
Iron-EDTA(b)	5ml	5ml	5ml	5ml	5ml

(a) Z-(Zenkeler 1973), G-(Gresshoff and Doy 1972), NA-(Nitsch and Nitsch 1969), NP-(Nitsch 1974), MS-(Murashige and Skoog 1962).

(b) A liter of stock solution composed of 7.45 g of Na₂EDTA and 0.557 g of FeSO₄·7H₂O in double distilled water.

Table 2. Agar, pH, and the Organic Components (mg/L) of Selected Media for the Growth of Azalea Tissues

Components	Media (a)									
	Z	G	NA	NP	NP+7aa (c)	1/2L	MS(d)	MS-Aaa (c)	MS-NA (c)	
pH	5.6	5.8	5.5	5.8	5.8	5.6	5.6	5.6	5.6	
agar	10 g/l	8 g/l	8 g/l	20 g/l	20 g/l	8 g/l	10 g/l	10 g/l	10 g/l	
Sucrose	30 g/l	20 g/l	20 g/l	20 g/l	20 g/l	20 g/l	30 g/l	30 g/l	30 g/l	
Kinetin	6	5			0.2		2			
Indoleacetic acid	4	2	0.1		2.0		5			
Nicotinic acid		0.01	5				0.5			
Thiamine-HCl	0.4	0.01	0.5		0.40		0.1	0.1	0.1	
Pyridoxine		0.01	0.5				0.5			
Myo-inositol	100	1.0	100	5 g/l	5 g/l	100	100	100	100	
Folic acid			0.5							
Biotin			0.05							
Glycine		0.04	2				2 (b)			
Glutamine				800				2.62	800	
OH-proline					2.62			3.60		
Threonine				100	3.60			6.30	100	
Serine					6.30			11.50		
Proline					11.50			3.10		
Histidine					3.10			3.50		
Arginine					3.50					

(a) Z-(Zenkteler 1973), G-(Gresshoff and Doy 1972), NA-(Nitsch and Nitsch 1969), NP-(Nitsch 1974), MS-(Murashige and Skoog 1962).

(b) Glycine filter sterilized (0.2 micron) and added after autoclaving to make MS-gly.

(c) The amino acids of these media were filter sterilized and added to the other components after they had been autoclaved (0.2 micron filter). The amino acids occur in relatively large concentrations in azalea (Mathes, unpublished data).

(d) 10%CM and 0.1 NAA/1.0 2,4-D are composed of MS basal salts with minimal organics plus 0.1 mg/ml naphthaleneacetic acid and 1 mg/ml 2,4 dichlorophenoxyacetic acid, with 100 ml/L coconut milk extract added for 10%CM.

Table 3. Summary of Preliminary Azalea anther culture studies.

Experimental Material (a)	Media	Petal length and color	Incubation treatment	Response
1. Preliminary Studies. Anthers excised from var. Hinodegiri	2 NA G	range from 5 to 14 mm green to red daily sample	Darkness at 25°C	Anthers, initially white turn brown after 7 days of culture. Some filaments and anthers became slightly swollen.
2. Liquid cultures. Anthers excised from var. Hinodegiri	NP NP+7aa	range from 5 to 16 mm green to red sample every 2 days	Darkness at 25°C or 12 hr photo-period.	White anthers start to brown in approx. 19 days. Some swelling of anthers and filaments. MS used as control. (b)
3. Anthers from var. Hinodegiri	MS MS-gly MS-NA MS-Aaa	range from 5 to 24 mm green to red sample every 3 days	Darkness at 25°C	Anthers started to turn brown after 7 days. Some anthers split open, while others slightly swelled.

(a) The pollen in the anthers ranged from microspore tetrads initially to fully developed pollen in the mature anthers.

(b) Two anthers developed small green protrusions, but failed to develop further.

cultures in darkness and those given a 12 hr photoperiod. The anthers did not develop plantlets or callus. In general, the response was a gradual color change of the anthers from an initial uniform tan color to a final progression from dark brown to black.

Wernicke and Kalenbach (1976) obtained plantlets using cultured Nicotiana anthers in a liquid medium, suggesting a possible treatment for azalea anthers. The media NP (Nitsch 1974) and NP+7aa (tables 1 and 2) were chosen for use as liquid medium because NP had been successfully employed in the culture of haploid tobacco cells. NP was modified by the addition of seven amino acids found to be present in relatively high concentrations in azalea anthers (Mathes unpublished data, table 4). A small number of anthers were placed on MS, a solid medium (Murashige and Skoog 1962), each day to serve as a standard to compare to the anthers in liquid culture (Gamborg et al. 1976). As in the first set of experiments, the anthers developed swollen filaments or became swollen but did not develop callus or plantlets (table 3). During a subsequent liquid culture series two anthers on MS produced small protrusions. This occurred on anthers placed in culture on the first and second days after the bush was removed from cold storage, where plants were kept in winter conditions to inhibit development beyond the tetrad stage. This observation was further explored using MS as the basal medium. Two considerations were examined in this series: density, that is, the number of anthers per dish, and the influence of amino acids on proliferation and development. MS was modified by the addition of amino acids (tables 1 and 2) which were filter sterilized to avoid

Table 4. Amino acid content of Azalea anthers in the tetrad stage and the composition of the Azalea amino acid supplement

Amino acid	Micro-moles per 100 grams fresh weight	Amino acid supplement ppm (a)
Cysteic	0.0240	-
OH-proline	0.1706	2.60
Aspartic	0.1133	-
Threonine	0.2717	3.60
Serine	0.5870	6.30
Glutamic	0.1795	2.90
Proline	1.0227	11.50
Glycine	0.0879	-
Alanine	0.1401	
Valine	0.1095	
Isoleucine	0.0799	
Leucine	0.0489	
Tyrosine	0.0541	
Phenylalanine	0.0329	
Ornithine	0.0071	-
Histidine	0.1584	3.1
Arginine	0.2068	3.5

(a) Arbitrarily, to ease media formulation, the decimal of the micro-moles was shifted right. The numbers greater than one, were rounded up to the nearest whole number and converted to milli-grams. These seven amino acids were incorporated into various media.

the harsh treatment of autoclaving. Four sets of dishes, each with 10.30 or 60 anthers per dish, were placed in culture.

Anther density was considered because anthers may condition the medium (Sunderland and Roberts 1977, Sopory 1977) thus making the medium more satisfactory for pollen proliferation. The numbers of anthers employed in this series did not induce the development of callus or plantlets. Once again, the general pattern of anther response was browning, with some random filament swelling (table 3).

MS-NA contained the amino acids as reported by Nitsch and Nitsch (1969) in the successful culture of Nicotiana anthers. MS-Aaa contained those amino acids which are naturally occurring in relatively high concentrations in azalea anthers containing pollen at the tetrad stage (as determined by amino acid analysis, table 4) MS-gly used the amino acids normally present in MS. The basal medium, MS, was autoclaved and filter sterilized amino acids were added at appropriate concentrations. The results indicated that little could be determined about the influence of amino acids on azalea anther culture since proliferation was not observed.

Nitsch's (1974) success with suspension cultures of Nicotiana pollen suggested additional treatments to explore the proliferation and development of azalea pollen. Pollen culture was proposed to more closely observe plantlet development. The influence of the anther wall would be replaced by media components. Successful pollen culture would provide tissue from an easily identified origin. Seven azalea pollen suspensions were established but they did not produce positive results.

In summary, tissue response was limited to random swelling of anther filaments. The basal medium MS produced the only observed anther response but this development was never repeated. The lack of response of the anthers indicates that Azalea anther-pollen culture will require further investigation.

Preliminary callus initiation. Stem segments from actively growing bushes placed in culture did not produce callus, and in general all the varieties tested, Pluto, Roadrunner and Hinodegiri, produced a gradual tissue necrosis and browning. A variety of media and conditions were employed. As in anther-pollen culture the influence of filter sterilized amino acid supplements was investigated but there was no stimulation of callus development. Dark and 12 hr photoperiods were utilized as well as various tissue sterilization techniques but again there was no callus production.

Appendix table 5 summarizes the various media and treatments used to stimulate callus production in the preliminary studies using seedlings. Liquid media (rotary shaker, on a 12 hr photoperiod) resulted in callus development on two treatments of MS basal salts plus growth regulators. One treatment, containing K at 1.0 ppm, produced callus at the cut surface of the seedling section, while callus also developed on both whole and cut seedlings on the treatment containing 0.02 ppm 2,4-D. This initial callus was friable. After 70 days in liquid culture the callus was transferred to agar medium containing the same concentration of growth regulators as in liquid culture. Subsequent transfers were to a maintenance medium, basal MS plus 10% CM, which was later found to

Table 5. Summary of the preliminary Azalea tissue explant studies.

<u>Azalea</u> variety	Explant source	Description and culture conditions	Growth regulators (a)	Response
Hinodegiri Roadrunner Pluto	Actively growing stem cuttings from field grown bushes.	10mm internode segments incubated in darkness or 12 hr photoperiod of low intensity light at 25C 20 pieces/treatment	K, IAA, 2,4-D at 0.01 0.1 1.0 2.0 mg/l	Gradual necrosis and browning of the tissue.
Glacier	Seedlings approx. 8 weeks of germination 9 to 15 mm	30 seedlings cut in half, 15 seedlings cut in thirds, 45 seedlings left uncut. Placed in flasks on rotary shaker, 12 hr photoperiod low intensity light	K, IAA, 2,4-D at 0.02 0.2 1.0 2.0 mg/l 1,5,10% CM	White callus on cut surface of segment with K at 1.0mg/l White callus on whole and segment at 0.02mg/l 2,4-D
Glacier	Seedlings germinated 40 to 45 days	5mm hypocotyl segments Rotary shake, liquid culture, 12 hr photoperiod low intensity light. 5 segments/flask. 2 flasks/treatment.	IAA, K, 2,4-D at 0.01 0.1 1.0 2.0 mg/l	After prolonged culture (9 weeks) white callus on 0.01 mg/l 2,4-D Grey-white callus on 1.0 mg/l IAA.

(a) Abbreviations: Kinetin(K), indoleacetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) benzylaminopurine(BA) and coconut milk(CM).

promote callus proliferation.

Isolated callus growth. A series of treatments were initiated, using comparable seedlings, to examine the effects of various auxins and cytokinins, alone and in combinations, on the proliferation of callus derived from excised seedling pieces, shoots, hypocotyls and roots. Both liquid and agar based media were examined with the concentration of the growth regulators IAA, K and 2,4-D at 0.01, 0.1, 1.0 or 2.0 ppm. After prolonged (9 weeks) culture in liquid media, callus developed on two treatments (1.0 ppm IAA and 0.01 ppm 2,4-D).

Isolated Azalea tissue was grown on basal MS medium either in the absence or the presence of selected growth regulators. Three aspects were studied initially: (1) concentrations of 0.01, 0.1, 1.0 and 2.0 ppm of the growth regulators (IAA, BA or 2,4-D) were employed to assess the influence on callus fresh weight; (2) selected combinations of auxin (IAA) and cytokinin (BA) each at concentrations of 0.01, 0.1, 1.0 and 2.0 ppm and all combinations were utilized to study the effects of auxin-cytokinin interaction on the increase in fresh weight of callus tissue; and (3) the reports (Ma and Wang 1977, Priel and Engelhardt 1977) indicating that reduced salt concentrations of the major and minor inorganic components of MS medium are beneficial to Azalea callus tissue culture. As a result of this suggestion, isolated Azalea tissue was grown on three salt concentrations, full strength, 1/2 strength and 1/10 strength, in the presence or absence of 10% CM and/or 2.0 ppm IAA. Each treatment in these series contained six replicate pieces

of tissue which had been cut from stock tissue and selected for uniformity. Fresh weight, recorded every four days for 28 days, indicated a large amount of variation between "uniform" tissue pieces within the treatments. However, it was established in series (1) that basal medium without growth regulators adequately supported the growth of isolated tissue and it was suggested that 2,4-D increased the rate of tissue growth at the 0.1 ppm level. Treatment with BA at the 1.0 ppm level also produced a possible stimulation of tissue growth.

Increases in fresh weight in series (3) indicated that treatments containing 1/2 strength salt (.5 MS) in the presence of 10% CM and 2.0 ppm IAA supported active cell proliferation. Similarly, 1/10 strength salt (.1 MS) in the presence of 10% CM and 2.0 ppm IAA stimulated callus tissue growth. The control treatments of this study did not support callus tissue growth. The first set of experiments indicated that the basal medium (full strength salt) supported tissue growth. This variance is the result of variation in tissue samples even though procedures were devised to provide randomly selected, "uniform" explants. Selection of callus on the basis of visual uniformity did not eliminate significant physiological variation.

The use of hypocotyl segments reduced the physiological variability as demonstrated using callus tissue. Comparable preliminary studies, using uniform hypocotyls from Azalea variety Mollis, and various hormones, CM, sucrose and salt concentrations, were designed to determine medium compositions which would initiate

the growth of Azalea callus tissue.

Hypocotyl studies hormone levels. The basal medium MS, with and without 10% CM, was combined with 0.01, 0.1, 1.0 and 2.0 ppm of the growth regulators IAA, 2,4-D and BA, either alone or in all combinations. After the initial weight of each set of three hypocotyls was recorded, fresh weight was recorded every four days for 28 days. Callus generally began at the cut ends of the segments and, in the presence of 2,4-D, the entire segment would swell. In general, the presence of CM favored callus growth (i.e., increases in fresh weight). Four treatments produced relatively large masses of callus: 0.1 ppm 2,4-D, friable callus, gold in color; in ppm, 0.1 IAA/0.1 BA, friable callus, a gold color; in ppm, 1.0 IAA/0.1 BA firm callus, a gold color; in ppm, 0.01 2,4-D/0.01 BA, friable callus, a gold color. All the above treatments contained 10% CM (table 6).

Salt, sucrose and coconut milk levels. This study was initiated to screen numerous combinations of salt, sucrose and CM concentrations for the determination of acceptable levels for callus initiation and growth. From earlier studies it was shown that 0.1 ppm 2,4-D would support callus growth so this treatment was used as a control for the assessment of relative tissue growth. Three salt strengths, MS (full strength), 0.5 MS (half strength) and 0.1 MS (tenth strength) were used as suggested by Ma and Wang (1977) and Priel and Engelhardt (1977). Each salt level had all combinations of sucrose (at 1%, 2% and 3%) and/or CM (0%, 10%, 20% and 30%).

The initial weight of three hypocotyl segments was recorded

Table 6. Influence of hormonal levels on the proliferation of Azalea callus derived from hypocotyl tissue.

Media Additive(b)	Basal medium (MS) with 10% CM			
	Hormone levels (mg/l)			
	0.01	0.1	1.0	2.0
2,4-D	SG(a)	SG	-	-
IAA	-	Gs	-	-
BA	-	-	-	-
IAA 0.01/BA	Se	-	-	-
IAA 0.1/BA	-	SG	-	-
IAA.1.0/BA	G1	-	Gs	Gs
IAA 2.0/BA	-	-	-	-
2,4-D 0.01/BA	SG1	SGs	Se	Se
2,4-D 0.1/BA	-	-	-	-
2,4-D 1.0/BA	-	-	-	-
2,4-D 2.0/BA	-	-	-	-
10% CM (no hormones)	-	-	-	-

	Basal medium (MS) without CM			
	Hormone levels (mg/l)			
	0.01	0.1	1.0	2.0
MS(no hormones)	-	-	-	-
2,4-D	-	-	-	-
IAA	-	-	-	-
BA	-	-	-	-
IAA 0.01/BA	-	Gs	-	-
IAA 0.1/BA	-	-	Gs	-
IAA.1.0/BA	-	-	-	-
IAA 2.0/BA	-	-	Se	-
2,4-D 0.01/BA	Gs	-	Gs	Se
2,4-D 0.1/BA	-	Gs	Se	Gs
2,4-D 1.0/BA	-	-	-	-
2,4-D 2.0/BA	Se	-	-	Se

- (a) SG soft gold callus
 Gs gold callus, small amount
 Se swollen ends of the hypocotyl segments
 G1 large amount of gold callus
 SG1 soft, gold, large amount of callus
 SGs soft, gold, small amount of callus

(b) Concentration expressed in ppm.

and the cultures were examined for the presence of callus every seven days. When callus was initially present the weight of the segment with callus attached was recorded and the fresh weight was determined every four days for a total period of 28 days. Callus developed using a wide variety of treatments (table 7) and the presence of 2,4-D enhanced growth, as measured by increases in fresh weight, at all three salt levels. The reduced salt concentrations (0.1 and 0.5 MS) with the sucrose-CM levels of 3-30, 3-20 and 2-30 produced the largest masses of callus in the presence of 2,4-D.

Using the information available from the previous preliminary studies, three growth regulator combinations were selected; in ppm, 1.0 IAA/0.01 BA, 0.1 IAA/0.1 BA and 0.01 2,4-D/0.01 BA. The appropriate CM level was not clearly delineated so both 20% and 30% CM were used in combination with 2% sucrose and 3% sucrose. These combinations were tested with full strength MS, half strength (.5 MS) and tenth strength (.1 MS). Results indicated that the three growth regulator combinations with the reduced salt strengths of .1 MS and .5 MS, in combination with 30% CM, at both 2% and 3% sucrose provided a nutrient media range which would initiate and support the active growth of Azalea callus tissue.

Another study based on previous results, was designed to further explore the influence of media components. Twelve of the most satisfactory media treatments were selected from the preceding studies. Additionally, two media reported to support Azalea tissue in culture (Ma and Wang 1977, Priel and Engelhardt 1977) were employed. They suggested reducing the salt and sucrose

Table 7. Influence of salt concentration, sucrose and coconut milk on Azalea callus initiation and proliferation.

		Full strength MS salts (1 MS)		
Sucrose (%)	CM %	1	2	3
	0	-	-	-
	10	Se(a)	-	-
	20	Se	Se	-
	30	-	-	-
		Full strength MS salts (MS) with 0.1 mg/l 2,4-D		
Sucrose (%)	CM %	1	2	3
	0	-	Se	Gs
	10	Gs	Gs	Gs
	20	-	Gs	Gs
	30	SGm	SGl	sgl
		Half strength (.5 MS) basal salts		
Sucrose (%)	CM %	1	2	3
	0	-	-	-
	10	-	Se	Se
	20	Se	Se	-
	30	-	Gs	-
		Half strength (.5 MS) basal salts with 0.1 mg/l 2,4-D		
Sucrose (%)	CM %	1	2	3
	0	Gs	Gs	Gs
	10	Gm	Gm	Gm
	20	Gm	SGl	SGl
	30	Gm.	SGl	SGm
		Tenth strength (.1 MS) basal salts		
Sucrose (%)	CM %	1	2	3
	0	-	-	-
	10	Gs	Gs	Gs
	20	Se	Se	Gs
	30	-	Se	-
		Tenth strength (.1 MS) basal salts with 0.1 mg/l 2,4-D		
Sucrose (%)	CM %	1	2	3
	0	Gs	Gs	Gs
	10	Gm	Gm	Gm
	20	Gm	Gm	Gl
	30	Gs	Gm	SGl

- (a) Se swollen ends on hypocotyl segments
 Gs gold callus, small amount
 SGm soft, gold callus, moderate amount
 SGl soft, gold callus, large amount
 Gm gold callus, moderate amount
 Gl gold callus, large amount

concentrations of the MS medium. Priel and Engelhardt (1977) found that shoot development can be induced in Azalea tip meristem explants by 2-5 ppm BA and reduced salt concentration. After shoot differentiation 2 ppm IAA in the absence of BA will induce root development. Ma and Wang (1977) used Azalea shoot tips which were explanted to modified MS (reduced salt concentration) containing 5 ppm K and 500 ppm salt extract. Shoot proliferation began with the emergence of axillary shoots. Regeneration of plants was accomplished by rooting the shoot clusters in a medium of low salt concentration and without auxin. In this study Azalea variety Mollis hypocotyls were used, with three to a set for a total of 20 sets per treatment, and the fresh weight of each set was determined every four days for at least 8 observation periods (table 8).

At the 0.5 MS salt concentration, growth was supported by, in ppm, 0.1 IAA/0.1 BA, 2% sucrose and 30% CM. The 0.1 MS salt concentration proved more appropriate for the initiation and growth of Azalea callus tissue. The .1 MS treatments, in ppm, 0.1 IAA/0.1 BA, 30% CM at both the 2% and 3% sucrose levels initiated early callus development while the most rapid callus growth was attained with, in ppm, 0.01 2,4-D/0.01 BA, 3% sucrose and 30% CM (table 9). Observations were continued for 10 sets of the original 20 sets of hypocotyls. From this information (table 9) it was shown that two treatments, one at .5 MS and one at .1 MS with, in ppm, 0.01 2,4-D/0.01 BA, 3% sucrose and 30% CM, will support long term callus growth.

The additional media, as suggested by Ma and Wang (1977) and

Table 8. The influence of selected modifications of basal MS on Azalea callus initiation and growth.

Salt(a)	Hormones ppm	Sucrose %	CM %	Days in culture (b)					(c)
				16	20	24	28	32	
.1	2,4-D 0.01 BA 0.01	2	30	-	10/0	15/10	15/10	15/10	(c)
.1	2,4-D 0.01 BA 0.01	3	30	-	40/20	45/30	55/40	50/75	
.1	IAA 0.1 BA 0.1	2	30	-	5/0	15/20	15/20	15/20	
.1	IAA 0.1 BA 0.1	3	30	-	15/0	25/20	40/20	40/20	
.1	IAA 1.0 BA 0.01	2	30	5/0	20/10	40/40	50/40	55/40	
.1	IAA 1.0 BA 0.01	2	30	5/0	15/0	40/10	40/50	45/55	
.5	2,4-D 0.01 BA 0.01	2	30	-			5/0	5/10	
.5	2,4-D 0.01 BA 0.01	3	30	-	15/0	15/0	15/10	15/35	
.5	IAA 0.1 BA 0.1	2	30	25/30	40/30	45/30	45/30	45/40	
.5	IAA 0.1 BA 0.1	3	30	-		5/0	15/0	15/0	
.5	IAA 1.0 BA 0.01	2	30	10/20	10/20	15/20	25/20	25/35	
.5	IAA 1.0 BA 0.01	3	30	-			20/0	20/0	
(PE)									
.1	BA 5.0	2							
(MW) (d)									
.33	K 5.0	2	20	-	-	-	-	-	

(a) Salt concentration. The major and minor inorganic salts of MS were reduced to half strength (.5) and tenth strength (.1).

(b) Days in culture. There was no growth of callus production until the 16th day, so the earlier observations were omitted.

(c) Percent of sections producing callus. This number was generated by counting all sets which produced callus even though in some sets with callus there was no increase in fresh weight. Growth index. Growth index was calculated by subtracting initial fresh weight (Wi) from fresh weight at the time of observation (Wo) divided by initial weight, (Wo - Wi)/Wi.

(d) MN also contains, in ppm, myo-Inositol 100, thiamine-HCl 0.4, and malt extract 500.

Table 9. Azalea callus growth on modified basal media.

Salt concentrations	(a) Hormones ppm (b)	Sucrose %	Days in culture (c)				36(d)	40	56
			16	20	24	28			
.1	2,4-D 0.01	3	5/0	20/10	45/30	55/40	50/75	60/70	60/80
	BA 0.01								
.1	IAA 1.0	2	5/0	20/10	40/40	50/40	55/45	50/30	50/30
	BA 0.01								
.1	IAA 1.0	3	5/0	15/0	40/10	40/50	45/50	30/20	40/20
	BA 0.01								
.5	2,4-D 0.01	3	15/0	15/0	15/0	15/10	15/35	30/60	30/70
	BA 0.01								
.5	IAA 0.1	2	25/30	40/30	45/30	45/30	45/40	30/20	30/20
	BA 0.1								

(a) Salt concentration. The major and minor inorganic salts as listed in MS were reduced to half strength (.5) and tenth strength (.1).

(b) Hormones. 2,4-dichlorophenoxyacetic acid (2,4-D), benzylaminopurine (BA), indoleacetic acid (IAA). In addition 30% coconut milk was present in each treatment.

(c) Days in culture. There was no growth or callus production until day 16 so the first three observations were omitted.

(d) Observations on days 36, 40 and 56 were based on only 10 sets of hypocotyls. The earlier observations were based upon 20 sets of hypocotyls.

(e) The first number is the Percent of sections producing callus. The second number is the Growth index, calculated by subtracting initial fresh weight (WI) from fresh weight at the time on observation (Wo) divided by initial weight, (Wo - Wi)/Wi.

Priel and Engelhardt (1977), did not initiate callus tissue. The potential for support of the growth of previously established callus by these media is unclear.

CONCLUSION

Azalea anther culture. Firm conclusions concerning the proliferation of Azalea anther cultures, other than the obvious lack of response, remain elusive. The proper conditions for androgenesis were not delineated despite the testing of an extensive series of anther developmental stages and a wide range of media additives. In the complete absence of a trend, it is difficult to ascertain that additional treatments and manipulations of Azalea anthers would result in positive response. However, certain areas may be applied to anther culture and outlined for further exploration. Media preparation with activated charcoal, as originally proposed by Anagnostakis (1974) for enhancement of haploid tobacco plantlet production, could induce and promote the development of Azalea microspores by removing inhibitory substances from the medium (Fridliorg et al. 1978). The initial anther culture work with Datura (Guha and Maheswari 1964) and Nicotiana (Nitsch and Nitsch 1969), however, did not depend on the addition of activated charcoal. Suggestions regarding the cold treatment of anther (Sang-Norrell 1977, Duncan and Herberle 1976) prior to culturing, may also warrant further work. There also remains the possibility that the anthers from the Azalea varieties tested are not capable of androgenesis. Cytological examination of Azalea pollen with

acetocarmine was inconclusive, although Sunderland and Wicks (1971) report that androgenic pollen can be recognized by differential staining. From work with acetocarmine stain, Horner and Street (1978) suggest that when excised Nicotiana tabacum anthers are cultured, pollen plants arise only from (S) grains, which are characterized as smaller than normal grains, occur at a lower frequency and have weakly staining cytoplasm. Jefferies (1977) has developed an assay which indicates plasmalemma integrity and nonspecific esterase activity in an assessment of pollen viability. Pollen viability, as assessed by enzymatically induced fluorescence resulting from hydrolysis of fluorescein diacetate (Heslop-Harrison and Heslop-Harrison 1970), could provide an insight concerning Azalea pollen viability and developmental potential.

It was thought that the response of Azalea anther and stem cultures to reduced nitrogen in the form of amino acids, would provide positive results. Exogenous aspartic and glutamic acids promoted tobacco tissue growth as much as the complex combination of amino acids derived from yeast extracts. Amino acids (5mM aspartic and glutamic acid) in media with NAA/BA combinations stimulated bud formation of Torenia fournieri L., while roots were stimulated in the presence of NAA alone (Kamada and Harada 1979c). Glutamic acid was the only amino acid present in significantly greater amounts in aqueous extracts of androgenic anthers as compared with nonandrogenic and mature anthers of Burley tobacco (Horner and Pratt 1979). Sangwan (1978a,b) noted that changes in amino acid levels can be associated with the developmental phases of Datura metel

pollen but the physiological significance of these changes is not understood. Kamada and Harada (1979) maintain that the addition of alanine accelerates cell division during earlier stages of somatic embryogenesis in carrot tissue cultures.

Aseptic cultures of actively growing Azalea stems were difficult to establish. As a result of pubescent stems, the sterilization treatment was necessarily harsh. Skirvin and Chu (1977) mention similar difficulties in the tissue culture of woody plants (peaches). Rao and Bapat (1978), using sandalwood, also found that isolated stem segments from mature plants failed to show a response to hormonal treatment. Their results, obtained with hypocotyl segments, were parallel to the results using Azalea hypocotyl segments, which responded to selected growth regulator treatments.

This study also included preliminary seedling work which was initiated after the unsuccessful attempt, due to contamination problems, to produce callus from Azalea stem segments. Initial experiments indicated slow growth of white callus in liquid shake cultures derived from Azalea variety Glacier seedlings. This preliminary experiment was repeated and expanded using seedlings. Seedling hypocotyl segments have been excised from cotton (Smith et al. 1977), Xanthium (Suthers 1978), Brassica (Zee and Hui 1977) and Lycopersicon (Gunay and Rao 1980) and have been employed as suitable tissue explants. Previous reports using hypocotyl sections coupled with their availability and easy aseptic manipulation prompted the use of Azalea hypocotyl segments in this study.

Growth was defined as an increase in fresh weight and was quantified by calculating a growth index (final weight minus initial weight divided by initial weight) as suggested by the work of Ogihara and Twunewaki (1978) and Suthers (1978).

Growth regulator combinations, as suggested using tobacco (Linsmaier and Skoog 1965) and Brassica species (Zee and Hui 1977), proved helpful in determining the hormonal levels for the stimulation of Azalea callus production. Coconut milk was used because it provides a balanced combination of natural substances, which promotes the growth of isolated tissues (Steward and Bleichert 1970, Steward 1976). The addition of 10% CM to MS medium with 1 ppm IAA and 0.1 ppm K, greatly increased the percentage of pimperlrel (Anagallis arvensis) hypocotyl cultures showing differentiation of roots and shoots (Bajaj and Mader 1974).

Incubating cultures in darkness, as suggested for Sedum species (Branda and Salema 1977) and cotton (Smith et al. 1977) was employed for the growth of Azalea callus.

The beneficial influence of reduced salt concentrations in Azalea tissue culture as proposed by Ma and Wang (1977) and by Priel and Engelhardt (1977), were confirmed for the Azalea variety Mollis. The importance of a favorable auxin-cytokinin balance was indicated in experiments with Azalea hypocotyl sections. This supports the earlier work (Skoog and Tsui 1948, Das et al. 1965, Skoog and Miller 1957) using isolated tobacco pith tissue. The growth regulators did not induce the development of Azalea roots or shoots but did stimulate the development and proliferation of soft

friable callus. Similar results were obtained using carrot tissue cultures (Kamada and Harada 1979) where the BA/IAA combinations induced only soft friable callus. In this study, the Azalea hypocotyl system was developed to establish a method to study organogenesis. If a developmental pattern can be established, then morphological and anatomical aspects of the development of plantlets from cultured tissues can be effectively studied.

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