SOMATIC EMBRYOGENESIS OF *PHALAENOPSIS*

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By

Se-Young Kim

Dissertation Committee:

Yoneo Sagawa, Chairman
Charles L. Murdoch
Richard M. Manshardt
Douglas J. C. Friend
James A. Silva

We certify that we have read this dissertation and that, in our opinion, it is satisfactory in scope and quality as a dissertation for the degree of Doctor of Philosophy in Horticulture.

DISSERTATION COMMITTEE

Yoneo Sagawa
Chairman

Douglas Senn

Richard M. Marshall

Charles L. Murdock

James A. Silva

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ABSTRACT

The growth and differentiation of a unique Phalaenopsis callus was characterized. The callus was determined to be friable and embryogenic producing plantlets which matured and flowered.

In contrast to other orchid calli reported previously, the callus was unique in its extreme friability and undifferentiation with no trace of organization. The callus was maintained on a modified Vacin and Went medium containing sucrose 20.0 $g \cdot liter^{-1}$ supplemented with coconut water (CW) 150 $ml \cdot liter^{-1}$ without any morphological change for more than five years. Analyses of callus growth (fresh weight) showed that the callus had an extremely low growth rate, which was not affected by sucrose levels between 2.5 to 30.0 $g \cdot liter^{-1}$. Although CW stimulated callus growth, IAA (indole-3-acetic acid) or BA (benzyl adenine) was not effective.

Somatic embryogenesis was induced by lowering sucrose level to 2.5 $g \cdot liter^{-1}$ or deletion of sucrose and supplementing with CW. Callus turned green and subsequently formed numerous embryoids at periphery of green callus. The development of embryoids into plantlets morphologically resembled germination of zygotic embryos of Phalaenopsis.

Sucrose (20.0 $g \cdot liter^{-1}$) inhibited greening and somatic embryogenesis. Sugar inhibition of greening and somatic

embryogenesis was not overcome by transfer of callus from auxin-enriched medium to auxin-free medium or increasing nitrogen level, but only by reduction of carbohydrates available in medium. Pretreatment with IAA or NAA (α-naphthaleneacetic acid), and supplement of CW, casein hydrolysate (CH), glutamic acid or glutamine with 2.5 $g \cdot liter^{-1}$ of sucrose were marginally effective in promoting embryogenesis.

Readily utilizable monosaccharides, glucose, fructose, mannose, ribose, xylose and disaccharide, sucrose at 20.0 $g \cdot liter^{-1}$ were inhibitory, while other disaccharides, multisaccharides and sugar-alcohols supported greening and somatic embryogenesis. Amylose (20.0 $g \cdot liter^{-1}$) was the most effective in induction of embryogenesis among carbohydrate sources.

Regeneration of plantlets through indirect somatic embryogenesis is shown to be a viable pathway for clonal propagation of recalcitrant Phalaenopsis orchids.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF TABLES (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

ABBREVIATIONS

ABA	Abscisic acid
7-AZA	7-Aza indole
BA	Benzyl adenine
cAMP	Cyclic adenosine 3′,5′-monophosphate
CH	Casein hydrolysate
CW	Coconut water, 150 ml·liter^{-1} was used in this study.
2,4-D	2,4-Dichlorophenoxyacetic acid
DNA	Deoxyribonucleic acid
GA	Gibberellic acid
HBN	5-Hydroxynitrobenzylbromide
IAA	Indole-3-acetic acid
IBA	Indolebutyric acid
IKI	Potassium iodide-iodine
2-iP	2-Isopentenyladenine
MS	Murashige and Skoog (1962) medium
MVW	Modified, Vacin and Went (1949) medium
NAA	α-Naphthaleneacetic acid
NOA	ß-Naphthoxyacetic acid
pCPB	p-Chlorophenoxyisobutyric acid
RNA	Ribonucleic acid
PLB	Protocorm-like body
0S	Deletion of supplemental sucrose from culture medium
20S	20 g·liter^{-1} of sucrose in culture medium

ABBREVIATIONS (continued)

SAS	Statistical Analysis System, a computer system for data analysis
TDZ	Thidiazuron (N-phenyl-N'-(1,2,3, thiadiazol-yl) urea)

CHAPTER I

INTRODUCTION

Somatic organogenesis and embryogenesis are the principal pathways for regeneration of plantlets in clonal propagation of plants. Through organogenesis from explants of vegetative shoot tips (shoot-tip culture) of cymbidiums, Morel (1960) and Wimber (1963) demonstrated the commercial potential of *in vitro* culture techniques for clonal propagation of orchids. Other orchids have subsequently been successfully propagated through similar methods (Arditti, 1977; Rao, 1977; Sagawa and Kunisaki, 1984; Arditti, 1993). However, two commercially important genera, *Phalaenopsis* and *Paphiopedilum* have, as yet, not responded to similar treatments and remain recalcitrant.

For some plant species, plantlets have been regenerated from explants which produce embryoids (direct embryogenesis) or callus which, in turn, produce embryoids (indirect embryogenesis) resulting in numerous uniform-sized plantlets. Direct embryogenesis is most successful when explants are from embryonic tissues such as cotyledons, epicotyl or hypocotyl. However, since orchids are highly heterozygous, there is no advantage in production of numerous plantlets from embryonic tissues prior to

selection. The alternative is to induce callus and somatic embryogenesis from tissues of mature selected plants. A friable callus is a prerequisite to successful indirect embryogenesis.

The occurrence of callus in orchids has been reported in _in vitro_ cultures during seed germination (Curtis and Nicole, 1948; Rao, 1963) and clonal propagation through organogenesis. Careful study of published reports and photographs indicates that these "calli" are disorganized masses of tissue enclosed by epidermis rather than callus. During investigations in our laboratory on induction of multiple vegetative shoots from _in vitro_ culture of inflorescence nodes of _Phalaenopsis_, a friable callus was isolated for the first time in orchids. Preliminary experiments indicated that this friable callus was embryogenic and merited further study.

Therefore, the objective of this dissertation is to determine whether indirect somatic embryogenesis is a viable pathway for clonal propagation of the recalcitrant genus, _Phalaenopsis_ by use of the unique friable callus isolated from _Phalaenopsis_. Initially its growth and differentiation from callus to plantlet will be documented. Thereafter, the factors which control callus growth and somatic embryogenesis will be defined.

CHAPTER II

LITERATURE REVIEW

Regeneration of plant from somatic tissue culture *in vitro* can follow one of two alternative pathways: somatic organogenesis or embryogenesis. Since clonal propagation through organogenesis of plants other than orchids has been well documented (Debergh and Zimmerman, 1991), only the literature relevant to orchids and indirect somatic embryogenesis will be included.

CLONAL PROPAGATION OF ORCHIDS THROUGH ORGANOGENESIS

Most orchids, being complex hybrids, are highly heterozygous (Arditti, 1992). Therefore, it is difficult to obtain uniform plants of desirable characteristics from seeds. By *in vitro* culture of vegetative shoot tips of *Cymbidium*, Morel (1960) and Wimber (1963) obtained numerous plantlets. Organogenesis was preceded by small protocorm-like bodies (PLBs) which morphologically resembled protocorms produced by germination of orchid seeds. Depending on the treatment, each PLB either proliferated or differentiated into plantlet. It was possible to obtain millions of *Cymbidium* plants in a year from a single shoot-

tip (Arditti, 1993). The technique has been successfully applied or modified for some other orchid genera. Procedures, media, environmental conditions and other requirements are specific for each genus, species or hybrid (Arditti, 1977; Rao, 1977; Sagawa and Kunisaki, 1984; Goh,1990; Arditti, 1993).

Shoot-tips and axillary buds have been explanted *in vitro* for propagation of *Aranda*, *Aranthera*, *Ascocenda*, *Calanthe*, *Cattleya*, *Cymbidium*, *Dendrobium*, *Miltonia*, *Odontoglossum*, *Odontonia*, *Oncidium*, *Phaius*, *Rhyncostylis*, *Vanda*, *Vuylertertekeara* and others (Arditti, 1977; Rao, 1977; Sagawa and Kunisaki, 1984; Arditti, 1993).

Other sources of explants have been leaf bases of *Cattleya* (Champagnat et al., 1970), young leaf-tips of seedlings of *Dendrobium*, *Epidendrum* and *Laeliocattleya* (Churchill et al., 1970; 1971), young leaves of mature plants of *Renantanda* (Goh and Tan, 1982), inflorescence primordia of *Ascofinetia*, *Neostylis* and *Vascostylis* (Intuwong and Sagawa, 1973), floral buds of *Mokara* (Lim-Ho et al., 1984) and *Vanda* (Valmayor, 1986), and root-tips of *Catasetum* (Kerbauy, 1984a) and *Cyrtopodium* (Sanchez, 1988).

CLONAL PROPAGATION OF PHALAENOPSIS

Within the past decade, *Phalaenopsis* hybrids have gained commercial acceptance and importance as a potted

plant and cut flower due to its tolerance and lush growth in low light, showy flowers and good keeping quality (Griesbach, 1985; Vasquez,1990).

Considerable difficulty has been encountered in clonal propagation of Phalaenopsis due to the limited source of explants; this group being monopodial has only a single terminal shoot-tip which when harvested may result in the demise of the plant. The genus is recalcitrant to shoot-tip culture. It releases high levels of phenolic oxidation products on cut surfaces of explant and into culture medium, which appear to be toxic to the culture (Fast, 1979).

Success in clonal propagation through organogenesis of Phalaenopsis by tissue culture was first reported by Rotor (1949) who cultured the basal nodes of flower stalk to activate vegetative buds and produce plantlets. This method has been modified by a number of workers (Sagawa, 1961; Kotomori and Murashige, 1965; Scully, 1965; Urata and Iwanaga, 1965; Tse et al, 1971; Intuwong et al., 1972; Reisinger et al., 1976; Fu, 1978; Zimmer and Pieper, 1978; Tanaka and Sakanishi, 1980; Ernst, 1986; Gil, 1987; Tanaka et al., 1988; Ichihashi, 1992). However, only a limited number of plants could be obtained by this method. PLBs were occasionally produced from thin disks of internode sections from tip of young inflorescence stalk (Lin, 1986).

Although no details on yield were reported, Intuwong and Sagawa (1974) succeeded in clonal propagation by culture

of terminal and axillary buds of Phalaenopsis. Explants were cultured in liquid medium, which was changed frequently and then transferred to solid medium with sucrose. Yellowish PLBs were produced on explants. Upon transfer to solid medium without sucrose, the PLBs turned green and plantlets developed.

Others have used as explants organs derived from vegetative shoots induced on inflorescence nodes cultured in vitro. These include basal sections of shoot (Griesbach, 1983), leaf tissues (Tanaka et al., 1975; Tanaka and Sakanishi, 1977; Tanaka and Sakanishi, 1980; Haas-von Schmude, 1983), parts of leaves, stems and roots (Pieper and Zimmer, 1976), and root-tips derived from inflorescence stalk (Tanaka et al., 1976). Most of these approaches, however, have resulted in limited numbers of plantlets and intensive labor.

CLONAL PROPAGATION THROUGH SOMATIC EMBRYOGENESIS

The initiation and development of embryoids from somatic tissues was demonstrated from carrot cell suspension cultures by Steward (Steward et al., 1958) and Reinert (1958; 1959). The developmental sequence of embryoids has been shown to be similar to that of zygotic embryos (Reinert, 1958; 1959). Since embryo-like structures are derived from somatic cells, the phenomenon is referred to as

somatic embryogenesis. A bipolar somatic embryoid shows no vascular connection with the maternal tissue or explant. Reports of somatic embryogenesis have been increasing in both angiosperms and conifers (Ammirato, 1983a; Rangaswamy, 1986; Wann, 1988).

Somatic embryogenesis offers some advantages to organogenesis for micropropagation. It has high proliferation rate. More than one million embryoids can be produced per liter of cell suspension culture in carrot (Drew, 1980; Janick, 1993). Each embryoid is separate, which can be handled without the physical separation required from organogenesis or axillary branching system in vitro. Developed embryoid contains a root and shoot meristem indicating that conversion to seedlings can be obtained in a single step.

FACTORS FOR INDUCTION OF SOMATIC EMBRYOGENESIS

Many factors such as genotype and physiological state of explant, choices of plant growth regulator, nitrogen source and cation in culture medium, state of medium, physical environment, culture vessels and others affect embryogenic development (Ammirato, 1983a; Rangaswamy, 1986; Wann, 1988; Ammirato, 1989). However, callus type has not been considered to any extent partly due to the lack of clear definition of the degree of dedifferentiation of

callus. Involvement of chlorophyll and/or chloroplast development is not clear, although the development of chlorophyll level is influenced by carbohydrate source in at least some plant tissue cultures. In this section, only selected factors which may affect somatic embryogenesis of Phalaenopsis callus are discussed.

Callus Type

A friable callus consists of loosely aggregated parenchyma cells without a delimiting epidermis. In carrot cell cultures, cell isolation and dedifferentiation occurred just prior to somatic embryogenesis (Steward et al., 1958; 1964). Release from constraints of neighboring maternal and endosperm tissue such as when a special callose wall was laid around zygote of angiosperms triggered embryogenesis (Williams et al., 1984). Specialized walls also developed around nucellar cells prior to development of somatic embryoids in Citrus. The thick walls within original primary walls, severed plasmodesmatal connections and isolated them from neighboring nucellar cells (Wilms et al., 1983). In wild carrot cells, somatic embryogenesis was enhanced by plasmolysis. Enhancement was interpreted as an increase in regeneration of cells which had become physiologically isolated from tissue of origin by rupture of plasmodesmata (Wetherell, 1984). In direct somatic embryogenesis from differentiated tissues, tissue explanting

was normally followed by at least a short period of proliferation as friable callus before the appearance of proembryogenic complexes in suspension cultures (Evans et al., 1981).

In a maize inbred line, friability of callus determined plant regeneration via somatic organogenesis or embryogenesis. An organogenic callus, which was characterized as compact, convoluted and covered by a well defined epidermis was selected from compact callus. Embryogenic callus tissue, which was composed of aggregates of small isodiametric cells with dense cytoplasm and which was distinguished by its friability, was selected from the organogenic tissue (Lowe et al., 1985). The friable embryogenic cell line was found to express isozymes of glutamate dehydrogenase and esterase characteristic of zygotic embryos developing in vitro. These isozymes could be used to distinguish between embryogenic and shoot-forming cultures (Everett et al., 1985). A friable oat callus established from a compact, organogenic callus by selection, was also found to be embryogenic (Bregitzer et al., 1989).

Disorganized Cell Masses in Orchids: Many highly organized cell masses in orchids have been described as calli regardless of degree of dedifferentiation or friability.

Disorganized cell masses derived from embryos of

Cymbidium and Vanda during seed germination appeared highly organized in illustrations by Curtis and Nicole (1948) and Rao (1963). Structure of so called callus from zygotic embryos of Vanda was similar to proliferating zygotic protocorms. Spherical calli developed bulging structures which were either cream yellow or dark green. Sections showed that they consisted of parenchymatous cells bounded by an epidermis-like layer with basal rhizoids. Differentiation of stomata and tracheids also occurred in the callus tissue. The callus masses developed into single or multiple protocorms through organogenesis in subsequent culture (Rao, 1963).

Table 1 is a summary of descriptions of calli derived from explants of organs other than embryos from various orchid genera. Calli as described were highly differentiated and organized. Calli were shown as rather compact or nodular calli (Loh, et al., 1975; Bapat and Narayanaswamy, 1977; Kerbauy, 1984a), or as calli without any descriptions (Churchill et al., 1970; Tse et al., 1971; Churchill et al., 1973; Stewart and Button, 1978; Lin, 1986; Sanchez, 1988). An Aranda callus was described as smooth dome shaped in outline consisting of homogeneous parenchyma cells and units which were distinctly separated from each other (Loh et al., 1975). A callus with numerous surface hairs from Vanilla bud (Gu et al., 1987) and light green callus from cattleya root-tip (Kerbauy, 1991) were reported

Table 1. Disorganized cell masses reported in orchids.

Genus	Explant	Initial medium (per liter)	Callus type description	Transfer medium (per liter)	Result	Reference
Aranda	Young leaf-tip	VW 20 g sucrose 200 ml CW	"Cream colored callus with distinctly separated smooth dome shapes"	VW 20 g sucrose 200 ml CW	Callus unit produced 1 or 2 protocorm-like bodies (PLBs)	Loh et al., 1975
Catasetum	Root-tip	Knudson 20 g sucrose 150 ml CW 5 mg NAA	Callus without description	Knudson 20 g sucrose 150 ml CW 5 mg NAA	Only a few of calli produced PLBs	Kerbauy, 1984b
Cattleya	Root-tip	Modified VW 20 g sucrose 0.4-1.6 mg 2,4-D 60 g banana pulp	"Friable light green callus"	Modified VW 20 g sucrose 0.1 mg 2,4-D 0.5 mg NAA 60 g banana pulp	Malformed roots, PLB and plantlets	Kerbauy, 1991
Cymbidium	Shoot-tip	White 20 g sucrose 100 ml CW 5 mg NAA	"Free cells and some globular cell clusters"	White 20 g sucrose 100 ml CW	Compactly arranged PLBs	Steward and Mapes, 1971
Cyrtopodium	Root-tip	Modified VW 20 g sucrose 0.5 NAA 0.1 BAP 1 g pepotne	Callus without description	Modified VW 20 g sucrose 0.5 NAA 1.0 BAP 1 g peptone	PLBs formed from calli or directly upon root meristem	Sanchez, 1988

Table 1. Disorganized cell masses reported in orchids (continued).

Genus	Explant	Initial medium (per liter)	Callus type description	Transfer medium (per liter)	Result	Reference
Doritaenopsis	Flower-stalk section	VW (x1.2) 20 g sucrose 5 mg BA	"Yellowish white callus-like tissue"	VW (x1.2) 20 g sucrose 1 mg BA	PLBs formed on epidermis and cut surface of the flower-stem section	Lin, 1986
Epidendrum	Young leaf-tip	MS 30 g sucrose 0.5 mg BA 1 mg 2,4-D	Callus without description	Knudson 20 g sucrose	Plantlets	Churchill et al., 1970
	Root-tip	SH 30 g sucrose 1 mg 2,4-D	"Disorganized growths and callus"	SH or Knudson 20 or 30 g sucrose	No further organization	Stewart and Button, 1978
Laeliocattleya	Young leaf-tip	Modified Heller 30 g sucrose 1 mg 2,4-D 0.5 mg BA	Callus without description	Knudson C 20 g sucrose	PLBs attached each other	Churchill et al., 1973
Oncidium	Root-tip	Modified VW 20 g sucrose 1.25-10 mg NAA	"Callus masses reminiscent of malformed roots"	Modified VW 20 g sucrose 150 ml CW	PLBs	Kerbauy, 1984a
Phalaenopsis	Injured bud	MS 30 g sucrose 2 mg NAA	Callus without description	Knudson C 20 g sucrose	Shoots	Tse et al., 1971

Table 1. Disorganized cell masses reported in orchids (continued).

Genus	Explant	Initial medium (per liter)	Callus type description	Transfer medium (per liter)	Result	Reference
Phalaenopsis	Flower-stalk section	VW (x1.2) 20 g sucrose 5 mg BA 0.5 mg NAA	"Yellowish white callus-like tissue"	VW (x1.2) 20 g sucrose 1 mg BA	PLBs formed on epidermis and cut surface of the flower-stem section	Lin, 1986
Spathoglottis	Young root stock	MS 20 g sucrose 150 ml CW 5 mg NAA 1 mg Kinetin	"Nodular callus"	MS 20 g sucrose 6 mg NAA 2 mg 2,4-D 2 mg Kinetin	Only rhizogenetic response	Bapat and Narayanaswamy, 1977
Vanilla	Intact bud	LS 30 g sucrose 1 g CH 0.5 mg BA 0.5 mg Kinetin	"Friable yellow mass with numerous surface hairs"	LS 15 g sucrose 1 g CH 0.1 mg BA	Shoots	Gu et al., 1987
	Young leaf	LS 30 g sucrose 150 ml CW	Callus without description	LS 15 g sucrose 1 g CH	Several shoots	Gu et al., 1987

Medium: VW, Vacin and Went (1949); Knudson, Knudson (1946); White, White (1949); MS, Murashige and Skoog (1962); SH, Schenk and Hildebrandt (1972); Heller, Heller (1953); LS, Linsmaier and Skoog (1965).

as friable calli without detailed descriptions or illustrations. These calli generally produced limited numbers of PLBs or shoots via organogenesis. Calli derived from Cattleya (Kerbauy, 1991) and Oncidium (Kerbauy, 1984) root cultures strongly reflected root-state and their limited morphogenetic expression. Low regeneration capacity in Oncidium callus was attributed to incomplete dedifferentiation-state of the callus (Kerbauy, 1984a). Reported somatic embryogenesis in suspension culture of free cells and some globular cell clusters of Cymbidium developed into compactly arranged protocorms (Steward and Mapes, 1971). The illustrations, however, showed that initial cell clusters were compact, and the arrangement of reported protocorms resembled the clusters of PLB produced through organogenesis.

Failure to induce somatic embryogenesis in earlier orchid tissue cultures may be due to calli which were not completely dedifferentiated. Embryogenic potential were limited by the compactness or organized state of these calli.

Carbohydrate

Most plant tissue cultures require an exogenous supply of carbohydrate. Carbohydrate requirement for callus cultures was initially investigated by White (1934) and Gautheret (1955). The superiority of sucrose as the

carbohydrate source was first reported by White (1940), Dormer and Street (1949), and has been confirmed by many workers. Sucrose at the concentration of 20 to 30 $g \cdot liter^{-1}$ is the most commonly used carbohydrate. Studies of altered carbohydrate sources and levels have been ignored because it was considered to be merely a carbon source in tissue culture systems.

In addition to the role of carbohydrate as a carbon source, there has been increasing evidence that it may act as a regulating factor for morphogenesis. Verma and Dougall (1977) examined the effect of carbohydrate sources on both growth and somatic embryogenesis in wild carrot suspension culture. They found that the cell suspension utilized not only sucrose but glucose, fructose, galactose, mannose, maltose, raffinose and stachyose as carbon sources. A positive correlation between growth and embryoid number was obtained regardless of carbohydrate source, suggesting the involvement of a common intermediate in the metabolism of various carbohydrates. Studies of a narrow range of carbohydrates in eggplant confirmed sucrose to be the best source of carbon for somatic embryogenesis (Gleddie et al., 1983).

Remarkable stimulation of somatic embryogenesis by the manipulation of carbohydrates has been obtained in nucellar calli of Citrus. Citrus cell lines derived from nucellar tissue have been initiated and maintained in a

nondifferentiated state in a sucrose-containing medium. Omission of sucrose for a single culture period caused cessation of growth but stimulated somatic embryogenesis in habituated ovular callus from Shamouti orange (Kochba and Button, 1974). Lactose and maltose stimulated somatic embryogenesis in cultures of Citrus ovular callus remarkably, though growth was promoted to the greatest extent on sucrose (Button, 1978). Substitution of galactose or galactose-yielding sugar, lactose for sucrose had a stimulatory effect on somatic embryogenesis of nucellar calli from several Citrus cultivars (Kochba et al., 1978). The most efficient initiation of embryogenesis was obtained with glycerol in several Citrus nucellar cell cultures (Ben-Hayyim and Neumann, 1983). By changing the carbon source in the culture medium from sucrose to glycerol, cell cultures were induced to undergo somatic embryogenesis forming embryoids. Glycoproteins from the proembryogenic cells suppressed further development on sucrose-containing medium (Gavish et al., 1991). The normal progression of embryoid development appeared to depend on the absence of these glycosylated extracellular proteins from the culture medium (Gavish et al., 1992).

Embryoid formation in wild carrot (Kinnersley and Henderson, 1988) and Digitalis lanata suspension culture (Kuberski et al., 1984) was enhanced by the use of maltose rather than sucrose. In alfalfa, maltose, maltotriose and

soluble starch improved embryoid yield when compared to sucrose (Strickland et al., 1987). The concentration of sucrose also had a pronounced effect on somatic embryogenesis (Lu et al., 1982).

Effect of Carbohydrate on Chlorophyll Formation: The chlorophyll content of cultured callus tissue may influence embryogenic capacity. Genes which were rapidly expressed at the onset of blue-light-induced chloroplast differentiation were also involved in expression of somatic embryogenesis of Chenopodium rubrum (Kaldenhoff and Richter, 1990) and carrot (Aleith and Richter, 1991) cultures. Viability and expression of a specific protein involved in early somatic embryogenesis was identified in chlorophyll content of pea protoplasts (Koonen and Jacobson, 1991). Stimulation of somatic embryogenesis in cultures of Citrus ovular callus on lactose was correlated with the formation of chlorophyll in the callus (Button, 1978).

Sucrose is related to the formation of chlorophyll in some plant tissue cultures. The level of chlorophyll was reduced by the commonly used levels of sucrose in callus cultures of endive, lettuce (Hildebrandt et al., 1963) and tobacco (Kaul and Sabharwal, 1971) and also in cell suspension cultures of carrot (Neumann and Rafaat, 1973), tobacco (Nato et al., 1977), spinach (Dalton and Street, 1977) and Atropa belladonna (Davey et al., 1971).

The greening of spinach cell cultures only occurred as the growth rate declined toward zero. Chlorophyll was synthesized only when the concentration of total sugars in the medium was at or below 2.5 $g \cdot liter^{-1}$. At the higher concentrations, chlorophyll synthesis was inhibited (Dalton and Street, 1977). A negative correlation between chlorophyll level and growth was obtained in suspension culture of Picea abies (Simola et al., 1992).

Substitution of sucrose by glucose or fructose did not enhance chlorophyll formation in spinach cells (Dalton, 1980b; Dalton and Street, 1977; Grob and Richter, 1982). In Lemna perpusilla culture, mannose inhibited chlorophyll synthesis whereas mannitol at the same molarity did not showing that the inhibition was not due to the osmotic effects of sugars (Posner, 1970). Slowly utilized sugars such as starch (Jaspars, 1965), inulin and raffinose (Dalton and Street, 1977) enhanced the levels of chlorophyll, which resulted from reduced availabilities of sugars in the media. Chlorophyll content in cultured cells of Asparagus offinalis showed fifteen times higher chlorophyll content when lactose replaced sucrose (Dalton and Peel, 1983).

Chlorophyll synthesis in a carrot callus strain was reduced by sucrose, whereas it was not suppressed by glucose. The effect was shown to be a inhibition of greening specifically by sucrose rather than a reducing sugar requirement for chlorophyll synthesis (Edelman and

Hanson, 1971a). Invertase in free space of the callus relieved the suppression of chlorophyll synthesis (Edelman and Hanson, 1971b). The sucrose effect was attributed to sucrose inhibition of aminolevulinic acid synthesis (Pimplin and Chapman, 1975). The suggestion that sucrose specifically inhibited greening of the culture seems to be confined to one particular strain of carrot.

In an attempt to explain the inhibitory effect of sugar on greening, it was simply suggested that the greening of some cell culture is inhibited in excess of sugar, but is promoted during sugar depletion independently of cell growth rate (Dalton, 1980a). The hypothesis has been supported from the uses of slow utilized-carbohydrates that promote chlorophyllous cultures.

Catabolite Repression: A similar inhibitory effect of sugar has been reported in the physiology of microorganisms, while the mechanism by which sugar inhibits greening in higher plant cells has not been explained. In bacteria as well as in simple eukaryotes, the high concentration of glucose or other readily utilizable carbon sources represses the synthesis of certain enzymes involved in the production of metabolites as well as entire organelles such as chloroplast and mitochondria (Mahler et al., 1981). The repression, called catabolite repression, has been reported to be mediated by cyclic adenosine 3′,5′-monophosphate

(cAMP) as primary messenger in case of bacteria. In culture of Escherichia coli, the concentration of cAMP varied inversely with the concentration of glucose in the medium. When glucose was added to the culture medium, there was a decrease in the intracellular concentration of cAMP. The addition of cAMP to the culture medium partially reversed the catabolite repression (Pastan and Perlan, 1970). Although catabolite repression in yeast is also mediated by cAMP (Mahler et al., 1981), the catabolite repression in yeast referred to a number of regulatory systems that regulated transcription of groups of genes (Entian, 1981).

Chloroplast formation in Euglena gracilis was inhibited by glucose (Monroy and Schwartzbach, 1984; Reinbothe, 1992). In a facultatively heterotrophic alga, functional chloroplast development did not occur until cells were starved for fixed carbon. The formation of an active photosynthetic apparatus was repressed by glucose (Handa et al., 1981). Cell differentiation in a moss was also regulated by the mechanism of catabolite repression (Handa and Johri, 1979).

Although the existence and function of cAMP in higher plant tissues have been controversial (Brown and Newton, 1981: 1986), attributions of physiological changes to sucrose or catabolite repression in higher plant have been increasing. Activity of glutamate dehydrogenase was suppressed by sucrose in root segment (Sahulka et al.,

1975), detached shoot of Pisum sativum (Nauen and Hartmann, 1980) and aseptic culture of Lemna perpusilla (Duke and Koukkari, 1977). The effects of glucose and fructose was equivalent to sucrose.

In cell cultures of Asparagus offinalis, cAMP derepressed the inhibitory effect of glucose on synthesis of glutamate dehydrogenase and acid phosphatase. The data supported the hypothesis that a regulatory mechanism similar to the catabolite repression may function in cells from higher plants (Tassi et al., 1984).

Catabolite repression may function to influence somatic embryogenesis. Key enzyme activities for glyoxysome such as isocitrate lyase and malate synthase were induced during early stages of somatic embryogenesis in Pimpinella anisum cultures when sucrose was withdrawn from culture medium (Kudielka and Theimer, 1983). In Digitalis lanata cell culture, the most suitable carbon source was maltose, which was slowly degraded. Sucrose yielded poor rates of embryogenesis and cardenolide formation. The inhibition was attributed to catabolite repression (Luckner and Diettrich, 1985).

In microbial cultures of industrial biotechnology, production of a large number of secondary metabolites is suppressed by glucose and other readily utilizable carbon sources. The problem has been solved by medium manipulation. One way is to use a low concentration of

readily utilizable carbon source and the other is to use a non-repressive, slowly utilizable carbon source.

Effect of Sucrose on Tissue Culture of Phalaenopsis: Morphogenesis in tissue culture of Phalaenopsis has been affected by the sucrose level usually used in many plant tissue cultures. Elimination of sucrose from culture medium was beneficial for tissue culture of Phalaenopsis when the medium was supplemented with coconut water. Yellowish PLBs were produced from shoot-tip culture in Vacin and Went medium (Vacin and Went, 1949) supplemented with 20 $g \cdot liter^{-1}$ sucrose and 15 percent coconut water. The PLBs turned green when subcultured to medium without sucrose (Intuwong and Sagawa, 1974).

Sucrose inhibited proliferation and chlorophyll formation in some sarcanthine orchids. Vigorous proliferation and green PLBs were obtained from shoot-tip culture of Vanda Miss Joaquim in a medium without sucrose (Kunisaki et al., 1972). Shoot-tips of a strap-leafed Vanda hybrid cultured in sucrose containing medium turned yellow then brown and eventually died (Teo et al.,1973). Similar observations were reported in inflorescence cultures of Vascostylis, Neostylis and Ascofinetia (Intuwong and Sagawa, 1973). Protocorms of a vandaceous orchid, Holttumara turned yellow and subsequently became necrotic in sucrose, glucose or fructose medium. The sugar effect was not attributed to

osmotic effect since non-detrimental effect of mannitol on chlorophyll formation (Teo and Wong, 1978).

Plant Growth Regulators

Auxin: Auxin has been one of the key inducers of somatic embryogenesis. Somatic embryogenesis has been successfully induced in callus and cell suspension by subculture from an auxin-enriched medium (2,4-D or NAA, generally at the range of 0.1 mg to 5.0 $mg \cdot liter^{-1}$) to an auxin-free medium, by lowering the concentration of auxin (generally at the range of 0.01 mg to 0.2 $mg \cdot liter^{-1}$), or by substitution of weaker or less stable auxins (such as IAA, IBA or NAA). Auxin, typically 2,4-D, has a crucial role in generating embryogenic competence (Ammirato, 1983a; Sung et al., 1984; Wann, 1988), but 2,4-D itself inhibits the progression of embryoid development (Borkird et al., 1986). Embryogenic culture must be subcultured to auxin-free or low auxin medium for embryogenesis to proceed. 2,4-D were essential for the production of embryogenic cultures of monocotyledonous plants including rice (Abdullah et al., 1986), wheat (Vasil et al., 1990) and maize (Morocz et al., 1990).

The concentration or the duration of auxin treatment required for inductive effect is different in various species, genotypes and tissue origins of explant (Brown and Atanassov, 1985; Bogre et al., 1990; Rao, 1992). The

inhibition or promotion of somatic embryogenesis by auxins, anti-auxins and auxin synthesis inhibitors depends on age and stage of tissue culture, and endogenous auxin present in the culture (Fujimura and Komamine, 1979). IAA was found to inhibit development of embryoid in a carrot suspension culture (Fujimura and Komamine, 1979; Schavone and Cooke, 1987), while continuous treatment of carrot hypocotyl segments with IAA, indolebutyric acid (IBA), NAA or ß-naphthoxyacetic acid (NOA) was found to favor somatic embryogenesis (Kamada and Harada, 1979a).

Endogenous auxin is involved in somatic embryogenesis of carrot suspension cells. Total endogenous IAA level was high on 2,4-D supplemented medium. Even after transferring the culture to 2,4-D free medium, there was little change in endogenous IAA level through the early stage of embryogenesis (Fujimura and Komamine, 1979; Michalczuk et al., 1992) while free and conjugated 2,4-D metabolites showed a rapid decline (Michalczuk et al., 1992). Addition of either auxin or anti-auxin at early stage inhibited embryogenesis (Fujimura and Komamine, 1979). A critical level of endogenous auxin in cell clusters was necessary for embryogenesis (Fujimura and Komamine, 1979). Most single cells or small clusters from carrot cell suspension culture did not develop directly to embryoids in auxin-free medium. The single cells divided to form cell clusters of a certain size even in an auxin-free medium before embryogenesis took

place (Fujimura and Komamine, 1980a). Auxin may be necessary for the development of single cells to cell clusters which are capable of embryoid formation in auxin-free medium. The IAA level progressively declined after the early stage.

A habituated ovular callus of Shamouti orange which has autonomous growth habit showed a marked stimulation of embryogenesis after treatment with inhibitors of auxin synthesis, 5-hydroxy nitrobenzylbromide (HNB) and 7-aza indole (7-AZA), while addition of even as low as 0.01 $mg \cdot liter^{-1}$ of IAA or NAA strongly inhibitory (Kochba and Spiegel-Roy, 1977a). In cultures from τ-irradiated callus, subcultured in the absence of IAA, embryogenesis increased with radiation dose. The inhibition caused by the presence of IAA was progressively removed by increasing the radiation dose. With certain doses, the addition of IAA stimulated embryogenesis. The auxin levels might be reduced to suboptimal levels for embryogenesis with certain radiation doses (Kochba and Spiegel-Roy, 1977b). Studies of the auxin metabolism of Shamouti orange callus lines showed that peroxidase activity (Kochba et al., 1977) and the capability to form conjugates of IAA with aspartate (Epstein et al., 1977) were notably higher in an embryogenic callus line and thereby lowered the auxin level to an embryogenesis-inductive level.

Procedure of somatic embryogenesis is involved in the

alteration of gene expression at molecular aspect as response to an external stimulus. The consequence of auxin binding to its receptor was depolarization of the membrane, which was mediated by a plasmalemma H^+-adenosine 5′-triphosphatase (Barbier-Brygoo et al., 1989). Auxin sensitivity was related to the amount of auxin-binding protein on protoplast plasmalemma (Barbier-Brygoo et al., 1990). Auxin increased pH in both cytoplasm and cell wall (Brummel and Hall, 1987). A culture medium containing 1-5 mM NH_4^+ at pH 4 initiated and maintained the early stage of somatic embryoid development from wounded carrot explants without any application of exogenous auxin (Smith and Krikorian, 1990). Auxins such as IAA or 2,4-D stimulated hydrolysis of phosphatidylinositol in cell membrane which was involved in transmembrane signalling in higher plants. The hydrolysis of phosphatidylinositol caused an increase in the concentration of cytosol free Ca^+ (Ettilinger and Lehle, 1988; Zbell and Walter-Back, 1988). The initiation of deoxyribonucleic acid (DNA) synthesis and cell division are key events in somatic embryogenesis accompanied by essential alterations in the expression of a set of genes and coordinated changes in cellular functions. Treatment and removal of 2,4-D altered expression of various proline- or glycine- rich genes (Aleith and Richter, 1990). Comparative analysis of differences between carrot cells grown in 2,4-D and somatic embryoids developed after removal of 2,4-D

indicated a limited number of embryo- specific proteins (Choi and Sung, 1984). Embryo-specific ribonucleic acid (RNA) (Choi et al., 1987) has also been identified.

Cytokinin: Cytokinins have frequently been added into culture media to induce somatic embryogenesis in many plant cultures. Progressive decrease in embryogenic potential in carrot cells over long periods was alleviated by the addition of kinetin (Wochok and Wetherell, 1972). Cytokinins are not involved in embryoid development directly, but promote somatic embryogenesis by rapid cell division in the early stage. Cytokinin, 2-isopentenyladenine (2-iP) promoted embryogenesis in carrot cells by stimulating cell division (Sung et al., 1979). Rate of cell division was rapidly increased during the first few days of embryogenesis in individual carrot cell cluster (Fujimura and Komamine 1980b). Zeatin was required for its promotive effect on embryogenesis of carrot during this period (Fujimura and Komamine, 1980a). Isopentyladenosine and 2-iP had the same effect in Pimpinella anisum cell culture. Endogenous maxima of cytokinins were correlated with the early stage of growth, not with a morphologically visible embryoid formation (Ernst and Oesterhelt, 1984).

Embryoid formation in Digitalis lanata was initiated by a step-by-step shift of the auxin-cytokinin ratio of nutrient medium toward a higher content of cytokinin

(Kuberski et al., 1984; Scheibner et al., 1989). Somatic embryogenesis was induced in seedlings of peanut (Saxena et al., 1992) and in hypocotyl explants of geranium (Visser et al., 1992) on media supplemented with thidiazuron (TDZ: N-phenyl-N′-(1,2,3, thiadiazol-yl) urea). With the substitution of auxin-cytokinin requirement for the induction by TDZ, the modulation of endogenous auxin-cytokinin balance by TDZ may constitute a inductive stimulus for somatic embryogenesis.

Gibberellin: Gibberellins have rarely been incorporated in culture media for induction of somatic embryogenesis. Gibberellic acid $(GA)_3$ inhibited somatic embryogenesis in carrot (Fujimura and Komamine, 1975; Kamada and Harada, 1979a) and Citrus (Kochba et al., 1982; Tisserat and Murashige, 1977). In carrot, development of embryoids was not influenced by exogenous GA_3 at the early stage, but was inhibited at a later stage of embryogenesis (Fujimura and Komamine, 1975). In anise and carrot cell cultures, high levels of endogenous GA_1 and a reduced capability to metabolize GA_1 were correlated with the inhibitions of embryoid development. Lowered levels of GA_1 and rapid metabolism of GA_1 into GA_8 and GA-conjugates were correlated with continued embryoid development. The 2,4-D suppression was accompanied by high levels of GA_1 and reduced metabolism of GA_1 (Noma et al., 1982).

GA_3 was markedly stimulatory for somatic embryogenesis in callus derived from mesophyll protoplast of rapeseed (Pelletier et al., 1981) and in callus and protoplasts of Brassica nigra (Jagannathan et al., 1990).

Abscisic Acid (ABA): ABA is involved in the late stages of somatic embryogenesis. Amount of endogenous ABA during somatic embryogenesis in carrot remained constantly low except for temporary increase during the development stage of embryoids. This temporary increase corresponded to the period at which globular embryoids developed into heart-shaped or torpedo-shaped embryoids (Kamada and Harada, 1981). Appearance of abnormal embryoids was suppressed by the application of ABA. In caraway cell culture, ABA effectively normalized development-inhibiting abnormal proliferation and precocious germination, and fostered normal maturation (Ammirato, 1974). ABA had a similar effect in carrot (Ammirato, 1983b; Kamada and Harada, 1981), Triticum aestivum (Carman, 1988) and Digitalis lanata (Reinbothe et al., 1990). ABA was involved in regulation of embryogenic genes, of which corresponding proteins functioned during desiccation and maturation phase of embryogenesis in carrot. Expression of embryogenic gene of carrot was dependent on ABA (Hatzopoulos et al., 1990). The addition of ABA at low concentration was essential for

somatic embryogenesis in Pennisetum americanum (Vasil and Vasil, 1981).

Nitrogen Source

Of all the mineral nutrients, nitrogen form supplied in culture medium has significantly influenced the induction of somatic embryogenesis. Ammonium in the medium was not essential for embryoid formation, but a certain level of intracellular ammonium was a prerequisite for the induction of somatic embryogenesis in carrot cell culture (Halperin and Wetherell, 1965; Reinert et al., 1967). This level could be reached by a moderately high level of nitrate in the medium (Tazawa and Reinert, 1969).

Reduced nitrogen is essential for high-frequency embryogenesis. In the presence of nitrate, reduced nitrogen supplied as ammonium or certain amino acids, particularly glutamine, stimulated embryoid formation in carrot (Kamada and Harada, 1979b), alfalfa (Stuart and Strickland, 1984) and soybean (Finer and Nagasawa, 1988). Glutamine was essential for embryogenesis in Gossypium klotzschianum suspension culture (Price and Smith, 1979). Ammonium supplemented with proline remarkably stimulated somatic embryogenesis in alfalfa (Stuart and Strickland, 1984). Alanine and glutamic acid in carrot (Kamada and Harada, 1979b), and asparagine in soybean (Finer and Nagasawa, 1988) were also stimulatory. High concentration of alanine,

arginine, glutamine or proline in culture medium without plant growth regulators stimulated embryogenic response in a clone of alfalfa (Skokut et al., 1985). When alanine was incorporated in nutrient medium, it was quickly transformed to glutamic acid and utilized as a nitrogen source (Kamada and Harada, 1984).

Polyamines are involved in somatic embryogenesis. Embryogenic cell of carrot synthesized putrescine from arginine at twice the rate of non-embryogenic cells (Montague et al., 1978), which was paralleled by increased arginine decarboxylase activity (Montague et al., 1979). The arginine decarboxylase activity was not changed in a non-embryogenic, mutant cell line (Fienberg et al., 1984). Polyamines may regulate somatic embryogenesis by blocking biosynthesis of ethylene which suppresses embryogenesis (Bradley et al., 1984).

Nitrogen source can be in the forms of complex additives such as CW and CH. Improved results were obtained with these additives (Chandler and Vasil, 1984; Gray et al., 1984).

CHAPTER III

GROWTH AND DIFFERENTIATION OF CALLUS

INTRODUCTION

Although regeneration of plants from somatic callus tissue culture *in vitro* can follow one of two alternative pathways of organogenesis and embryogenesis, friability or dedifferentiation of callus may affect mode of regeneration from culture (Steward et al., 1958; 1964). Friability was the crucial factor in induction of somatic embryogenesis in callus cultures of inbred lines of maize (Lowe et al., 1985) and oat (Bregitzer et al., 1989).

The occurrence of callus in orchids has been reported in *in vitro* cultures during seed germination (Curtis and Nicole, 1948; Rao, 1963) and clonal propagation through organogenesis (Table 1). These calli from various explants were highly differentiated and organized, and generally produced limited numbers of PLBs or shoots via organogenesis.

In contrast to the orchid calli reported, a callus of a *Phalaenopsis* hybrid isolated from inflorescence-stem culture has been maintained. This chapter contains a morphological

and histological description of the growth and differentiation of the callus on a basal medium of modified Vacin and Went supplemented with 150 ml·liter^{-1} CW and after deletion of sucrose (20.0 g·liter^{-1}). A numerical score system for developmental stages is established for use in subsequent experiments.

MATERIALS AND METHODS

Callus of a Phalaenopsis hybrid isolated from previous experiments by Sagawa was maintained on basal medium consisting of modified Vacin and Went (MVW) (Sagawa and Kunisaki, 1984) containing 20 g·liter^{-1} of sucrose and supplemented with 150 ml·liter^{-1} of CW (MVW+20S+CW) in 150 ml flask, under continous illumination from G.E. Power Groove cool white fluorescent lamps at approximately 2 klux and 26 ± 3 °C and subcultured every 4 weeks. All components of media were autoclave at 121 °C for 1 kg·cm^{-2} for 15 minutes.

For histological studies, callus fixed in Craf III (Berlyn and Miksche, 1976) was dehydrated through a graded series of tertiary butyl alcohol, embedded in Paraplast and sectioned (10 microns) on a Spencer rotary microtome and stained with safranin-fast green (Berlyn and Miksche, 1976). Starch was traced by staining section overnight with

safranin and transferred to potassium iodide-iodine (IKI) solution. Total carbohydrate was determined by the periodic acid-Schiff reaction (Berlyn and Miksche, 1976), total RNA by methyl-green pyronine method (Brachet, 1973) and total protein by ninhydrin Schiff's method (Yasuma and Ichikawa, 1973). Observations and photographs were made with a Zeiss Stereomicroscope with MC 63 camera and Zeiss photomicroscope.

To characterize callus growth on MVW+20S+CW, peripheral yellow callus pieces between 40 and 45 mg were excised from stock calli at the end of a subculture period. Five callus pieces were randomly distributed on medium in each 100 x 15 mm Fisher disposable petri dish. Fifteen petri dishes were prepared and cultured in a completely randomized design. Fresh weights of 15 callus pieces from three petri dishes were measured every week for 5 weeks. Growth indices were calculated by (Final fresh weight - Initial fresh weight) $\cdot$ Initial fresh weight^{-1}.

Numerical scores ranging from -1 to 4 were assigned to developmental stages for use in future studies.

RESULTS AND DISCUSSION

On basal medium with 150 ml$\cdot$liter^{-1} CW and 20.0 g$\cdot$liter^{-1} sucrose (MVW+20S+CW) under continous

illumination the callus was yellow and granular as shown in Figures 1 and 2. Growth curve in terms of fresh weight showed approximately 2.3 fold increase in 5 weeks as shown in figure 3.

Four to 6 weeks after subculture to medium without sucrose (MVW+0S+CW), friable yellow callus turned green and rough without any conspicuous structure as shown in Figures 4 and 5.

Twelve weeks after subculture, numerous green globular-shaped proembryonic structures with rough surfaces developed at the periphery of the green callus as shown in Figures 6 and 7. Axial lengths of the embryoids ranged from 0.3 to 0.8 mm with diameters ranging from 0.2 to 0.6 mm. The ratio of axial length to diameter rarely exceeded 1.2 at this globular embryoid stage.

Sixteen weeks after subculture, embryoids developed into oblong-shaped embryoids with noticeable elongation as shown in Figures 8 and 9. Axial lengths of the embryoids ranged from 0.8 to 2.6 mm with diameters ranging from 0.4 to 1.0 mm. The ratio of axial length to diameter of embryoid frequently exceeded 2.0 at this oblong embryoid stage.

Thirty-two to 36 weeks after subculture, epidermis differentiated in transition from oblong to protocorm stage. Axial symmetry of the embryoid-structure was often distorted with differentiation at this intermediate stage (Figure 10;

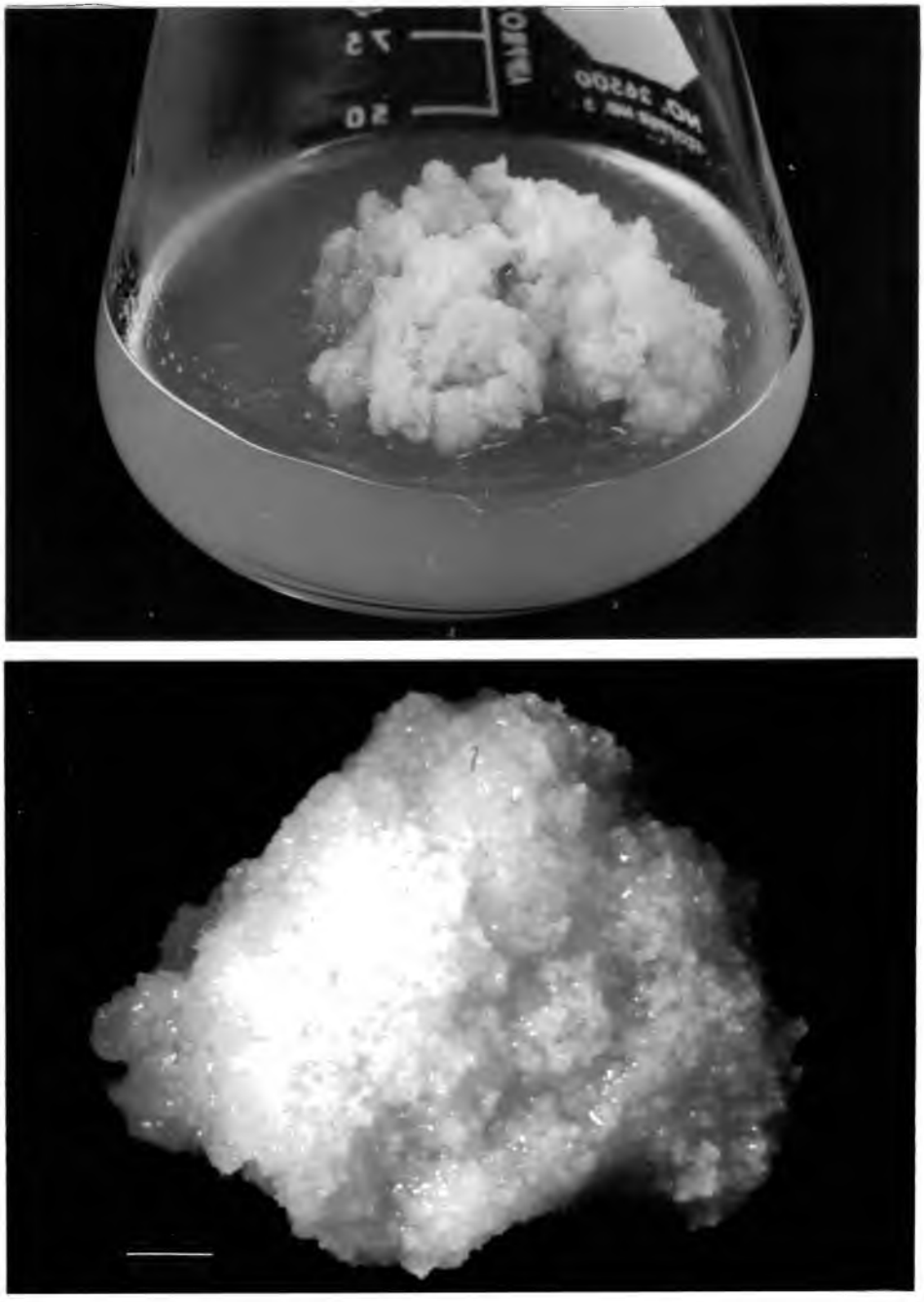


Fig. 1. A yellow and granular callus of Phalaenopsis maintained on MVW+20S+CW for three years.

Fig. 2. Enlargement of yellow and granular callus maintained on MVW+20S+CW showing no organization. Bar represents 1.0 mm.

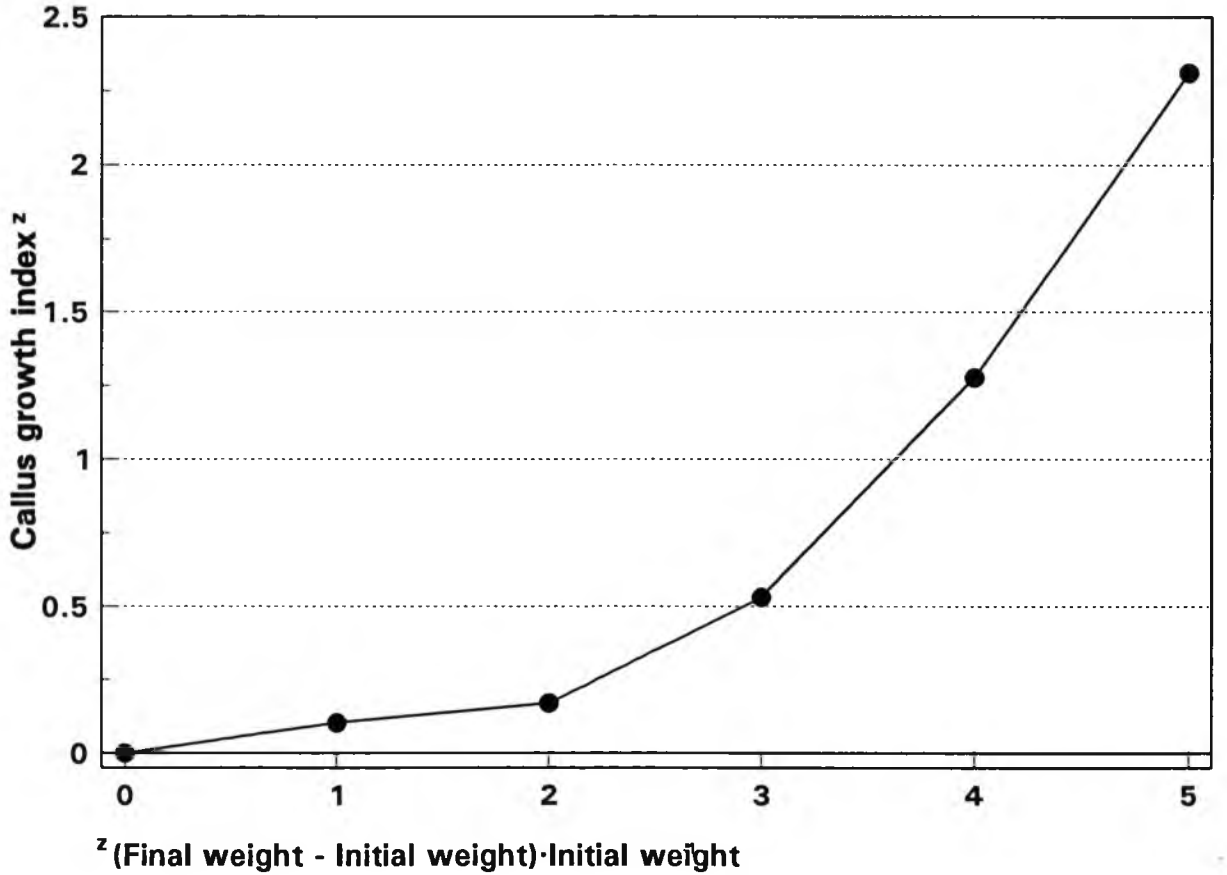


Fig. 2. Callus growth on media MVW + 20S + CW.

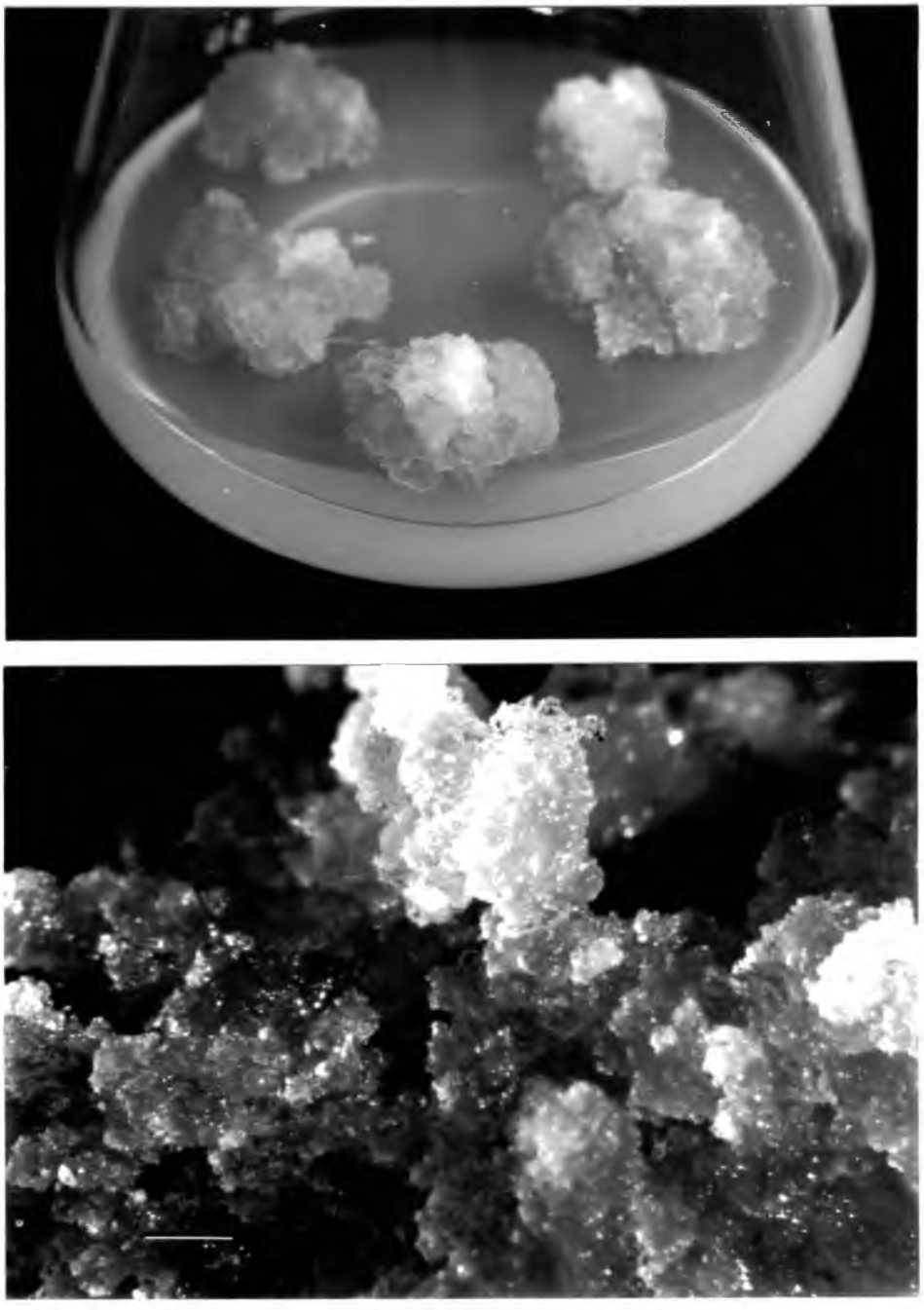


Fig. 4. Green calli 4 weeks after subculture to MVW+0S+CW.

Fig. 5. Enlargement of green callus showing its rough surface. Bar represents 1.0 mm.

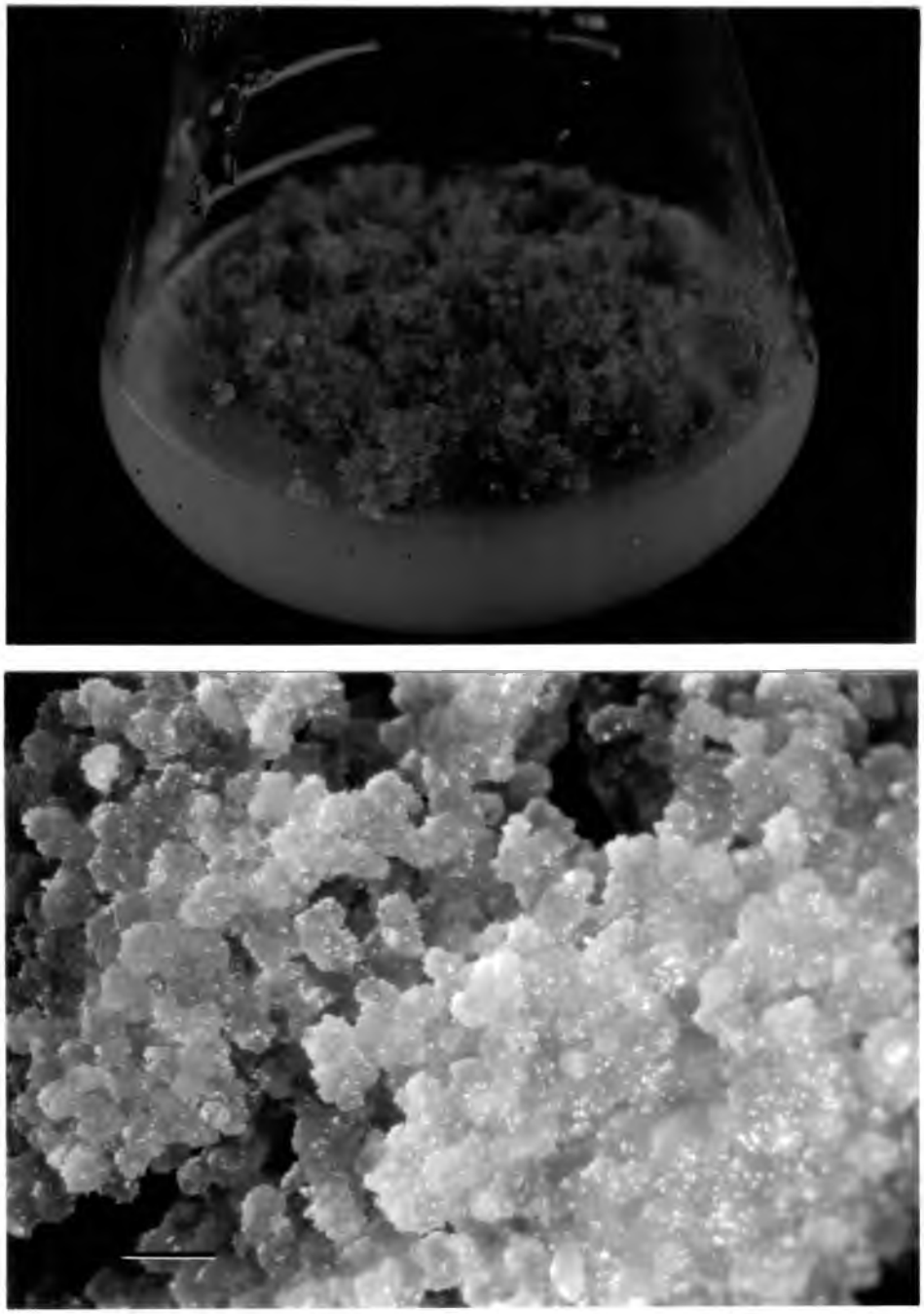


Fig. 6. Globular proembryoids developed at periphery of green callus 12 weeks after subculture to MVW+0S+CW.

Fig. 7. Enlargement of globular proembryoids developed at periphery of green callus showing their rough surface. Bar represents 1.0 mm.

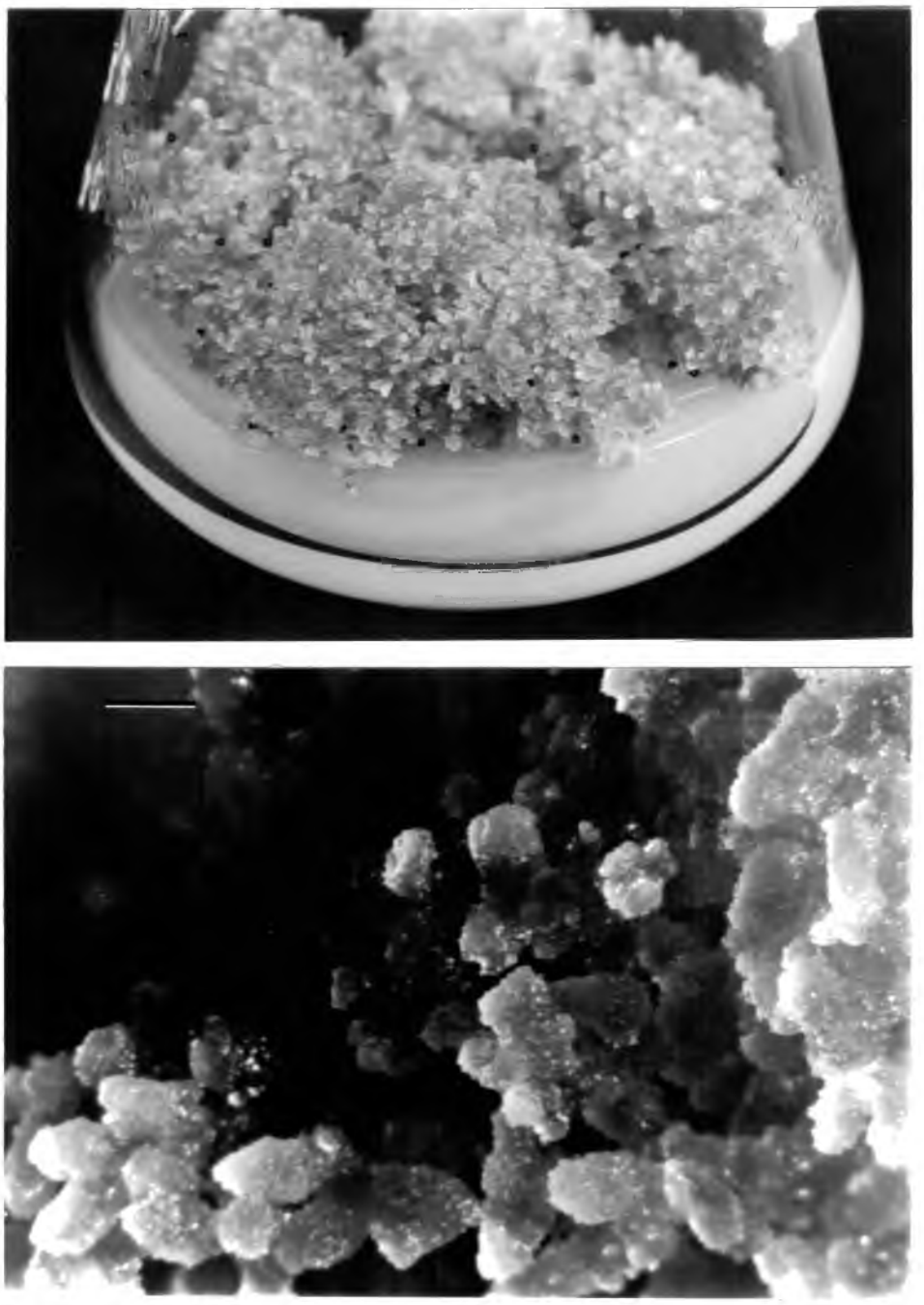


Fig. 8. Oblong embryoids developed from globular proembryoids 16 weeks after subculture to MVW+0S+CW.

Fig. 9. Enlargement of oblong embryoids, showing their elongated sizes. Bar represents 1.0 mm.

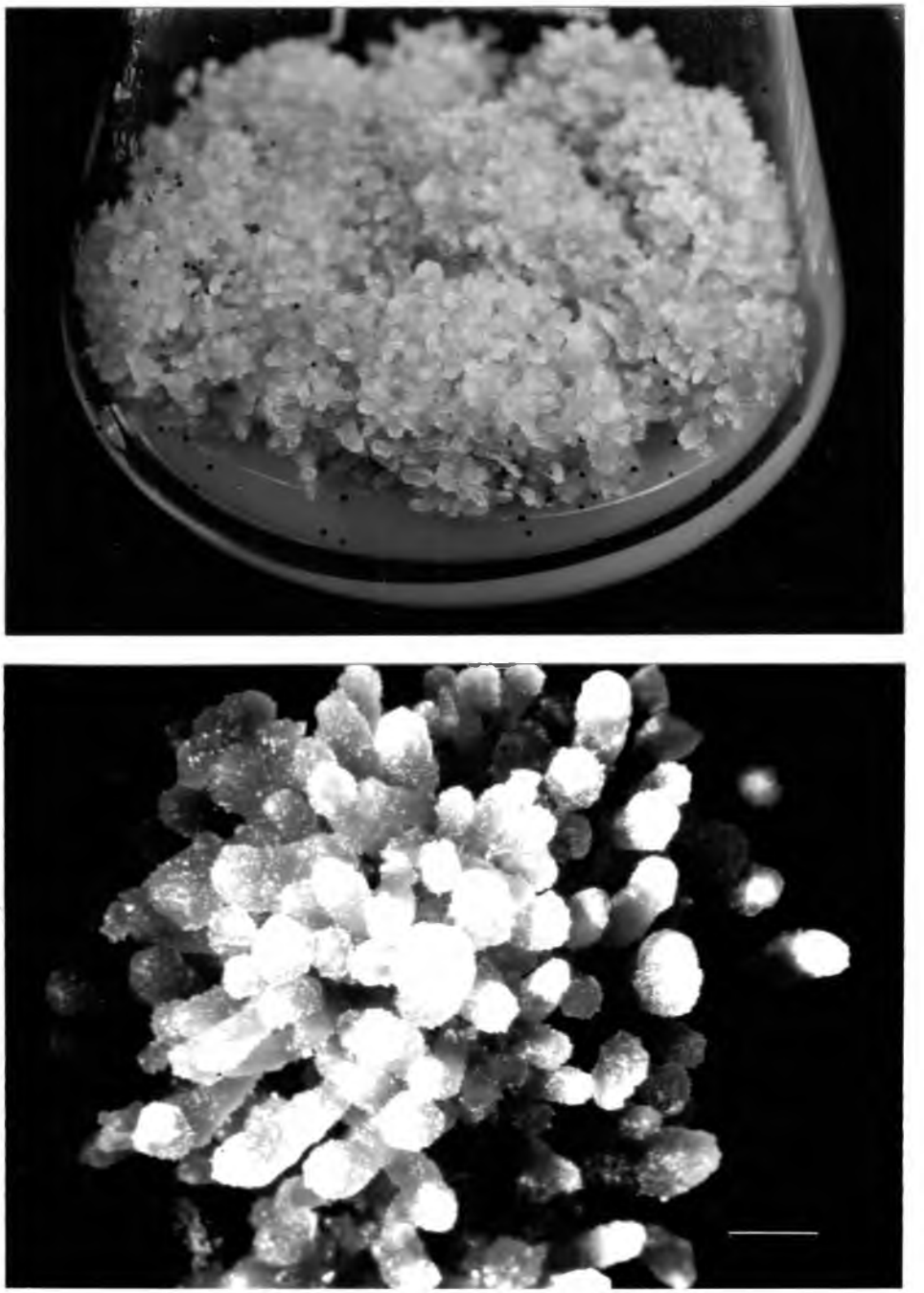


Fig. 10. Embryoids in intermediate stage developed from oblong embryoids 36 weeks after subculture to CW+0S+CW.

Fig. 11. Enlargement of embryoids in intermediate stage developed from oblong embryoids. Bar represents 1.0 mm.

11). Axial lengths of embryoids ranged from 1.4 to 6.4 mm and diameters ranged from 0.5 to 2.4 mm.

Fifty-two weeks after subculture, mature embryoids were similar in appearance to protocorms from zygotic embryos as shown in Figures 12 and 13. Epidermis and rhizoids were present on the protocorm-like embryoids. Axial lengths of embryoids ranged from 1.9 to 8.4 mm and diameters ranged from 0.7 to 2.0 mm.

Sixty-four weeks after subculture, leaves and roots were present on the protocorm-like embryoids as shown in Figures 14 and 15.

Approximately two years after subculture, they were fully developed into plantlets (Figure 16). Plantlets were transferred to community pots as shown in Figure 17.

Approximately three and a half years after subculture, plants from somatic embryoids flowered as shown in Figure 18.

When green callus or callus at early developmental stages up to the oblong stage was subcultured to medium with sucrose, it dedifferentiated to the yellow callus state. Brown colored callus was not generally observed in the medium, but occasionally occurred when culture conditions were not favorable to support the callus.

Vertical sections through yellow callus maintained on MVW+20S+CW medium stained with safranin-fast green showed undifferentiated, isodiametric cell aggregates randomly

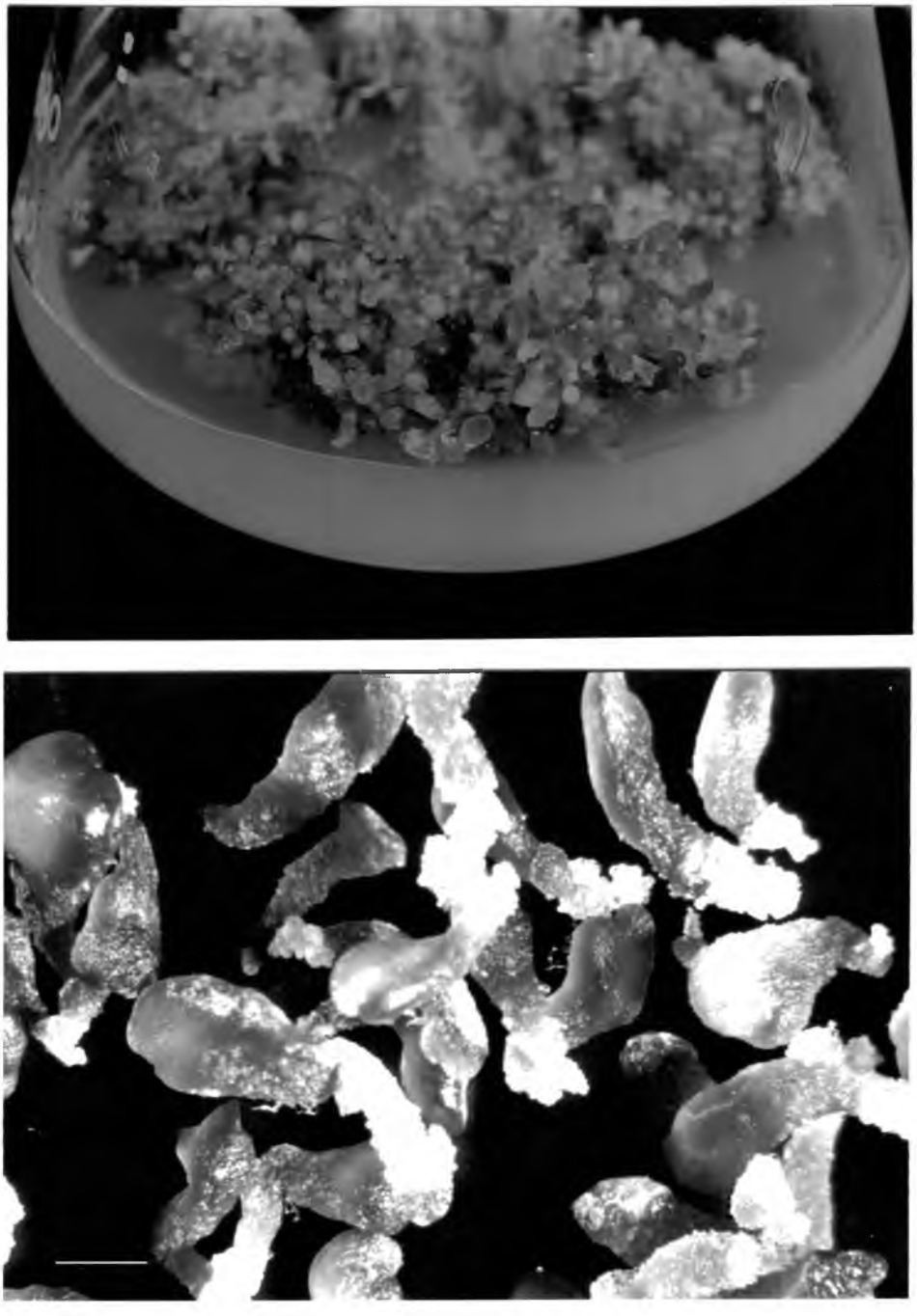


Fig. 12. Embryoids in protocorm stage after transitional stage 52 weeks after subculture to CW+0S+CW.

Fig. 13. Enlargement of embryoids in protocorm stage, showing their smooth surfaces with rhizoids. Bar represents 1.0 mm.

Fig. 14. Protocorm-like structures with leaves and roots 64 weeks after subculture to CW+0S+CW.

Fig. 15. Enlargement of protocorm-like structures with leaves and roots.

Fig. 16. Plantlets ready for transferring to community pot approximately two years after subculture of callus to CW+0S+CW.

Fig. 17. Plantlets in community pot.

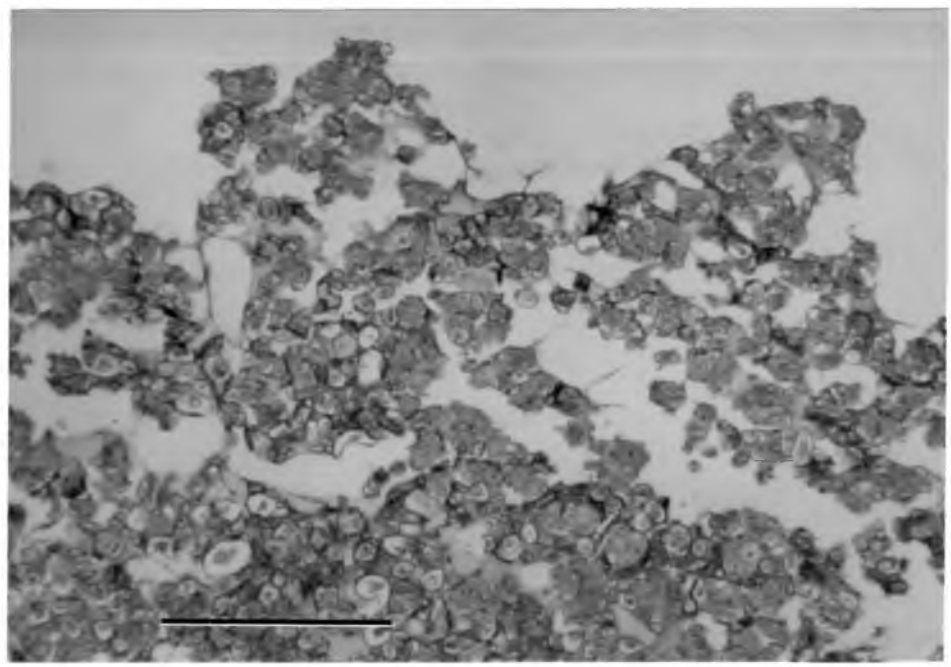


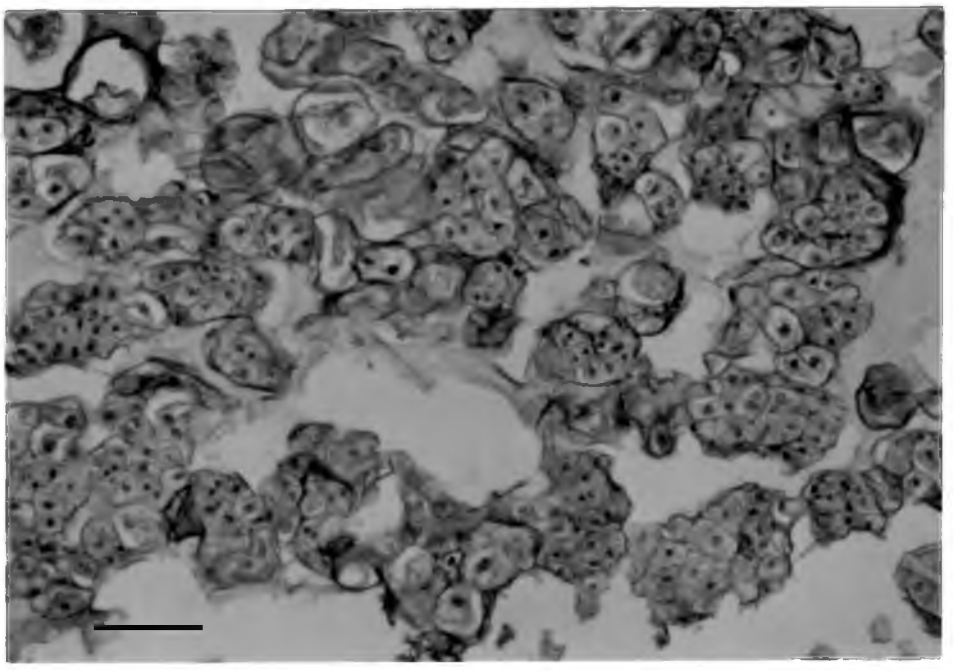
Fig. 18. Matured plants with flowers approximately three and a half years after subculture to CW+0S+CW.

Fig. 19. Vertical section of yellow callus stained with safranin-fast green, showing dispersed cells with large intercellular space. Bar represents 500 μm.

dispersed with large intercellular spaces, no vascular elements and no epidermis (Figure 19; 20). The cells had dense cytoplasm and small vacuoles. When tested for starch by safranin-IKI, most cells were heavily stained and dispersed throughout the callus (Figure 21). Upon transfer to liquid medium, callus was readily dissociated into small cell aggregates which consisted of small round, opaque cells (Figure 22) with small numbers of chloroplasts.

Vertical section of green callus 4 weeks after subculture to MVW+0S+CW stained with safranin-fast green showed that callus consisted of expanded and highly vacuolated cells and much smaller meristematic cells around the large cells (Figure 23). IKI staining was concentrated in the regions of small meristematic cell aggregates as shown in Figure 24. Periodic acid-Schiff staining for total carbohydrate showed the same patterns as IKI in yellow and green callus sections. Repeated staining with methyl green and pyronine for total RNA and ninhydrin-Schiff for total protein was too weak to compare. When suspended in liquid medium, larger cells were translucent and smaller cells were opaque as shown in Figure 25. Large numbers of chloroplasts were also present in the cells.

Suspension of green callus 12 weeks after the subculture in liquid medium showed globular-shaped proembryonic structures consisting of a meristematic region



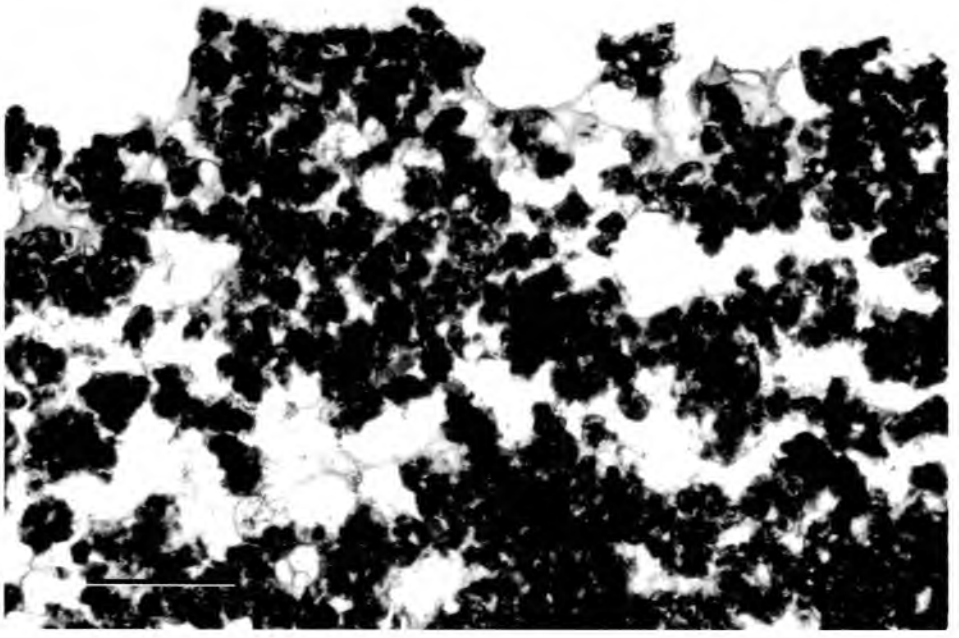


Fig. 20. Enlargement of vertical section of yellow callus stained with safranin-fast green, showing undifferentiated, isodiametric cells. Bar represents 100 μm.

Fig. 21. Vertical section of yellow callus stained with safranin-IKI, showing starch grains in cells were stained, which were dispersed throughout callus. Bar represents 500 μm.

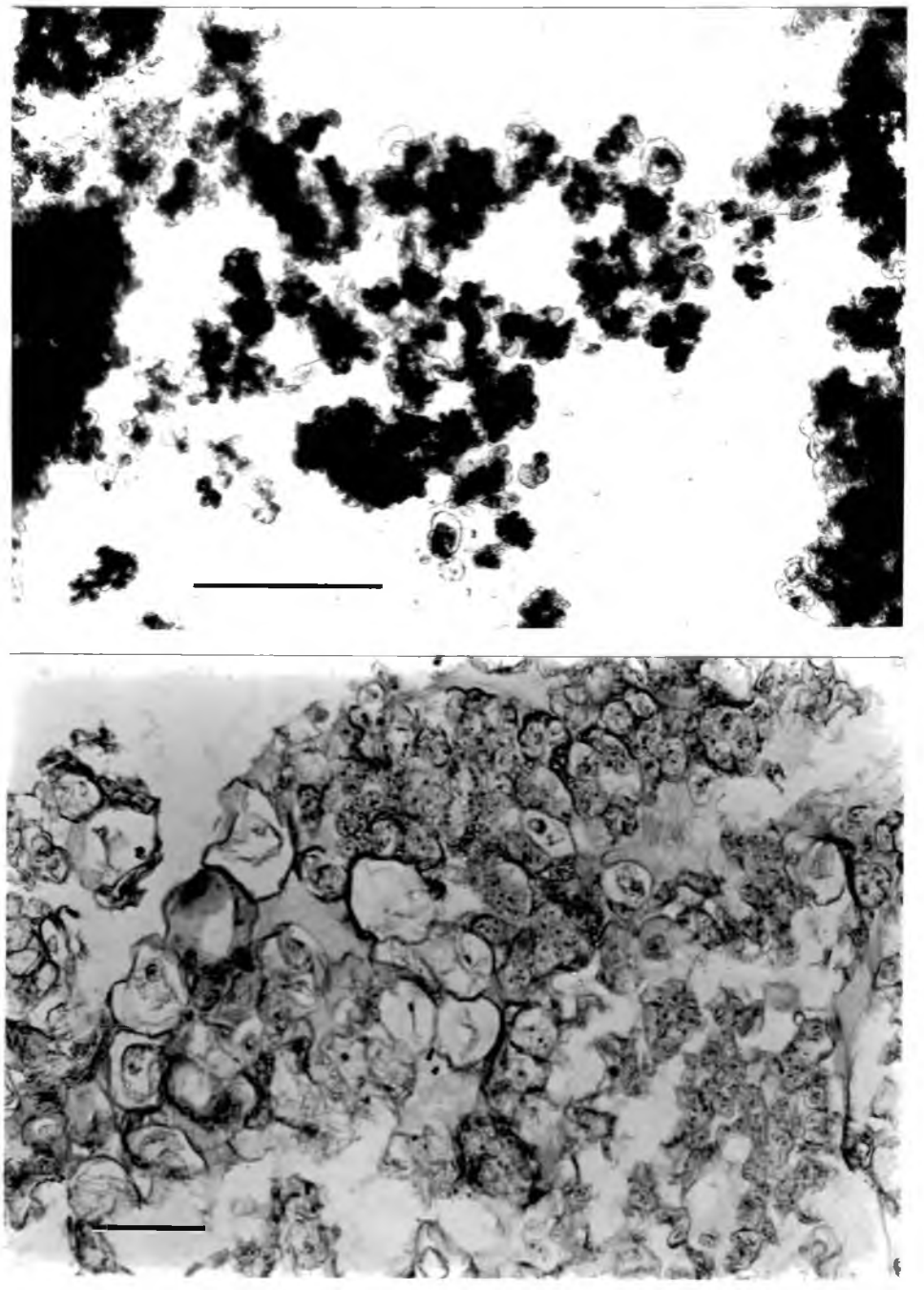


Fig. 22. Opaque cell aggregates dissociated from yellow callus upon transfer to liquid medium. Bar represents 500 μm.

Fig. 23. Vertical section of green callus 4 weeks after subculture to MVW+0S+CW, stained with safranin-fast green, showing small meristematic cells. Bar represents 100 μm.

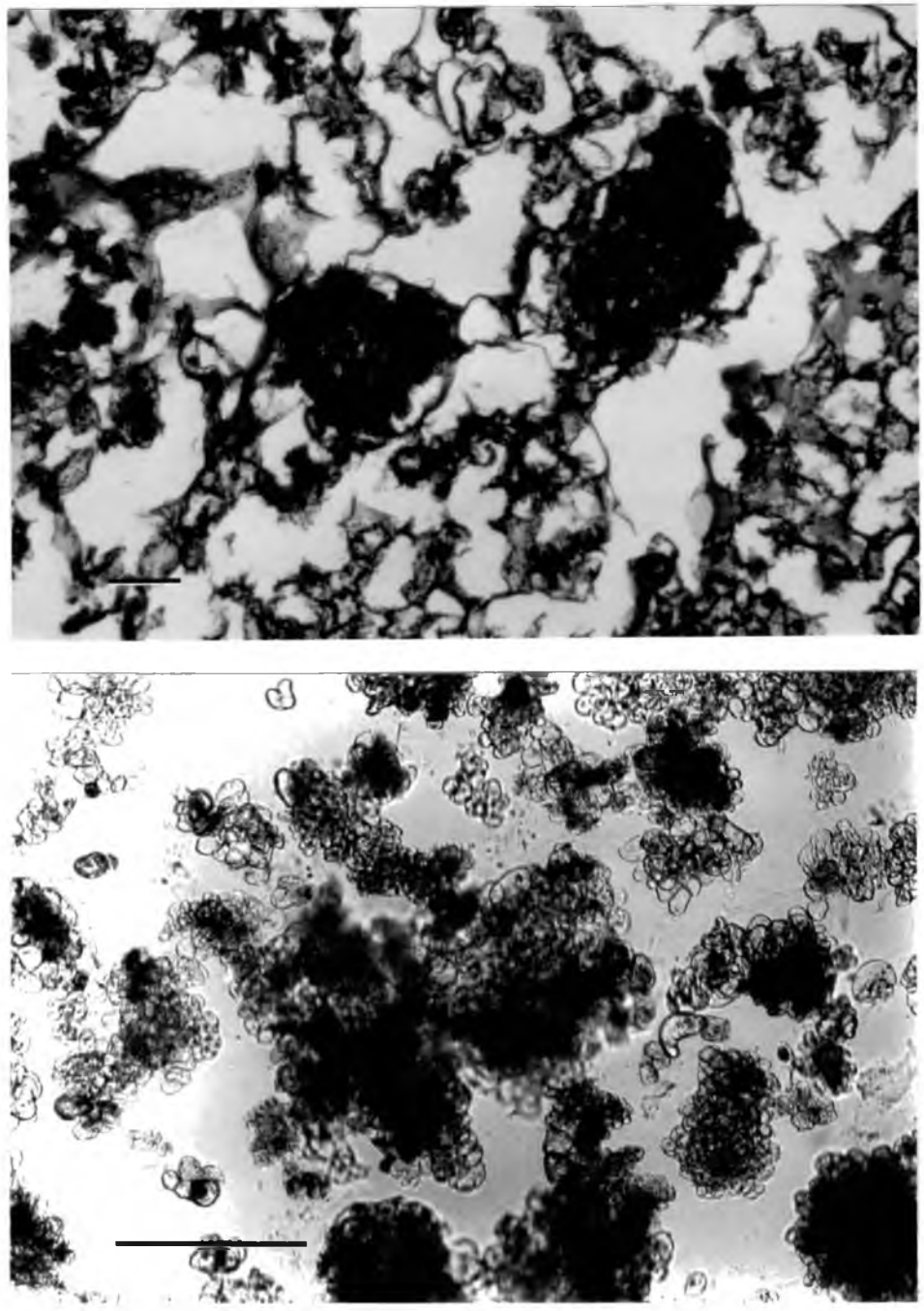


Fig. 24. Vertical section of green callus 4 weeks after transfer to MVW+0S+CW, stained with safranin-IKI, showing IKI staining localized on small meristematic cells. Bar represents 100 μm.

Fig. 25. Cell aggregates dissociated from green callus upon transfer to liquid medium showing various sizes of cells. Bar represents 500 μm.

of small cells at the tip and relatively large cells at the base (Figure 26).

Longitudinal sections of embryoid 32 to 36 weeks after subculture showed presence of epidermis and procambial tissue in transition from oblong to protocorm stage. No trace of vascular differentiation was found on maternal tissue.

Evidence of callus consisting of the morphologically granular appearance and histologically undifferentiated, isodiametric cell aggregates randomly dispersed with large intercellular spaces, no vascular elements and no epidermis clearly establishes this callus as friable and undifferentiated. The small cell size, dense cytoplasm, small vacuoles and profusion of starch grains suggest active metabolism in cells which is common to meristematic cells. This type of callus has not been previously described or reported in orchids. Cells in suspension culture of Cymbidium were in clusters and consisted of compactly arranged cells (Steward and Mapes, 1971).

The sequential development from friable callus to growth resembling embryos which differentiated into plantlets confirms the process as indirect somatic embryogenesis. Embryogenic potential in callus of maize inbred line was shown to be limited by compactness or differentiation-state of callus (Everett et al., 1985; Lowe

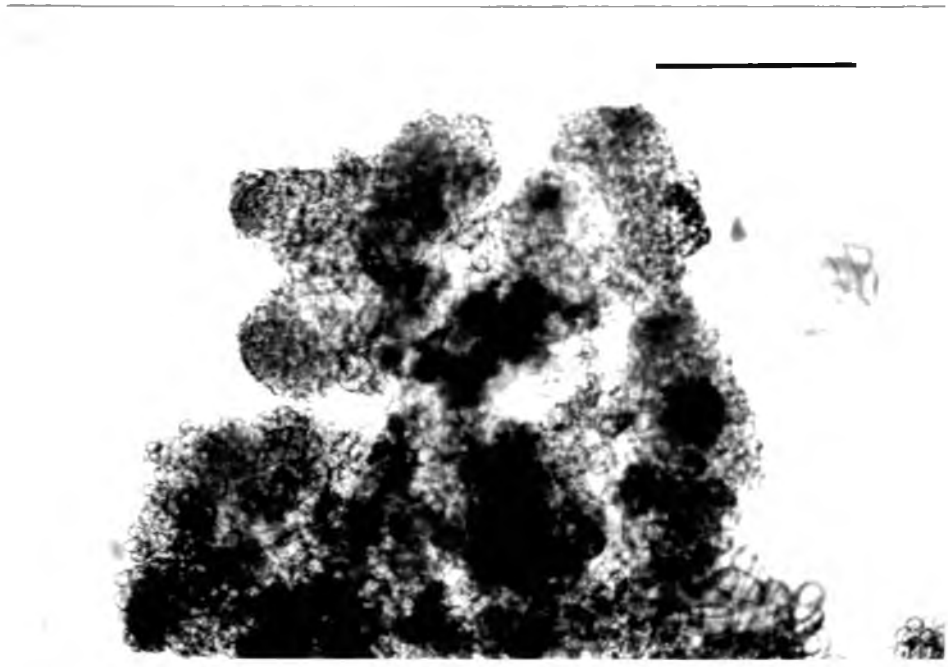


Fig. 26. Globular proembryoids with meristematic region of small cells and relatively large cells at base. Bar represents 500 μm.

et al., 1985). Previous reports of orchid calli indicated that those were masses of differentiated cells bordered by epidermis (Rao, 1963) rather than friable callus. This may account for the absence of reports on somatic embryogenesis in previous orchid *in vitro* culture.

The stages of development from callus to plantlet have been assigned numerical scores as shown Table 2 ranging from -1 to 4 for use in subsequent experiments.

Changes in distribution of starch within cells from random in initial culture and accumulation in meristematic regions and disappearance from peripheral areas upon subculture to medium without sucrose were similar to that observed in embryoid forming cell lines of *Corylus avellana* and *Poulownia tomentosa* (Radojevic et al., 1979) and shoot forming tobacco callus (Thorpe and Murashige, 1970; Thorpe, 1980).

From this study, it can be concluded that with a friable callus indirect somatic embryogenesis is a viable pathway for clonal propagation of *Phalaenopsis*.

Table 2. Summary of description and numerical score for developmental stages of somatic embryogenesis in Phalaenopsis callus culture[z].

Developmental stage	Numerical score	Description
Brown callus	-1	Callus turned brown.
Yellow callus	0	Friable yellow callus proliferating without morphogenesis.
Green Callus	1	Friable green callus, without conspicuous structures developed at periphery of callus.
Globular embryoid	2	Multicellular aggregates resembling proembryonic structure developed at periphery of green callus; Axial length, 0.47 ± 0.012 mm with range of 0.3 - 0.8 mm[z]; Diameter, 0.38 ± 0.008 mm with range of 0.2 - 0.6 mm.
Oblong embryoid	3	Elongated embryonic structure developed from globular embryoid; Axial length, 1.54 ± 0.049 mm with range of 0.8 - 2.6 mm; Diameter, 0.63 ± 0.048 mm with range of 0.4 - 1.0 mm.
Intermediate stage	4	Transitional stage from oblong to protocorm stage, epidermis and procambial tissue begin to develop; Axial length, 2.80 ± 0.096 mm with range of 1.4 - 6.4 mm; Diameter, 0.90 ± 0.030 mm with range of 0.5 - 2.4 mm.
Protocorm stage	5	Protocorm-like structure with epidermis, procambial strand and rhizoids developed; Axial length, 4.08 ± 0.126 mm with range of 1.9 - 8.4 mm; Diameter, 1.39 ± 0.044 mm with range of 0.7 - 2.0 mm.

[z]Means ± s.e. and range of 100 measurements for each developmental stage.

CHAPTER IV

EFFECT OF PLANT GROWTH REGULATOR, SUCROSE AND COCONUT WATER ON CALLUS

INTRODUCTION

Morphogenesis of Phalaenopsis tissue has been affected by the deletion of supplemental sucrose at levels generally used in many plant tissue cultures (Intuwong and Sagawa, 1974). The inhibitory effect of sucrose on proliferation and organogenesis of shoot-tip explants has been demonstrated in sarcanthine orchids (Kunisaki et al., 1972; Intuwong and Sagawa, 1973; Teo et al.,1973; Sagawa and Kunisaki, 1984). Deletion of supplemental sucrose from culture medium was shown beneficial for promotion of chlorophyllous culture of Phalaenopsis (Intuwong and Sagawa, 1974).

The effect of auxins and cytokinins on callus growth of other plants has been extensively reviewed by Geroge and Sherrington (1984). It has also been demonstrated that the ratio of auxin to cytokinin plays an important role in organogenesis from callus (Thorpe, 1980).

The objective of this chapter is to present experiments

designed to study the effects of different concentrations of growth regulators, sucrose and CW on a friable Phalaenopsis callus.

MATERIALS AND METHODS

Callus of a Phalaenopsis hybrid maintained and described in Chapter III was cultured on various media and maintained conditions described in Chapter III.

Basal medium of Murashige and Skoog medium (MS) (1962) was supplemented with IAA (0.1, 1.0 and 5.0 $mg \cdot liter^{-1}$), BA (0.1, 1.0 and 5.0 $mg \cdot liter^{-1}$), sucrose (0, 2.5, 5.0, 10.0, 20.0, 30.0 and 50.0 $g \cdot liter^{-1}$) or mannitol (58.4 mM). Media with 20.0 $g \cdot liter^{-1}$ sucrose (MS+20S) and supplemented with 150 $ml \cdot liter^{-1}$ of CW (MS+20S+CW) were used as controls.

Peripheral yellow callus pieces between 40 and 45 mg were excised from stock calli at the end of a subculture period. Five callus pieces were randomly placed on medium in each 100 x 15 mm Fisher disposable petri dish. Fifteen petri dishes for each treatment were prepared and cultured in a completely randomized design. Fresh weights of fifteen callus pieces from 3 petri dishes were measured every week for 5 weeks. To compare callus growth, growth indices were calculated for fresh weights on media, MS+20S and MS+20S+CW. The callus growth index was expressed as (Final fresh weight

- Initial fresh weight) · Initial fresh weight^{-1}. Data were analyzed by Statistical Analysis System (SAS). Analysis of variance and Duncan's multiple range test at the five percent level were conducted.

To identify the effects of sucrose and CW, five callus pieces weighing 30 to 40 mg each were transferred to MS medium in each 150 ml Erlenmeyer flask. Two flasks were prepared for each treatment and placed in a completely randomized design. Sucrose at the levels of 2.5 and 20.0 g·liter^{-1} was incorporated into MS medium.

A numerical score as established in Chapter III was assigned based on prevalent developmental stage on the periphery of each callus every 4 weeks for 16 weeks. Data were analyzed by SAS. Analysis of variance and Duncan's multiple range test at the 5 percent level were conducted.

RESULTS AND DISCUSSION

Effect of Plant Growth Regulator and Sucrose on Callus Growth: Figure 27 shows that there was no significant increase in callus growth when 0.1 to 5.0 mg·liter^{-1} of IAA was added to medium through the duration of experiment. The means of callus fresh weights at 1.0 and 5.0 mg·liter^{-1} of IAA were significantly less than the control (MS+20S) after

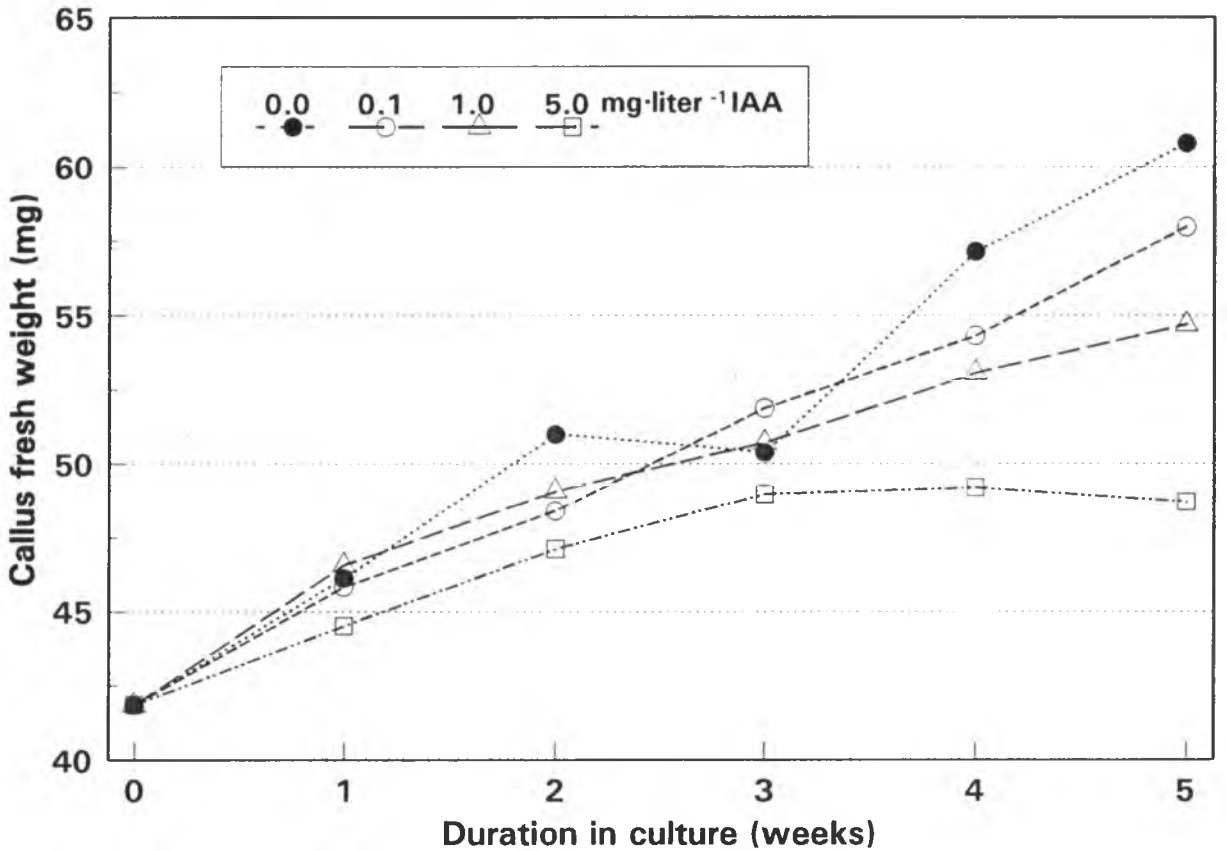


Fig. 27. Effect of IAA concentration on callus growth of *Phalaenopsis.*

5 weeks due to the occasional appearance of brown colored and hardened calli on the media.

The fresh weight increases of callus cultures on BA media in the range of 0 to 5.0 $mg \cdot liter^{-1}$ showed no significant differences throughout the 5 weeks (Figure 28).

Callus growth on the medium supplemented with CW was significantly stimulated when compared to callus growths on the rest of media.

Growth of the callus was not affected by sucrose levels of 2.5 to 30.0 $g \cdot liter^{-1}$ throughout the experiment (Figure 29). Callus growth on the medium without sucrose was very limited. Fresh weight was significantly lower than callus grown with sucrose throughout the culture duration. Calli grown on medium without sucrose and 2.5 $g \cdot liter^{-1}$ of sucrose turned green after 5 weeks. The mean fresh weight of calli on medium with 50.0 $g \cdot liter^{-1}$ of sucrose was the least among the group with sucrose after 5 weeks.

Growth curves (Figure 30) of mean weights on MS+20S+CW and MS+20S media show that growth increases were 2.5-fold and 0.4-fold in 5 weeks respectively.

Although auxin and/or cytokinin have been reported as required for proliferation of most plant callus tissues, this friable callus did not respond to either IAA or BA, while coconut water stimulated growth significantly. The callus may be auxin and cytokinin autotrophic. The stimulation of callus growth by CW was attributed to its

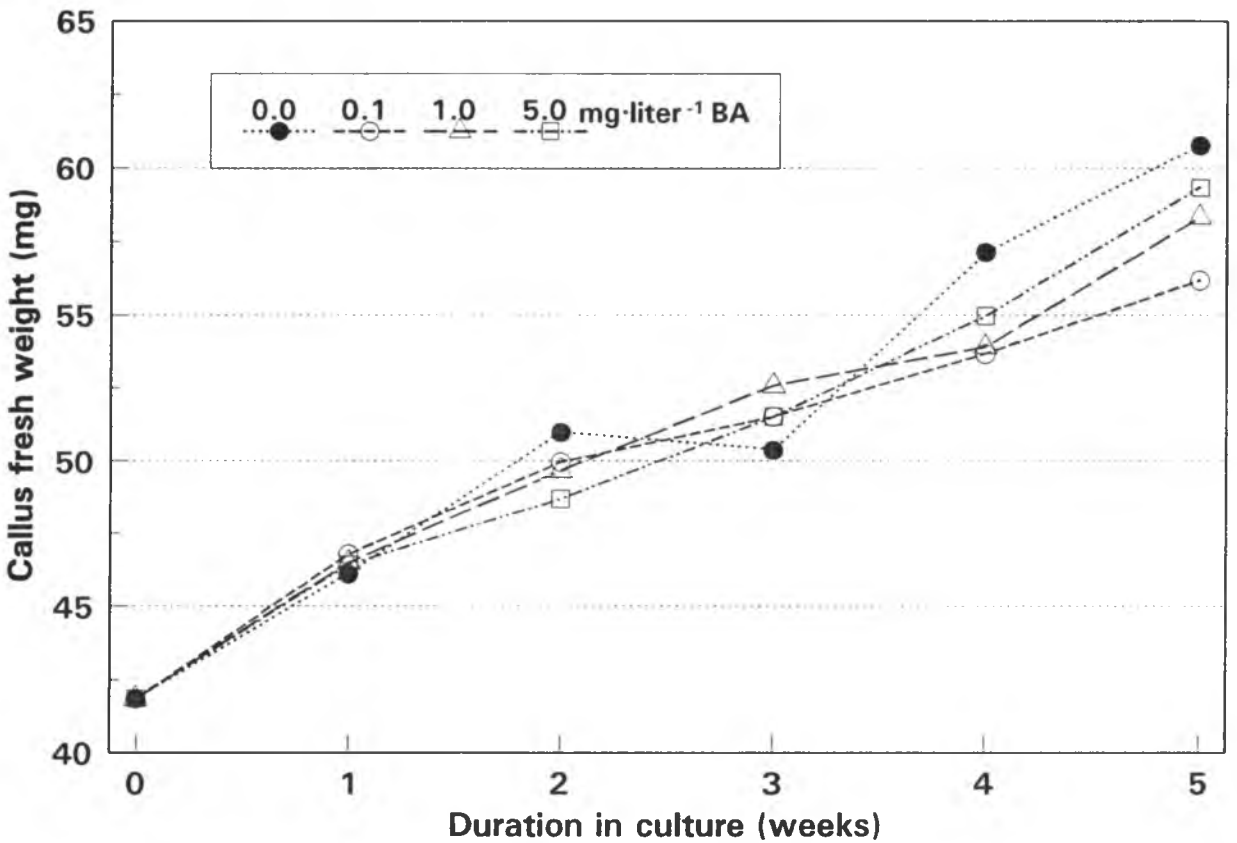


Fig. 28. Effect of BA concentration on callus growth of *Phalaenopsis*.

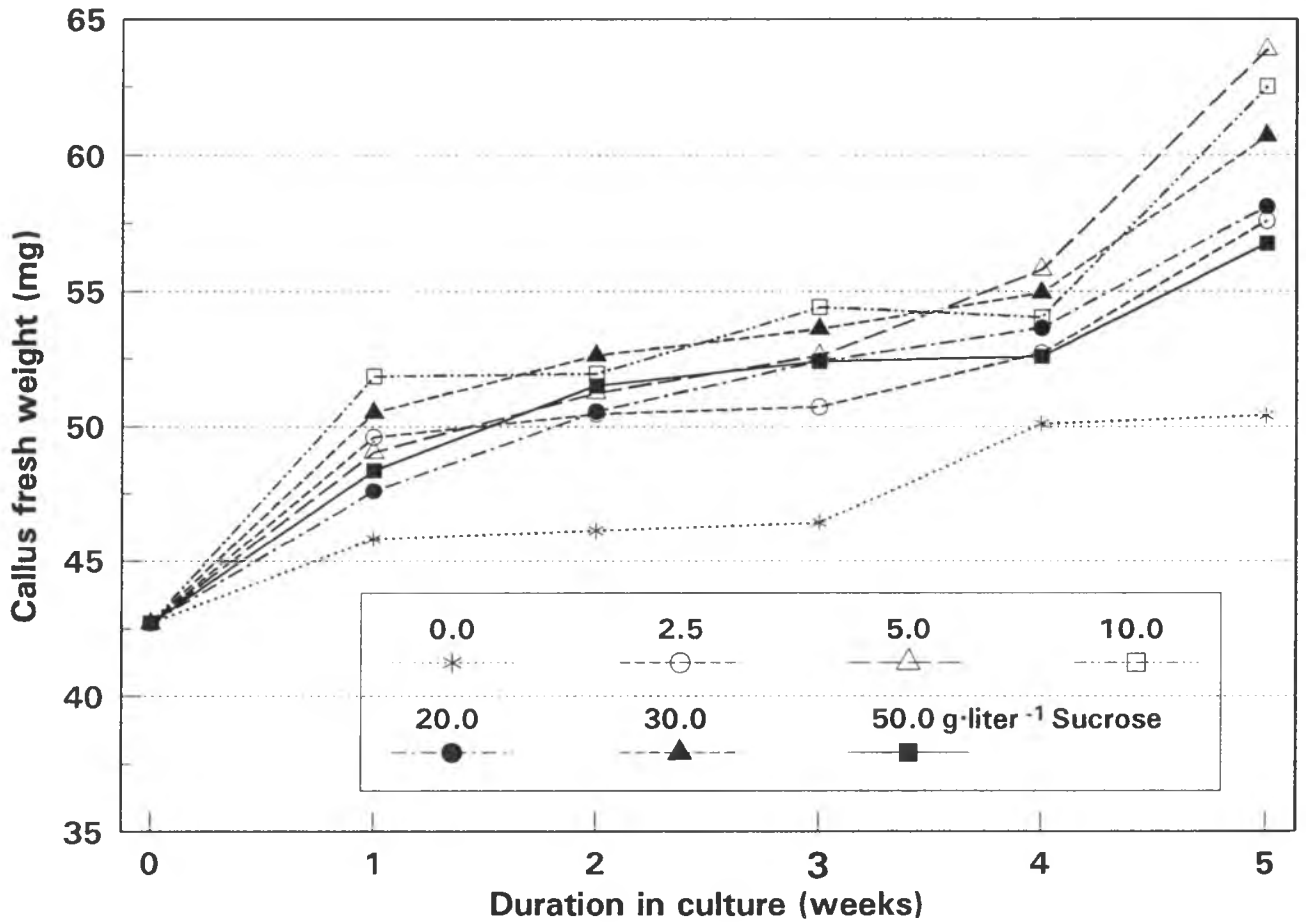
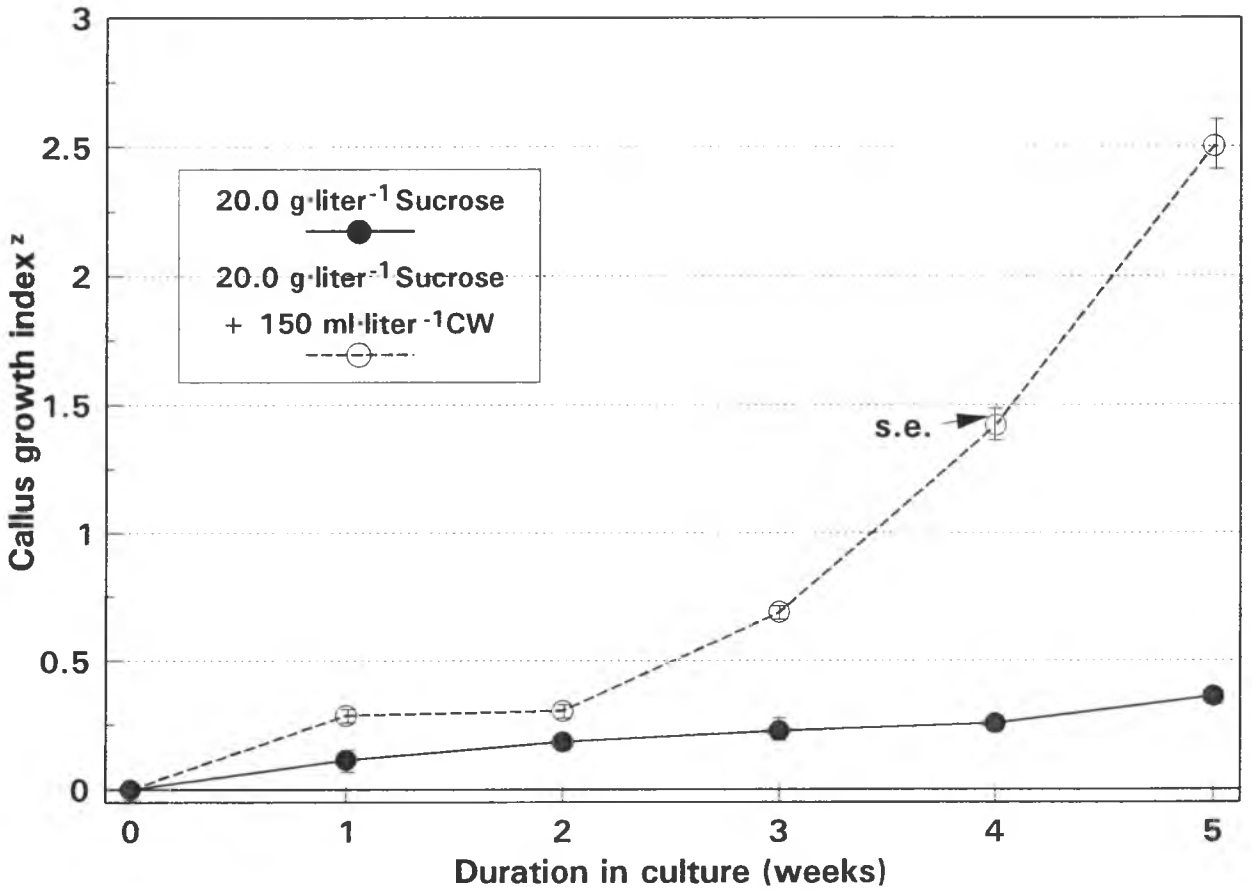


Fig. 29. Effect of sucrose concentration on callus growth of *Phalaenopsis*.



z(Final weight - Initial weight)·Initial weight^{-1}

Fig. 30. Callus growth rates of on media MS + 20S and MS + 20S + CW.

nutritional content rather than its growth regulating components. Callus tissue of Oncidium also showed to be cytokinin autonomous (Kerbauy, 1984a).

The range of 20 to 30 g·liter^{-1} of sucrose is the most commonly used levels incorporated in plant tissue culture media. These levels of sucrose were significantly more effective in supporting many plant callus tissues. However, callus growth of Phalaenopsis was not affected by sucrose concentration in the relatively wide range of 2.5 to 30.0 g·liter^{-1}. The result was consistent with the growth response of Holttumara protocorm, which was not affected by different concentrations of sucrose as well as glucose and fructose in the range of 0.015 M to 0.058 M (Tse and Wong, 1978).

Fresh weight increase of many plant callus cultures occurred exponentially for most of the culture period. A classical growth curve of carrot cells in a culture medium with coconut milk (water) showed that initial fresh weight was approximately doubled after 1 week (Steward and Caplin, 1954). An average 3-fold increase in fresh weight per passage of 1 week was reported in tobacco cell culture (White, 1939). Fresh weight increase of monocot Lilium longiflorum cell culture was about 5-fold after 30 days on a medium containing major and minor salts, thiamin, inositol and 40 g·liter^{-1} of sucrose (Sheridan, 1968).

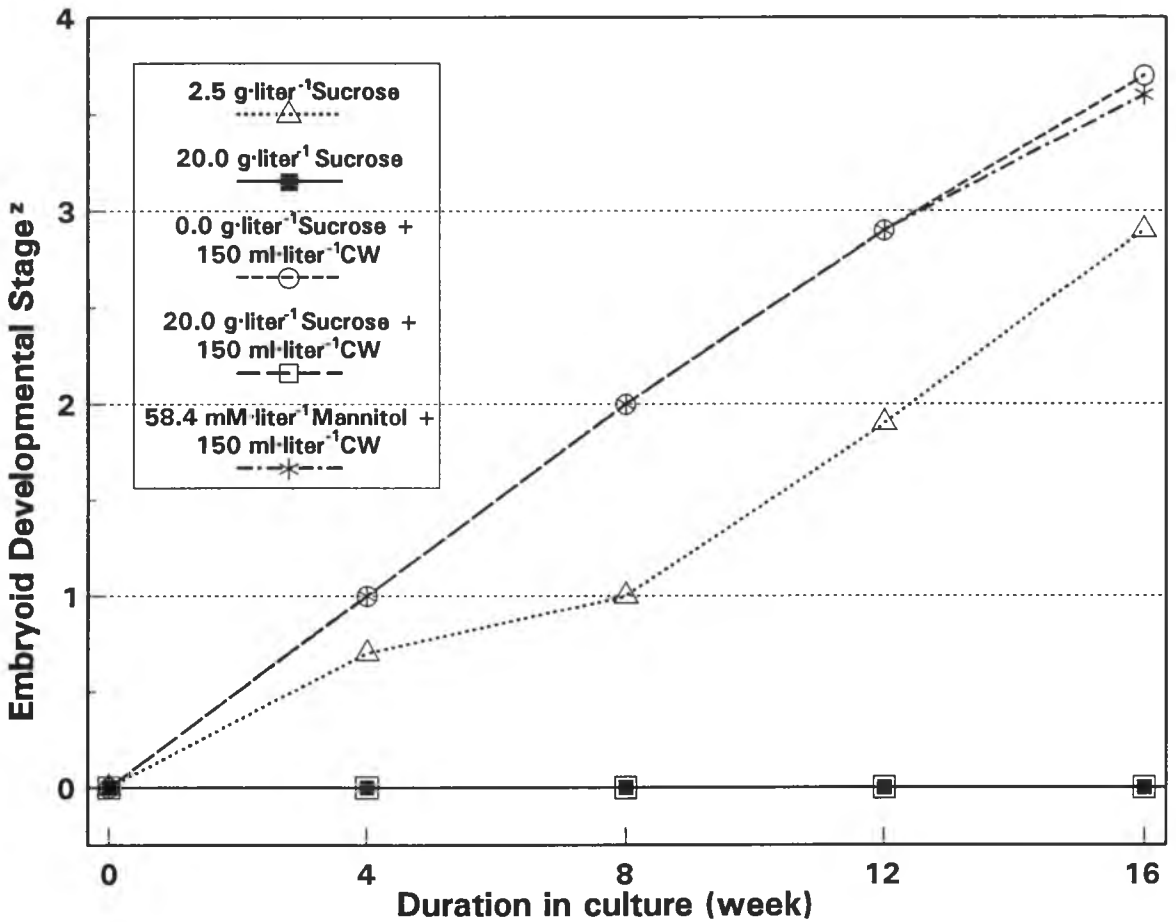
Callus growth curves exhibited extremely low growth

rates in the present study compared with other classical cell cultures. The culture duration may have been too short for the callus to show exponential phase because of the slow growth rate. Callus growths of Cattleya (Kerbauy, 1991) and Oncidium (Kerbauy, 1984a) were also slow. Callus culture derived from root-tip of Cattleya reached 1-2 cm in diameter after 6 months of culture.

Effect of Sucrose and Coconut Water on Somatic Embryogenesis: When sucrose level was lowered or omitted, somatic embryogenesis was successfully induced. Figure 31 shows that sucrose level was the most effective factor in the induction of somatic embryogenesis from the callus. There was no trace of morphogenetic change of calli cultured on media with 20.0 $g \cdot liter^{-1}$ of sucrose regardless of the presence of coconut water. Lowering sucrose level to 2.5 $g \cdot liter^{-1}$ in MS basal medium without any supplements induced embryogenesis. Most embryoids at the periphery of the callus developed into intermediate stage with mean numerical score of 2.9 after 16 weeks.

MS medium without sucrose but supplemented with CW significantly enhanced the development of embryoids. The mean developmental numerical score was 3.7 on MS+0S+CW medium.

On CW medium in which 20.0 $g \cdot liter^{-1}$ of sucrose was replaced with the equivalent molar concentration of mannitol



z-1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5,Protocorm stage.

Fig. 31. Effect of sucrose concentration and coconut water on somatic embryogenesis of *Phalaenopsis* over 16 weeks.

(58.4 mM), mean embryogenic development was not significantly different from that on MS+0S+CW.

Better callus growth did not correspond to callus greening and somatic embryogenesis.

Coconut water is a rich source of amino acids such as glutamic acid, glutamine, alanine, serine and asparagine, nucleic acid and other organic acids. The sugar content of coconut water which is composed of mainly sucrose, glucose and fructose ranged from 9.16 to 21.68 $g \cdot liter^{-1}$ varied with the maturity of coconut (Telex et al., 1961). Since 150 $ml \cdot liter^{-1}$ of coconut water was incorporated into the culture medium, the final sugar concentration of the medium, MS+0S+CW would be between 1.37 and 3.25 $g \cdot liter^{-1}$. Assuming that the effects of sugar concentrations in the media of MS+0S+CW and MS+2.5S were the same, the other nutritional contents of CW other than sugar might enhance the development of embryoids.

Mannitol has often been used as an osmoticum in plant tissue culture media (Gamborg et al.,1979) since it was reported to rarely support growth (Jones and Veliky, 1980). Since MS+20S+CW medium did not induce somatic embryogenesis while induction and development occurred upon replacement of sucrose with mannitol, the reduction of osmotic pressure in the medium is not the cause of induction.

The results of present studies showed that sucrose

inhibits greening of callus and somatic embryogenesis, and excludes the possibility that stimulation on medium MS+0S+CW is due to reduction of osmotic pressure by omitting sucrose. Protocorms of Holttumara also showed the inhibitory effect of sucrose on chlorophyll formation (Teo and Wong, 1978). The sugar effect cannot be attributed to osmotic effect because of non-detrimental effect of mannitol on chlorophyll formation. The inhibitory effect of sucrose on proliferation and chlorophyll formation has been demonstrated in sarcanthine orchids. Green PLBs were obtained from the shoot-tip cultures of Phalaenopsis (Intuwong and Sagawa, 1974) and Vanda Miss Joaquim (Kunisaki et al., 1972) in medium without sucrose. The shoot-tip culture of a strap-leafed Vanda hybrid (Teo et al.,1973), and the inflorescence cultures of Vascostylis, Neostylis and Ascofinetia (Intuwong and Sagawa, 1973) in sucrose containing media turned yellow then brown and eventually died.

CHAPTER V

EFFECTS OF PLANT GROWTH REGULATOR, NITROGEN SOURCE AND CARBOHYDRATE-SUBSTITUTION ON SOMATIC EMBRYOGENESIS

INTRODUCTION

The most decisive step in the induction of somatic embryogenesis has been transferring cultures grown in an auxin-enriched medium to an auxin-free medium.

The concentration or the duration of auxin treatment required for inductive effect is different in various species, genotypes and tissue origins of explant (Brown and Atanassov, 1985; Bogre et al., 1990; Rao, 1992). The inhibition or promotion of somatic embryogenesis by auxins, antiauxins and auxin synthesis inhibitors depends on age and stage of tissue culture, and endogenous auxin present in the culture (Fujimura and Komamine, 1979).

Cytokinins have promoted induction indirectly by their stimulatory effect on cell division (Sung et al., 1979; Fujimura and Komamine, 1980a; b; Ernst and Oesterhelt, 1984). Although gibberellins have rarely been incorporated in culture media for induction of somatic embryogenesis,

they stimulate the synthesis of α-amylase in plant tissue (Hardie, 1975).

Reduced nitrogen is essential for high-frequency embryogenesis. Reduced nitrogen supplied as ammonium or certain amino acids, particularly glutamine (Finer and Nagasawa, 1988; Price and Smith, 1979), stimulated embryoid formation. Alanine (Kamada and Harada, 1979b), asparagine (Finer and Nagasawa, 1988) and proline (Stuart and Strickland, 1984), were also stimulatory. Nitrogen source can be incorporated in culture medium in a form of complex supplements such as coconut milk (water) or CH which contains a mixture of amino acids.

Although a number of factors are known to affect embryogenic development, involvement of chlorophyll and/or chloroplast development in somatic embryogenesis is not clear, although the development of chlorophyll level is influenced by the carbohydrate source in at least some plant tissue cultures. Sucrose concentrations of 20 to 30 $g \cdot liter^{-1}$ are the most commonly used carbohydrate in plant tissue culture. It has been postulated that carbohydrate incorporated in culture medium is utilized as merely a carbon source regardless of its source. In addition to the role of carbohydrate as a carbon source, however, there has been increasing evidence that it may act as a regulating factor in morphogenesis.

Remarkable stimulation of somatic embryogenesis by the

manipulation of carbohydrates has been reported in nucellar calli of Citrus. Citrus cell lines have been maintained in a nondifferentiated state in sucrose-containing medium. Somatic embryogenesis was stimulated by omission of sucrose for a single culture period (Kochba and Button, 1974) or by substitution of sucrose by glycerol (Ben-Hayyim and Neumann, 1983), galactose (Kochba et al.; 1978) and lactose (Button, 1978; Kochba et al., 1978).

Embryoid formation in wild carrot (Kinnersley and Henderson, 1988) and Digitalis lanata suspension culture (Kuberski et al., 1984) were enhanced by the use of maltose rather than sucrose. In alfalfa, maltose, maltotriose and soluble starch improved somatic embryogenesis while glucose and sucrose did not have similar effects (Strickland et al., 1987).

Embryogenic capacity may be influenced by the chlorophyll content of cultured callus tissue. Genes which were rapidly expressed at chloroplast differentiation were also involved in expression of somatic embryogenesis of Chenopodium rubrum (Kaldenhoff and Richter, 1990) and carrot (Aleith and Richter, 1991) cultures. It has also been reported that the level of chlorophyll is reduced by the commonly used levels of sucrose in some plant tissue cultures such as lettuce (Hildebrandt et al., 1963), tobacco (Kaul and Sabharwal, 1971; Nato et al., 1977), carrot (Neumann and Rafaat, 1973), spinach (Dalton and Street,

1977) and Atropa belladonna (Davey et al., 1971). Sucrose inhibited chlorophyll formation in sarcanthine orchids (Kunisaki et al., 1972; Intuwong and Sagawa, 1973; Teo et al.,1973; Intuwong and Sagawa, 1974; Sagawa and Kunisaki, 1984).

This study was conducted to identify the effects of sucrose levels, several types of plant growth regulators, nitrogen sources and carbohydrate-substitutions on somatic embryogenesis.

MATERIALS AND METHODS

Friable yellow Phalaenopsis callus as maintained and described in Chapter III was used.

Basal medium was MS (Murashige and Skoog, 1962). Five callus pieces of 30 to 40 mg each from the surface of yellow callus were transferred to the medium in 150 ml Erlenmeyer flask. Two flasks for each treatment were prepared and arranged in a completely randomized design.

Cultures were placed under continuous illumination from G. E. Power Groove cool white fluorescent lamps at approximately 2 klux and 26 ± 3 °C and were subcultured every 4 weeks.

For growth regulator experiments, IAA, NAA and 2,4-D for auxins, Kinetin, BA and 2-iP for cytokinins,

p-chlorophenoxyisobutric acid (pCPB), 7-AZA and GA_3 at concentrations of 0.1, 1.0 and 5.0 $mg \cdot liter^{-1}$ were incorporated into MS basal medium. GA_3 was cold filter sterilized. Pretreatment of auxin was conducted by culture of callus pieces on auxin medium with 20.0 $g \cdot liter^{-1}$ of sucrose for two weeks before transfer to either medium with the lower or higher level of sucrose without any supplement.

Nitrogen sources at several levels combined with sucrose levels were incorporated in MS basal medium. As an inorganic nitrogen source, NH_4 and NO_3 mixture at 1:2 ratio of NH_4/NO_3 which was the same as in MS medium was added. The total concentrations of 60 and 180 mM were incorporated in the medium resulting in nitrogen concentrations that were two and four times of MS medium. Casein hydrolysate was added at the levels of 0.5, 1.0 and 2.0 $g \cdot liter^{-1}$. L-glutamine, L-glutamic acid, L-alanine, L-asparagine and L-proline at concentrations of 1.0, 10.0 and 50.0 mM were incorporated in MS basal medium as amino acids. The media MS+20S and MS+2.5S were used as controls.

Sucrose was substituted by a wide range of carbohydrates at the levels of 2.5 and 20.0 $g \cdot liter^{-1}$ in the MS medium. Monosaccharides including D-glucose, D-fructose, D-mannose, D-ribose, L-arabinose, D-xylose and D-galactose, disaccharides including sucrose, melibiose, trehalose, maltose, cellobiose and lactose, multisaccharides, raffinose, amylose and starch were used as substitutes. As

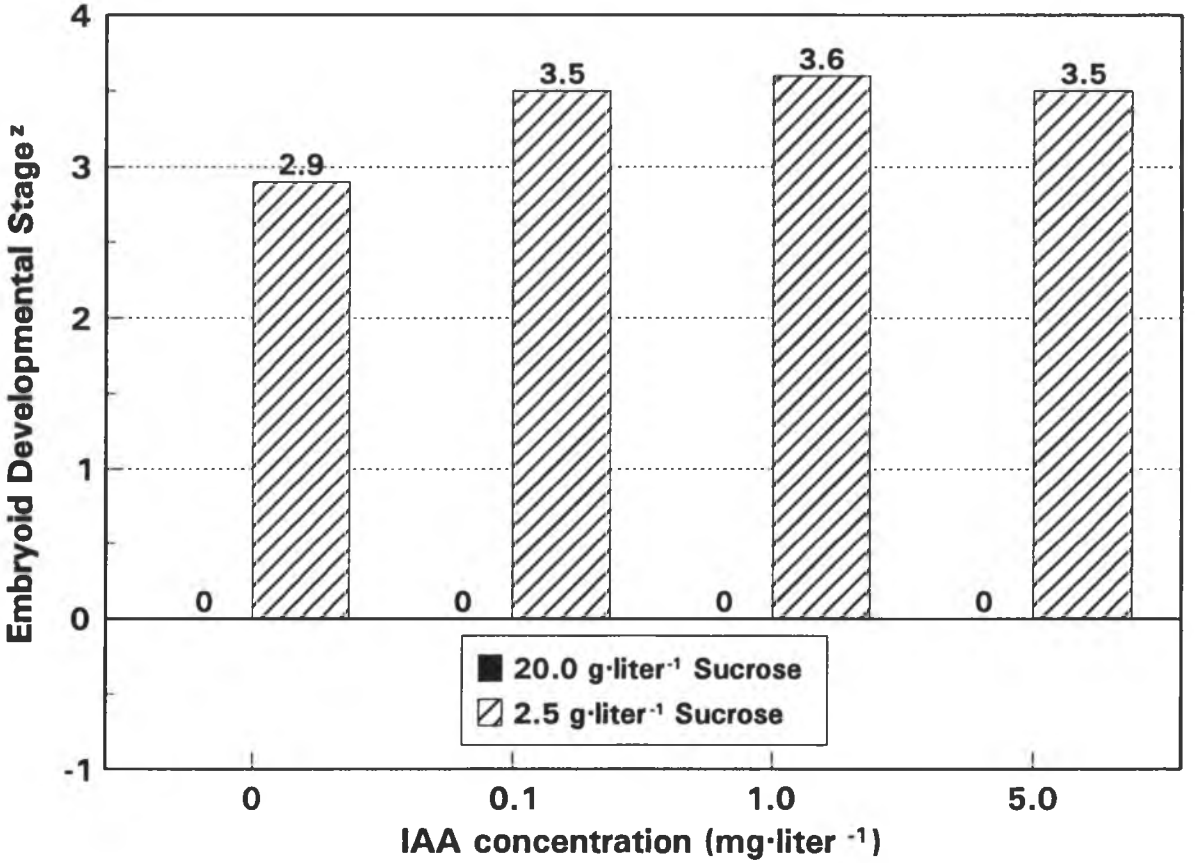
sugar-alcohols, sorbitol, mannitol and glycerol were also included.

A numerical score was given according to Table 2 for the most prevalent developmental stage of callus or embryoids on the periphery of each callus after 16 weeks. Data were analyzed by SAS. Analysis of variance and Duncan's multiple range test at five percent level were conducted.

RESULTS AND DISCUSSION

Effect of Plant Growth Regulator: Induction and development of somatic embryogenesis was observed only on media with 2.5 $g \cdot liter^{-1}$ of sucrose. Embryogenesis did not occur with any plant growth regulator incorporated into the medium with 20.0 $g \cdot liter^{-1}$ of sucrose regardless of type or concentration.

Among the treatments with growth regulator, the pretreatment of IAA was the most effective in stimulating embryogenesis after transfer of callus to medium with 2.5 $g \cdot liter^{-1}$ of sucrose. Figure 32 shows that IAA pretreatments stimulated embryogenesis above control after transfer of callus to medium with 2.5 $g \cdot liter^{-1}$ of sucrose, while treatments were not effective when callus was transferred to medium with 20.0 $g \cdot liter^{-1}$ of sucrose.



z-1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5,Protocorm stage.

Fig. 32. Effect of IAA pretreatment and subsequent sucrose level on somatic embryogenesis of *Phalaenopsis* after 16 weeks.

Stimulation was also significant with NAA pretreatment (Figure 33).

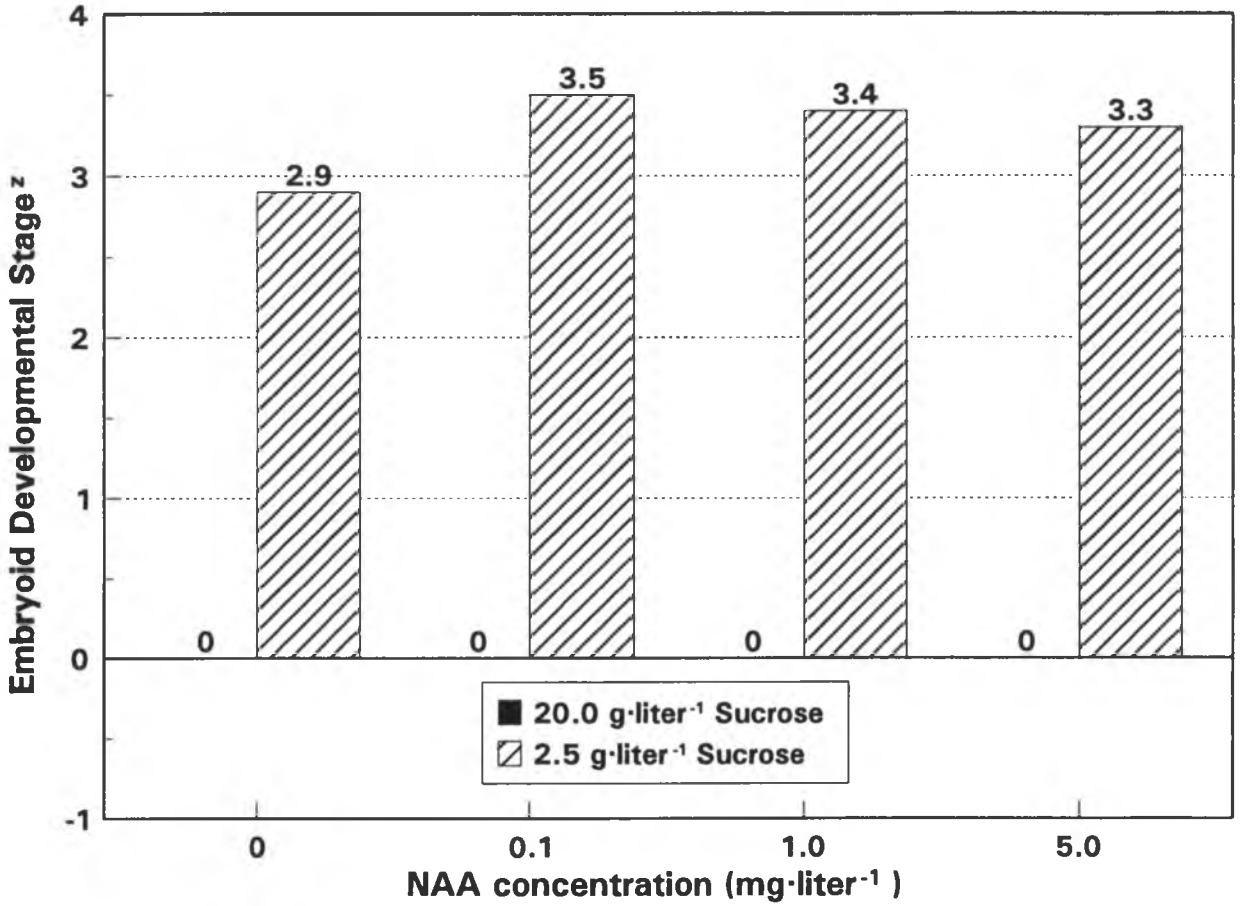
Occasional brown colored and hardened calli were observed with pretreatment with 2,4-D, especially at the higher concentrations. Enhancement with the continuous treatment of IAA at the lower level of sucrose was not statistically significant.

Results indicated that 20.0 $g \cdot liter^{-1}$ of sucrose inhibited greening of callus and embryogenesis, and showed no morphological change in most of the cultures. Cultures with 2.5 $g \cdot liter^{-1}$ of sucrose supported embryogenesis. The mean effect of 2.5 $g \cdot liter^{-1}$ of sucrose was 2.17 and that of 20 $g \cdot liter^{-1}$ was -0.15 in the numeric score. With partitioning variances, the analysis of variance showed that the most significant effect was the sucrose level, accounting for over 52 percent of the total treatment variation.

The enhanced results from pretreatment of IAA or NAA before transfer to medium with lower level of sucrose, confirmed the classical protocol for somatic embryogenesis to transfer culture from auxin-enriched medium to auxin-free medium.

2,4-D was observed to be toxic to the callus in both continuous treatment and pretreatment. Little enhancement was found with continuous treatment of auxin or cytokinin.

GA_3 was tested since starch degradation and



z-1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5,Protocorm stage.

Fig. 33. Effect of NAA pretreatment and subsequent sucrose level on somatic embryogenesis of *Phalaenopsis* after 16 weeks.

translocation seemed to be required for embryogenesis from observations in Chapter III. There was increased α-amylase activity in shoot-forming callus tissue as compared to nonshoot-forming tissue of tobacco. Gibberellin-treated tissues which had lower starch content did not produce shoot (Leung and Thorpe, 1985). Newly synthesized starch was not immediately utilized but the timing of utilization could be correlated with the intensity of metabolism occurring at different stages of shoot formation (Thorpe et al., 1986). The results of the present study showed that GA treatment had a negative effect on embryogenesis.

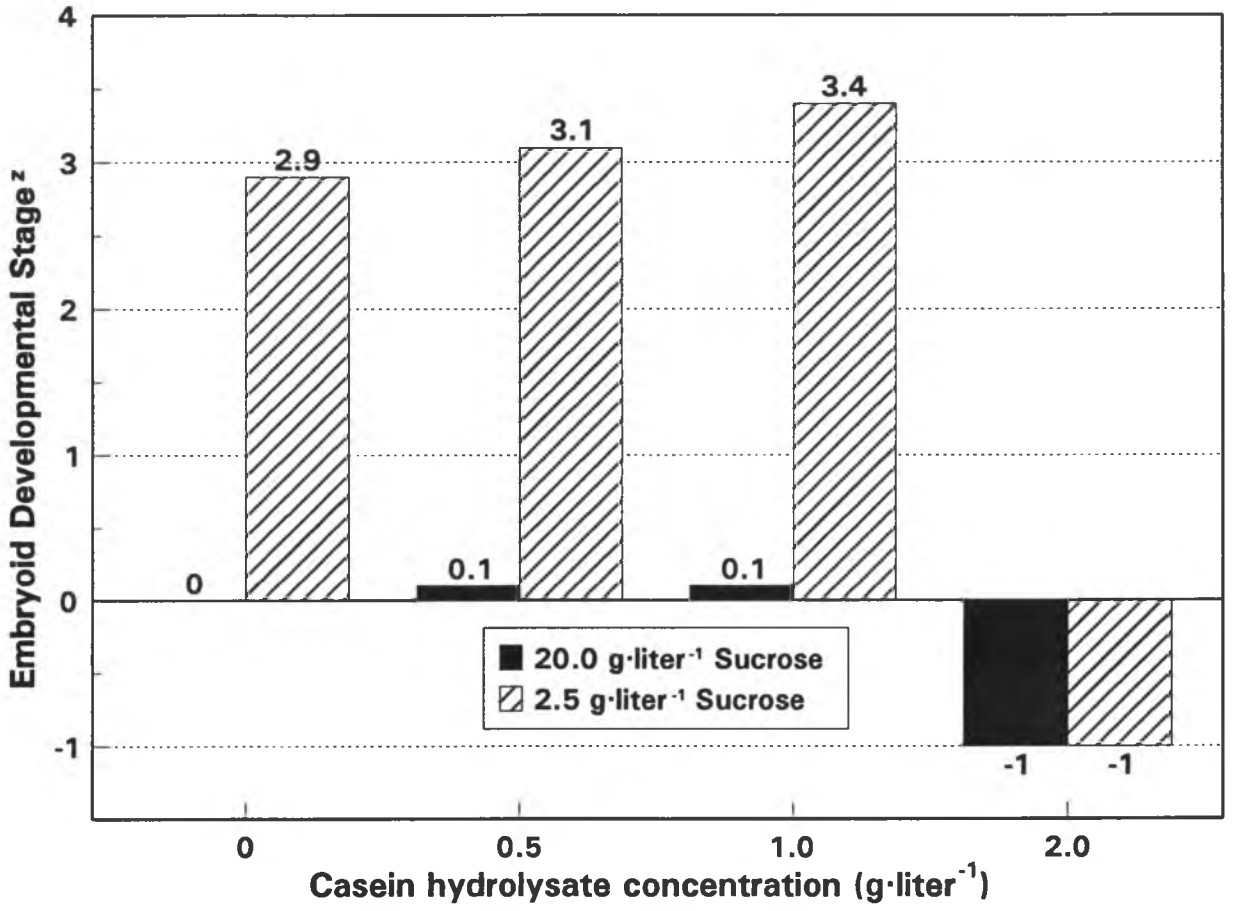
Embryogenesis was stimulated on habituated ovular callus of Shamouti orange which has autonomous growth habit by treatment with inhibitors of auxin synthesis, HNB and 7-AZA, while addition of even a low concentration of IAA or NAA strongly inhibited embryogenesis (Kochba and Spiegel-Roy, 1977a). pCPB also stimulated embryogenesis in carrot tissue culture (Kamada and Harada, 1979a). However, the present results showed that they had no stimulatory effects on somatic embryogenesis.

Effect of Nitrogen Source: Somatic embryogenesis was observed only on medium with 2.5 $g \cdot liter^{-1}$ of sucrose. The nitrogen source incorporated in medium with 20.0 $g \cdot liter^{-1}$ of sucrose did not induce embryogenesis regardless of type or concentration.

Only three treatments significantly enhanced embryogenesis above control with lower level of sucrose: 1.0 $g \cdot liter^{-1}$ of CH (Figure 34), 1.0 mM of glutamic acid (Figure 35) and 1.0 mM of glutamine (Figure 36) combined with 2.5 $g \cdot liter^{-1}$ of sucrose.

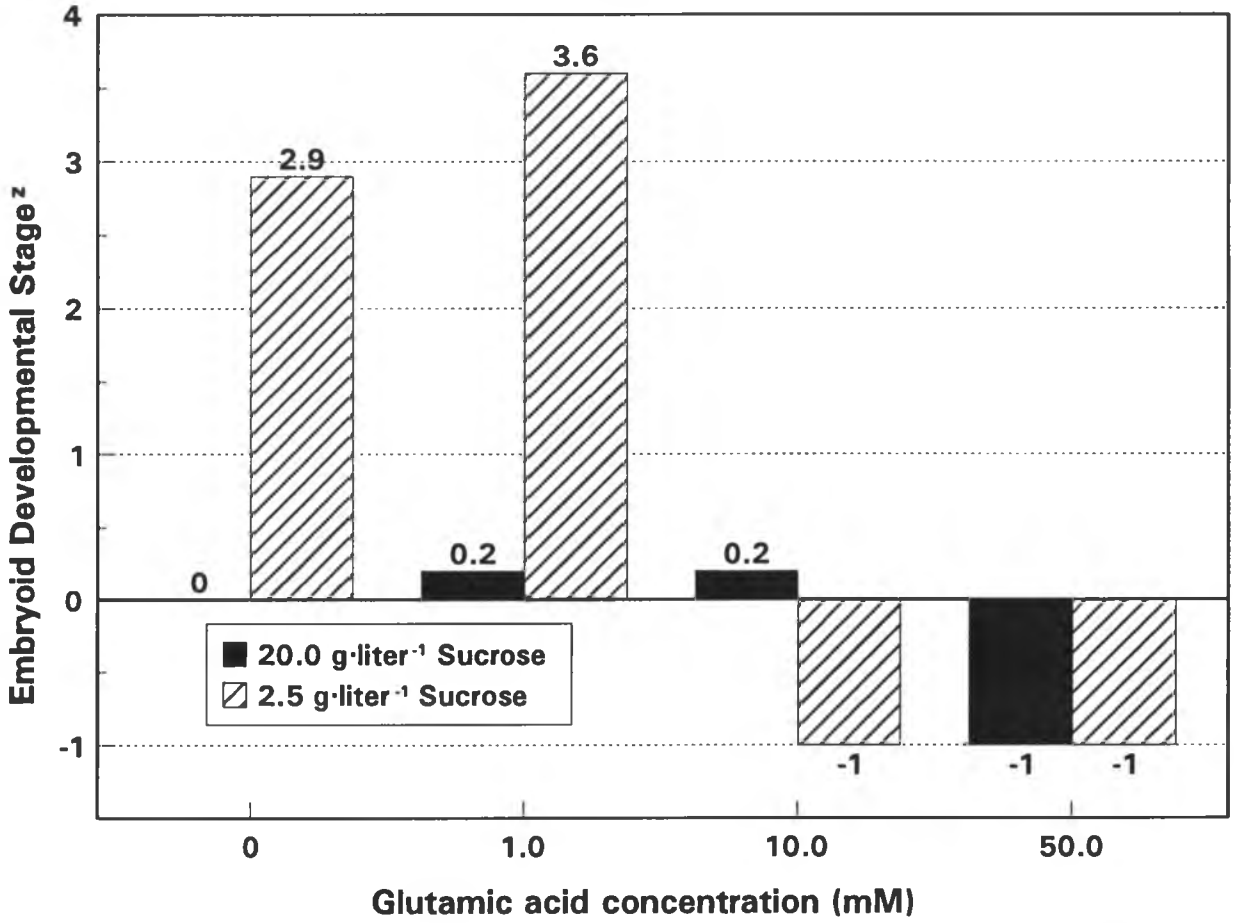
Inorganic or other organic nitrogen supplements were not effective in promoting embryogenesis above controls. Sucrose effect in the nitrogen supplements was calculated to be less evident than that in the treatments of growth regulator, because many treatments at the higher levels resulted in brown calli. The mean effect of 2.5 $g \cdot liter^{-1}$ of sucrose was 0.90 while that of 20.0 $g \cdot liter^{-1}$ was -0.35 in the numerical score. Sum of squares partitioned for sucrose effect was approximately 10.5 percent of the total treatment variation.

Besides the sucrose effect, the results confirmed the importance of a supplement of the proper nitrogen source. Since glutamine and glutamic acid are primary products of ammonium assimilation in plants, their promoting effect was expected. Glutamine has been reported to stimulate embryoid formation in carrot (Kamada and Harada, 1979b), alfalfa (Stuart and Strickland, 1984) and soybean (Finer and Nagasawa, 1988). Price and Smith (1979) reported that glutamine was essential for embryogenesis in Gossypium klotzschianum suspension culture.



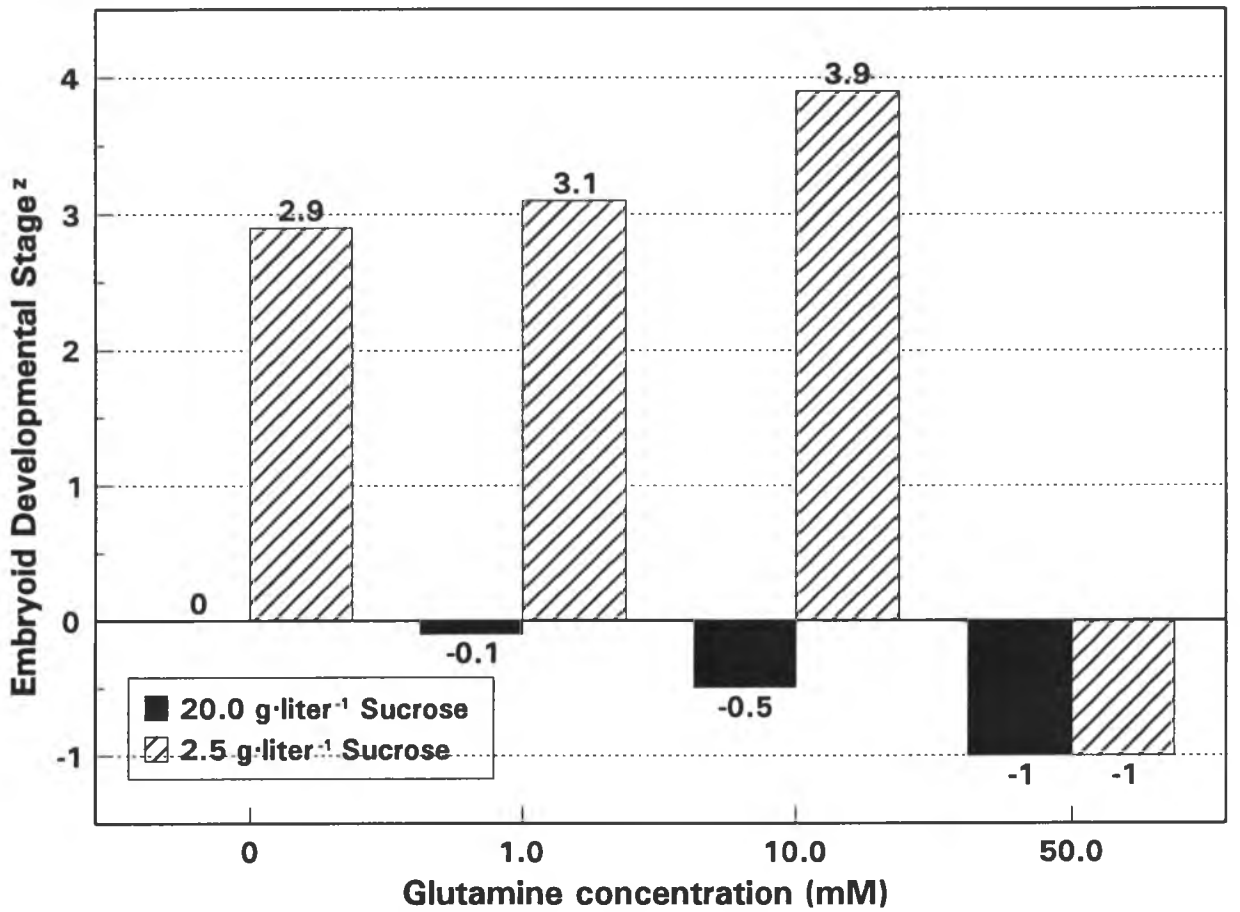
z-1,Brown callus; 0,Yeliow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5,Protocorm stage.

Fig. 34. Effect of casein hydrolysate with sucrose level on somatic embryogenesis of *Phalaenopsis* after 16 weeks.



z-1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5,Protocorm stage.

Fig. 35. Effect of glutamic acid with sucrose level on somatic embryogenesis of *Phalaenopsis* after 16 weeks.



z-1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5,Protocorm stage.

Fig. 36. Effect of glutamine with sucrose level on somatic embryogenesis of *Phalaenopsis* after 16 weeks.

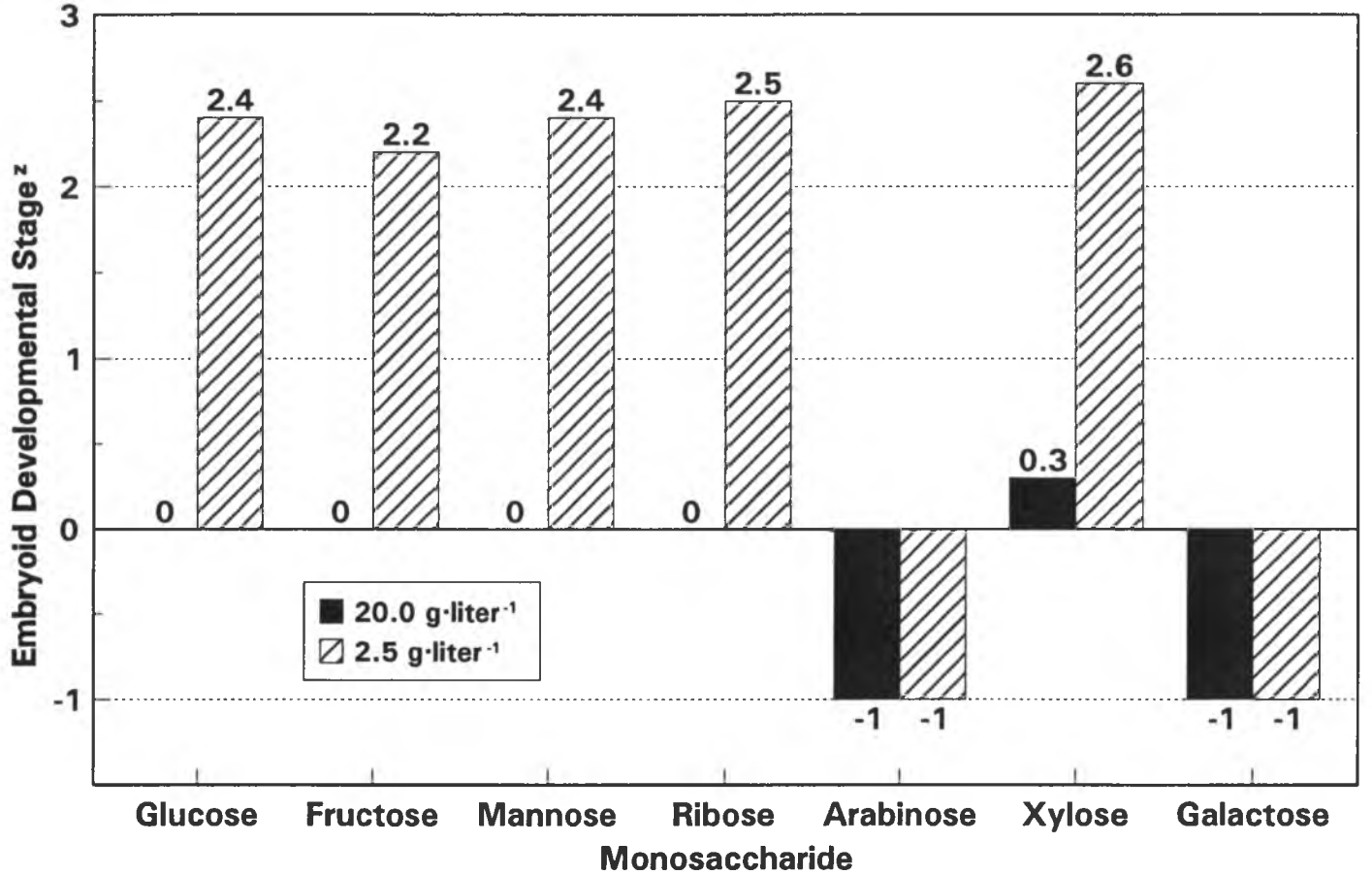
Effect of carbohydrate: The results showed that monosaccharides inhibited greening and somatic embryogenesis at the level of 20.0 $g \cdot liter^{-1}$ except for arabinose and galactose which were detrimental to callus growth. Figure 37 shows growth-supportive monosaccharides such as glucose, fructose, mannose, ribose and xylose at the level of 2.5 $g \cdot liter^{-1}$ induced embryogenesis, even though most of them were not as effective as sucrose at the same level. No induction occurred at the level of 20.0 $g \cdot liter^{-1}$.

Among disaccharides, sucrose was the only carbon source that showed the inhibition of greening and somatic embryogenesis at the higher level. Other disaccharides at both levels were as effective as 2.5 $g \cdot liter^{-1}$ of sucrose except for the detrimental effect of melibiose and lactose on callus growth as shown in Figure 38.

Figure 39 shows that multisaccharides did not show the inhibition of greening and somatic embryogenesis at either level. Amylose at the level of 20.0 $g \cdot liter^{-1}$ was the most effective in promoting embryogenesis in the experiment of carbohydrate substitution.

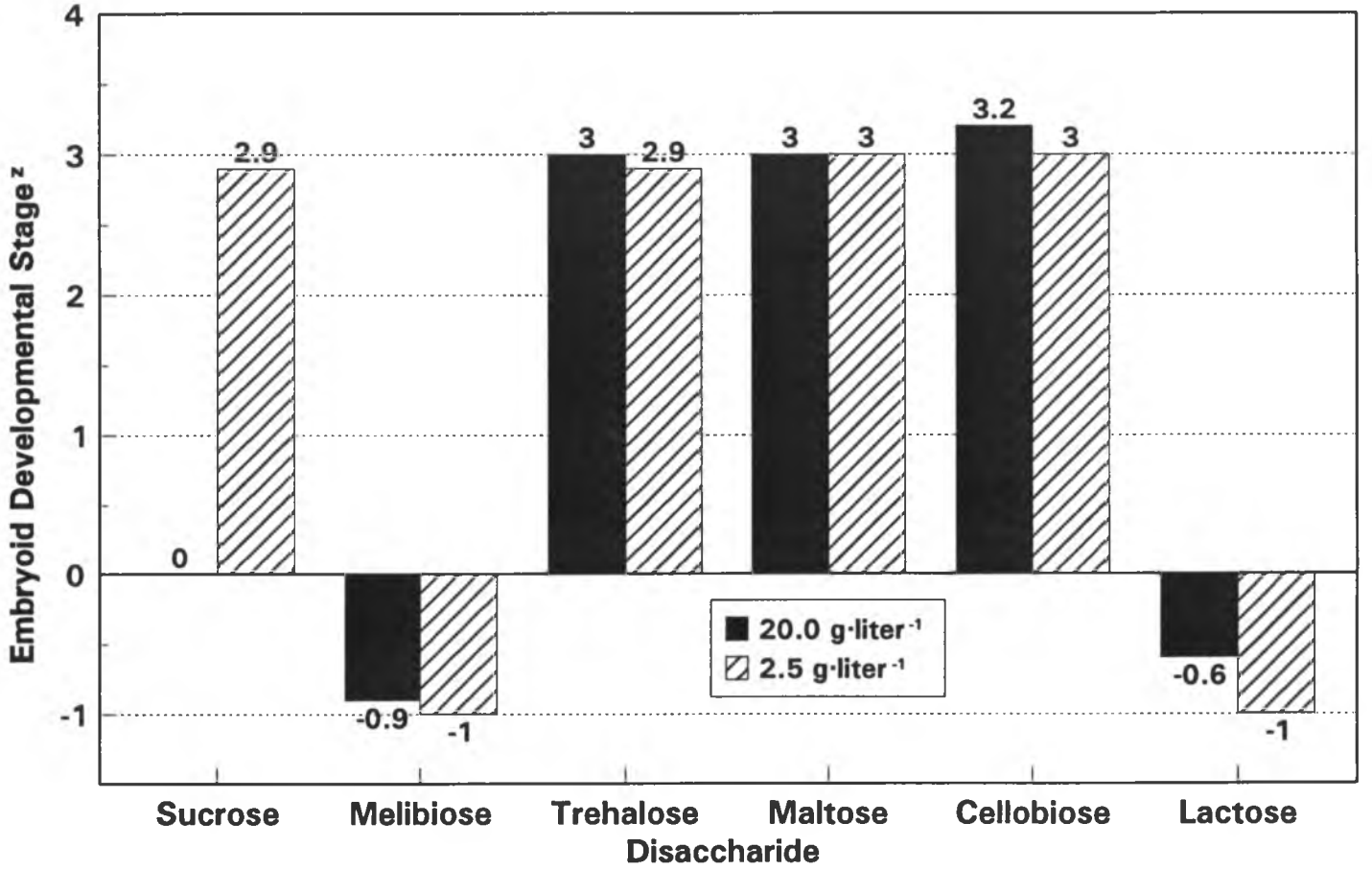
Sugar-alcohol including sorbitol and mannitol induced embryogenesis while glycerol was detrimental to callus growth (Figure 40).

The results indicated that arabinose and galactose were detrimental to callus and caused callus to brown. Disaccharides and multisaccharides which consisted of



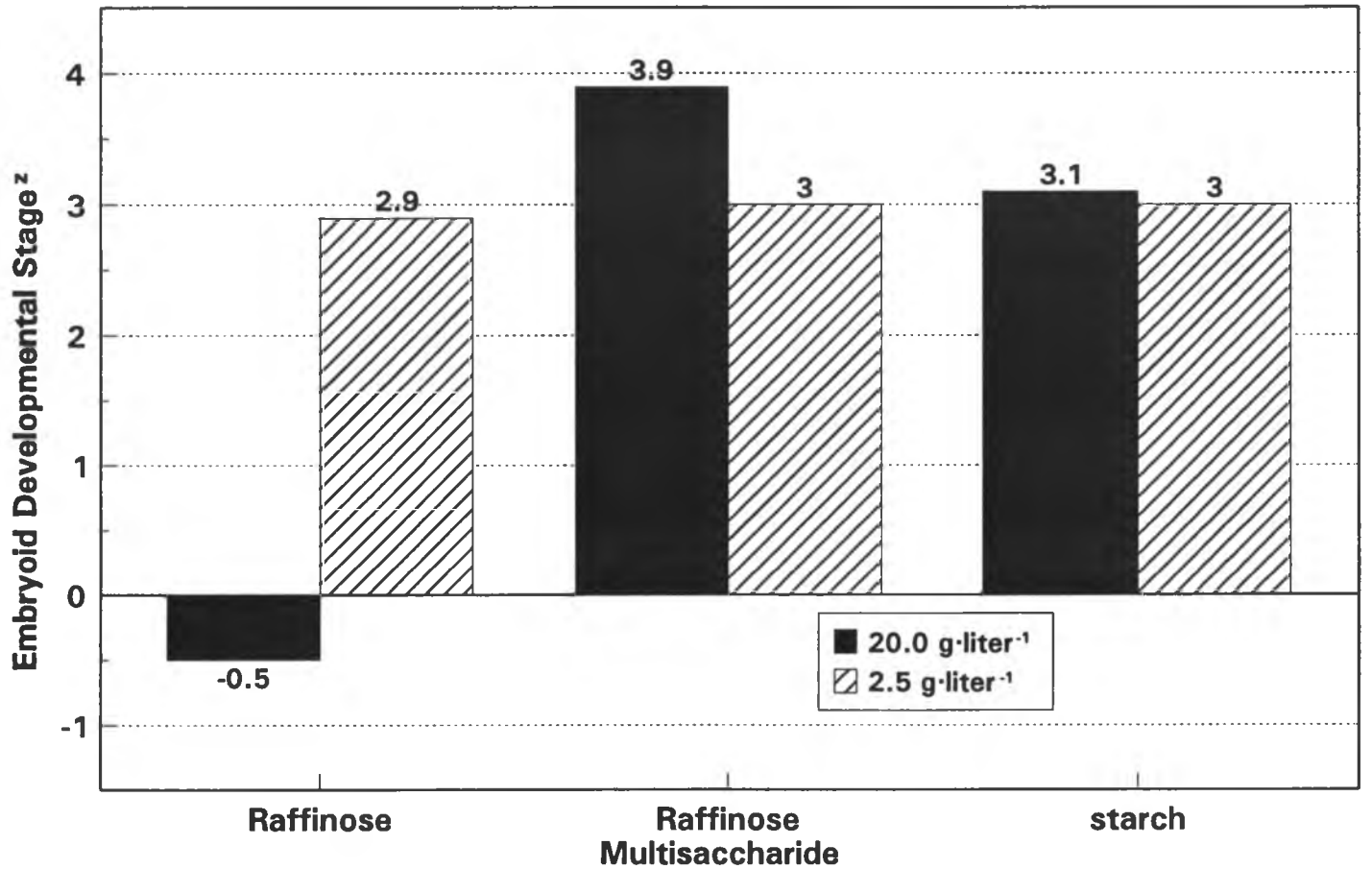
z-1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5,Protocorm stage.

Fig. 37. Effect of monosaccharide concentration on somatic embryogenesis of *Phalaenopsis* after 16 weeks.



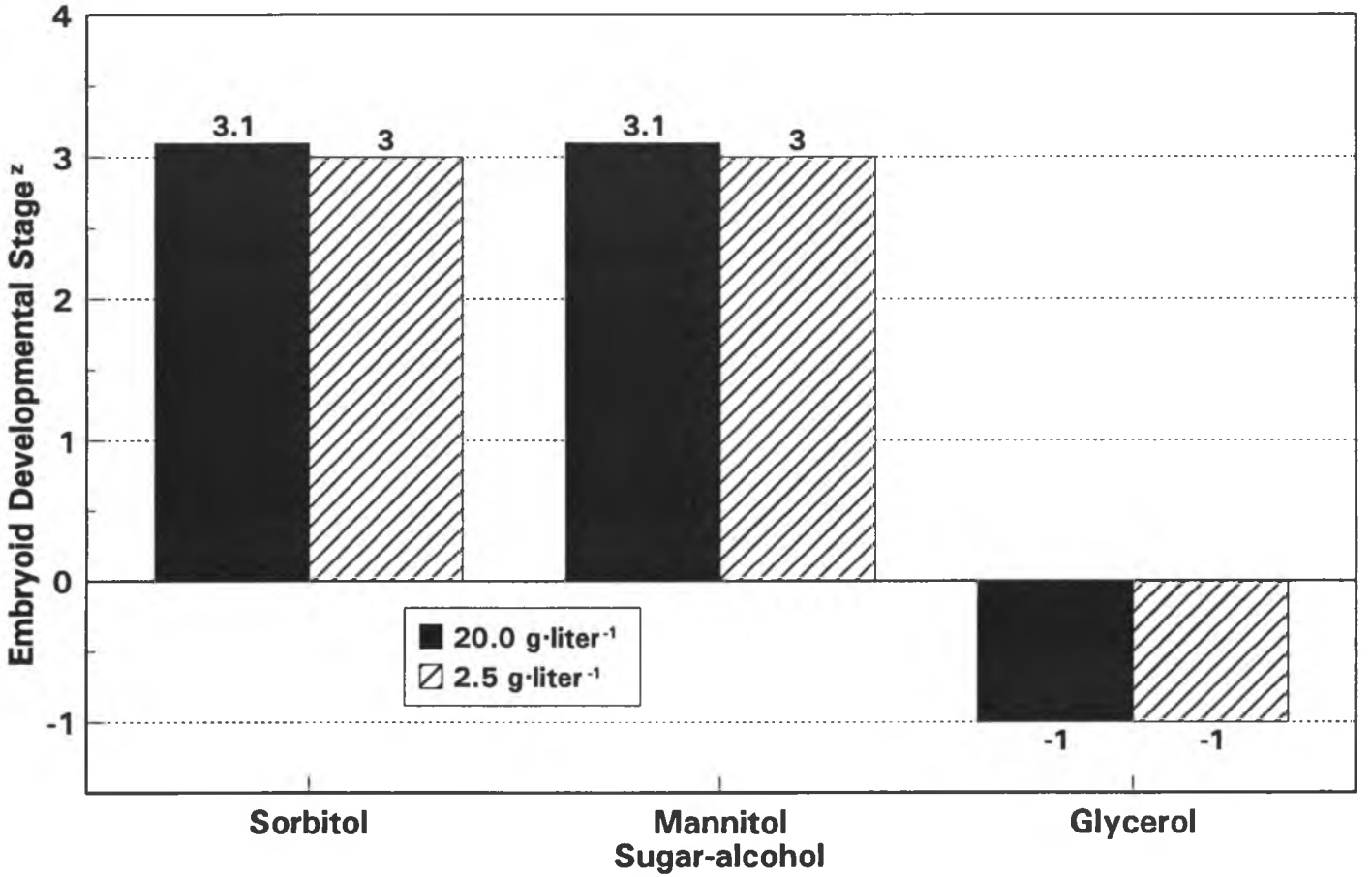
z-1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5,Protocorm stage.

Fig. 38. Effect of disaccharide concentration on somatic embryogenesis of *Phalaenopsis* after 16 weeks.



z-1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5,Protocorm stage.

Fig. 39. Effect of multisaccharide concentration on somatic embryogenesis of *Phalaenopsis* after 16 weeks.



z-1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5,Protocorm stage.

Fig. 40. Effect of sugar-alcohole concentration on somatic embryogenesis of *Phalaenopsis* after 16 weeks.

galactose unit, melibiose, lactose and raffinose were also toxic to callus growth revealing the detrimental effect of galactose. Glycerol was also detrimental to callus growth.

The inhibition of callus greening and somatic embryogenesis at the higher level was observed only with monosaccharides and one disaccharide, sucrose. The greening of callus and somatic embryogenesis did not correspond to better callus growth, but were independent of callus growth.

The results suggest that carbohydrate source or level in the culture medium regulates the greening of callus and somatic embryogenesis of Phalaenopsis in addition to its role as a carbon source. The greening of callus may be involved in the embryogenic capacity of the present callus tissue. The observation that greening and somatic embryogenesis was independent of the callus growth, supports the hypothesis suggested by Dalton (1980a). To explain the inhibitory effect of sugar on greening of some plant cell cultures, he postulated that greening of cell culture is inhibited in excess of sugar, but is promoted during sugar depletion independent of growth rate of culture. Although, the mechanism of inhibition of greening and somatic embryogenesis by readily utilizable monosaccharides and sucrose can not be clearly explained, the results showed that the low level of monosaccharide or sucrose, or the substitution with slow releasing sugars overcame the inhibition of greening and embryogenesis. This suggests

that the callus culture of Phalaenopsis is controlled by a mechanism similar to catabolite repression.

Some evidence supports the involvement. Key enzyme activities for glyoxysomes such as isocitrate lyase and malate synthase which are known to be subject to the catabolite repression, were induced during the early stages of somatic embryogenesis in Pimpinella anisum cultures when sucrose had been withdrawn from culture medium (Kudielka and Theimer, 1983). In Digitalis lanata cell culture, the most suitable carbon source was maltose, which was slowly degraded. Sucrose yielded poor rates of embryogenesis and cardenolide formation. The inhibition was attributed to catabolite repression (Luckner and Diettrich, 1985).

The induction of somatic embryogenesis by lowering the availability of carbohydrate in culture medium may be discussed in some other ways. Somatic embryogenesis may be the result of active chloroplasts developed by the response to depletion of carbohydrate. Active chloroplasts may integrate nitrogen assimilation, which facilitate protein syntheses required for embryogenesis, since chloroplast is one of the primary sites for ammonium assimilation in higher plants.

In another way, genes involved in the expression of somatic embryogenesis may be expressed by chloroplast differentiation as suggested in the somatic embryogenesis of

Chenopodium rubrum (Kaldenhoff and Richter, 1990) and carrot (Aleith and Richter, 1991) cultures.

Somatic embryogenesis may be induced independent of callus greening. Greening of callus and somatic embryogenesis may be induced by the enzyme syntheses specific to greening and embryogenesis respectively by the responses in gene expressions to depletion of carbohydrate. The depletion may play a role as a stimulus for the induction of both greening and somatic embryogenesis.

It is necessary to examine with the present system whether somatic embryogenesis is suppressed by glycoproteins released on sucrose or other readily utilizable carbohydrate-containing medium, independent from callus greening, which has been reported in system of Citrus nucellar cell cultures (Gavish et al., 1991; Gavish et al., 1992). The embryogenic responses of these cell cultures, however, have been reported as very different from the present callus. The most efficient embryogenesis was obtained with glycerol in the Citrus cell cultures (Ben-Hayyim and Neumann, 1983), while the callus tissue of the present study could not tolerate glycerol.

CHAPTER VI

CONCLUSIONS

To determine whether indirect somatic embryogenesis is a viable pathway for clonal propagation of Phalaenopsis, the growth and differentiation of a unique callus was characterized. The callus was determined to be friable and embryogenic producing plantlets which matured and flowered. The factors which affect callus growth and somatic embryogenesis were defined and discussed.

In contrast to other orchid calli previously reported, the callus was unique in its extreme friability and undifferentiation with no trace of organization. The callus could be maintained and proliferated for more than five years on a MVW medium with 20.0 $g \cdot liter^{-1}$ of sucrose and 150 $ml \cdot liter^{-1}$ of CW without any morphological changes (MVW+20S+CW). The callus retained its embryogenic competence after the long term of maintenance.

Analyses of growth responses in terms of fresh weight showed that the callus had an extremely low growth rate, which was not affected by the level of sucrose in the range of 2.5 to 30.0 $g \cdot liter^{-1}$. Although the addition of CW to the culture medium stimulated callus growth, IAA of BA was

not effective in promoting callus growth suggesting the callus was autotrophic for auxin and cytokinin.

Somatic embryogenesis was observed after transfer of callus to the MVW medium without sucrose (MVW+0S+CW). The callus turned green with increased numbers of chloroplasts. Numerous embryoids developed at the periphery of the green callus. The embryoids matured into protocorm-like structures. The sequential stages of embryoid development were reminiscent of the germination of zygotic embryo of Phalaenopsis. It was also observed that the induction and development of somatic embryogenesis in early stage was reversible in the system. Returning the green callus to sucrose medium caused the cultured callus to dedifferentiate and turned back to the yellow callus state.

Evaluations of the effects of various types of plant growth regulators and nitrogen sources combined with sucrose levels in MS medium showed that the most dominant factor for somatic embryogenesis was sucrose level. Greening of callus and somatic embryogenesis occurred only on media with 2.5 $g \cdot liter^{-1}$ of sucrose. Sucrose level of 20.0 $g \cdot liter^{-1}$ inhibited greening of callus and somatic embryogenesis. Pretreatment with IAA or NAA, and supplement of CW, CH, glutamine or glutamic acid were effective in stimulating embryogenesis only when combined with 2.5 $g \cdot liter^{-1}$ of sucrose in the medium.

Readily utilizable monosaccharides, glucose, fructose,

mannose, ribose, xylose and the only disaccharide, sucrose inhibited greening and somatic embryogenesis of callus at 20.0 $g \cdot liter^{-1}$. The other disaccharides, trehalose, maltose and cellobiose, multisaccharides, amylose and starch and sugar-alcohols, sorbitol and mannitol supported greening and somatic embryogenesis. Among carbohydrate sources, amylose at the level of 20.0 $g \cdot liter^{-1}$ was the most effective in inducing embryogenesis.

The callus of Phalaenopsis in the present study was extremely friable and the constituent cells were in an undifferentiated state. This type of callus has not, as yet, been reported in orchids. Somatic embryogenesis was successfully induced by lowering sucrose level or sucrose substitution by slow releasing carbohydrate in the culture medium. The absence of reports on somatic embryogenesis in earlier orchid callus cultures might be due to the type of callus cultured. Embryogenic potential might be limited by the compactness or differentiation-state of callus as in callus cultures of maize inbred line (Everett et al., 1985; Lowe et al., 1985).

The sugar inhibition of callus greening and somatic embryogenesis of Phalaenopsis was not overcome by any conventional method of medium manipulation such as transfer from auxin-enriched to auxin-free media or increasing nitrogen level in medium, but only by reducing availability of carbohydrate in the culture medium. The results suggest

that carbohydrate source in culture medium regulated greening of callus and somatic embryogenesis of Phalaenopsis in addition to its role as a carbon source. Chloroplast development may be involved in the embryogenic capacity of the present callus tissue.

Further experiments to ensure the induction of friable embryogenic callus and to test somaclonal variation are necessary to completely assess the the clonal propagation of Phalaenopsis through somatic embryogenesis.

Friability or dedifferentiated state of callus may be a crucial factor for the successful induction of somatic embryogenesis. Choice of carbohydrate source with its level in culture medium may also influence the expression of somatic embryogenesis. The results may be applied to induce somatic embryogenesis of other sucrose-sensitive plant tissue cultures, especially sarcanthine orchids which are recalcitrant to shoot-tip culture. It may also be useful for germination of sarcanthine orchid seeds and manipulation of chlorophyllous culture.

APPENDIX

Table 3. Callus growth on medium MVW + 20S + CW.

Callus fresh weight (mg)[z]					
0 Week	1 Week	2 Weeks	3 Weeks	4 Weeks	5 Weeks
42.34 ± 0.62	46.72 ± 0.87	49.54 ± 1.46	64.78 ± 1.31	96.45 ± 2.30	140.20 ± 4.74

[z]Means ± s.e. of 15 measurements

Table 4. Effect of IAA or BA concentration on callus growth of *Phalaenopsis*.

Conc.[y]	Callus fresh weight (mg)[z]				
	1 Week	2 Weeks	3 Weeks	4 Weeks	5 Weeks
150 CW	53.92 ± 0.86 [x]a[w]	54.68 ± 0.79 a	70.80 ± 0.97 a	101.37 ± 2.40 a	146.75 ± 3.74 a
0.0	46.13 ± 0.87 b	51.00 ± 0.64 b	50.39 ± 1.03 b	57.15 ± 1.84 b	60.79 ± 1.26 b
0.1 BA	46.80 ± 0.78 b	49.98 ± 0.79 b	51.54 ± 1.67 b	53.68 ± 1.59 bc	56.19 ± 0.52 bc
1.0 BA	46.52 ± 0.51 b	49.69 ± 0.85 bc	52.59 ± 1.08 b	53.91 ± 1.07 bc	58.34 ± 0.93 bc
5.0 BA	46.45 ± 0.76 b	48.71 ± 0.69 bc	51.53 ± 0.93 b	54.97 ± 1.21 b	59.35 ± 1.50 bc
0.1 IAA	45.84 ± 1.03 b	48.41 ± 0.96 bc	51.89 ± 1.15 b	54.32 ± 0.94 b	57.98 ± 1.46 bc
1.0 IAA	46.57 ± 1.09 b	49.08 ± 1.07 bc	50.72 ± 0.98 b	53.07 ± 1.36 bc	54.69 ± 1.79 c
5.0 IAA	44.52 ± 1.03 b	47.12 ± 0.76 c	48.98 ± 1.39 b	49.20 ± 2.00 c	48.70 ± 1.63 d

[z]Initial weight, 41.87 ± 0.38

[y]BA and IAA, mg·liter^{-1}; CW, ml·liter^{-1}

[x]Means ± s.e. of 15 measurements

[w]Means in the same column followed by the same letter are not significantly different at the 5 % level according to Duncan's multiple range test.

Table 5. Analysis of variance for effect of IAA or BA on callus growth of Phalaenopsis after 1 week.

Dependent variable: fresh callus weight

Source	DF	Sums of squares	F value	Pr > F
Total	119	2167.5346		
Treatments	7	853.9813	10.40	0.0001
CW vs. others	1	800.4762	68.25	0.0001
Among others	6	53.5051	0.76	0.6029
Error	112	1313.5533		

Table 6. Analysis of variance for effect of IAA or BA on callus growth of Phalaenopsis after 2 weeks.

Dependent variable: fresh callus weight

Source	DF	Sums of squares	F value	Pr > F
Total	119	1161.9320		
Treatments	7	541.9313	7.46	0.0001
CW vs. others	1	402.8273	38.83	0.0001
Among others	6	139.1040	2.23	0.0453
Error	112	1161.9320		

Table 7. Analysis of variance for effect of IAA or BA on callus growth of Phalaenopsis after 3 weeks.

Dependent variable: fresh callus weight

Source	DF	Sums of squares	F value	Pr > F
Total	119	8354.0983		
Treatments	7	5526.2979	36.15	0.0001
CW vs. others	1	5099.5929	246.95	0.0001
Among others	6	126.7050	1.02	0.4161
Error	112	2312.8613		

Table 8. Analysis of variance for effect of IAA or BA on callus growth of Phalaenopsis after 4 weeks.

Dependent variable: fresh callus weight

Source	DF	Sums of squares	F value	Pr > F
Total	119	34685.9480		
Treatments	7	30276.2133	109.85	0.0001
CW vs. others	1	29757.1430	755.78	0.0001
Among others	6	519.0703	2.20	0.0481
Error	112	4409.7347		

Table 9. Analysis of variance for effect of IAA or BA on callus growth of Phalaenopsis after 5 weeks.

Dependent variable: fresh callus weight

Source	DF	Sums of squares	F value	Pr > F
Total	119	113798.3993		
Treatments	7	108177.2926	309.92	0.0001
CW vs. others	1	106733.6663	2122.66	0.0001
Among others	6	1443.6263	4.79	0.0003
Error	112	5621.1067		

Table 10. Effect of sucrose concentration on callus growth of *Phalaenopsis*.

Suc. conc.	Callus fresh weight (mg)[z]				
(g·liter $^{-1}$)	1 Week	2 Weeks	3 Weeks	4 Weeks	5 Weeks
0.0	45.82 ± 1.02[y] b[x]	46.13 ± 1.37 b	46.43 ± 0.91 b	50.10 ± 1.29 b	50.40 ± 1.10 c
2.5	49.61 ± 1.67 ab	50.46 ± 1.43 a	50.72 ± 1.22 a	52.71 ± 1.60 ab	57.58 ± 1.61 ab
5.0	49.05 ± 1.43 ab	51.23 ± 1.50 a	52.61 ± 1.61 a	55.79 ± 1.42 a	63.88 ± 1.95 a
10.0	51.84 ± 1.51 a	51.94 ± 1.39 a	54.40 ± 1.52 a	54.03 ± 1.57 ab	62.51 ± 2.42 ab
20.0	47.61 ± 1.72 ab	50.55 ± 1.59 a	52.41 ± 1.77 a	53.64 ± 1.02 ab	58.11 ± 0.96 ab
30.0	50.51 ± 1.03 a	52.62 ± 1.51 a	53.60 ± 1.50 a	54.94 ± 1.50 a	60.71 ± 2.75 ab
50.0	48.36 ± 1.20 ab	51.50 ± 1.37 a	52.39 ± 0.95 a	52.58 ± 1.51 ab	56.75 ± 2.99 b

[z]Initial weight, 42.70 ± 0.97

[y]Means ± s.e. of 15 measurements

[x]Means in the same column followed by the same letter are not signigicantly different at the 5 % level according to Duncan's multiple range test.

Table 11. Analysis of variance for effect of sucrose concentration on callus growth Phalaenopsis after 1 week.

Dependent variable: fresh callus weight

Source	DF	Sums of squares	F value	Pr > F
Total	104	3287.3733		
Treatments	6	354.5373	1.97	0.0766
0 sucrose vs. others	1	184.4648	6.16	0.0147
Among others	5	170.0725	1.14	0.3445
Error	98	2932.8360		

Table 12. Analysis of variance for effect of sucrose concentration on callus growth Phalaenopsis after 2 weeks.

Dependent variable: fresh callus weight

Source	DF	Sums of squares	F value	Pr > F
Total	104	3515.8263		
Treatments	6	406.5743	2.14	0.0559
0 sucrose vs. others	1	355.1254	11.19	0.0012
Among others	5	51.4489	0.32	0.8999
Error	98	3109.2520		

Table 13. Analysis of variance for effect of sucrose concentration on callus growth Phalaenopsis after 3 weeks.

Dependent variable: fresh callus weight

Source	DF	Sums of squares	F value	Pr > F
Total	104	3466.3676		
Treatments	6	621.4303	3.57	0.0031
0 sucrose vs. others	1	504.3773	17.37	0.0001
Among others	5	117.0530	0.81	0.5453
Error	98	2844.9373		

Table 14. Analysis of variance for effect of sucrose concentration on callus growth of Phalaenopsis after 4 weeks.

Dependent variable: fresh callus weight

Source	DF	Sums of squares	F value	Pr > F
Total	104	3301.9099		
Treatments	6	308.7579	1.68	0.1327
0 sucrose vs. others	1	190.4650	6.24	0.0142
Among others	5	118.2929	0.77	0.5736
Error	98	2993.1520		

Table 15. Analysis of variance for effect of sucrose concentration on callus growth of Phalaenopsis after 5 weeks.

Dependent variable: fresh callus weight

Source	DF	Sums of squares	F value	Pr > F
Total	104	8196.2224		
Treatments	6	1793.7531	4.58	0.0004
0 sucrose vs. others	1	1166.3363	17.85	0.0001
Among others	5	627.4168	1.92	0.0978
Linear	1	6.1916	0.09	0.7648
Quadratic	1	493.9824	7.55	0.0071
Deviation	4	1293.5791	5.64	0.0004
Error	98	6402.4693		

Table 16. Effect of sucrose concentration and coconut water on somatic embryogenesis of Phalaenopsis after after 4 weeks.

Medium	Mean developmental stage[z] and range
MS + 2.5S	0.7 ± 0.15[y] b[x] 0 - 1
MS + 20S	0.0 ± 0.00 c 0 - 0
MS + 0S + CW	1.0 ± 0.00 a 1 - 1
MS + 20S + CW	0.0 ± 0.00 c 0 - 0
MS + CW + 58.4 mM MANNITOL	1.0 ± 0.00 a 1 - 1

[z]Developmental stage: -1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5;Protocorm stage.

[y]Means ± s.e. of 10 observations.

[x]Mean separation by Duncan's multiple range test, 5 % level.

Table 17. Analysis of variance for effect of sucrose concentration and coconut water on somatic embryogenesis of Phalaenopsis after 4 weeks.

Dependent variable: embryoid developmental stage

Source	DF	Sums of squares	F value	Pr > F
Total	49	12.4200		
Treatments	4	10.3200	55.29	0.0001
Error	45	2.1000		

Table 18. Effect of sucrose concentration and coconut water on somatic embryogenesis of Phalaenopsis after 8 weeks.

Medium	Mean developmental Stage[z] and range
MS + 2.5S	1.0 ± 0.00[y] b[x] 1 - 1
MS + 20S	0.0 ± 0.00 c 0 - 0
MS + 0S + CW	2.0 ± 0.00 a 2 - 2
MS + 20S + CW	0.0 ± 0.00 c 0 - 0
MS + CW + 58.4 mM MANNITOL	2.0 ± 0.00 a 2 - 2

[z]Developmental stage: -1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5;Protocorm stage.

[y]Means ± s.e. of 10 observations.

[x]Mean separation by Duncan's multiple range test, 5 % level.

Table 19. Analysis of variance for effect of sucrose concentration and coconut water on somatic embryogenesis of Phalaenopsis after 8 weeks.

Dependent variable: embryoid developmental stage

Source	DF	Sums of squares	F value	Pr > F
Total	49	40.0000		
Treatments	4	40.0000		0.0000
Error	45	0.0000		

Table 20. Effect of sucrose concentration and coconut water on somatic embryogenesis of Phalaenopsis after after 12 weeks.

Medium	Mean developmental Stage[z] and range		
MS + 2.5S	1.9 ± 0.10[y]	b[x]	1 - 2
MS + 20S	0.0 ± 0.00	c	0 - 0
MS + 0S + CW	2.9 ± 0.10	a	2 - 3
MS + 20S + CW	0.0 ± 0.00	c	0 - 0
MS + CW + 58.4 mM MANNITOL	2.9 ± 0.10	a	2 - 3

[z]Developmental stage: -1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5;Protocorm stage.

[y]Means ± s.e. of 10 observations.

[x]Mean separation by Duncan's multiple range test, 5 % level.

Table 21. Analysis of variance for effect of sucrose concentration and coconut water on somatic embryogenesis of Phalaenopsis after 12 weeks.

Dependent variable: embryoid developmental stage

Source	DF	Sums of squares	F value	Pr > F
Total	49	88.4200		
Treatments	4	85.7200		0.0000
Error	45	2.7000		

Table 22. Effect of sucrose concentration and coconut water on somatic embryogenesis of Phalaenopsis after after 16 weeks.

Medium	Mean developmental stage[z] and range			Callus growth[y]
MS+2.5S[x]	2.9 ± 0.10[w]	b[v]	2 - 3	++
MS+20S	0.0 ± 0.00	c	0 - 0	++
MS+0S+CW[u]	3.7 ± 0.15	a	3 - 4	++++
MS+20S+CW	0.0 ± 0.00	c	0 - 0	++++
MS+CW+58.4 mM MANNITOL	3.6 ± 0.16	a	3 - 4	++++

[z]Developmental stage: -1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5;Protocorm stage.

[y]0,no growth; +,limited to ++++,excellent growth.

[x]Concentration of sucrose: 2.5S, 2.5 g·liter^{-1}; 20S, 20.0 g·liter^{-1}.

[w]Means ± s.e. of 10 observations.

[v]Mean separation by Duncan's multiple range test, 5 % level.

[u]Cocentration of coconut water, 150 ml·liter^{-1}.

Table 23. Analysis of variance for effect of sucrose concentration and coconut water on somatic embryogenesis of Phalaenopsis after 16 weeks.

Dependent variable: embryoid developmental stage

Source	DF	Sums of squares	F value	Pr > F
Total	49	147.9200		
Treatments	4	142.5200	296.92	0.0001
Error	45	5.4000		

Table 24. Effect of plant growth regulator and sucrose concentration on somatic embryogenesis of Phalaenopsis after 16 weeks.

Suc conc.[z]	PGR and conc.[y]		Medium abbr.[x]	Mean developmental stage[w] and range			Callus growth[v]
20.0	-		MS+20S	0.0 ± 0.00[u]	l[t]	0	++
2.5	-		MS+2.5S	2.9 ± 0.10	efgh	2,3	++
20.0	IAA	0.1	MS+20S+0.1IAA	0.0 ± 0.00	l	0	++
		1.0	MS+20S+1.0IAA	0.0 ± 0.00	l	0	++
		5.0	MS+20S+5.0IAA	-0.1 ± 0.10	l	-1,0	++
2.5		0.1	MS+2.5S+0.1IAA	3.2 ± 0.13	bcde	3,4	++
		1.0	MS+2.5S+1.0IAA	3.1 ± 0.10	cdef	3,4	++
		5.0	MS+2.5S+5.0IAA	2.8 ± 0.13	fghi	2,3	++
20.0	NAA	0.1	MS+20S+0.1NAA	0.0 ± 0.00	l	0	++
		1.0	MS+20S+1.0NAA	0.0 ± 0.00	l	0	++
		5.0	MS+20S+5.0NAA	0.0 ± 0.00	l	0	++
2.5		0.1	MS+2.5S+0.1NAA	3.0 ± 0.00	defg	3	++
		1.0	MS+2.5S+1.0NAA	2.7 ± 0.15	ghij	2,3	++
		5.0	MS+2.5S+5.0NAA	2.6 ± 0.16	hijk	2,3	++
20.0	2,4-D	0.1	MS+20S+0.124D	0.0 ± 0.00	l	0	++
		1.0	MS+20S+1.024D	-1.0 ± 0.00	o	-1	0
		5.0	MS+20S+5.024D	-1.0 ± 0.00	o	-1	0
2.5		0.1	MS+2.5S+0.124D	-1.0 ± 0.00	o	-1	0
		1.0	MS+2.5S+1.024D	-1.0 ± 0.00	o	-1	0
		5.0	MS+2.5S+5.024D	-1.0 ± 0.00	o	-1	0

Table 24. Effect of plant growth regulator and sucrose concentration on somatic embryogenesis of Phalaenopsis after 16 weeks (continued).

Suc conc.	PGR and conc.		Medium abbr.	Mean developmental stage and range			Callus growth
20.0	Pre-IAA	0.1	MS+20S+0.1PIAA	0.0 ± 0.00	l	0	+++
		1.0	MS+20S+1.0PIAA	0.0 ± 0.00	l	0	+++
		5.0	MS+20S+5.0PIAA	0.0 ± 0.00	l	0	+++
2.5		0.1	MS+2.5S+0.1PIAA	3.5 ± 0.17	ab	3,4	++
		1.0	MS+2.5S+1.0PIAA	3.6 ± 0.16	a	3,4	++
		5.0	MS+2.5S+5.0PIAA	3.5 ± 0.17	ab	3,4	++
20.0	Pre-NAA	0.1	MS+20S+0.1PNAA	0.0 ± 0.00	l	0	+++
		1.0	MS+20S+1.0PNAA	0.0 ± 0.00	l	0	+++
		5.0	MS+20S+5.0PNAA	0.0 ± 0.00	l	0	+++
2.5		0.1	MS+2.5S+0.1PNAA	3.5 ± 0.17	ab	3,4	++
		1.0	MS+2.5S+1.0PNAA	3.4 ± 0.16	abc	3,4	++
		5.0	MS+2.5S+5.0PNAA	3.3 ± 0.15	abcd	3,4	++
20.0	Pre-2,4-D	0.1	MS+20S+0.1P24D	0.0 ± 0.00	l	0	++
		1.0	MS+20S+1.0P24D	-1.0 ± 0.00	o	-1	0
		5.0	MS+20S+5.0P24D	-1.0 ± 0.00	o	-1	0
2.5		0.1	MS+2.5S+0.1P24D	-0.7 ± 0.30	no	-1,2	0
		1.0	MS+2.5S+1.0P24D	-1.0 ± 0.00	o	-1	0
		5.0	MS+2.5S+5.0P24D	-1.0 ± 0.00	o	-1	0
20.0	Kinetin	0.1	MS+20S+0.1KN	0.0 ± 0.00	l	0	+++
		1.0	MS+20S+1.0KN	0.0 ± 0.00	l	0	+++

Table 24. Effect of plant growth regulator and sucrose concentration on somatic embryogenesis of Phalaenopsis after 16 weeks (continued).

Suc conc.	PGR and conc.	Medium abbr.	Mean developmental stage and range			Callus growth
20.0	Kinetin 5.0	MS+20S+5.0KN	0.0 ± 0.00	l	0	+++
2.5	0.1	MS+2.5S+0.1KN	2.9 ± 0.10	efgh	2,3	++
	1.0	MS+2.5S+1.0KN	2.9 ± 0.10	efgh	2,3	++
	5.0	MS+2.5S+5.0KN	2.9 ± 0.10	efgh	2,3	++
20.0	BA 0.1	MS+20S+0.1BA	0.0 ± 0.00	l	0	+++
	1.0	MS+20S+1.0BA	0.0 ± 0.00	l	0	+++
	5.0	MS+20S+5.0BA	0.0 ± 0.00	l	0	+++
2.5	0.1	MS+2.5S+0.1BA	2.9 ± 0.10	efgh	2,3	++
	1.0	MS+2.5S+1.0BA	2.4 ± 0.16	jk	2,3	++
	5.0	MS+2.5S+5.0BA	-0.3 ± 0.32	lm	-1,2	++
20.0	2-iP 0.1	MS+20S+0.1IP	0.0 ± 0.00	l	0	+++
	1.0	MS+20S+1.0IP	0.0 ± 0.00	l	0	+++
	5.0	MS+20S+5.0IP	0.0 ± 0.00	l	0	+++
2.5	0.1	MS+2.5S+0.1IP	2.9 ± 0.10	efgh	2,3	++
	1.0	MS+2.5S+1.0IP	2.9 ± 0.10	efgh	2,3	++
	5.0	MS+2.5S+5.0IP	2.8 ± 0.13	fghi	2,3	+
20.0	GA_3 0.1	MS+20S+0.1GA	-0.1 ± 0.10	l	-1,0	+++
	1.0	MS+20S+1.0GA	-0.3 ± 0.15	lm	-1,0	+++
	5.0	MS+20S+5.0GA	-0.5 ± 0.17	mn	-1,0	+++
2.5	0.1	MS+2.5S+0.1GA	2.6 ± 0.16	hijk	2,3	+

Table 24. Effect of plant growth regulator and sucrose concentration on somatic embryogenesis of Phalaenopsis after 16 weeks (continued).

Suc conc.	PGR and conc.		Medium abbr.	Mean developmental stage and range			Callus growth
2.5	GA_3	1.0	MS+2.5S+1.0GA	2.5 ± 0.17	ijk	2,3	+
		5.0	MS+2.5S+5.0GA	2.5 ± 0.17	ijk	2,3	+
20.0	7-AZA	0.1	MS+20S+0.1AZA	-0.2 ± 0.13	lm	-1,0	++
		1.0	MS+20S+1.0AZA	0.0 ± 0.00	l	0	++
		5.0	MS+20S+5.0AZA	-0.2 ± 0.13	lm	-1,0	++
2.5		0.1	MS+2.5S+0.1AZA	2.9 ± 0.10	efgh	2,3	+
		1.0	MS+2.5S+1.0AZA	2.9 ± 0.10	efgh	2,3	+
		5.0	MS+2.5S+5.0AZA	2.3 ± 0.21	k	1,3	+
20.0	p-CPB	0.1	MS+20S+0.1CPB	0.0 ± 0.00	l	0	++
		1.0	MS+20S+1.0CPB	0.0 ± 0.00	l	0	++
		5.0	MS+20S+5.0CPB	0.0 ± 0.00	l	0	++
2.5		0.1	MS+2.5S+0.1CPB	2.3 ± 0.15	k	2,3	+
		1.0	MS+2.5S+1.0CPB	3.0 ± 0.00	defg	3	+
		5.0	MS+2.5S+5.0CPB	2.6 ± 0.16	hijk	2,3	+

[z]$g \cdot liter^{-1}$. [y]$mg \cdot liter^{-1}$. [x]MS medium.

[w]Developmental stage: -1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5;Protocorm stage.

[v]0,no growth; +,limited to ++++,excellent growth.

[u]Means ± s.e. of 10 observations.

[t]Means followed by the same letter are not significantly different at the 5 percent level according to Duncan's Multiple Range Test.

Table 25. Analysis of variance for effect of plant growth regulator and sucrose concentration on somatic embryogenesis of Phalaenopsis after 16 weeks.

Dependent variable: developmental stage

Source	DF	Sums of squares	Percent of total var.	F value	Pr > F
Total	719	1933.8611			
Treatment	71	1853.8611	100	211.50	0.0001
Sucrose level	1	975.3388	52.61	7897.48	0.0001
Type of PGR	11	517.5611	27.92	380.98	0.0001
Level of PGR	2	12.0861	0.65	48.93	0.0001
Suc. x Type	11	279.6277	15.08	205.84	0.0001
Suc. x Level	2	3.2527	0.18	13.17	0.0001
Type x Level	22	31.4138	1.69	11.56	0.0001
S x T x L	22	34.5809	1.87	12.73	0.0001
Error	648	80.0000			

Table 26. Effect of nitrogen source and sucrose concentration on somatic embryogenesis of Phalaenopsis after 16 weeks.

Suc conc.[z]	Source of N and conc.	Medium abbr.[y]	Mean developmental stage[x] and range		Callus growth[w]
20.0	-	MS+20S	0.0 ± 0.00[v]	g[u] 0	++
2.5	-	MS+2.5S	2.9 ± 0.10	d 2,3	++
20.0	NH_4/NO_3[t] 60	MS+20S+60N	0.3 ± 0.15	fg 0,1	++
	180	MS+20S+180N	-1.0 ± 0.00	i -1	0
2.5	60	MS+20S+60N	2.9 ± 0.10	d 2,3	++
	180	MS+20S+180N	-0.9 ± 0.10	i -1,0	0
20.0	CH[s] 0.5	MS+20S+0.5CH	0.1 ± 0.10	fg 0,1	++
	1.0	MS+20S+1CH	0.1 ± 0.10	fg 0,1	++
	2.0	MS+20S+2CH	-1.0 ± 0.00	i -1	0
2.5	0.5	MS+2.5S+0.5CH	3.1 ± 0.10	cd 3,4	++
	1.0	MS+2.5S+1CH	3.4 ± 0.50	bc -1,4	++
	2.0	MS+2.5S+2CH	-1.0 ± 0.00	i -1	0
20.0	ALA[r] 1.0	MS+20S+1ALA	0.3 ± 0.15	fg 0,1	++
	10.0	MS+20S+10ALA	0.3 ± 0.15	fg 0,1	+++
	50.0	MS+20S+50ALA	-0.6 ± 0.16	hi -1,0	+
2.5	1.0	MS+2.5S+1ALA	3.1 ± 0.10	cd 3,4	++
	10.0	MS+2.5S+10ALA	-1.0 ± 0.00	i -1	0
	50.0	MS+2.5S+50ALA	-1.0 ± 0.00	i -1	0
20.0	ASPG 1.0	MS+20S+1ASPG	0.5 ± 0.17	f 0,1	++

Table 26. Effect of nitrogen source and sucrose concentration on somatic embryogenesis of *Phalaenopsis* after 16 weeks (continued).

Suc conc.	Source of N and conc.		Medium abbr.	Mean developmental stage			Callus growth
20.0	ASPG	10.0	MS+20S+10ASPG	-0.7 ± 0.15	hi	-1,0	0
		50.0	MS+20S+50ASPG	-1.0 ± 0.00	i	-1	0
2.5		1.0	MS+2.5S+1ASPG	3.2 ± 0.13	cd	3,4	++
		10.0	MS+2.5S+10ASPG	-1.0 ± 0.00	i	-1	0
		50.0	MS+2.5S+50ASPG	-1.0 ± 0.00	i	-1	0
20.0	GLMA	1.0	MS+20S+1GLMA	0.2 ± 0.13	fg	0,1	++
		10.0	MS+20S+10GLMA	0.2 ± 0.20	fg	-1,1	+++
		50.0	MS+20S+50GLMA	-1.0 ± 0.00	i	-1	0
2.5		1.0	MS+2.5S+1GLMA	3.6 ± 0.16	ab	3,4	++
		10.0	MS+2.5S+10GLMA	-1.0 ± 0.00	i	-1	0
		50.0	MS+2.5S+50GLMA	-1.0 ± 0.00	i	-1	0
20.0	GLMN	1.0	MS+20S+1GLMN	-0.1 ± 0.10	g	-1,0	++
		10.0	MS+20S+10GLMN	-0.5 ± 0.17	h	-1,0	++
		50.0	MS+20S+50GLMN	-1.0 ± 0.00	i	-1	0
2.5		1.0	MS+2.5S+1GLMN	3.1 ± 0.10	cd	3,4	++
		10.0	MS+2.5S+10GLMN	3.9 ± 0.10	a	3,4	++
		50.0	MS+2.5S+50GLMN	-1.0 ± 0.00	i	-1	0
20.0	PRLN	1.0	MS+20S+1PRLN	-0.1 ± 0.10	g	-1,0	++
		10.0	MS+20S+10PRLN	-1.0 ± 0.00	i	-1	0
		50.0	MS+20S+50PRLN	-1.0 ± 0.00	i	-1	0

Table 26. Effect of nitrogen source and sucrose concentration on somatic embryogenesis of Phalaenopsis after 16 weeks (continued).

Suc conc.	Source of N and conc.		Medium abbr.	Mean developmental stage			Callus growth
2.5	PRLN	1.0	MS+2.5S+1PRLN	2.5 ± 0.17	e	2,3	++
		10.0	MS+2.5S+10PRLN	-1.0 ± 0.00	i	-1	0
		50.0	MS+2.5S+50PRLN	-1.0 ± 0.00	i	-1	0

Amino acid abbreviation: ALA, alanine; ASPG, asparagine; GLMA, glutamic acid; GLMN, glutamine; PRLN, proline.

[z]Sucrose concentration, g·liter^{-1}.

[y]Murashige and Skoog medium was used as basal medium.

[x]Developmental stage: -1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5;Protocorm stage.

[w]0,no growth; +,limited to ++++,excellent growth.

[v]Means ± s.e. of 10 observations.

[u]Means followed by the same letter are not significantly different at the 5 percent level according to Duncan's Multiple Range Test.

[t]Concentration of inorganic nitrogen (NH_4/NO_3 mixture at ratio of 1:2), mM.

[s]Concentration of casein hydrolysate, g·liter^{-1}.

[r]Concentration of amino acids; mM.

Table 27. Analysis of variance for effect of nitrogen source and sucrose concentration on somatic embryogenesis of Phalaenopsis after 16 weeks.

Dependent variable: embryoid developmental stage

Source	DF	Sums of squares	F value	Pr > F
Total	119	1159.2975		
Treatments	39	1100.3975	172.45	0.0001
Error	360	58.9000		

Table 28. Analysis of variance for effect of inorganic nitrogen and sucrose concentration on somatic embryogenesis of Phalaenopsis after 16 weeks.

Dependent variable: developmental stage

Source	DF	Sums of squares	Percent of total var.	F value	Pr > F
Total	39	102.7750			
Treatments	3	98.8750	100	304.23	0.0001
Sucrose level	1	18.2250	18.43	168.23	0.0001
N level	1	65.0250	65.76	600.23	0.0001
Suc. x N	1	15.6250	15.80	144.23	0.0001
Error	36	3.9000			

Table 29. Analysis of variance for effect of casein hydrolysate and sucrose concentration on somatic embryogenesis of Phalaenopsis after 16 weeks.

Dependent variable: developmental stage

Source	DF	Sums of squares	Percent of total var.	F value	Pr > F
Total	59	220.1833			
Model	5	195.0833	100	83.94	0.0001
Sucrose level	1	66.1500	33.91	142.31	0.0001
CH level	2	95.6333	49.02	102.87	0.0001
Suc. x CH	2	33.3000	17.07	35.82	0.0001
Error	54	25.1000			

Table 30. Analysis of variance for effect of amino acid and sucrose concentration on somatic embryogenesis of Phalaenopsis after 16 weeks.

Dependent variable: developmental stage

Source	DF	Sums of squares	Percent of total var.	F value	Pr > F
Total	299	816.9966			
Treatments	29	787.0966	100	245.09	0.0001
Sucrose level	1	84.2700	10.71	717.80	0.0001
Type of A. A.	4	32.2133	4.09	68.60	0.0001
Level of A. A.	2	353.0867	44.86	1594.79	0.0001
Suc. x Type	4	42.9467	5.46	69.99	0.0001
Suc. x Level	2	134.5400	17.09	607.68	0.0001
Type x Level	8	69.6467	8.85	78.64	0.0001
S x T x L	8	70.3932	8.94	79.49	0.0001
Error	270	29.9000			

Table 31. Effect of carbohydrate substitution and its concentration on somatic embryogenesis of Phalaenopsis after 16 weeks.

Carbohydrate and conc. ($g \cdot liter^{-1}$)		Medium abbr.[z]	Mean developmental stage[y] and range			Callus growth[x]
Monosaccharide						
Glucose	20.0	MS+20GLU	0.0 ± 0.00[w]	f[v]	0	++
	2.5	MS+2.5GLU	2.4 ± 0.16	de	2,3	++
Fructose	20.0	MS+20GLU	0.0 ± 0.00	f	0	++
	2.5	MS+2.5FRU	2.2 ± 0.25	e	1,3	++
Mannose	20.0	MS+20MANSE	0.0 ± 0.00	f	0	+++
	2.5	MS+2.5MANSE	2.4 ± 0.16	de	2,3	++
Ribose	20.0	MS+20RIB	0.0 ± 0.00	f	0	++
	2.5	MS+2.5RIB	2.5 ± 0.17	de	2,3	++
Arabinose	20.0	MS+20ARA	-1.0 ± 0.00	h	-1	0
	2.5	MS+2.5ARA	-1.0 ± 0.00	h	-1	0
Xylose	20.0	MS+20XYL	0.3 ± 0.15	f	0,1	+++
	2.5	MS+2.5XYL	2.6 ± 0.16	cd	2,3	++
Galactose	20.0	MS+20GAL	-1.0 ± 0.00	h	-1	0
	2.5	MS+2.5GAL	-1.0 ± 0.00	h	-1	0
Disaccharide						
Sucrose	20.0	MS+20S	0.0 ± 0.00	f	0	++
	2.5	MS+2.5S	2.9 ± 0.10	bc	2,3	++
Melibiose	20.0	MS+20MEL	-0.9 ± 0.10	h	-1,0	+
	2.5	MS+2.5MEL	-1.0 ± 0.00	h	-1	+

Table 31. Effect of carbohydrate substitutions and its concentration on somatic embryogenesis of Phalaenopsis after 16 weeks (continued).

Carbohydrate and conc. ($g \cdot liter^{-1}$)		Medium abbr.	Mean developmental stage and range			Callus growth
Disaccharide						
Trehalose	20.0	MS+20TRE	3.0 ± 0.00	b	3	++++
	2.5	MS+2.5TRE	2.9 ± 0.10	bc	2,3	++
Maltose	20.0	MS+20MAL	3.0 ± 0.00	b	3	++
	2.5	MS+2.5MAL	3.0 ± 0.00	b	3	++
Cellobiose	20.0	MS+20CEL	3.2 ± 0.13	b	3,4	++++
	2.5	MS+2.5CEL	3.0 ± 0.00	b	3	++
Lactose	20.0	MS+20LAC	-0.6 ± 0.16	g	-1,0	++
	2.5	MS+2.5LAC	-1.0 ± 0.00	h	-1	+
Multisaccharide						
Raffinose	20.0	MS+20RAF	-0.5 ± 0.17	g	-1,0	++
	2.5	MS+2.5RAF	2.9 ± 0.18	bc	2,4	+
Amylose	20.0	MS+20AMY	3.9 ± 0.10	a	3,4	+++
	2.5	MS+2.5AMY	3.0 ± 0.00	b	3	+
Starch	20.0	MS+20STA	3.1 ± 0.10	b	3,4	++++
	2.5	MS+2.5STA	3.0 ± 0.00	b	3	++
Sugar-alcohol						
Sorbitol	20.0	MS+20SOR	3.1 ± 0.10	b	3,4	+++
	2.5	MS+2.5SOR	3.0 ± 0.15	b	2,4	+
Mannitol	20.0	MS+20MANOL	3.1 ± 0.10	b	3,4	+++

Table 31. Effect of carbohydrate substitutions and its concentration on somatic embryogenesis of Phalaenopsis after 16 weeks (continued).

Carbohydrate and conc. ($g \cdot liter^{-1}$)		Medium abbr.	Mean developmental stage and range			Callus growth
Sugar-alcohol						
Mannitol	2.5	MS+2.5MANOL	3.0 ± 0.00	b	3	+
Glycerol	20.0	MS+20GLY	-1.0 ± 0.00	h	-1	0
	2.5	MS+2.5GLY	-1.0 ± 0.00	h	-1	0

[z]Murashige and Skoog medium was used as basal medium.

[y]Developmental stage: -1,Brown callus; 0,Yellow callus; 1,Green callus; 2,Globular embryoid; 3,Oblong embryoid; 4,Intermediate stage; 5;Protocorm stage.

[x]0,no growth; +,limited to ++++,excellent growth.

[w]Means ± s.e. of 10 observations.

[v]Means followed by the same letter are not significantly different at the 5 percent level according to Duncan's Multiple Range Test.

Table 32. Analysis of variance for effect of carbohydrate-substitution and its concentration on somatic embryogenesis of Phalaenopsis after 16 weeks.

Dependent variable: developmental stage

Source	DF	Sums of squares	Percent of total var.	F value	Pr > F
Total	379	1241.0395			
Treatments	37	1205.9395	100	317.57	0.0001
Type of sugar	18	916.2895	75.98	520.36	0.0001
Level of sugar	1	68.2132	5.66	664.64	0.0001
Type x Level	18	176.4368	14.63	95.51	0.0001
Error	342	35.1000			

Table 33. Analysis of variance for effect of monosaccharide and its concentration on somatic embryogenesis of Phalaenopsis after 16 weeks.

Dependent variable: developmental stage

Source	DF	Sums of squares	Percent of total var.	F value	Pr > F
Total	139	301.6000			
Model	13	284.2000	100	158.31	0.0001
Type of sugar	6	144.7000	50.91	174.64	0.0001
Level of sugar	1	99.4571	35.00	720.21	0.0001
Type x Level	6	40.0429	14.09	48.33	0.0001
Error	126	17.4000			

Table 34. Analysis of variance for effect of disaccharide and its concentration on somatic embryogenesis of Phalaenopsis after 16 weeks.

Dependent variable: developmental stage

Source	DF	Sums of squares	Percent of total var.	F value	Pr > F
Total	119	413.7917			
Treatments	11	407.0917	100	596.55	0.0001
Type of sugar	5	363.9417	89.40	1173.30	0.0001
Level of sugar	1	3.6750	0.90	59.24	0.0001
Type x Level	5	39.4750	9.70	127.26	0.0001
Error	108	6.7000			

Table 35. Analysis of variance for effect of multisaccharide and its concentration on somatic embryogenesis of Phalaenopsis after 16 weeks.

Dependent variable: developmental stage

Source	DF	Sums of squares	Percent of total var.	F value	Pr > F
Total	59	126.7333			
Treatments	5	119.5333	100	179.30	0.0001
Type of sugar	2	57.6333	48.21	216.12	0.0001
Level of sugar	1	9.6000	8.03	72.00	0.0001
Type x Level	2	52.3000	43.75	196.12	0.0001
Error	54	7.2000			

Table 36. Analysis of variance for effect of sugar-alcohol and its concentration on somatic embryogenesis of Phalaenopsis after 16 weeks.

Dependent variable: developmental stage

Source	DF	Sums of squares	Percent of total var.	F value	Pr > F
Total	59	222.6000			
Treatments	5	218.8000	100	621.85	0.0001
Type of sugar	2	218.7000	99.95	1553.92	0.0001
Level of sugar	1	0.0667	0.03	0.95	0.3347
Type x Level	2	0.0333	0.02	0.24	0.7899
Error	54	3.8000			

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