MICROPROPAGATION OF *Phalaenopsis*

By

JYOTHI BHASKAR

THESIS

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DECLARATION

I hereby declare that the thesis entitled "**Micropropagation of *Phalaenopsis***" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship, associateship or other similar title, of any other University or society.

Vellanikkara
3-12-1996

Jyothi Bhaskar

JYOTHI BHASKAR

Dr.P.K.RAJEEVAN
Professor and Head i/c
Department of Pomology & Floriculture
College of Horticulture

3-12-1996
Vellanikkara

CERTIFICATE

Certified that this thesis entitled "**Micropropagation of *Phalaenopsis***" is a record of research work done independently by **Mrs.Jyothi Bhaskar**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

Dr.P.K.Rajeevan
Chairman
Advisory Committee

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Mrs.Jyothi Bhaskar**, a candidate for the degree of **Doctor of Philosophy in Horticulture**, agree that the thesis entitled "**Micropropagation of *Phalaenopsis***" may be submitted by Mrs.Jyothi Bhaskar in partial fulfilment of the requirements for the degree.

Dr.P.K.Rajeevan
Professor and Head (i/c)
Dept. of Pomology and Floriculture
College of Horticulture
Vellanikkara
(Chairman)

Dr.P.K.Valsalakumari
Associate Professor
College of Horticulture
Vellanikkara
(Member)

Dr. P.A.Nazeem
Associate Professor
College of Horticulture
Vellanikkara
(Member)

Sri.V.K.G.Unnithan
Associate Professor
College of Horticulture
Vellanikkara
(Member)

Dr.P.A.Wahid
Associate Dean
College of Agriculture
Pilicode
(Member)

EXTERNAL EXAMINAR

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ABBREVIATIONS

AC	-	Activated charcoal
BA	-	Benzyladenine
CH	-	Casein hydrolysate
CW	-	Coconut water
2,4-D	-	2,4-Dichlorophenoxy acetic acid
EDTA	-	Ethylene diamine tetra acetic acid
GA	-	Gibberellic acid
HCl	-	Hydrochloric acid
IAA	-	Indole-3-acetic acid
IBA	-	Indole-3-butyric acid
2iP	-	2-isopentenyl adenine
KC	-	Knudson's C (1975) medium
KIN	-	Kinetin, N^6-furfurl adenine
MS	-	Murashige and Skoog's (1962) medium
NAA	-	1-Napththalene acetic acid
NaOCl	-	Sodium hypochlorite
NaOH	-	Sodium hydroxide
PLB	-	Protocorm like body
ppm	-	Parts per million; mg l^{-1}
PVP	-	Poly vinyl pyrrollidene
VW	-	Vacin and Went's (1949) medium
v/v	-	Volume/volume
w/v	-	weight/volume

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Introduction

INTRODUCTION

Orchids are among the most beautiful flowers in nature. With their exotic shapes and hues and the added advantage of longevity, these flowers of rare beauty have become increasingly popular as flowers of the 21st century. The family of orchids Orchidaceae, comprises about 600-800 genera and 25,000-35,000 species (Chadha, 1992).

Entry of orchids has drastically changed the attitude towards commercial flower cultivation in Kerala. The humid tropical forests of Kerala is reported to be the home of about 200 species of orchids, both terrestrial and epiphytic (Maheshwari, 1980). The genera of orchids which are popular in our State are *Arachnis, Dendrobium* and *Vanda*.

The genus *Phalaenopsis* which is popularly known as the "moth orchid" is one of the important epiphytic orchids. Because of the high cost and strong apical dominant character, which limits the availability of planting material, only very few hobbyists are engaged in growing of the genus *Phalaenopsis*. The name *Phalaenopsis*, meaning moth-like, was given by a Dutch botanist, Dr.Karl Blume, in 1825, when he established the genus (Moses, 1981). It was so named because the white and pink species growing on trees bear many flowers on long, arching sprays and at twilight, resemble flights of moths (Freed, 1976).

Plants belonging to this genus are monopodial in nature and the inflorescence arises on alternate sides between the leaves. The flowers are the most graceful and in addition, are of the most beautiful and spectacular of all orchids.

The present day hybrids, when mature, generally bear from twelve to twenty or even more flowers, attractively spaced and the spikes have a tendency to branch as they grow older and larger. In addition, the fact that many last for two to five months and that bloom succession keeps them in flower for much longer period makes them all the more desirable.

Mature plants of *Phalaenopsis* retain an average of five to six leaves, although a few individuals may retain more. The foliage is attractive and long, broad, shiny or leathery, plain green on upper surface and often purple underneath. They are 10 to 35 cm long, so that a mature plant covers fairly a broad area. The plant increases in height only very slowly and added only one or two leaves a year. The robust flattened roots develop from the stem between mature leaves and grow down into the pot or outside of it to wander to some lengths.

Phalaenopsis does not prefer strong sunlight and requires 50 per cent shade. It also cannot withstand water stagnation and requires 60 to 70 per cent relative humidity. It prefers cool night and warm day for better growth and development.

The number of propagules available in *Phalaenopsis* is very limited as compared to other orchids. Usually mature plants produce two side shoots from the base, when the main apex of the mature plant is damaged. These shoots can be used for planting after they have developed good root system. Another method is by dividing a tall plant having a good array of roots at the base, leaving a basal stump with a few living roots. Often a young plant will develop at the top of the old part and this can be separated and planted. Yet another method commonly employed is inducing young plants to develop from the nodes of the flower spike. Though pod

culture is also employed for producing plants, the seedlings may not be true to type. Thus the number of propagules produced is very much limited in *Phalaenopsis*.

Though a number of orchids are routinely propagated through tissue culture, *Phalaenopsis* is one among the few recalcitrant genera of economic interest producing difficulties in rapid clonal propagation (Reuter, 1983). Hence it would be desirable to develop a technique which would help in rapid multiplication of *Phalaenopsis* hybrids so that plants can be made available to growers at a reasonable rate in sufficient number. Majority of the works on micropropagation techniques in *Phalaenopsis* was carried out abroad. The multiplication rate achieved in the widely followed aseptic culture of flower stalks is low as only a few buds are available on each flower stalk. A faster method of multiplication well established in orchids is through the production of PLB's.

The present study was undertaken with the objective to study the response of the floral tissues and meristems of *Phalaenopsis* in *in vitro* culture and to assess the establishment and growth of plantlets *ex vitro*.

Review of Literature

REVIEW OF LITERATURE

The orchids are highly heterozygous and their vegetative propagation through division, splitting and use of shoots and keikis is very slow and yields only a meagre number of plants even after five to six years. Although orchid seeds are produced in large numbers, i.e., two to three millions per capsule, as they lack metabolic machinery and functional endosperm, they germinate very poorly in nature.

The propagation and cultivation of orchids was revolutionised by the discovery of Knudson (1922) who showed that orchid seeds can germinate on a relatively simple medium containing sucrose. This became the standard procedure for germinating orchid seeds. The earliest report of using tissue culture techniques in the clonal propagation of orchid was that of Rotor (1949). Observing that plantlets could develop from buds of inflorescences in *Phalaenopsis*, he cultured flower stalk nodes *in vitro* and obtained some plantlets. The tremendous development made in orchid industry through the wide applications of tissue culture techniques owes its credit to Morel (1960), who initiated meristem tip culture in *Cymbidium*.

The orchids represent the first floricultural crop successfully mass propagated through tissue culture technique. At present, more than 69 genera and their hybrids are being multiplied through tissue culture techniques in countries like Thailand, Singapore and Hawaii (Singh, 1992).

2.1 Seed/embryo culture

2.1.1 Pollination

In the family Orchidaceae it is possible to hybridise between different genera and this has made possible the creation of new plants, combining the genes of three or four genera (Abraham, 1980) or even more.

Devi and Deka (1992) observed, the percentage of pollen viability and duration of stigma receptivity in some of the terrestrial (*Spathoglottis plicata, Phaius tankervilliae*) and epiphytic species (*Aerides odoratum, Dendrobium amoemum, D. aphyllum*). They reported that the pollen viability in terrestrial orchids declined gradually after anthesis, whereas in the epiphytes it improved during the first three days after anthesis and declined thereafter.

Cross compatibility studies involving about four orchid genera and six related species resulted in 0-100 per cent fruit set in interspecific crosses and 0-75 per cent in intergeneric crosses (Devi and Deka, 1992). In most of the successful interspecific and intergeneric crosses, both the parents have usually uniform chromosome number.

Hegde and Ingalhalli (1986) also reported about easy interspecific cross-ability among the genera *Coelogyne, Cymbidium* and *Paphiopedilum*, when species with identical chromosome numbers were used as parents.

2.1.2 Immature/green pod/embryo culture

A major advancement in germination of orchid seeds was the development of the green pod culture (Tsuchiya, 1954).

Selfing the flowers of various species and germinating the immature seeds have revealed that the embryos are ready for sowing within half the time taken for full seed development. *Dendrobium* seeds could be harvested after 100-120 days of pollination, *Phalaenopsis* after 90 days, *Vanda* after 50 days, *Spathoglottis plicata* after 15 days and *Phaius wallichii* after 27 days of pollination (Singh, 1987).

Duncan and Curtis (1942) studied the growth of seed pods of *Phalaenopsis schilleriana* and correlated the changes in growth rates with the internal development of the ovules. They found that fertilization occurred 63 to 70 days after pollination and the fruit matured in 180 to 190 days.

Immature ovules of *Phalaenopsis* were cultured in nutrient media by Tsuchiya (1958) and Poddubnaya Arnoldi (1959). Tsuchiya reported that ovules from 2½ months and older pods will grow and produce seedlings. Poddubnaya Arnoldi (1959) found that excised ovules with zygotes or embryos will grow into seedlings but those in pre fertilization stages will not develop further in the culture medium.

The maturity of the pod or the number of days after pollination is very important in getting seedlings from green pod culture of *Phalaenopsis* (Niimoto and Sagawa, 1962). Seedlings were obtained only from the 75 days and older ovules in *P. pulcherrima* and 85 days and older ovules in *P.* x *Cheiftain* but not from younger stages. The longer maturity period of *P.* x *Cheiftain* was probably due to its tetraploidy.

2.2 Factors influencing *in vitro* propagation

2.2.1 Explant

The type of explant, its size, age and the type of culture affect the induction of morphogenesis. Sagawa and Kunisaki (1982) after studying orchids of 12 genera, reported that apical shoots or axillary buds were the best source of explants for successful clonal propagation, followed by inflorescences, leaves and roots.

2.2.1.1 Type of explant

The earliest report of using tissue culture technique in orchids goes back to 1949 when it was demonstrated that *Phalaenopsis* plantlets could develop from the buds of inflorescence stalks (Rotor, 1949). The shoot meristem culture techniques (Morel, 1960) revolutionised commercial propagation of orchids. However, as the technique entails the sacrifice of the mother plant or its entire new growth, organ culture (leaves, roots, inflorescence stalks) is fast emerging as an ideal method for cloning elite orchids (Arditti, 1977).

2.2.1.1.1 Meristem/shoot tip

The credit for achieving mass clonal propagation of orchids goes to Morel (1960) who was successful in culturing *Cymbidium* shoot apices on nutrient media. It has been estimated that more than four million plants could be produced in a year from a single explant (Morel, 1964).

Apical meristem of a young shoot is the most commonly used explant, both in monopodial and sympodial orchids. Shoot tip culture was successfuly

standardised in *Vanda* (Kunisaki *et al.*, 1972), *Phalaenopsis* (Intuwong and Sagawa, 1974), *Paphiopedilum* (Stewart and Button, 1976) and *Renantanda* (Karim *et al.*, 1992).

Rhizome formation from shoot tip culture of *Cymbidium* was observed by Hasegawa (1992). In *Phalaenopsis* and *Doritaenopsis*, by culturing shoot tips of flower stalk buds, green protocorm like bodies with high multiplication capacity were induced using New Dogashima Medium (NDM) containing 0.1 mg l^{-1} NAA and 1 mg l^{-1} BA. When shoot tips from *in vitro* grown plants of *Arundina bambosifolia* and *Phaius tankervilleae* were cultured (Nagaraju and Parthasarathy, 1993) heaviest plantlets were produced on Raghavan and Torrey medium.

2.2.1.1.2 Inflorescence stalk

In certain orchids like *Phalaenopsis* and *Doritis* the inflorescence stalks are capable of producing plantlets. Young inflorescence stalk nodal explants is the preferred explant for such orchids as well as their hybrids (Rotor, 1949; Tse *et al.*, 1971; Intuwong *et al.*, 1972; Arditti *et al.*, 1977; Tanaka *et al.*, 1988; Ichihashi, 1992; Xiong and Yazawa, 1995). *Ascofinetia* (Intuwong and Sagawa, 1973), *Aranda* (Goh and Wong, 1990) *Dendrobium* (Singh and Sagawa, 1972), *Vanda* (Sagawa and Sehgal, 1967) and *Cymbidium* (Kim and Kako, 1984). Multiple shoots were reported to form from the flower stalk cuttings in *Thunia alba* (Singh and Prakash, 1984).

2.2.1.1.3 Leaves

The advantage of using leaves or other organs as explant is that only very little portion of the plant is sacrificed in the process.

Young leaves and leaf tips of *Aranda, Epidendrum, Rhyncostylis, Cattleya* and *Phalaenopsis* have been successfully cultured *in vitro* on VW or MS medium (Goh, 1989; Vij and Pathak, 1990). Tanaka and Sakanishi (1977) successfully demonstrated the formation of protocorm like bodies and plantlets from young *Phalaenopsis* leaves under the influence of NAA and BA in the medium.

Development of callus, PLB's and plantlets from leaf segments (leaf base, leaf top) depends upon the cultivar and medium used. Callus formation from the leaf base was observed in *Cattleya* (Fu, 1978) and regeneration of plantlets was observed in *Aranda, Ascocendra* and *Cattleya* (Fu, 1979).

Rapid multiplication of *Phalaenopsis* through leaf tissue culture was achieved by Latha and Seeni (1991) using Knudson C. (1946) medium supplemented with minor elements of Mitra *et al.* (1976), coconut water, peptone, casein hydrolysate, NAA and BA.

2.2.1.1.4 Root

The inherent regeneration potential of roots have been exploited in the *in vitro* culture of orchids like *Epidendrum, Phalaenopsis* and *Rhyncostylis*. Direct shoot buds were found in medium supplemented with IAA in root culture of *Rhyncostylis* (Sood and Vij, 1986). Aerial roots have yielded callus and plantlets in *Epidendrum* (Stewart and Button, 1978) and *Dendrobium* (Sagawa and Kunisaki, 1982). *Bletilla striata* is the first terrestrial orchid to have plantlets induced from its root tips (Yam and Weatherhead, 1991).

Root tip culture was also attempted for clonal propagation of *Phalaenopsis* (Tanaka *et al.*, 1976). A practical method was developed using aerial

root tips from adult plants (Kobayashi *et al.*, 1991). Latha and Seeni (1991) developed a rapid method of clonal propagation using *in vitro* grown roots of the hybrid *Phalaenopsis* Mab x Eagle.

2.2.1.2 Size of the explant

The size of the explant determines the survival and establishment of culture. Hasegawa *et al.* (1985) demonstrated that the longest shoots were formed from the rhizome tip of 3 mm length.

In *Phalaenopsis*, the length of flower stalk cuttings has got pronounced influence on shoot development and multiplication (Tanaka *et al.*, 1988). Shoot production was enhanced when shorter cuttings were used. Maximum shoot formation (87%) occurred when 1 cm cuttings were cultured. Also the number of buds remaining dormant decreased with shorter cuttings.

2.2.1.3 Position of explant

Besides the type and the size of the explant, its relative position on the plant determines the performance *in vitro*.

Urata and Iwanaga (1965) showed that secondary flower stalks were developed on the cuttings excised from the upper position on the flower stalk, while shoots were developed on those obtained from the lower position in *Phalaenopsis*. Similarly, Tanaka and Sakanishi (1978), while culturing *Phalaenopsis* flower stalks, observed that buds on the upper position had a tendency to remain dormant while in the basal sections they grow vigorously.

Protocorm like bodies were formed on more than 50 per cent of the sections taken from the top 3 cm of the flower stalk in *Phalaenopsis* (Homma and Asahira, 1985). Sections from near the tip of the flower stalk gave best results for adventitious bud development in both *Phalaenopsis* and *Doritaenopsis* (Lin, 1986).

2.2.1.4 Age of the mother plant

Age of the mother plant is an important factor determining the success of the tissue culture method. In majority of the orchids, mature plant parts did not respond at all to *in vitro* culture.

Lin (1986) reported that the most suitable stage of the stalk for the induction of PLB from internodes is following its formation and before the first flower becomes visible.

The percentage of shoot formation was higher in younger scapes (fully formed flower bud stage) than in scapes from full bloom flower stalks or in scapes with intact flower buds. The full bloom inflorescence had old scapes. Thus the percentage of shoot formation was low and scapes from intact flower buds were too young to produce shoots in *Oncidium* and *Phalaenopsis* (Nuraini and Shaib, 1992).

2.2.2 Culture medium

The media used in orchid seed culture range from simple three salt solutions to complex ones which contain 20 or more salts (Singh, 1992). Many proliferate very easily on a very simple medium such as Knudson C (1946) or the more stable Vacin and Went medium (1949). Orchids like *Cattleyas* have shown good response in somewhat more concentrated Murashige and Skoog medium (1962)

and media like Burgeff (N_3f) are used exclusively for *Paphiopedilum* seeds. The suitability of any medium is in turn governed by the physical and chemical state of the medium.

2.2.2.1 Mineral salts present in the medium

In trials with 12 media, Knudson C medium modified with chelated iron, microelements, coconut milk, banana and charcoal gave satisfactory seed germination and seedling growth with five genera namely, *Cattleya, Cymbidium, Paphiopedilum, Phalaenopsis* and *Eulophidium* (Rosa *et al.*, 1977).

Baker *et al.* (1987) observed germination and protocorm development of *Phalaenopsis* were the best in VW medium. Hinnen *et al.* (1989) observed that increased NH_4^+ and NO_3^- concentration promoted shoot growth but decreased root growth. High Cl^- and Na^+ concentration had a positive effect while $MgSO_4$, $Na_2H_2PO_4$ and $CaCl_2$ hardly affected the growth of *Phalaenopsis*.

In *Dendrobium fimbriatum* highest seed germination (91%) was obtained in Nitsch (1972) followed by MS medium (85%), whereas earlier formation of PLB's and their greatest volume were obtained on MS medium (Kumaria and Tandon, 1991).

Culture of *Dendrobium phalaenopsis* on modified MS with nitrate and ammonium (forming a constant level of total nitrogen 30 mM) was much better than in 5 mM or 10 mM of the total nitrogen given in the form of ammonium. Root formation was greatest with ammonium at 5 mM in combination with nitrate (Gandawidjaja, 1980). MS medium was generally better than Knudson C or White's (1943) medium (Fu, 1978; Lee *et al.*, 1984; Huang, 1984).

2.2.2.2 Physical state of the medium

Better growth and development of more shoots are found in a culture medium solidified using 8 g l^{-1} of agar than in a more solid one of 14 g l^{-1} (Kim *et al.*, 1979). According to Singh (1987), liquid medium is more suitable for growing mature embryo than solid medium. Adelberg *et al.* (1992) observed that proliferation of mericlones on the polypropylene membrane (Celgard) resulted in more harvestable plants than on agar medium. Membrane grown plants were also larger and unlike agar based cultures, membrane cultures did not require routine transfers to fresh medium. Kerbauy (1993) discovered that high levels of agar and sucrose promoted longitudinal root growth and lateral branching in particular, whereas relatively low concentration of both the compounds favoured the formation of PLB's.

The effect of liquid media on the orchid embryo germination may be due to various factors like better aeration, increased surface area and dilution of inhibitors.

Use of liquid media for the culture of immature embryos not only accelarates, the early stages of development but also saves 8-10 months of the time interval between pollination and flowering plant production in *Epidendrum radicans* (Singh and Prakash, 1985).

2.2.2.3 pH of the medium

The pH of the medium is found to influence the growth of the cultures as it regulates, the solubility and availability of nutrients to the plantlets.

Plant cells in culture require an acidic pH and an initial pH of 5.5 to 5.8 is optimum (Gamborg and Shyluck, 1981). They also reported that, pH changes during the growing cycle of a cell suspension culture.

In seed culture of *Paphiopedilum* it is reported that optimal germination occurred at a pH of 6.0. In the presence of light, the growth was clearly better at a low pH and a pH of 7.0 strongly inhibited growth (Pierik *et al.*, 1988).

There is little literature concerning the effects of the pH on orchid seed germination. Withner (1959) was of the opinion that the pH does not appear to have a definite effect. However, Reyburn (1978) observed in *Cypripedium* that dark germination was optimal at pH 5.5 to 6.0 whereas a pH of 7.0 was strongly inhibitory. In most studies on *Paphiopedilum*, germination was obtained at a pH of 5.0 to 5.6 (Flamee, 1978; Ernst, 1980; Fast, 1971; Thomale, 1957).

2.2.2.4 Carbon source

Most of the orchid seeds utilize disaccharides (sucrose) as a carbon source. Sucrose supports initial germination and subsequent growth and its effects, in both autoclaved and filter sterilized conditions, depend on its concentration. In clonal propagation of *Phalaenopsis* by shoot tip culture, yellow coloured PLB's developed on medium containing 2 per cent sucrose. The PLB's turned green when subcultured on to medium without sucrose and it also resulted in the production of numerous PLB's (Intuwong and Sagawa, 1974). Sucrose promotes organogenesis at suboptimal concentrations and protocorm proliferation at supraoptimal concentrations (Arditti, 1979). Some species of *Cymbidium* however, respond more favourably to glucose, while those of *Phalaenopsis* prefer, fructose to glucose.

In *Paphiopedilum*, germination of seeds did not occur without sugar in the medium. However, the highest germination occurred on a medium with the lowest sugar concentration, and decreased with further increase in the sugar concentration (Pierik *et al.*, 1988).

Good growth of *Phalaenopsis* seedlings was obtained in a medium containing 1.5 per cent sucrose (Hinnen *et al.*, 1989).

2.2.2.5 Vitamins

Much literature is not available on the role of vitamins in the performance of orchids *in vitro*. A study on the vitamin requirement of protocorms of *Vanda* hybrids showed that nicotinic acid was indispensable, for its absence in the medium affected protocorm multiplication considerably (Mathews and Rao, 1980).

The addition of L-glutamine (500-1200 mg l^{-1}) in MS medium was found to be necessary for the speedy proliferation of axillary buds in rosewood micropropagation (Raghavaswamy *et al.*, 1992).

According to Thorpe and Patel (1984) thiamine is the most often added vitamin, followed by nicotinic acid and pyridoxine.

2.2.2.6 Plant growth regulators

According to Krikorian (1982) the success of an *in vitro* system is directly influenced by the correct growth regulator used and its optimum concentration. The commonly used plant growth regulators belong to auxin and cytokinin groups. Small amounts of hormones are also provided by organic additives used in the media.

2.2.2.6.1 Auxins

Auxins generally promote callus growth, growth of meristem, and the morphogenesis of cells in interaction with cytokinin. Most commonly used auxins are 2,4-D, IBA, NAA, IAA, 2,4,5-T and PCPA.

Soediono (1983) observed that proliferation of *Dendrobium* PLB's and plantlet formation were rapid when the medium contained 10 mg l^{-1} NAA. Regeneration of *Cattleya* PLB's occurred only in media containing NAA (Kerbauy, 1991). Devi and Deka (1992) found that IAA and NAA at 1 mg l^{-1} enhances the growth of hybrid seedlings of the cross *D. moschatum* x *D. amoenum*. Addition of 2 mg l^{-1} IBA to the medium was necessary for the root formation of *Phalaenopsis* and *Dendrobium* (Nuraini and Shaib, 1992). IBA at 0.1 mg l^{-1} was the best for producing many tall rooted shoots in *Dendrobium* (Lim *et al.*, 1993).

2.2.2.6.2 Cytokinins

Cytokinins generally promote cell division, adventitious bud and axillary bud formation and inhibits root formation. The most commonly used cytokinins are BA, Kinetin and 2ip.

Flower stalk sections of *Phalaenopsis* and *Doritaenopsis* when cultured by Lin (1986) on modified VW medium with BA 1 or 5 mg l^{-1}, resulted in formation of PLB's on epidermis and cut surface in 30 days. Adventitious buds were formed and grew into plantlets after 60 days of culture.

According to Jambor-Benezer *et al.* (1986), buds from inflorescence stem of *Phalaenopsis amabilis* soaked in 100 ppm BA for 1-3 hours and cultured *in vitro* gave best results.

Kim and Kako (1982) found that the addition of BA encouraged the formation of PLB's and development of shoots. High level of BA at the rate of 10 mg l^{-1} prevents the shoot growth from rhizome in *Cymbidium faberi* (Hasegawa *et al.*, 1985).

In rhizome culture derived from the germinated seeds, 10 mg l^{-1} BA was effective in promoting organogenesis and 1 mg l^{-1} BA resulted in the production of shoots and rhizomes in *Cymbidium* (Paek *et al.*, 1987). Better shoot production was observed from rhizomes when exposed to 10 mg l^{-1} BA for 10 or 20 days followed by 0.5 mg l^{-1} BA,then transfer to a medium with no BA,or exposure to 10 mg l^{-1} BA for 30 or 40 days (Paek *et al.*, 1990).

Leaves of Laeliocattleya cultured in medium with 0.5 to 1.0 mg l^{-1} BA gave the best yield of 67 shoots per explant than in a medium without BA (Matos and Garcia, 1991).

From foliar peels of *Rhyncostylis* only non-organogenetic callus was produced with KIN at 2 mg l^{-1} + IBA, where as KIN alone at 2 mg l^{-1} gave excellent results with many PLB's each of which developed into a complete plantlet (Vij and Kaur, 1992).

2.2.2.6.3 Auxin-cytokinin combinations

The type and extent of organogenesis is determined by auxin to cytokinin

ratio (Skoog and Miller, 1957). Shoot induction is promoted when cytokinin level is higher than the auxin. Root induction can be achieved by increasing auxin level relatively over cytokinin level. Intermediate concentrations of these will tend to produce unorganised tissue.

Vij and Pathak (1989) observed that when 5 mg l^{-1} BA + 1 mg l^{-1} NAA were added to the medium, 65 per cent of *Phalaenopsis* flower stalk stem segments formed adventitious buds and 17 per cent formed PLB's.

Highest rate of PLB formation (60%) occurred with 0.1 mg l^{-1} NAA and 20 mg l^{-1} BA in *Phalaenopsis* and *Doritaenopsis* flower stalk buds and PLB proliferation into plantlets occurred in a medium without growth regulators (Tokuhara and Mii, 1993).

Kim and Kako (1984) observed that the adventitious bud formation from floral parts of *Cymbidium* was enhanced by BA but inhibited by NAA. Leaf elongation, differentiation and the formation of PLB's from shoot apices of *Cymbidium* were enhanced by BA 0.1 mg l^{-1} and inhibited by NAA 0.5 mg l^{-1}.

Homma and Asahira (1985) reported that in *Phalaenopsis*, plantlets were successfully obtained from internodal sections of flower stalk in a medium with 5 mg l^{-1} NAA and 20 mg l^{-1} BA.

Widiastoety (1986) reported that the addition of 1 mg l^{-1} BA or NAA to the growing medium inhibited the formation of PLB's from shoot tips in Aranda. Addition of 2,4-D 2 mg l^{-1} or IAA 2 mg l^{-1} had little or no effect on PLB and shoot formation in *Cymbidium* (Gu *et al.*, 1987).

Rhizome formation in shoot tip cultures of *Cymbidium goeringii* was best with KIN 1 mg l^{-1} + NAA 0.1 mg l^{-1} and with KIN 0.1 mg l^{-1} + NAA 0.1 mg l^{-1} (Choi *et al.*, 1989). A low kinetin : NAA ratio gave good rhizome growth and a high ratio gave shoot formation and growth. A very high ratio of KIN 3 mg l^{-1} + NAA 0.3 mg l^{-1} induced *in vitro* flowering from rhizomes of *Cymbidium* (Paek *et al.*, 1989).

In *Cymbidium* cultures, the presence of auxins retarded leaf formation and development, while the cytokinins (2ip, KIN, BA) generally reduced rhizome growth and number of branches,except BA which induced shoot formation (Paek and Yeung, 1991).

Shimasaki and Uemoto (1991) found that MS medium containing 0.1 mg l^{-1} BA and 10 mg l^{-1} NAA was optimal for initiating rhizome development and subsequent plant regeneration from the culture of apical flower buds of *Cymbidium*. They also reported the production of multiple shoots from rhizome branches when transferred to the same media.

2.2.2.7 Medium supplements

Apart from the inorganic constituents of the media which give consistent results, certain complex organic additives are added to the media, which is found to influence the establishment and growth of *in vitro* cultures and these organic additives are termed as medium supplements. The organic medium supplements often do not give any definite results. Some of the complex substances commonly used are coconut water, tomato juice, adenine, peptone etc. The salient works on which are reviewed under broad groups.

2.2.2.7.1 Coconut water

The liquid endosperm of the coconut promotes growth and differentiation. A number of cell division factors are present in CW including diphenyl urea (Shantz and Steward, 1952), 9-B-ribofuranozyl zeatin (Letham, 1974) and a compound which co-chromatographs with zeatin riboside (Van Staden and Drewes, 1974). It also contains a large number of free amino acids including phenylalanine which has the cell division activity in soybean assays (Van Staden and Drewes, 1974).

According to Homma and Asahira (1985) internodal sections of *Phalaenopsis* flower stalk produced PLB's on a medium containing Thomale's macroelements, Ringe and Nitsch's minor elements and organic supplements together with 10 per cent coconut milk, 5 mg l^{-1} NAA and 20 mg l^{-1} BA. It was observed by Lam *et al* (1991) that XE medium containing coconut water alone or with 2ip were equally effective in inducing the protocorm sections of a hybrid *Phalaenopsis* (cv. Michelle x Michelle) to form new protocorms. The importance of coconut water for inducing PLB formation from shoot tip explants of *Phalaenopsis* without using plant growth regulators was reported by Ichihashi (1992).

Kusumoto (1980) reported that PLB's proliferated and the highest increase in fresh weight was attained with 10 per cent CW. For the initial culture of shoot tips of *Dendrobium* VW medium containing CW 15 per cent (v/v) was suitable (Soediono, 1983).

Shoot meristems of *Dendrobium* initiated callus in liquid medium especially with 15 per cent CW (Sharon and Vasundhara, 1990). Seeds from mature

pods of *Cypripedium calceolus* var. *parviflorum* were germinated on medium with 10 per cent CW (Chu and Mudge, 1992). Sharon *et al.* (1992) demonstrated that the protocorms of *Dendrobium* were raised *in vitro* from immature seeds using VW medium with 15 per cent CW.

2.2.2.7.2 Tomato juice

It was found that in *Aranda* Deborah culture both leaf and root formation was inhibited by tomato juice and only a slight increase in fresh weight was observed in cultures on 15 per cent tomato juice enriched medium (Loh *et al.*, 1978). Arditti (1967) reported that eventhough orchid seed germination percentage is high on tomato juice media, the mortality rate is also high. The protocorms which survive only very few grow and develop normally at a very fast rate. The rest fail to differentiate, forming instead, very large mass of undifferentiated tissue possessing multiple meristem.

2.2.2.7.3 Adenine

Adenine and adenine sulphate are known as cytokinin synergists. They increase the activity of cytokinins like BAP, Kinetin etc. and their effect is more pronounced when they are present along with other hormones.

Tanaka and Sakanishi (1985) reported that leaf segments of *Phalaenopsis amabilis* produced PLB's when they were excised from proximal unexpanded parts of the youngest leaf and cultured on a Murashige and Skoog medium supplemented with 1 ppm NAA, 10 ppm adenine and 10 ppm BA.

According to Sudeep (1994) adenine and adenine sulphate did not have any significant influence on shoot proliferation of axillary bud culture of *Dendrobium nobile*. However, adenine influenced the production of leaves and length of shoots in MS medium, in combination with NAA 2.0 ppm and BA 5.0 ppm. Adenine sulphate when added to the medium can enhance growth and shoot formation (Skoog and Tsui, 1948).

2.2.2.7.4 Peptone

Peptone is an organic compound which is composed (Arditti, 1977) of vitamins and amino acids as well as other compounds. In *Paphiopedilum*, growth and development of seedlings completely stoped on media without peptone in light and a concentration of 2.0 g l^{-1} was optimal (Pierik *et al.*, 1988).

Peptone was usefully employed in inducing PLB's from root cultures of *Rhyncostylis retusa* (Sood and Vij, 1986), *Vanda cristata* and *Cymbidium pendulum* (Vij, 1993), *Catasetum, Oncidium* and *Cyrtopodium* cultures (Kerbauy, 1984a, b; Sanchez, 1988) and in *Phalaenopsis* (Latha and Seeni, 1991). In an earlier study, Churchill *et al.* (1972) also suggested that orchid roots specifically require peptone which is a rich source of amino acids.

The addition of peptone greatly promoted the growth of PLB in *Doritaenopsis*, but more than 4 g l^{-1} increased the rate of death of PLB segments (Amaki and Higuchi, 1989).

Vij *et al.* (1991) reported on the morphogenetic response of floral buds of *Dendrobium crepidatum* and *D. pierardi* when cultured *in vitro* in Mitra *et al.* (1976) medium, supplemented with 2 g l^{-1} peptone/yeast extract, 1-2.5 mg l^{-1}

IAA/NAA and 1 mg l^{-1} BAP/Kinetin, the older buds matured into flowers, whereas the younger ones reverted to vegetative growth.

In *Dendrobium nobile*, it was observed that the best medium for multiple shoot production was peptone 40 ppm, NAA 2.0 ppm and BA 5.0 ppm in VW medium (Sudeep, 1994).

2.2.2.7.5 Activated charcoal

The addition of activated charcoal to plant tissue culture medium may have either beneficial or harmful effects. These effects may be attributed to at least four factors: (1) establishing polarity by darkening of the medium (2) irreversible adsorption of inhibitory compounds (3) irreversible adsorption of plant growth hormones and other organic compounds and (4) improving aeration of the culture medium (Constantin *et al.*, 1977; Fridborg and Eriksson, 1975; Fridborg *et al.*, 1978; Johansson, 1983; Weatherhead *et al.*, 1978). The effect of AC also depends upon the species, the stage of development and the concentration.

The addition of AC is beneficial for those species with a high release of phenolic compound in the culture medium. The higher the release of phenolic compounds the better the development is on a medium with a higher concentration of AC. In contrast, for other orchids without a visible release of inhibitory compounds AC is harmful or not stimulating (Waes, 1987).

Phalaenopsis seedlings generally exude phytotoxic phenolics, which diffuse into the medium. For that reason, activated charcoal has often been added to the medium (Ernst, 1975).

2.2.2.7.6 Triazoles

Triazoles are sterol inhibiting fungicides, which have got both fungicidal and plant growth regulator activity. The plant growth regulating properties of sterol inhibiting fungicides was reviewed by Fletcher in the year 1985. Triadimefon, one of the triazole derivative, was found to protect plants from injury due to drought, chilling and ozone (Fletcher and Nath, 1984).

2.2.2.7.7 Anti contaminants

One major problem in *Phalaenopsis* tissue culture was explant sterilization. Older tissue such as buds from basal parts of flower stalks, were especially difficult to decontaminate (Zimmer and Pieper, 1978). However, the difficulty was eliminated through the use of younger parts of flower stalks. The use of antimicrobials is another means of decreasing contamination rate (Arditti *et al.*, 1981; Tanaka *et al.*, 1988). However the use of antimicrobials caused inhibition of plant growth.

A method for cent per cent survival of flower stalk nodal culture of *Phalaenopsis* was developed (Johnson *et al.*, 1982) using an anticontaminant containing media. The media contained the following anticontaminants, Benlate (50 ppm), Nystatin (25 ppm) (two broad spectrum fungicide) and Pencillin G (100 ppm), Gentamycin (100 ppm) (two bactericides). But graphite should be added as the darkening agent instead of charcoal to get the effect.

2.3 PLB development

A method of rapid multiplication in orchids is through PLB formation. When shoot apices of *Cymbidium* was cultured on nutrient media, the explant first

turned green and then enlarged slowly as a small bulblet similiar to a protocorm which develop from an embryo. The PLB's (somatic embryos) further proliferated into clumps of protocorms each in turn developing into a new plantlet. When cut into small pieces and subcultured, these PLB's regenerate more protocorms and it has been estimated that it is possible to obtain more than four million plants in a year from a single explant (Morel, 1964).

In *Phalaenopsis*, PLB's were induced from shoot tips (Intuwong and Sagawa, 1974; Ichihashi, 1992), stalk tips of flower stalks (Homma and Asahira, 1985), internodal section of flower stalk (Homma and Asahira, 1985; Lin, 1986; Vij and Pathak, 1989) *in vitro* leaf (Tanaka and Sakanishi, 1977, 1985; Latha and Seeni, 1991) and *in vitro* roots (Tanaka *et al.*, 1976; Latha and Seeni, 1991).

Green PLB's with high multiplication capacity were induced by culturing shoot tips of flower stalk buds using New Dogashima Medium (NDM) containing 0.1 mg l^{-1} NAA and 1 mg l^{-1} BA. These PLB's when subcultured on the same medium, more than 10,000 PLB's were obtained from a few buds on a single flower stalk within one year. After transfer on to NDM containing no plant growth regulator, the PLB developed into plantlets (Tokuhara and Mii, 1993).

In *Doritaenopsis*, it was observed that segments from dorsal parts of PLB had a tendency to form plantlets, while segments from basal parts produced no plantlets (Amaki and Higuchi, 1989).

In *Dendrobium*, wounded protocorms proliferated to form a mass of callus and then differentiated into somatic embryos, whereas the intact protocorms developed directly into seedlings (Sharon *et al.*, 1992).

Recently Su (1995) reported the development of hyperhydric (vitrified) protocorm like bodies (h PLB's) in *Doritaenopsis*. The h PLB's had a translucent and turgid appearance which was due to lack of air volume in the intercellular spaces. The h PLB's had a lower capacity for shoot formation but a higher capacity for differentiation of new PLB's than the normal PLB's.

2.4 Culture environment

Light, temperature and humidity conditions provided inside the culture room plays a significant role in the success of any tissue culture medium.

2.4.1 Light

Murashige (1974, 1977) observed that light intensity, quality and duration are the three major factors affecting the growth of *in vitro* culture. He found that the optimum light intensity for shoot formation in a large number of herbaceous species to be around 1000 lux. The optimum day light period was considered to be 16 hours for a wide range of plants.

Different day lengths (8, 16 or 24 h) had no effect on the production of shoots from *Phalaenopsis* flower stalk cuttings cultured *in vitro*. However, shoot production was promoted when light intensity was decreased (Tanaka *et al.*, 1988).

In *Paphiopedilum ciliolare*, best germination occurred in darkness under a photoperiod of 16 h an increase in irradiance resulted in a decrease in germination and later caused high mortality and growth inhibition (Pierik *et al.*, 1988).

In darkness, a higher percentage of leaf tips formed shoots than those kept in light in *Pholidota*. Explants cultured in the light turned brown very quickly and a dark exudate (probably phenolic oxidates) appeared on the surface of the medium. It was known that phenolic compounds were secreted from cut ends of plant tissues and that the production of such compounds was increased by high intensity blue or near UV light. These compounds interfere with growth regulator activity (Anderson and Kasperbauer, 1973).

In *Bletilla striata*, a terrestrial orchid root tips tended to elongate in darkness, whereas callus formation occurred in the light (Yam and Weatherhead, 1991).

2.4.2 Temperature

Yeoman (1986) reported that the environmental temperature at the original habitat of a particular species should be taken into consideration while fixing the culture temperature under *in vitro* condition.

Tanaka and Sakanishi (1978) showed that in *Phalaenopsis* flower stalk culture, sprouting buds placed at 20°C or 25°C showed reproductive growth except for some on the basal sections which grew vegetatively. At 28°C, all buds developed vegetatively independent of their original position on the stalk.

Kerbauy (1984a) cultured micro inflorescence of *Oncidium varicosum* on liquid Knudson medium supplemented with 0.5 mg/l NAA under 16 hour photo-period and 25°C and obtained PLB's.

In *Paphiopedilum ciliolare*, highest germination occurred at 25°C temperature (Pierik *et al.*, 1988).

2.4.3 Aeration

Cuttings cultured in vessels plugged with rubber stoppers tended to produce flower stalks rather than shoots, as compared to those cultured in vessels covered with aluminium foil or plugged with a silicon stopper. Aeration was found to be important for getting high shoot production from flower stalk cutting culture, otherwise secondary flower stalks developed (Tanaka *et al.*, 1988).

2.4.4 Humidity

Humidity is rarely a problem except in arid climates, where rapid drying of the medium occurs. This can be reduced by the use of tightly closed containers, covering closures such as foam or cotton wool plugs with aluminium foil. In climates with high humidity, dehumidifier in the culture room may be advantageous. In some urban environments, it may be necessary to filter the air entering the culture room (Yeoman, 1986).

2.5 Hardening and planting out

Orchids are grown in special media, since roots need plenty of air around them at all times. The media used should provide support to the plant, supply water and nutrients to the roots and enough air for the roots to breathe.

Wainwright (1988) observed that the environment in a tissue culture container is that of very high humidity, low light levels and usually a constant

temperature. Plantlets leaving the environment are as a result very poorly adapted to resist the low relative humidity, high light levels and more variable temperature found *in vivo*.

Langford and Wainwright (1987) observed that physiologically the leaves grown *in vitro* are incapable of significant photosynthesis. The stomata are unable to close and as cuticular wax in the leaf surface is minimal, are unable to control water loss. Improper development of vascular connections between the shoot and roots may cause poor establishment of the plantlets.

A period of humidity acclimatization was suggested for the newly transferred plantlets to make them adapted to the outside environment (Hu and Wang, 1983). Light, temperature and relative humidity are the major factors to be controlled during acclimatization.

Sutter *et al.* (1985) found that the survival of the plantlets depended as much on the vigorous growth of the newly produced leaves as on the adoption of the leaves present at the time of planting out.

Ziu (1986) reported that the success in acclimatization of *in vitro* cultured plants is dependant not only on the post-transfer growth conditions, but also on the pre-transfer culture condition.

Kim *et al.* (1988) found that the survival rate of *Cymbidium kanran* plantlets transferred to pots in the greenhouse was increased from 53 to over 80 per cent by soaking the moss peat compost for 30 minutes in a disinfectant solution of 0.1 per cent Benlate. Best growth of two year old seedlings was obtained in forest top soil followed by forest top soil + shredded pine bark (1:1). In another trial, one

year old seedlings growing in shreded sphagnum moss and fed with MS nutrient solution grew better than those fed with Knudson C solution.

Lakshmidevi (1992) used brick and charcoal in equal proportions as a potting medium for *Dendrobium fimbriatum* and *D. moschatum* and obtained 70 and 40 per cent survival of plantlets respectively. Sharma and Tandon (1992) reported that a survival rate of 65 per cent was observed when the plantlets were transferred to a potting substrate comprising of charcoal fragments, brick bats and coconut fibres, under glasshouse condition.

Kumar (1992) observed that *in vitro* seedlings of *Dendrobium* recorded best survival per cent in pure charcoal followed by fern root and rubber seed husk when kept at a temperature between 29 and 35°C with RH between 70 and 90 per cent and illumination between 1000 and 1500 lux at pot level. Seedlings were watered daily and sprayed with a NPK nutrient solution on alternate days.

Thomas and Thomas (1992) transplanted *Odontoglossums* and their hybrids into perlite and rockwool media and found that orchids in perlite made slower growth initially, but after a year started to grow more rapidly with extensive root system.

Agrawal *et al.* (1992) showed that the plantlets of *Vanilla walkeriae* were hardened for four weeks and then successfully planted out in sand : soil : brick (4:1:1) mixture. Irrigation with Knudson C nutrient medium on alternate days during the hardening stage was helpful in the establishment of plants and their further growth.

Acclimatization was best done in peat moss for improving stem diameter and plantlet height although perlite was best for increasing root number (Lim *et al.*, 1993).

One important work on the post-transplantation growth of *Phalaenopsis* hybrid seedlings was reported by Seeni and Latha (1990) from TBGRI, Palode. They observed that axenic seedlings of the hybrid cultivar Fire Water Ponce recorded 100 per cent survival even without any acclimatization or pretreatment of the seedlings. The medium which was found satisfactory was broken tiles, followed by charcoal, cassava pith, rubber seed husk and coconut husk. The foliar nutrient solution which was found to be the most effective in terms of rapid leaf and root growth was a combination of commercial diammonium phosphate and potassium nitrate (20:10:10 NPK).

Material and Methods

MATERIALS AND METHODS

The studies on the micropropagation of *Phalaenopsis* were carried out at the Plant Tissue Culture Laboratory attached to All India Co-ordinated Floriculture Improvement Project, Department of Pomology and Floriculture, College of Horticulture, Vellanikkara, during 1993-96. The details pertaining to the experimental material, methodology of experiments and analytical techniques adopted are presented stepwise in this chapter.

In the present study, green pod culture of *Phalaenopsis* and the response of various explants to rapid cloning were attempted through enhanced release of axillary buds and through the induction of protocorm like bodies.

3.1 Explant

Details of different types of explants used for the study are given below:

1. Enhanced release of axillary buds	
a) Field grown plant	Inflorescence stalk node
b) *In vitro* plantlet	Apical bud, shoot node and basal portion
2. Induction of protocorm like bodies	
a) Field grown plant	Inflorescence stalk tip, stalk node, stalk internode, flower bud, sepal, petal, flower stalk, gynostemium and pollinia
b) *In vitro* plantlet	Shoot node, leaf and root.

3.1.1 Source of explants and pretreatment

The inflorescence stalk explants were collected from the plants in the orchidarium of AICFIP and from the private growers. Leaf and aerial roots were collected from the plants in the orchidarium.

Plantlets of 'Diana Pinky' was used in *Phalaenopsis* for carrying out axillary bud, leaf and root culture studies.

The field grown plants which were used as explant source were regularly sprayed with fungicides and antibiotics.

3.1.2 Collection and preparation of explants

The method of preparation of explants for inoculation varied with the source and type of explants.

3.1.2.1 *In vivo* plants

Inflorescence stalk explants were collected from mature stalk with all the flowers faded and dropped off. Due to the shortage of inflorescence stalks, trials were not conducted with young stalks. Mature stalks were severed at the base after retaining the basal node on the stalk for the development of keiki. The stalks were swabbed with cotton dipped in 70 per cent alcohol, then cut into nodal and internodal sections, washed in teepol water followed by distilled water. The bracts covering the buds were then removed and the sections were subjected to fungicidal and antibiotic treatments (Table 1 and 2). Using sterile blotting paper, the materials were made free of water and then wiped with cotton dipped in 70 per cent alcohol and then transferred to sterile room.

The flower buds collected were washed and subjected to various sterilization treatments as detailed in Table 1 and 2. The different parts of the flower bud, viz., sepal, petal, gynostemium, flower stalk, pollinia and flower bud as a whole were used for culturing.

Leaf explants were collected from young emerging leaves of length 3.0 cm and root explants from young emerging aerial roots of 2.0 cm length from the field grown plants. These explants were then washed in tap water, then in teepol water, followed by distilled water wash, wiped with tissue paper and then swabbed with cotton dipped in 70 per cent alcohol and moved to the sterile room. Square pieces of size 2.0 cm made out of the leaves and root piece of 1.5 cm length were used for culturing.

3.1.2.2 Pod culture

Capsules of *Phalaenopsis* hybrid 'Red Lip' were used for the study. The flowers were artificially pollinated about 10 days after the opening of flowers. The fruits were harvested at 70, 80, 90 and 100 days maturity. Capsules were rinsed with tap water and then with teepol water and rinsed with distilled water. The capsules were surface sterilised using 0.1 per cent mercuric chloride for 30 minutes, then washed with distilled water and then wiped dry using blotting paper and moved to the laminar flow chamber. Just before inoculation, the capsules were flamed twice after dipping in absolute alcohol and then the capsules were split open and the seeds inoculated.

3.1.2.3 *In vitro* plantlets

Different parts of the *in vitro* plantlet, viz., axillary shoot, leaf and root were used for the study. Since grown *in vitro*, the tedious task of sterilization could be avoided. Apical bud, first node, shoot node (second and third node) and basal portion (fourth node) of the plantlets were used. Whole leaf and leaf segments, whole root and root segments were also used as explants.

3.1.3 Standardisation of surface sterilization methods

Surface sterilization of explants was done in order to make the explants free of contaminations and micro-organisms. Details of the sterilants and anti-contaminants used for surface sterilization are given in Tables 1 and 2.

Observations were made on the percentage of cultures bleached, blackened, infected by fungi, bacteria and survived on twenty cultures after 2 weeks for nodal and internodal explants and after 4 weeks for flower bud, leaf and root explants. For this purpose, MS medium with half strength inorganic salts containing 3.0 per cent sucrose and optimum level of growth regulator was used.

The explants after surface sterilization were rinsed four times with sterile distilled water and dried by carefully transferring them onto sterile filter paper pieces on sterile petridish. The end portions from both sides of the inflorescence segments were removed and made to 2.0 cm long pieces. The leaf pieces were trimmed on the four sides and size reduced to 1.0 cm. Root pieces were also trimmed to a size of 1.0 cm.

Table 1. Sterilants used for surface sterilization

Sterilants	Concentration (%)	Duration of treatment (min)	Explants
Mercuric chloride	0.1	5, 10, 15	Inflorescence stalk node, internode, flower bud, leaf and root
Mercuric chloride	0.2	5, 10, 15	Inflorescence stalk node, internode and flower bud
Mercuric chloride	0.01	20, 30	Infloresence stalk node and internode
+			
Mercuric chloride	0.10	10	
Sodium hypochlorite	10.00	10, 20	Inflorescence stalk node and internode
Alcohol	50.00	1	Flower bud

Table 2. Anticontaminants used for surface sterilization

Anticontaminants	Concentration (%)	Duration of treatment (min)	Explants
Fungicides			
1. Emisan	0.10	30	Inflorescence stalk node and internode
2. Emisan	1.00	15, 30	Inflorescence stalk node, internode, flower bud, leaf and root
3. Bavistin	0.10	30	Inflorescence stalk node and internode
4. Bavistin	1.00	30	Inflorescence stalk node and internode
Bactericides			
1. Norfloxacin	1.00	10, 20, 30	Inflorescence stalk node, internode, leaf and root
2. Streptomycin	0.01	60, 90	Inflorescence stalk node and internode
3. Pencillin	0.01	60, 90	Inflorescence stalk node and internode
4. Roscillin	1.00	10	Leaf and root

3.1.4 Type of explant

To study the response of explants on multiple shoot production *in vitro*, three types, viz., apical bud, shoot node and basal portion, taken from *in vitro* grown plantlets were cultured on MS medium with half strength of inorganic salts, 1.5 per cent sucrose and containing optimum level of growth regulators. Observations were recorded for the number of shoots, leaves and roots produced per culture at the time of inoculation and at an interval of 3 weeks, for a total culture period of 12 weeks in MS medium containing half strength of inorganic salts, 1.5 per cent surcrose and optimum level of growth regulators.

Gradation of phenolic exudates

A trial was conducted to know the change in the quantity of phenolic exudates using five different explants, namely, apical bud, first node, shoot node and basal portion. The explants were cultured on 1/4 MS medium containing CW 5 per cent and sucrose 1.5 per cent and visual observations were recorded after two weeks of culturing.

3.1.5 Position of explant

Trials were conducted with explant from five positions, ie., apical bud, first node, second node, third node and fourth node, to know whether the position of explant is having any influence on the elongation of buds formed from the node. Observations were recorded on the length of the bud at 20, 40 and 60 days interval in MS medium containing half strength of inorganic salts, 1.5 per cent sucrose and optimum level of growth regulator.

3.2 Culture media

Three media were employed for the study, namely, MS (Murashige and Skoog, 1962), VW (Vacin and Went, 1949) and modified KC (Rowe and Richardson, 1975). The composition of the media are given in Appendix-I, II and III.

3.2.1 Media preparation

MS medium was prepared according to the standard procedures given by Gamborg and Shyluck (1981). Stock solutions were prepared by dissolving required quantities of major and minor nutrients in distilled water and were stored in amber coloured bottles under refrigerated conditions. The chemicals used for the preparation of the media were of analytical grade from British Drug House (BDH), Sisco Research Laboratory (SRL), Merck or Sigma. The nutrient stock solutions were prepared fresh monthly and the vitamins, amino acids and phytohormones were prepared fresh weekly. Specific quantities of the stock solutions and phytohormones were pipetted out into a 1000 ml standard flask. To this, the required amount of sucrose and inositol were added and the volume was made up with double glass distilled water. Then the pH of the made up solution was checked and adjusted to 5.8. For adjustment of pH one per cent NaOH/HCl was employed. After bringing the solution to the desired pH, agar was added and the final volume of the medium was made upto 1000 ml.

Agar was then dissolved in the medium by heating the medium at a temperature of 90-95 °C. When all the agar had completely dissolved the medium

was poured hot into clean dry culture vessels. The culture vessels were then closed tightly using sterilized non-absorbant cotton wool plugs.

Knudson's C medium was prepared by the same procedure as described for MS medium. For preparing VW medium the required quantity of chemicals were taken and dissolved fresh in distilled water. Sucrose and phytohormones were then added and the volume was made up after which pH was adjusted. Agar was then added and melted.

After preparation, the media was heats sterilised at a pressure of 1.1 kg/cm^2 at 121°C for 20-30 minutes. After sterilization, the culture vessels were immediately transferred to an air conditioned culture room.

3.3 Explant inoculation

The explants were inoculated under aseptic conditions in a 'Klenzaids' laminar air flow cabinet. For this, the neck of the culture vessel was brought near the flame from a gas burner, cotton plug was removed and the neck flamed over the gas burner. The surface sterilised explants were then quickly transferred to the heat sterilised medium in the culture vessel and then plugged tightly with cotton plug. Utmost care was taken to avoid contamination at the time of inoculation.

In order to provide the optimum conditions for growth and establishment of the explant, the culture vessels were transferred to a culture room where the temperature was maintained at 27 $\pm$ 2°C. White fluorescent tube lights were provided to meet the light requirements (2000 lux) of the explant. Photoperiod was fixed as 16 hours per day which was regulated by a diurnal timer. For the trial on the effect of serial subculturing the subculture interval was one week whereas for all

the other experiments, subculturing was carried out only after the period of study of each of the experiments.

3.4 The routes adopted for micropropagation

3.4.1 Enhanced release of axillary buds

The explants used for enhanced release of axillary buds were inflorescence stalk node, apical bud, shoot node and basal portion of *in vitro* grown plantlets.

3.4.1.1 Effect of mineral salts in the media

To identify the best mineral salt composition favouring multiple shoot production, a trial was conducted using media with six different mineral salt composition. They were

1/4 MS - MS medium containing one fourth concentration of inorganic salts and full strength of organic compounds.

1/2 MS - MS medium containing half the concentration of inorganic salts and full strength of organic compounds.

3/4 MS - MS medium containing three fourth concentration of inorganic salts and full strength of organic compounds.

MS - MS medium containing full strength of both inorganic and organic compounds.

VW - Vacin and Went medium containing full strength of inorganic and organic salts.

KC - Modified Knudson's C medium containing full strength of inorganic and organic salts.

Optimum level of growth regulator was used in all the media and observations were recorded on the number of shoots, leaves and roots produced per culture after 4 and 8 weeks of culturing.

3.4.1.2 Effect of physical state of the media

Physical state of the media was found to influence the growth of the cultures and so a trial was conducted using the best basal medium identified from the earlier studies. Two states of the media, namely liquid (with filter paper bridge) and semi solid (solidified with 0.7% agar) were tried using shoot node. Observations were recorded on the number of shoots, leaves and roots produced per culture at the end of 4 and 8 weeks of culturing. The medium used was 1/4 MS with 12 treatment combinations involving growth regulators and medium supplements.

3.4.1.3 Effect of carbon source

Sucrose was used as the carbon source in the media tried. Level of sucrose was reported to influence the growth of shoots *in vitro* and so a trial was conducted with sucrose at 6 levels (0, 1.5, 3.0, 4.5, 6.0 and 7.5 %) in 1/2 MS with optimum level of growth regulators. Observations were recorded on number of shoots, leaves and roots per culture at the end of 4 and 8 weeks of culturing.

3.4.1.4 Effect of vitamins

It was reported that among the different types of vitamins, Thiamine-HCl

is the most important one in favouring the growth of the cultures, and so a trial was conducted with Thiamine-HCl at 6 levels (0, 5, 10, 15, 20 and 25 ppm) in 1/2 MS with optimum level of growth regulators. Observations were recorded for the number of shoots, leaves and roots produced per culture at the end of 4 and 8 weeks of culturing.

3.4.1.5 Effect of growth regulators

Growth regulators present in the media were found to have a profound influence on the growth of the cultures. Different treatments were tried for inflorescence stalk node and shoot node explants.

Inflorescence stalk node

Trials were carried out on 1/2 MS medium containing 3 per cent sucrose. The details are given below.

Treatment combinations	Culture period
3 x 2 levels of BA (5, 10 and 20 ppm) and NAA (1 and 2 ppm)	8 weeks
3 x 2 levels of BA (5, 10 and 20 ppm) and 2,4-D (1 and 2 ppm)	8 weeks
3 x 1 x 1 levels of BA (5, 10 and 20 ppm), NAA (2 ppm) and 2,4-D (2 ppm)	8 weeks
3 x 3 x 1 levels of BA (5, 10 and 20 ppm), adenine (10, 20 and 40 ppm) and NAA (1 ppm)	12 weeks

Observations recorded

1. Number of days taken for the swelling of nodes
2. Number of days taken for bud emergence
3. Number of days taken for the buds to attain a size of 0.5 cm

Shoot node

The best basal medium and sucrose combination identified earlier were selected (1/4 MS, 1/2 MS and 1.5% sucrose). Trials were carried out either on 1/4 MS or 1/2 MS medium containing 1.5 per cent sucrose. The details of the treatment combinations are presented below.

Treatment combinations	Media	Culture period
6 x 1 x 1 levels of NAA (0, 1, 2, 3, 4 and 5 ppm), BA (10 ppm) and adenine (10 ppm)	½ MS	4 weeks 8 weeks
6 x 1 x 1 levels of BA (0, 5, 10, 15, 20 and 25 ppm), adenine (10 ppm) and NAA (1 ppm)	½ MS	4 weeks 8 weeks
5 x 3 levels of BA (1, 5, 10, 15 and 20 ppm) and NAA (0, 0.1 and 1.0 ppm)	1/4 MS	4 weeks 8 weeks
4 x 3 levels of BA (5, 10, 15 and 20 ppm) and 2,4-D (0, 2.5 and 5.0 ppm)	1/4 MS	4 weeks 8 weeks
5 x 1 x 3 levels of BA (1, 5, 10, 15 and 20 ppm), NAA (2 ppm) and 2,4-D (0, 2 and 4 ppm)	1/4 MS	4 weeks 8 weeks
6 levels of KIN (0, 10, 20, 30, 40 and 50 ppm)	1/4 MS	4 weeks
1 x 6 levels of KIN (20 ppm) and NAA (0, 1, 2, 3, 4 and 5 ppm)	1/4 MS	4 weeks
6 x 1 x 1 levels of KIN (0, 5, 10, 15, 20 and 25 ppm), NAA (2 ppm) and 2,4-D (2 ppm)	1/4 MS	4 weeks
6 x 1 x 1 x 1 levels of KIN (0, 5, 10, 15, 20 and 25 ppm), BA (5 ppm), adenine (20 ppm) and 2,4-D (5 ppm)	1/4 MS	4 weeks

Observations recorded

1. Number of shoots/culture
2. Number of leaves/culture
3. Number of roots/culture

3.4.1.6 Effect of medium supplements

Trials were conducted with defined medium supplements (peptone and adenine) and undefined medium supplements (coconut water and tomato juice) for enhancing the growth of the cultures. The details of the trials are presented below.

Coconut water

To study the influence of coconut water on axillary bud release from inflorescence stalk node, six treatment combinations were tried. The experiment was conducted with 1/2 MS basal medium with 3.0 per cent sucrose as the control and the details are as follows.

T_1 - BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm

T_2 - BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm + CW 15%

T_3 - BA 20 ppm + NAA 1 ppm + adenine 10 ppm

T_4 - BA 20 ppm + NAA 1 ppm + adenine 10 ppm + CW 15%

T_5 - Control

T_6 - CW 15%

Observations were recorded on the number of days taken for nodal swelling, bud development and the buds to attain a size of 0.5 cm, during 10 weeks of culture period.

Trials were conducted with different explants viz., apical bud, first node, shoot node and basal portion of plantlet at six levels of coconut water (0, 5, 10, 15, 20 and 25 %) on 1/4 MS medium containing 1.5 per cent sucrose. Observations were recorded on the number of shoots, leaves, roots produced and length of longest leaf per culture after 4, 8 and 12 weeks of culturing.

A trial was conducted to know whether any difference exist between the usage of tender and mature coconut water and also to observe the difference, if any, by the usage of fresh, 2 days old, 4 days old and 6 days old coconut water in the medium. Coconut water stored in the refrigerator was used for the study. Observations were recorded on the number of shoots, leaves and roots produced per culture at the end of 8 weeks.

Tomato juice

Trials were conducted with four levels of tomato juice (0, 3.0, 6.0 and 9.0%) for the induction of multiple shoots using shoot explants on 1/4 MS medium containing BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm and sucrose 1.5 per cent. Observations recorded (Culture period : 4 and 8 weeks).

1. Number of shoots/culture
2. Number of leaves/culture
3. Number of roots/culture

Adenine

Trials were conducted to study the influence of adenine in inducing

multiple shoots using shoot explants, on 1/4 MS medium containing sucrose 1.5 per cent and the details of treatment are given below.

Treatment combinations

6 levels of BA (0, 4.0, 8.0, 12.0, 16.0 and 20.0 ppm)

5 x 1 levels of adenine (4,0, 8.0, 12.0, 16.0 and 20.0 ppm) and BA (16.0 ppm)

6 x 1 x 1 levels of adenine (0, 5.0, 10.0, 15.0, 20.0 and 25.0 ppm), BA (10.0 ppm) and NAA (1.0 ppm)

Observations recorded (culture period 4 and 8 weeks)

1. Number of shoots/culture
2. Number of leaves/culture
3. Number of roots/culture

Peptone

Trials were conducted to study the influence of peptone in inducing multiple shoots using shoot node in 1/4 MS medium containing BA 20.0 ppm and NAA 1.0 ppm with sucrose at 1.5 per cent level. Five levels were tried (0, 500, 1000, 1500 and 2000 ppm) for three culture periods namely 4, 8 and 12 weeks.

Observations recorded

1. Number of shoots/culture
2. Number of leaves/culture
3. Number of roots/culture

3.4.1.7 Control of discolouration of media and explants

To reduce media and explant discolouration, different treatments were tried for explants such as inflorescence stalk node and shoot node of plantlet.

Inflorescence stalk node

Five treatments to reduce phenolic blackening of inflorescence stalk node were tried in 1/2 MS medium containing BA 10.0 ppm and NAA 1.0 ppm with 3.0 per cent sucrose.

Treatment

T_1 - Adding AC (0.1%) in the media

T_2 - Rinsing the explants with PVP 1000 ppm before inoculation

T_3 - Rinsing the explants with ascorbic acid 1000 ppm before inoculation

T_4 - Keeping the cultures in the dark

T_5 - Use of liquid media with filter paper bridge

Observations recorded (Culture period : 4 weeks)

Percentage culture bleached

Percentage culture blackened

Percentage culture infected by fungi

Percentage culture infected by bacteria

Percentage culture survived

Shoot node

Trials were conducted by the addition of different antioxidants at

different levels, as follows in 1/4 MS medium containing BA 10.0 ppm and NAA 0.1 ppm with sucrose at 1.5 per cent level for a period of 8 weeks.

Antioxidants	Levels (ppm)
Citric acid	100, 200, 300, 400, 500
Ascorbic acid	-do-
PVP	-do-
Cysteine-HCl	-do-

Observations recorded

1. Percentage cultures blackened
2. Percentage cultures survived
3. Percentage cultures dead
4. Number of shoots/culture
5. Number of leaves/culture
6. Number of roots/culture

3.4.1.8 *In vitro* rooting

Since *Phalaenopsis* is a monopodial orchid a separate rooting stage is not essential as with the elongation of normal shoots, roots are found to develop simultaneously from the axils. However, trials were conducted to study the effect of incorporating activated charcoal and triadimefon, as follows in the culture medium. Three culture periods, namely, 4, 8 and 12 weeks, were tried.

Additive	Levels	Media	Observations recorded
AC	0, 0.025, 0.05, 0.10, 0.20, 0.40 and 0.80%	1/4 MS + BA 5 ppm	1. Number of shoots/culture 2. Number of leaves/culture 3. Number of roots/culture
Triadimefon	0, 5, 10, 15 and 20 ppm	1/4 MS + BA 5 ppm + AC 0.40%	1. Number of shoots/culture 2. Number of leaves/culture 3. Number of roots/culture 4. Length of the longest root (cm)

3.4.1.9 Control of bacterial contamination

From the trials conducted earlier it was found that inflorescence stalk nodes are highly infected with bacteria. So a trial was conducted using an antibiotic streptopencillin at six levels (0, 40, 80, 120, 160 and 200 ppm) using 1/2 MS liquid medium with filter paper bridge containing BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm and another medium with same growth regulator combination containing CW 15 per cent. Since streptopencillin is thermolabile filter sterilised chemical dissolved in sterile water was incorporated into the media after heat sterilisation. The temperature of the bulk medium after heat sterilisation was brought down to 40°C for adding the antibiotic and was then quickly dispensed into culture vessels.

Observations recorded (Culture period : 4 weeks)

1. Percentage cultures bleached
2. Percentage cultures blackened
3. Percentage cultures infected by fungus
4. Percentage cultures infected by bacteria
5. Percentage cultures survived
6. Number of days taken for nodal swelling
7. Number of days taken for bud development

3.4.1.10 Effect of serial subculturing on multiple shoot formation

This trial was conducted for four generation on 1/4 MS medium supplemented with BA (10 and 20 ppm), adenine (0, 10, 15 and 20 ppm) and NAA (1 ppm) with sucrose at 1.5 per cent level. Serial subculturing was carried out at an interval of 4 weeks for a period of 16 weeks and observations were recorded after each subculture.

3.4.2 Induction of protocorm like bodies

Inflorescence stalk tip, stalk node, internode, flower bud, sepal, petal, flower stalk, gynostemium, pollinia, shoot node of plantlet, leaf and root were used as explants to initiate protocorm like bodies.

3.4.2.1 Effect of carbon source

The level of sucrose used in the media was found to influence the initiation of PLB's and hence an experiment was conducted using shoot node as the explant in 1/2 MS medium containing BA 5 ppm, NAA 2 ppm and 2,4-D 2 ppm with sucrose at six levels (0, 1.5, 3.0, 4.5, 6.0 and 7.5%).

Observations recorded (Culture period : 14 weeks)

1. Number of days taken for PLB formation
2. Number of PLB's developed per culture

3.4.2.2 Effect of Thiamine-HCl

Trials were conducted with Thiamine-HCl at six levels (0, 5, 10, 15, 20 and 25 ppm) in 1/2 MS medium containing BA 5 ppm, NAA 2 ppm and 2,4-D

2 ppm supplemented with 1.5per cent sucrose, for a culture period of 8 weeks using shoot node as the explant.

Observations recorded

1. Number of days taken for PLB formation
2. Number of PLB's developed

3.4.2.3 Effect of growth regulators

Trials were conducted with different growth regulator combinations in 1/4 and 1/2 MS medium containing sucrose at 1.5 per cent level. Response of different explants were also observed. The details of treatment combinations and observations recorded are as follows.

Explant	Combinations	Media	Observations recorded	Culture period
1	2	3	4	5
1. Shoot node	1x4 levels of BA (20 ppm) and 2,4-D (1, 2, 3 and 4 ppm)	½ MS	1. No. of days taken for PLB formation 2. No. of PLB's developed	8 weeks
	4x1x1 levels of BA (1, 5, 10 and 15 ppm), NAA (2 ppm) and 2,4-D (2 ppm)	½ MS	1. No. of days taken for PLB formation 2. No. of PLB's developed	8 weeks
2. *In vitro* leaf	6x1x1 levels of BA (0, 5, 10, 15, 20 and 25 ppm), adenine (10 ppm) and NAA (1 ppm)	½ MS	1. No. of days taken for PLB formation 2. No. of PLB's developed 3. Percentage of cultures that developed PLB's 4. Size of PLB's developed	8 weeks

Contd.

1	2	3	4	5
3. *In vitro* root	6x1x1 levels of BA (0, 5, 10, 15, 20 and 25 ppm), adenine (10 ppm) and NAA (1 ppm)	1/4 MS	1. No. of days taken for root tip bulging 2. No. of days taken for PLB formation 3. Percentage of cultures that developed PLB's 4. No. of PLB's developed	8 weeks
	2x2x2 levels of BA (5 and 20 ppm), NAA (0 and 2 ppm) and 2,4-D (0 and 2 ppm)	1/4 MS	1. No. of days taken for root tip bulging 2. No. of days taken for PLB formation 3. Percentage of cultures that developed PLB's 4. No. of PLB's developed	12 weeks
4. PLB's	5x2 levels of BA (1, 5, 10, 15 and 20 ppm) and NAA (0 and 1 ppm)	1/4 MS	1. No. of PLB's developed additionally 2. No. of plantlets developed	4 weeks 8 weeks 12 weeks
5. Stalk node, inter-node, flower bud,	6 x 3 levels of BA (1, 5, 10, 15, 20 and 25 ppm) and NAA (1, 2 and 3 ppm)	½ MS	1. No. of days taken for PLB formation 2. No. of PLB's developed	8 weeks
sepal, petal, flower stalk, gynost-enium,	2x2x2 levels of BA (5 and 10 ppm), NAA (1 and 2 ppm) and 2,4-D (1 and 2 ppm)	½ MS	1. No. of days taken for PLB formation 2. No. of PLB's developed	8 weeks
pollinia, leaf and root	3x1x1 levels of BA (1, 3 and 5 ppm), NAA (2 ppm) and 2,4-D (2 ppm)	½ MS	1. No. of days taken for PLB formation 2. No. of PLB's developed	8 weeks
	2x2x2 levels of BA (15 and 20 ppm), NAA (1 and 2 ppm) and 2,4-D (1 and 2 ppm)	½ MS	1. No. of days taken for PLB formation 2. No. of PLB's developed	8 weeks

Contd.

1	2	3	4	5
	3x1x1 levels of BA (1, 3 and 5 ppm), NAA (20 ppm) and adenine (10 ppm)	½ MS	1. No. of days taken for PLB formation 2. No. of PLB's developed	8 weeks
	3x1x1 levels of KIN (5, 10 and 15 ppm), NAA (2 ppm) and 2,4-D (2 ppm)	½ MS	1. No. of days taken for PLB formation 2. No. of PLB's developed	8 weeks
	2x2x1x2 levels of BA (5 and 10 ppm), KIN (5 and 10 ppm) 2,4-D (5 ppm) and adenine (10 and 20 ppm)	½ MS	1. No. of days taken for PLB formation 2. No. of PLB's developed	8 weeks
	3x1x1x2 levels of BA (5, 10 and 15 ppm), NAA (2 ppm), 2,4-D (2 ppm) and adenine (2 and 4 ppm)	½ MS	1. No. of days taken for PLB formation 2. No. of PLB's developed	8 weeks

3.4.2.4 Effect of medium supplements

Trials were conducted using coconut water, tomato juice, adenine and peptone so as to enhance the PLB formation in *Phalaenopsis*.

Coconut water

Study was conducted using inflorescence stalk tip, shoot node and PLB's as explants. Coconut water at different concentration were incorporated into the media identified earlier. Response of different explants were also observed. The details are as follows.

Explant	Level of coconut water (%)	Media	Observations recorded	Culture period
1. Inflore-scence stalk tip	15	½MS supplemented with 2x2x2 levels of BA (5 and 20 ppm), NAA (2 and 5 ppm) and 2,4-D (0 and 2 ppm)	1. No. of days taken for stalk tip bulging 2. No. of days taken for PLB development 3. No. of PLB's developed	10 weeks
		½MS suspplemented with 1x1x2 levels of BA (20 ppm), NAA (1 ppm) and adenine (10 and 20 ppm)	1. No. of days taken for stalk tip bulging 2. No. of days taken for PLB development 3. No. of PLB's developed	10 weeks
2. Shoot node	0,5,10 15,20 and 25	½MS supplemented with BA 5 ppm, NAA 2 ppm and 2,4-D 2 ppm	1. No. of days taken for PLB formation 2. No. of PLB's developed	8 weeks
3. PLB	0,5,10, 15,20 and 25	1/4 MS	1. PLB growth 2. No. of plantlets developed	3 weeks 6 weeks 12 weeks

Tomato juice

Four levels of tomato juice (0, 3, 6 and 9 %) were tried using shoot node as the explants on 1/2 MS medium supplemented with BA 5 ppm, NAA 2 ppm and 2,4-D 2 ppm containing 1.5 per cent sucrose. Observations were recorded after 8 weeks on the number of days taken for PLB formation and number of PLB's developed.

Adenine

Shoot node, *in vitro* leaf and PLB's were the explants used for the study.

Explant	Combinations	Media	Observations recorded	Culture period
1. Shoot node	4x1 levels of adenine (4, 8, 12 and 16 ppm) and BA (16 ppm)	½ MS	1. No. of days taken for PLB formation 2. No. of PLB's developed	14 weeks
	1x1x4 levels of adenine (10 ppm), BA (10 ppm) and NAA (1, 2, 3 and 4 ppm)	½ MS	1. No. of days taken for PLB formation 2. No. of PLB's developed	14 weeks
2. *In vitro* leaf	6x1x1 levels of adenine (0, 5, 10, 15, 20 and 25 ppm), BA (10 ppm) and NAA (1 ppm)	1/4 MS	1. No. of days taken for PLB formation 2. No. of PLB's developed 3. Percentage cultures that developed PLB's 4. Size of the PLB's developed	8 weeks
3. PLB's	5x1 levels of adenine (4, 8, 12, 15 and 20 ppm) and BA (16 ppm)	1/4 MS	1. No. of PLB's developed additionally 2. No. of plantlets developed	4 weeks 8weeks 12 weeks
	1x6 levels of adenine (0 ppm) and BA (0, 4, 8, 12, 16 and 20 ppm)	1/4 MS	1. No. of PLB's developed additionally 2. No. of plantlets developed	4 weeks 8 weeks 12 weeks
4. Stalk node, inter-node, flower bud, sepal, petal, flower stalk, gynoste-nium, pollinia leaf and root	5x5x2 levels of adenine (5, 10, 15, 20 and 25 ppm), BA (5, 10, 15, 20 and 25 ppm) and NAA (1 and 2 ppm)	½ MS	1. No. of days taken for PLB formation 2. No. of PLB's developed	8 weeks

Peptone

Trials were conducted with peptone at five levels (0, 500, 1000, 1500 and 2000 ppm) in 1/2 MS medium containing BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm with 1.5 per cent sucrose. Observations were recorded for the days taken for PLB formation and number of PLB's developed after 8 weeks of culturing.

3.5 Pod culture

Pods of *Phalaenopsis* at four stages of maturity (70, 80, 90 and 100 days after pollination) were cultured in 1/2 MS medium with 1.5 per cent sucrose. Growth regulator combinations tried for seed germination and plant growth are as follows.

Treatment combinations

1. 1 x 2 levels of BA (10 ppm) and NAA (1 and 2 ppm)
2. 1 x 2 levels of KIN (5 ppm) and 2,4-D (1 and 2 ppm)
3. 2 x 1 x 1 levels of BA (5 and 15 ppm), NAA (2 ppm) and 2,4-D (2 ppm)
4. 2 x 1 x 1 levels of KIN (5 and 15 ppm), NAA (2 ppm) and 2,4-D (2 ppm)
5. 2 x 1 x 1 levels of BA (10 and 20 ppm), NAA (2 ppm) and adenine (10 and 20 ppm)

Observations recorded

1. No. of days taken for greening of seeds
2. No. of days taken for protocorm formation
3. No. of days taken for leaf formation
4. No. of days taken for root formation

3.6 Culture environment

Trials were conducted to study the effect of light and type of culture vessel used on the growth of the cultures.

Light

Light was found to influence the growth of the culture and to observe the effect, six types of explant [leaf, root, cluster of 10 PLB, one globular PLB, axillary bud (0.5 cm long) and shoot node] were cultured on 1/4 MS medium supplemented with BA 10 ppm, adenine 10 ppm and NAA 1 ppm with 1.5 per cent sucrose. Half of the cultures were kept under dark and half under light and observations were recorded on the growth character for a period of 4 months.

Culture vessel

A trial was conducted to study the effect of type and volume of culture vessel on *in vitro* response on *Phalaenopsis*. Plantlets with three leaves and two roots were cultured on 1/2 MS medium supplemented with BA 10 ppm and NAA 1 ppm. Sucrose was added at 3.0 per cent level and AC at 0.4 per cent level. The different types of culture vessels utilised for the study were conical flasks of 50 ml, 100 ml and 250 ml capacity and test tubes of size 150 x 25 mm and 210 x 40 mm. Observations were recorded on the nature of the growth of the plantlets after a period of 4 months.

3.7 Planting out and acclimatization

Plantlets with a minimum height of 0.5 cm (measured from the base of the plant to the topmost leaf base), having minimum of four leaves (length 3.5 cm

and width 1.2 cm) and six roots (length 3.5 cm) were used for the planting out studies.

The cotton plugs of the culture vessels were removed and distilled water was added to the vessels. The bottles were shaken and kept as such for 10 to 15 minutes. Then the rooted plantlets were taken out from culture vessels with the help of forceps. The agar adhering to the roots was completely removed by thorough washing with running tap water. Then just before planting, the plantlets were kept immersed in Dithane M-45 0.1 per cent for 20-30 minutes and then spread over a blotting paper to remove the excess moisture and then planted out.

3.7.1 Influence of potting media

In order to study the effect of potting media on the growth of plantlets, the following media were tried.

1. Charcoal
2. Charcoal + Brick pieces (1:1)
3. Charcoal + Brick pieces + Coconut fibre (1:1:1)
4. Charcoal + Brick pieces + Sand (1:1:1)
5. Charcoal + Vermiculite (1:1)
6. Cocoapeat + Vermiculite (1:1)
7. Vermiculite
8. Sphagnum moss
9. Coconut fibre
10. Coconut husk

The container used was egg tray and observations on the percentage of

survival of plantlets were recorded after 4 weeks. Coconut fibre and coconut husk were used for planting out without containers.

3.7.2 Influence of containers

To study the influence of containers on the survival of planted out plantlets, six types of container were used for planting with charcoal + brick pieces (1:1) as potting medium. The containers used for the study were,

1. Jam bottle
2. Clay pot
3. Black plastic pot
4. White plastic (ice cream) cup
5. Polysachet
6. Egg tray

Observations were recorded for the percentage survival of plantlets after 4 weeks.

3.7.3 Influence of triadimefon added in the culture medium

Triadimefon was found to influence the growth of the plantlets, with respect to the root number and length of the roots. And so a trial was conducted using different levels of triadimefon, (0, 5, 10, 15 and 20 ppm) added in the culture medium for culturing the plantlets for about two months before planting out. The plantlets were then grown on coconut husk. Observation was recorded on the percentage of plantlets survived after 4 and 8 weeks of planting out.

3.7.4 Standardisation of hardening treatments

In order to acclimatise the plantlets produced *in vitro*, a trial to standardise the hardening treatment was also carried out. The plantlets were subjected to various pre-transfer and post-transfer hardening treatments.

3.7.4.1 Pre transfer treatments

The treatments to which the plantlets were subjected to in order to harden the plants before planting are given below.

In vitro treatment

1. Liquid media with filter paper bridge
2. Retaining the plantlets inside the the culture vessels till the drying up of media
3. Growing the plantlets in media containing 20 ppm triadimefon for 2 months, just before planting out

Ex vitro treatment

1. Keeping the plantlets in distilled water (3 h)
2. Dipping the whole plantlets in 50% glycerol (1 min)
3. Smearing paraffin wax over the leaf surface
4. Spraying triadimefon 20 ppm at fortnightly intervals
5. Spreading the plantlets over sterile charcoal pieces for 2 weeks

The plantlets after subjecting to the various *in vitro* and *ex vitro* hardening treatments were then planted in coconut husk and kept over wire net with water beneath and sprayed with water to maintain humidity. Observation was recorded on the percentage survival of planted out plantlets after 4 and 8 weeks of planting.

3.7.4.2 Post-transfer treatments

To attain maximum survival percentage of plantlets, the plantlets after subjecting to the best pre-transfer treatment were again subjected to post-transfer hardening treatments. The treatments given were

1. Keeping the plantlets in the culture room
2. Covering the plantlets with polythene bag
3. Keeping under microscope cover
4. Keeping in water in a tray
5. Keeping over wire net with water beneath
6. Hanging in the orchidarium with high humidity

Observations were recorded on the survival rate after 4 and 8 weeks of planting.

3.8 Field evaluation

The healthy, uniform sized plantlets after subjecting to the best among the pre-transfer and post-transfer hardening treatments and having a height of 0.7 cm with atleast 5 leaves (length 4.0 cm and width 1.5 cm) and 8 roots (length 4.0 cm), after 4 weeks of planting out were used for the study.

3.8.1 Effect of nutrient solutions

For carrying out the nutritional study, the following five nutrient solutions were used which were applied at fortnightly intervals.

1. MS (.25%) - MS medium containing one fourth strength of all inorganic and organic compounds except inositol and sucrose
2. MS (.50%) - MS medium containing half the strength of all inorganic and organic compounds except inositol and sucrose
3. 30:10:10 (.50%) - Afil's green care nutrient solution (500 mg/100 ml)
4. 17:17:17 (.10%) - Complex fertiliser mixture (100 mg/100 ml)
5. Hoagland (1.0%) - Full strength of Hoagland solution

Observations recorded

1. Percentage of plantlet survived
2. Plant height
3. Number of leaves per plant
4. Length of the longest leaf
5. Width of the longest leaf
6. Number of roots per plant
7. Length of the longest root

Observations were recorded after 8 and 12 weeks of planting.

3.8.2 Biometric observations of the plants

Plants of uniform size (the plants grown under best nutrient treatment) were selected and used for the study. The growth performance of the plantlets were observed for a period of 8 months after planting out and observations recorded at an interval of 1 month period, starting from the third month.

Observations recorded

1. Percentage of plantlets survived
2. Plant height
3. Number of leaves per plant
4. Length of the longest leaf
5. Width of the longest leaf
6. Number of roots per plant
7. Length of the longest root

3.9 Statistical analysis

The data generated from the various experiments were subjected to statistical analysis in randomised block design wherever necessary as per Panse and Sukhatme (1985).

Results

RESULTS

The results generated from the study on the micropropagation of *Phalaenopsis*, conducted at the Plant Tissue Culture Laboratory attached to the All India Co-ordinated Floriculture Improvement Project, College of Horticulture, are presented in this chapter.

4.1 Explant

The explants tried were inflorescence stalk node, internode, flower bud, leaf and root taken from field grown plants and also apical bud, shoot node, basal portion, leaf and roots of *in vitro* plantlets.

4.1.1 Standardisation of surface sterilisation of explants

The results of the experiments on surface sterilization of *Phalaenopsis* explants using different chemicals are presented in Tables 3, 4, 5 and 6.

Inflorescence stalk node and internode

Among the 22 treatments tried none of the treatments recorded total survival. The maximum survival per cent for nodal explants was 40 and for internodal explants 55 at the sterilant combination involving mercuric chloride (0.01%) for 30 min, streptomycin + pencillin (0.01%) for 90 min followed by final sterilization using mercuric chloride (0.10%) for 10 min.

In another sterilant combination, involving bavistin (1.00%) for 30 min, streptomycin + pencillin (0.01%) for 90 min followed by sterilization using

mercuric chloride (0.10%) for 10 min and yet another combination involving mercuric chloride (0.01%) for 30 min, streptomycin + pencillin (0.01%) for 60 min followed by final sterilization using mercuric chloride (0.10%) for 10 min, a survival percentage of 35 for nodal explants and 50 for internodal explants was recorded.

The treatment involving mercuric chloride alone did not result in survival of explants, whereas a two step sterilization procedure involving mercuric chloride at 0.01 per cent for 30 min followed by that at 0.10 per cent for 10 min recorded a survival per cent of 30 in nodal explants (Table 3) and 45 in internodal explants (Table 4).

Flower bud

In flower bud, among the seven treatments tried, the treatment combination involving emisan (1.00%) for 30 min followed by alcohol (50.00%) for 1 min recorded 55 per cent survival and a survival per cent of 45 was recorded in the treatment combination involving emisan (1.00%) for 30 min followed by mercuric chloride (0.20%) for 10 min (Table 5).

Leaf and root

Leaf explants recorded 50 per cent survival in the combination involving emisan (1.0%) for 30 min, norfloxacin (1.0%) for 30 min followed by final sterilization using mercuric chloride (0.1%) for 10 min.

Root explants, however, did not record survival in any of these combinations (Table 6).

Table 3. Effect of surface sterilants on nodal explants

Medium: 1/2 MS + BA 10.0 ppm + NAA 1.0 ppm
Culture period: 2 weeks

Treatment	Concentration (%)	Duration (min)	Percentage culture: Node: Bleached	Blackened	Fungal infected	Bacterial nfected	Survived
1	2	3	4	5	6	7	8
Mercuric chloride	0.10	5	--	--	80	20	--
		10	10	10	60	20	--
		15	25	20	45	10	--
	0.20	5	10	10	70	10	--
		10	25	25	40	10	--
		15	40	30	20	10	--
Emisan + Mercuric chloride	0.10 + 0.10	30 + 10	10	10	50	30	--
Emisan + Mercuric chloride	1.00 + 0.10	30 + 10	20	15	40	25	--
Bavistin + Mercuric chloride	0.10 + 0.10	30 + 10	10	15	45	30	--
Bavistin + Mercuric chloride	1.00 + 0.10	30 + 10	15	20	20	20	25
Mercuric chloride + -do-	0.01 + 0.10	20 + 10	10	20	30	25	15

Contd.

Table 3. Continued

1	2	3	4	5	6	7	8
Mercuric chloride +	0.01	30	15	25	10	20	30
-do-	0.10	10					
Emisan +	1.00	15	--	10	75	15	--
Sodium hypochlorite	10.00	10					
Emisan +	1.00	15	10	10	60	20	--
Sodium hypochlorite	10.00	20					
Emisan +	1.00	30					
Norfloxacin +	1.00	10	10	15	60	15	--
Mercuric chloride	0.10	10					
Emisan +	1.00	30					
Norfloxacin +	1.00	20	10	15	60	15	--
Mercuric chloride	0.10	10					
Bavistin +	1.00	30					
(S + P) +	0.01	60	20	15	20	15	30
Mercuric chloride	0.10	10					
Bavistin +	1.00	30					
(S + P) +	0.01	90	20	15	20	10	35
Mercuric chloride	0.10	10					

Contd.

Table 3. Continued

1	2	3	4	5	6	7	8
Mercuric chloride	0.01	20					
+							
(S + P)	0.01	60	20	10	30	20	20
+							
Mercuric chloride	0.10	10					
Mercuric chloride	0.01	20					
+							
(S + P)	0.01	90	20	10	30	15	25
+							
Mercuric chloride	0.01	10					
Mercuric chloride	0.01	30					
+							
(S + P)	0.01	60	20	20	10	15	35
+							
Mercuric chloride	0.10	10					
Mercuric chloride	0.01	30					
+							
(S + P)	0.01	90	10	30	10	10	40
+							
Mercuric chloride	0.10	10					

(S + P) - Streptomycin + Pencillin
Values were taken as the average of 20 observations

Table 4. Effect of surface sterilants on internodal explants

Medium: 1/2 MS + BA 10.0 ppm + NAA 1.0 ppm
Culture period: 2 weeks

Treatment	Concentration (%)	Duration (min)	Percentage culture: Node: Bleached	Blackened	Fungal infected	Bacterial nfected	Survived
1	2	3	4	5	6	7	8
Mercuric chloride	0.10	5	--	20	70	10	--
		10	10	20	60	10	--
		15	40	30	20	10	--
	0.20	5	15	10	50	25	--
		10	30	25	35	10	--
		15	40	40	20	--	--
Emisan + Mercuric chloride	0.10 + 0.10	30 + 10	10	20	50	20	--
Emisan + Mercuric chloride	1.00 + 0.10	30 + 10	25	25	40	10	--
Bavistin + Mercuric chloride	0.10 + 0.10	30 + 10	20	30	35	15	--
Bavistin + Mercuric chloride	1.00 + 0.10	30 + 10	10	20	10	20	40
Mercuric chloride + -do-	0.01 + 0.10	20 + 10	10	25	20	15	30

Contd.

Table 4. Continued

1	2	3	4	5	6	7	8
Mercuric chloride +	0.01	30	20	30	--	5	45
-do-	0.10	10					
Emisan +	1.00	15	--	10	60	30	--
Sodium hypochlorite	10.00	10					
Emisan +	1.00	15	5	20	50	25	--
Sodium hypochlorite	10.00	20					
Emisan +	1.00	30					
Norfloxacin +	1.00	10	20	25	40	15	--
Mercuric chloride	0.10	10					
Emisan +	1.00	30					
Norfloxacin +	1.00	20	20	25	40	15	--
Mercuric chloride	0.10	10					
Bavistin +	1.00	30					
(S + P) +	0.01	60	10	20	10	15	45
Mercuric chloride	0.10	10					
Bavistin +	1.00	30					
(S + P) +	0.01	90	15	15	10	10	50
Mercuric chloride	0.10	10					

Contd.

Table4 . Continued

1	2	3	4	5	6	7	8
Mercuric chloride	0.01	20					
+							
(S + P)	0.01	60	10	20	20	10	40
+							
Mercuric chloride	0.10	10					
Mercuric chloride	0.01	20					
+							
(S + P)	0.01	90	15	15	20	5	45
+							
Mercuric chloride	0.01	10					
Mercuric chloride	0.01	30					
+							
(S + P)	0.01	60	15	30	--	5	50
+							
Mercuric chloride	0.10	10					
Mercuric chloride	0.01	30					
+							
(S + P)	0.01	90	20	20	--	5	55
+							
Mercuric chloride	0.10	10					

(S + P) - Streptomycin + Pencillin
Values were taken as the average of 20 observations

Table 5. Effect of surface sterilants on mature flower bud

Medium: ½MS + BA 10.0 ppm + NAA 1.0 ppm
Culture period: 4 weeks

Treatment	Concentration (%)	Duration (mn)	Percentage of cultures				
			Bleached	Blackened	Fungal infected	Bacterial infected	Surviving
Mercuric chloride	0.10	5	-	-	80	20	-
		10	20	-	60	20	-
Mercuric chloride	0.20	5	20	-	60	20	-
		10	25	-	40	35	-
Emisan + Mercuric chloride	1.00 + 0.20	30 + 10	25	-	15	15	45
Emisan + Alcohol	1.00 + 50.00	30 + 1	-	-	45	-	55
Mercuric chloride + Alcohol	0.01 + 50.00	20 + 1	30	-	40	10	20

Values were taken as the average of 20 observations

Table 6. Effect of surface sterilants on leaf and root explants

Medium: ½MS + BA 10.0 ppm + NAA 1.0 ppm
Culture period: 4 weeks

Treatment	Concentration (%)	Duration (min)	Percentage of cultures									
			Leaf					Root				
			Bleached	Blackened	Fungal infected	Bacterial infected	Survived	Bleached	Blackened	Fungal infected	Bacterial infected	Survived
Mercuric chloride	0.10	5	--	--	90	10	--	20	--	70	10	--
		10	10	--	70	20	--	40	--	40	20	--
		15	40	--	50	10	--	60	--	20	20	--
Emisan + Mercuric chloride	1.00 + 0.10	30 + 5	--	--	90	--	10	20	--	70	10	--
Emisan + Norfloxacin + Mercuric chloride	1.00 + 1.00 + 0.10	30 + 30 + 10	10	10	20	10	50	50	--	50	--	--
Emisan + Roscillin + Mercuric chloride	1.00 + 1.00 + 0.10	30 + 10 + 10	10	--	40	20	30	40	--	60	--	--

Values were taken as the average of 20 observations

4.1.2 Type of explant

Results are presented in Table 7.

Number of shoots

When apical bud was used, there was no increase in the shoot number for the period under observation. But in the case of shoot node, there was a progressive increase in the number of shoots and the rate of increase at 3, 6, 9 and 12 weeks were 1.67, 3.00, 5.33 and 6.50 respectively. Similarly, when basal portion was used, the rate of increase at 3, 6, 9 and 12 weeks were 1.67, 3.33, 6.00 and 8.00 respectively. With respect to shoot production, the explant basal portion was found to be the best explant (Plate 1) followed by shoot node, in the specific combination identified.

Number of leaves

The leaf number was found to increase gradually when apical bud was used. In the case of shoot node the leaf number per culture increased at a rate of 2.00 at 3 week interval for 3 and 6 weeks period and thereafter showed a gradual decline. When basal portion was used, the leaf number increased at a rate of 1.00, 3.33, 6.50 and 7.67 at 3, 6, 9 and 12 weeks period respectively. Here also, basal portion was found to be better than shoot node which was far better than apical bud.

Number of roots

Roots were found induced in the shoot multiplication medium while the shoots attain optimum growth. When apical bud was used, it showed a gradual

Table 7. Response of explants for multiple shoot formation

Medium : 1/2 MS + BA 16.0 ppm + Adenine 8.0 ppm

Period (weeks)	Apical bud			Shoot node			Basal portion		
	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No.of roots
0	1.0	2.00	0.00	1.00	3.00	1.00	1.00	3.00	1.00
3	1.0	2.83	0.67	2.67	5.00	1.33	2.67	4.00	1.67
6	1.0	4.17	1.67	4.00	7.00	2.00	4.33	6.33	3.50
9	1.0	5.00	2.83	6.33	8.33	4.17	7.00	9.50	4.50
12	1.0	5.50	3.33	7.50	9.67	7.67	9.00	10.67	5.67
CD(0.05)		0.85	1.37	1.26	1.52	1.68	1.81	1.49	1.02
SEm±		0.29	0.46	0.42	0.51	0.56	0.60	0.50	0.34

Mean of 6 observations

increase in the root number produced at the rate of 0.67, 1.67, 2.83 and 3.33 at 3, 6, 9 and 12 weeks period respectively. When shoot node was used, it showed a very slow increase in root number at 3 and 6 weeks period (0.33 and 1.00) and thereafter showed a drastic increase (3.17 and 6.67) at 9 and 12 weeks respectively. In the case of basal portion, the root number increased at the rate of 0.67, 2.50, 3.50 and 4.67 at 3, 6, 9 and 12 weeks period respectively. In the long run (ie., at 12 weeks period) shoot node recorded the highest root number.

Discolouration of media and explants

Results are presented in Table 8. The different explants showed difference in phenolic exudation. Apical bud showed the highest phenolic exudation and the lowest amount by the basal portion (Plate 2).

4.1.3 Position of explant

Data presented in Table 9 indicated that apical bud did not produce any bud during the period of 60 days, whereas first, second, third and fourth nodes produced buds and showed an increase in their length (Fig. 1). The length of the bud produced by the first node increased at a faster rate and was significantly superior to explants from the other position of nodes at 20, 40 and 60 days period. The rate of increase was 1.13, 2.00 and 2.83 at 20, 40 and 60 days period, respectively, whereas for second and third nodes, the rate of increase was 0.58, 0.96 and 1.79 at 20, 40 and 60 days period, respectively. In the case of fourth node (basal portion), the rate was still lower.

Plate 1. Multiple shoot induction using basal portion of the plantlet as explant

Plate 2. Gradation of phenolic exudation in apical bud, first node, shoot node and basal portion

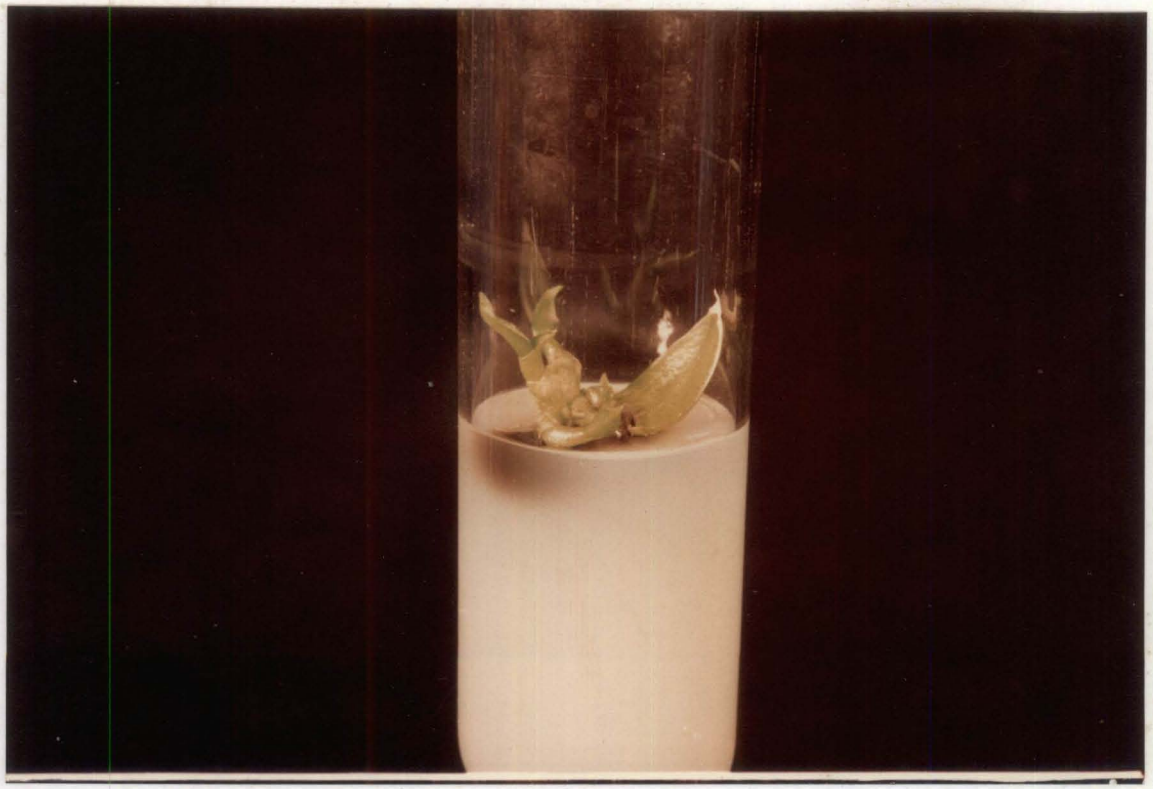

Fig.1. Effect of position of nodes on elongation of buds

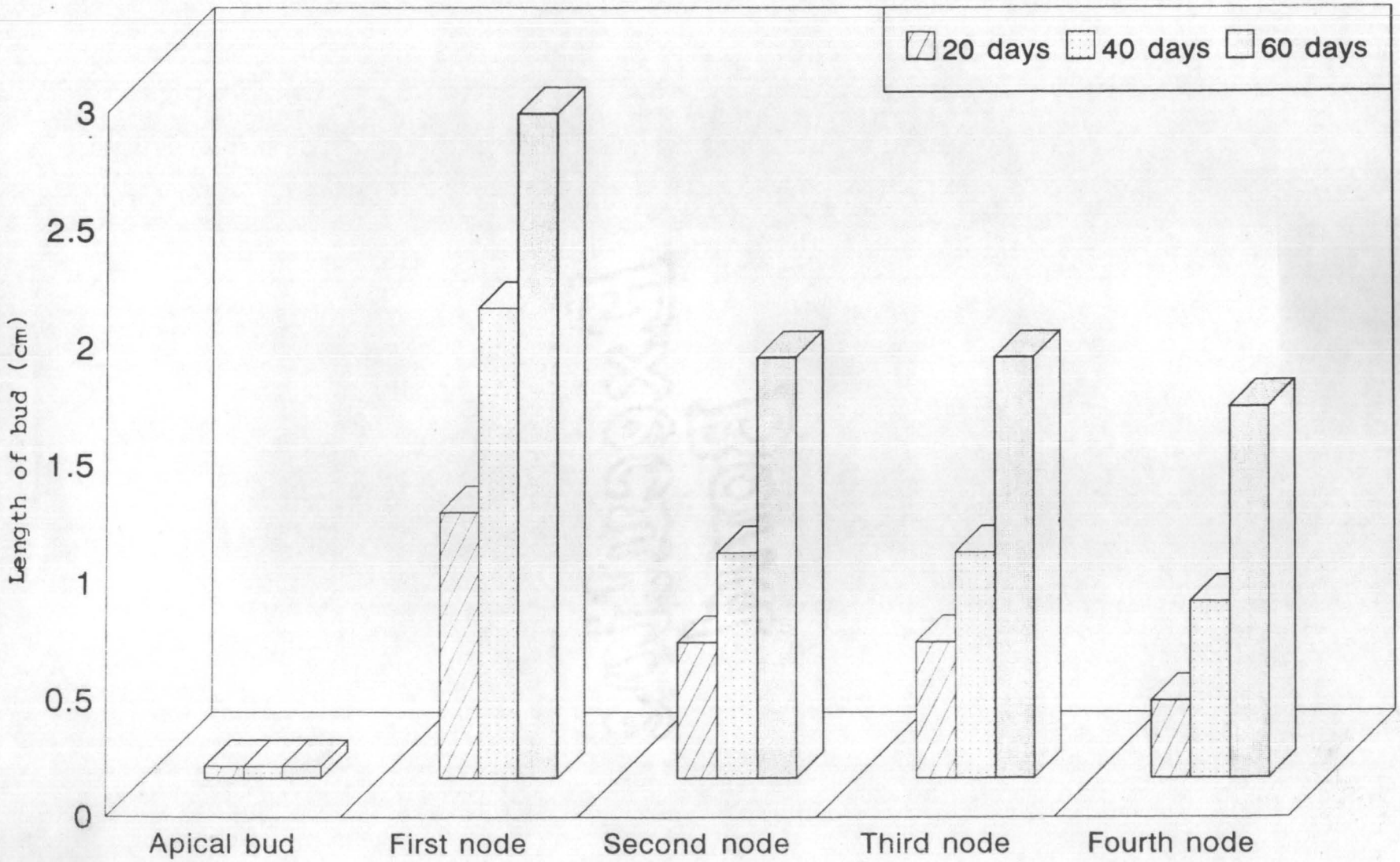

Table 8. Gradation of phenolic exudation in explants

Medium: 1/4 MS + CW 5% (Semi solid)
Culture period: 2 weeks

Explants	Phenolic exudation	Remarks
Apical bud	++++	Very high blackening
First node	+++	High blackening
Shoot node	++	Medium blackening
Basal portion	+	Low blackening

Table 9. Effect of position of nodes on elongation of buds

Medium : ½ MS + BA 16.0 ppm + Adenine 8.0 ppm

Explant	Length of the bud in 20 days (cm)	Length of the bud in 40 days (cm)	Length of the bud in 60 days (cm)
Apical bud	-	-	-
First node	1.13	2.00	2.83
Second node	0.58	0.96	1.79
Third node	0.58	0.96	1.79
Fourth node	0.33	0.75	1.58
CD (0.05)	0.17	0.25	0.27
SEm±	0.06	0.08	0.09

Mean of 6 observations

4.2 Culture medium

4.2.1 Effect of mineral salts present in the medium

When MS medium containing full strength of mineral salts was utilised, the shoots emerged showed yellowing and were dried up. So a trial was conducted to find out a suitable basal medium which favours the growth of the cultures. The data are presented in Table 10.

Number of shoots

The largest number of shoots (3.00 and 5.00) were recorded in 1/4 MS and 1/2 MS during 4 and 8 weeks period of culture respectively and were significantly superior to MS and KC media which was on par with VW and 3/4 MS medium (Fig. 2). The lowest number of shoots was recorded for full strength MS medium (0.33) at 8 week period.

Number of leaves

The largest number of leaves (5.83) during 4 weeks of culture was recorded on ½ MS and it was on par with 1/4 MS, 3/4 MS and VW media. During 8 weeks of culture, the highest number of leaves (7.67) was recorded for 1/4 MS and it was on par with ½ MS and VW. The lowest number of leaves was recorded for full strength MS medium (0.50) at 8 week period.

Number of roots

During 4 and 8 weeks of culture, the number of roots produced was highest (1.67 and 2.33, respectively) for ½ MS and VW media and it was on par

Fig.2. Effect of basal media on the induction of multiple shoots *in vitro*

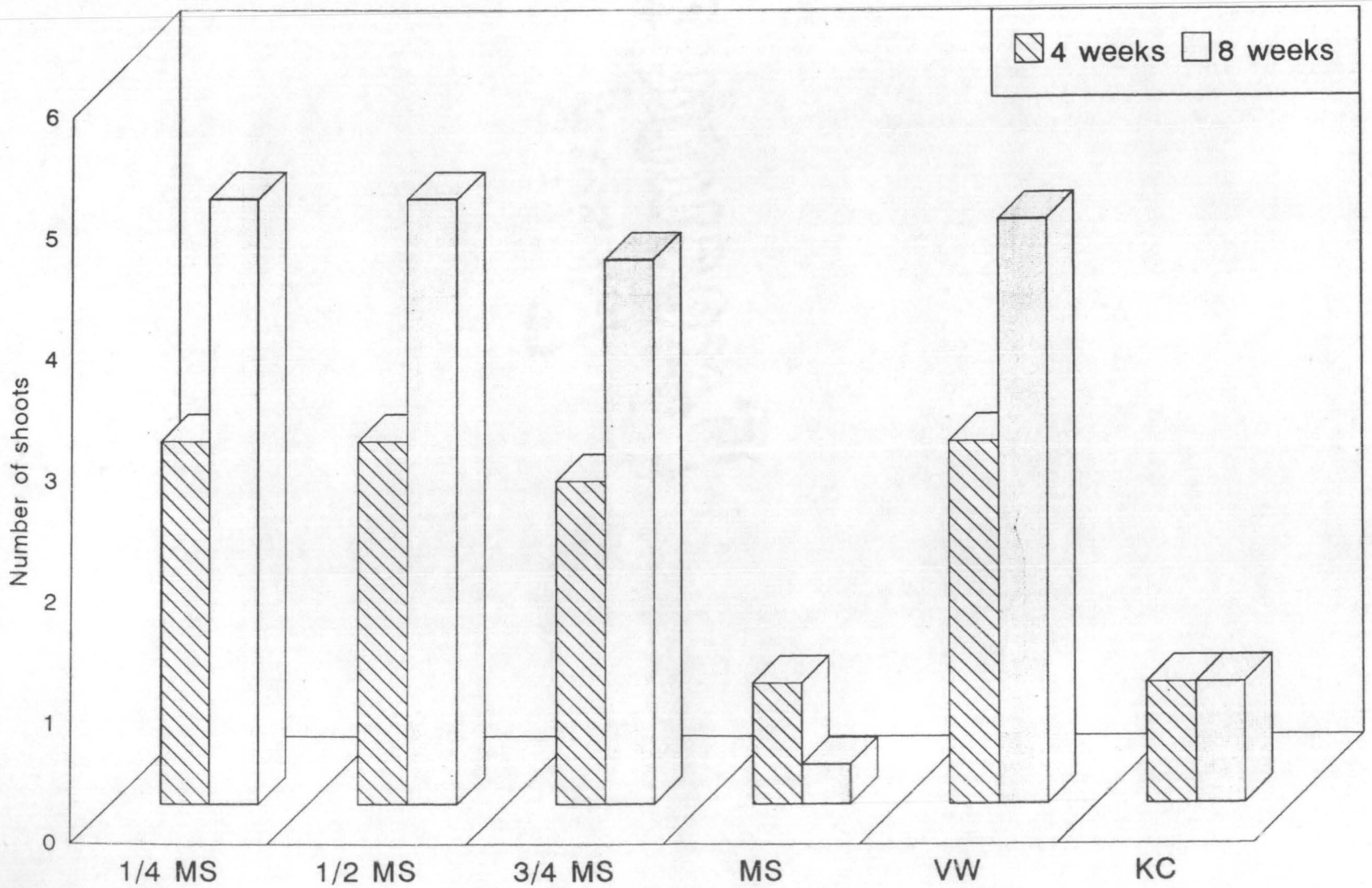

Table 10. Effect of basal media on the induction of multiple shoots *in vitro*

Medium: Basal medium + BA 16.0 ppm + Adenine 8.0 ppm
Explant: Shoot node

Media	4 weeks			8 weeks		
	Number of shoots	Number of leaves	Number of roots	Number of shoots	Number of leaves	Number of roots
1/4 MS	3.00	5.50	1.50	5.00	7.67	2.17
1/2 MS	3.00	5.83	1.67	5.00	7.33	2.33
3/4 MS	2.67	5.67	1.67	4.50	7.00	1.83
MS	1.00	3.00	1.00	0.33	0.50	1.00
VW	3.00	5.67	1.67	4.83	7.67	2.33
KC	1.00	3.00	1.00	1.00	3.17	1.00
CD(0.05)	0.72	1.26	0.54	0.35	0.54	0.74
SEm±	0.25	0.42	0.18	0.12	0.18	0.25

Mean of 6 observations

with 1/4 MS and 3/4 MS. The lowest number of roots (1.00) was recorded for MS and KC media.

4.2.2 Effect of physical state of the medium

Data pertaining to the growth of cultures in two different states of culture medium ie., liquid and semi-solid, are presented in Table 11.

Number of shoots

There was no significant difference among the treatments with respect to the number of shoots in both liquid and solid media during the 4 weeks period. At the end of 8 weeks period, the largest number of shoots (8.17) was recorded in T_{12} (BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm + CW 15% liquid) which was on par with T_4 (BA 16.0 ppm + adenine 8.0 ppm + CW 15% semi-solid), T_6 (BA 5.00 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm + CW 15% semi-solid), T_8 (BA 20.0 ppm + NAA 1.0 ppn + CW 15%), T_{10} (BA 16.0 ppm + adenine 8.0 ppm + CW 15%) and T_{11} (BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm).

Number of leaves

When observations were recorded after 4 weeks of culture, significant difference was found to exist in the number of leaves. The highest number of leaves (8.33) was recorded for T_6 (BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm - semi-solid) and it was on par with T_2, T_4, T_8, T_{10} and T_{12}. After 8 weeks of culture, no significant difference was observed among the treatments with respect to the number of leaves.

Table 11. Growth of *in vitro* shoots in liquid and solid media

Medium: 1/4 MS
Explant: Shoot node

Treatment	4 weeks			8 weeks		
	No. of shoots	No. of leaves	No. of roots	No. of shoots	No.of leaves	No. of roots
Semi solid medium + AC 0.4%						
T_1 - BA 20.0 ppm + NAA 1.0 ppm	3.00	5.67	2.00	4.83	7.17	3.33
T_2 - BA 20.0 ppm + NAA 1.0 ppm + CW 15%	3.83	7.33	2.83	6.00	8.33	4.17
T_3 - BA 16.0 ppm + Adenine 8.0 ppm	3.00	6.00	1.50	5.00	7.67	2.17
T_4 - BA 16.0 ppm + Adenine 8.0 ppm + CW 15%	4.17	8.00	2.67	6.50	9.17	3.17
T_5 - BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm	3.33	6.33	2.17	5.33	7.83	2.33
T_6 - BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm + CW 15%	4.67	8.33	3.17	7.50	9.33	3.50
Liquid						
T_7 - BA 20.0 ppm + NAA 1.0 ppm	3.17	5.50	2.00	5.83	6.83	3.00
T_8 - BA 20.0 ppm + NAA 1.0 ppm + CW 15%	4.17	6.83	2.67	6.67	8.00	3.83
T_9 - BA 16.0 ppm + Adenine 8.0 ppm	3.17	5.83	1.50	6.00	7.17	1.67
T_{10} - BA 16.0 ppm + Adenine 8.0 ppm + CW 15%	4.33	7.33	2.33	7.17	8.50	2.67
T_{11} - BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm	3.67	6.33	2.17	7.00	7.83	2.00
T_{12} - BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm + CW 15%	5.00	8.17	3.00	8.17	9.33	3.17
CD(0.05)	NS	1.92	1.02	1.92	NS	1.13
SEm±	0.54	0.68	0.36	0.68	0.76	0.40

Mean of 6 observations

Number of roots

After 4 weeks of culturing, highest number of roots (3.17) was recorded for T_6 (BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm + CW 15% - semi-solid) which was on par with T_2, T_4, T_5, T_8, T_{10}, T_{11} and T_{12}. The lowest number of roots (1.50) was recorded for T_3 and T_9 ie., BA 16.0 ppm + adenine 8.0 ppm, in both the semi-solid and liquid media.

The highest number of roots (4.17) was recorded for T_2 (BA 20.0 ppm + NAA 1.0 ppm + CW 15% - semi-solid) at the end of 8 weeks period and it was found on par with T_1, T_4, T_6, T_8 and T_{12}. The lowest number of roots (1.67) was recorded for T_9 (BA 16.0 ppm + adenine 8.0 ppm - liquid).

4.2.3 Effect of sucrose

The influence of sucrose on induction of multiple shoots (Fig. 3) was studied and the results are presented in Table 12.

Number of shoots

Four weeks after culturing, sucrose (1.5%) produced the maximum number (3.00) of shoots and was significantly superior to all other treatments except sucrose (3.0%). The lowest number of shoots (1.00) was recorded in media without sucrose and this was on par with sucrose 4.5 per cent, 6.0 per cent and 7.5 per cent.

After 8 weeks of culturing, the highest number of shoots (5.00) was recorded for sucrose (1.5%) and was significantly superior to all other treatments. The lowest number of shoots (1.17) was recorded for media containing no sucrose and this was on par with sucrose 6.0 per cent and 7.5 per cent.

Fig.3. Influence of sucrose on the induction of multiple shoots *in vitro*

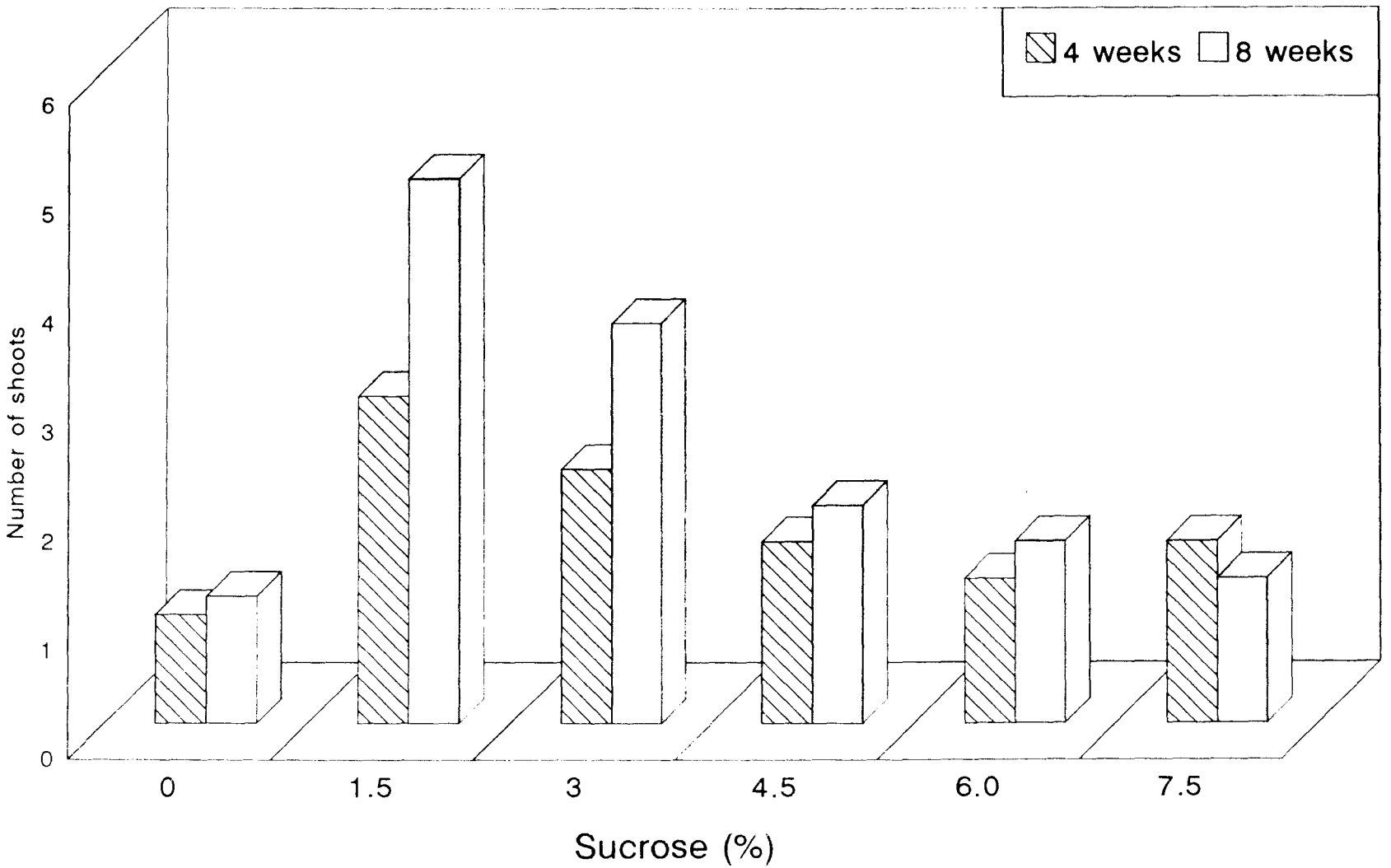

Table 12. Influence of sucrose on the induction of multiple shoots *in vitro*

Medium: ½ MS + BA 16.0 ppm + Adenine 8.0 ppm
Explant: Shoot node

Sucrose (%)	4 weeks			8 weeks			Remarks
	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No. of roots	
0.0	1.00	3.00	1.50	1.17	1.67	1.33	Callusing of leaf base
1.5	3.00	6.00	1.50	5.00	7.33	2.17	Very good growth and PLB development
3.0	2.33	3.67	2.00	3.67	5.17	3.00	Good growth
4.5	1.67	3.33	1.00	2.00	3.00	1.00	Yellowing
6.0	1.33	3.00	1.00	1.67	2.67	0.33	Bleaching
7.5	1.67	3.00	1.00	1.33	2.50	0.00	Blackening
CD (0.05)	0.75	0.61	0.48	0.93	1.08	0.39	
SEm±	0.26	0.21	0.16	0.32	0.37	0.13	

Mean of 6 observations

Number of leaves

When the number of leaves were considered after 4 weeks of culturing, sucrose (1.5%) produced the maximum number (6.00) and was significantly superior to all the other treatments. The lowest number (3.00) of leaves was observed for media containing no sucrose, sucrose 6.0 per cent and 7.5 per cent.

Eight weeks after culturing, highest number of leaves (7.33) was recorded for sucrose (1.5%) and was significantly superior to all the other treatments. The lowest number (1.67) of leaves was recorded for media containing no sucrose and this was on par with sucrose 6.0 per cent and 7.5 per cent.

Number of roots

With regard to root number, the highest number (2.00) was recorded for sucrose (3.0%) at the end of 4 weeks of culturing and was significantly superior to all other treatments.

After 8 weeks of culturing also the highest root number (3.00) was recorded for sucrose (3.0%) and was significantly superior to all other treatments.

4.2.4 Effect of Thiamine-HCl

A trial was conducted to study the effect of Thiamine-HCl on the growth of shoots *in vitro*, the results of which are presented in Table 13.

Number of shoots

Four weeks after culturing, no significant difference was observed among the different levels of Thiamine-HCl.

Table 13. Effect of Thiamine-HCl on the growth of shoots *in vitro*

Medium : ½MS + BA 16.0 ppm + Adenine 8.0 ppm

Thiamine-HCl (ppm)	4 weeks			8 weeks			Remarks
	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No. of roots	
0	3.00	6.00	1.50	5.00	7.67	2.17	Development of PLBs
5	3.17	6.33	1.50	5.17	8.00	2.00	Development of PLBs
10	3.33	6.67	1.33	5.50	8.33	1.50	Development of PLBs
15	3.50	7.00	1.17	5.67	8.67	1.33	Development of PLBs
20	4.17	7.83	1.00	6.50	9.17	1.17	Very good and early development of PLBs
25	3.67	7.33	1.00	5.67	8.50	1.17	Development of PLBs
CD (0.05)	NS	1.13	NS	0.70	NS	0.70	
SEm±	0.34	0.39	0.18	0.24	0.42	0.24	

Mean of 6 observations

At the end of eight weeks of culturing, highest number (6.50) of shoots was produced in media incorporated with by Thiamine-HCl (20 ppm) and was significantly superior to all the other treatments.

Number of leaves

The highest number of leaves (7.83) was recorded for Thiamine-HCl (20 ppm) after 4 weeks of culturing and was on par with Thiamine-HCl 15 ppm and 25 ppm.

Eight weeks after culturing, significant difference could not be observed among the treatments.

Number of roots

At the end of 4 weeks of culturing, no significant difference was observed among the treatments with regard to the number of roots produced.

Eight weeks after culturing, the highest number (2.17) of roots was recorded for media containing no Thiamine-HCl and this was on par with Thiamine-HCl 5 ppm and 10 ppm. The lowest number of roots (1.17) was observed in media with Thiamine-HCl 20 ppm and 25 ppm and was on par with Thiamine-HCl 10 and 15 ppm.

4.2.5 Effect of plant growth regulators

Trials were conducted involing auxins (NAA and 2,4-D) and cytokinins (BA and KIN) to study their effect on the induction of axillary buds and shoots *in vitro*. The data collected are presented below.

4.2.5.1 Effect of BA, NAA and 2,4-D on axillary bud development

Trials were conducted to induce axillary buds from nodal explants in media with different combinations of BA, NAA and 2,4-D, the results of which are presented in Table 14.

Among the 15 treatments tried, the time taken for swelling of the nodes was minimum (7.80 days) for the treatment combination involving BA 20 ppm + NAA 2 ppm and this was on par with treatment combination involving BA 10 ppm + NAA 2 ppm, BA 10 ppm + 2,4-D 2 ppm, BA 20 ppm + 2,4-D 2 ppm and BA 10 ppm + NAA 2 ppm + 2,4-D 2 ppm. The time taken for swelling of the nodes was maximum (18.00 days) for the treatment combination involving BA 5 ppm + 2,4-D 1 ppm and this was on par with BA 5 ppm + NAA 1 ppm and BA 10 ppm + 2,4-D 1 ppm.

The time taken for the development of buds was found to be minimum (17.00 days) for treatment combination involving BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm and this was significantly superior to all the other treatments.

With regard to the time taken for the buds to attain a size of 0.5 cm, the minimum number of days (39.80) was observed for the treatment combination involving BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm (Plate 3) and this was significantly superior to all the other treatments.

4.2.5.2 Effect of BA, NAA and adenine on axillary bud development

Data pertaining to the results of trials conducted to induce axillary buds from nodal explants, by using combinations of BA, NAA and adenine are presented in Table 15.

Table 14. Effect of BA, NAA and 2,4-D on axillary bud development from nodal explants

Medium : ½MS + Sucrose 3%
Culture period : 8 weeks

Treatments			Time taken for swelling of the nodes (days)	Time taken for bud development (days)	Time taken for the buds to attain a size of 0.5 cm (days)	Remarks
BA (ppm)	NAA (ppm)	2,4-D				
5	1	-	17.80	-	-	Only nodal swelling, no bud development
10	1	-	15.00	-	-	-do-
20	1	-	11.20	19.00	44.00	Bud development good, but blackened
5	2	-	11.80	22.20	52.60	Bud development good
10	2	-	8.20	-	-	Only nodal swelling, no bud development
20	2	-	7.80	-	-	-do-
5	-	1	18.00	-	-	-do-
10	-	1	16.20	-	-	-do-
20	-	1	11.40	-	-	-do-
5	-	2	11.60	21.20	51.80	Bud development good
10	-	2	9.00	-	-	Only nodal swelling, no bud development
20	-	2	8.80	-	-	-do-
5	2	2	10.00	17.00	39.80	Very good bud development
10	2	2	9.80	22.80	-	Bud initiation, no elongation
20	2	2	11.40	27.60	-	-do-
CD (0.05)			2.11	1.89	1.95	
SEm±			0.75	0.64	0.63	

Mean of 5 observations
- No response

Table 15. Effect of BA, NAA and adenine on axillary bud development from nodal explants

Explant : Shoot node
Medium : ½MS + NAA 1 ppm
Culture period: 12 weeks

Treatment		Time taken for swelling of nodes (days)	Time taken for bud emergence (days)	Time taken for the buds to attain a size of 0.5 cm (days)	Remarks
BA (ppm)	Adenine (ppm)				
5	10	20.4	-	-	Only nodal swelling, no bud development
	20	15.6	-	-	-do-
	40	11.0	-	-	-do-
10	10	15.0	-	-	-do-
	20	19.6	-	-	-do-
	40	20.0	-	-	-do-
20	10	11.0	23.2	56.2	Very good development of bud
	20	14.0	29.8	79.0	Bud development good
	40	15.0	-	-	Only nodal swelling
CD (0.05)		1.58	2.99	3.66	
SEm±		0.55	0.76	0.93	

Mean of 5 observations
- No response

Among the nine treatment combinations tried, minimum number of days (11.0) for swelling of nodes was observed for two treatment combinations, viz., BA 5 ppm + NAA 1 ppm + adenine 40 ppm and BA 20 ppm + NAA 1 ppm + adenine 10 ppm. These treatments were significantly superior to all the other treatments.

With regard to the number of days taken for bud development seven treatments out of the nine treatment combinations did not show any sign of bud development. The treatment combination involving BA 20 ppm + NAA 1 ppm + adenine 10 ppm recorded 23.2 days for bud development and was significantly superior to BA 20 ppm + NAA 1 ppm + adenine 20 ppm.

The time taken for the buds to attain a size of 0.5 cm was also minimum (56.2 days) for the treatment combination involving BA 20 ppm + NAA 1 ppm + adenine 10 ppm.

4.2.5.3 Effect of BA, NAA, 2,4-D and adenine on the growth of pollinia

Data relating to the effect of BA, NAA, 2,4-D and adenine on the growth of pollinia are presented in Table 16.

During the 4 weeks culture period, the pollinia started to callus and the time taken for callusing was minimum (2.0 days) for the treatment combination involving BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm and it was significantly superior to all the other treatments.

Table 16. Effect of BA, NAA, 2,4-D and adenine on the growth of pollinia

Medium: ½ MS
Culture period : 4 weeks

Treatments				Time taken for callusing (days)	Remarks
BA	NAA	2,4-D	Adenine		
1	2	2	--	15.6	Size doubled within three weeks
3	2	2	--	8.6	Size doubled within two weeks
5	2	2	--	2.0	Size increased to five times within two days
1	20	-	10	6.8	Size increased to five times within one week
3	20	-	10	16.6	Size doubled within four weeks
5	20	-	10	21.4	-do-
CD (0.05)				1.65	
SEm±				0.56	

Mean of 5 observations

4.2.5.4 Effect of BA, KIN, NAA, 2,4-D and adenine on the growth of seeds

The data are presented in Table 17. When 70 day old pod was used for culturing, the embryos did not turn green during the culture period of 15 weeks, in any of the 10 treatment combinations tried.

When pod of 80 day maturity was used, the time taken for greening of embryo was minimum (26.0 days) in the treatment combination involving BA 10 ppm + NAA 1 ppm. This was significantly superior to all the other treatments. With regard to protocorm formation, none of the treatments showed any response during the period under study.

In the case of 90 day old pod, the days taken for greening of embryo was minimum (14.6) for treatment combination involving BA 10 ppm + NAA 1 ppm and KIN 5 ppm + 2,4-D 2 ppm. These treatments were significantly superior to all the other treatments. When the number of days taken for protocorm formation was considered, the treatment combination KIN 5 ppm + 2,4-D 2 ppm took 30.2 days (Plate 4) was on par with BA 10 ppm + NAA 1 ppm (30.8 days). No other treatments recorded protocorm formation.

When 100 days old pod was used for culturing, the number of days taken for greening of embryo was minimum (45.8) for the treatment combination with BA 10 ppm + NAA 1 ppm. This was significantly superior to all other treatments except KIN 5 ppm + 2,4-D 2 ppm. None of the treatments using 100 day old pod recorded protocorm formation.

Plate 3. Infloresence stalk node with a bud developed in 1/2 MS medium + BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm

Plate 4. Protocorm development from seeds in 1/2 MS medium + KIN 5 ppm + 2,4-D 2 ppm

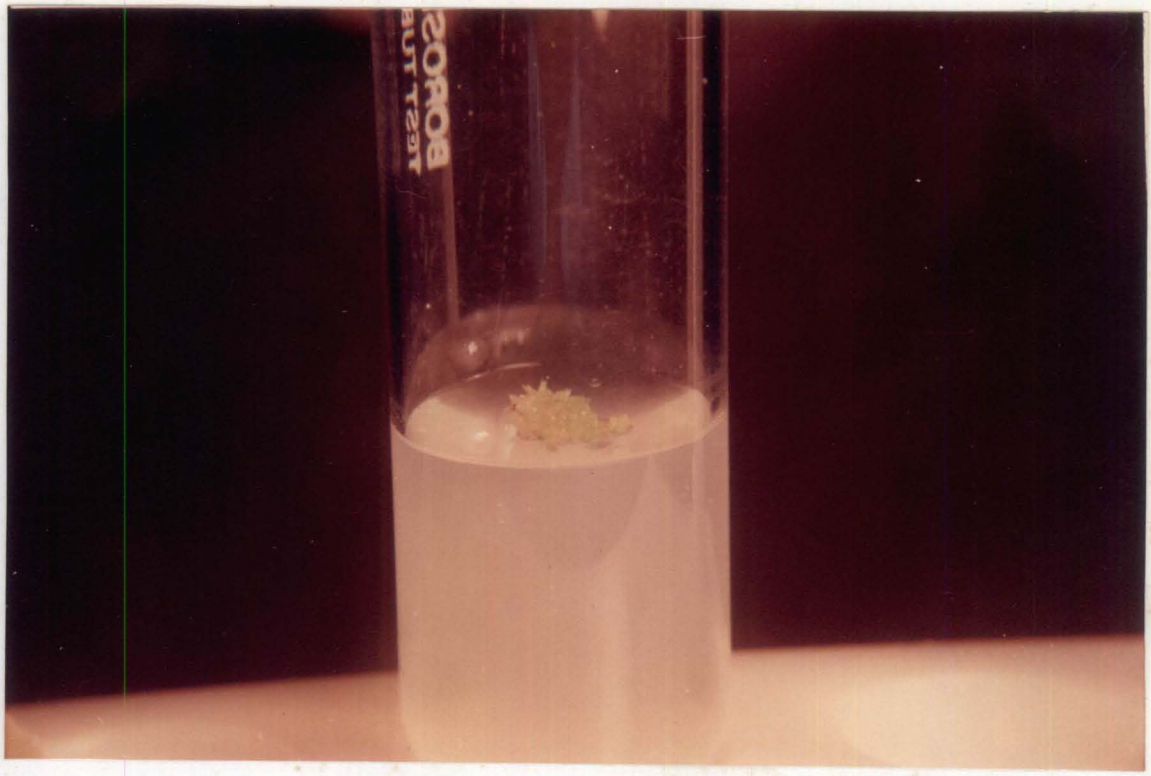
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Table 17. Effect of cytokinins (BA and KIN), auxins (NAA and 2,4-D) and adenine on the growth of seeds

Medium: 1/2 MS
Culture period : 15 weeks

Treatments					Maturity of pod					
					80 days		90 days		100 days	
BA	KIN	NAA (ppm)	2,4-D	Adenine	Time taken for					
					Greening of seeds (days)	Protocorm formation (days)	Greening of seeds (days)	Protocorm formation (days)	Greening of seeds (days)	Protocorm formation (days)
10	-	1	-	-	26.0	-	14.6	30.8	45.8	-
10	-	2	-	-	66.2	-	43.2	-	95.4	-
-	5	-	1	-	-	-	-	-	-	-
-	5	-	2	-	28.0	-	14.6	30.2	47.6	-
5	-	2	2	-	42.6	-	24.2	-	61.8	-
15	-	2	2	-	52.8	-	37.4	-	84.4	-
-	5	2	2	-	41.6	-	25.4	-	63.2	-
-	15	2	2	-	59.2	-	39.2	-	87.6	-
10	-	2	-	10	42.8	-	25.8	-	61.0	-
20	-	2	-	20	55.4	-	38.0	-	84.4	-
CD(0.05)					2.09		1.66	NS	3.07	
SEm±					0.72		0.57	0.17	0.78	

Mean of 5 observations
- No response

4.2.5.5 Effect of NAA on the induction of multiple shoots

The results of the trials conducted to study the effect of NAA on the induction of multiple shoots, in ½ MS medium containing BA 10 ppm and adenine 10 ppm are presented in Table 18.

Number of shoots

No significant difference was noticed among the different levels of NAA either after 4 weeks or after 8 weeks of culturing.

Number of leaves

In this case also, no significant difference was noticed among the different levels of NAA after 4 and 8 weeks of culturing.

Number of roots

At 4 and 8 weeks of culturing, the number of roots was found to increase with the increase in NAA level. Four weeks after culturing, the maximum number of roots (3.67) was produced by the treatment involving NAA 5 ppm which was on par with NAA 3 and 4 ppm. After 8 weeks of culturing, the maximum number (4.50) was recorded for the treatment having NAA 5 ppm and was on par with NAA 4 ppm. In the absence of NAA, the lowest number of roots (1.50 and 1.67) was observed.

4.2.5.6 Effect of BA on the induction of multiple shoots

The results of the trials carried out to study the effect of BA on inducing shoots in medium containing adenine and NAA are presented in Table 19.

Table 18. Effect of NAA on the induction of multiple shoots *in vitro*

Medium: ½ MS + BA 10 ppm + Adenine 10 ppm

Treatment	4 weeks			8 weeks			Remarks
NAA (ppm)	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No. of roots	
0	2.17	5.33	1.50	4.33	7.00	1.67	No development of PLBs
1	2.67	5.67	2.00	4.17	7.33	2.50	Good development of PLBs
2	2.33	5.33	2.17	4.00	7.00	2.83	-do-
3	2.00	5.00	3.00	3.67	6.50	3.50	PLB development
4	2.00	5.00	3.33	3.33	6.17	4.17	Slow PLB development
5	1.83	4.33	3.67	2.83	5.50	4.50	-do-
CD (0.05)	NS	NS	0.84	NS	NS	0.79	
SEm±	0.39	1.58	0.29	0.64	0.56	0.27	

Mean of 6 observations

Table 19. Effect of BA on the induction of multiple shoots *in vitro*

Explant : Shoot node
Medium: ½ MS + Adenine 10 ppm + NAA 1 ppm

Treatment	4 weeks			8 weeks			
BA (ppm)	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No. of roots	Remarks
0	1.33	3.33	1.67	2.67	5.67	2.17	No PLB development
5	2.17	4.33	1.67	3.67	6.33	2.17	Tendency to develop PLBs
10	2.67	5.67	2.00	4.67	7.33	2.50	Good development of PLBs
15	2.83	5.83	1.67	4.83	7.67	2.33	-do-
20	3.00	6.17	1.50	4.83	8.00	2.33	-do-
25	3.33	6.17	1.50	5.00	7.67	2.00	-do-
CD (0.05)	0.76	1.54	NS	1.44	NS	NS	
SEm±	0.26	0.53	0.22	0.49	0.66	0.27	

Mean of 6 observations

Number of shoots

The number of shoots was found to increase with increase in the level of BA in the medium (Plate 5). After 4 weeks of culturing, the maximum number of shoots (3.33) was recorded at BA 25 ppm and it was on par with BA 10 ppm, 15 ppm and 20 ppm. The maximum number (5.00) of shoots after 8 weeks of culturing was recorded at BA 25 ppm and it was on par with all the other treatments except for media devoid of BA.

Number of leaves

The number of leaves was also found to increase with increase in the level of BA, reaching a peak at BA 20 ppm. The maximum number of leaves (6.17) was recorded after 4 weeks of culturing at BA 20 ppm and 25 ppm and it was on par with BA 10 ppm and 15 ppm. After 8 weeks of culturing no significant difference was recorded among the different levels of BA.

Number of roots

No significant differences existed among the different levels of BA, with regard to root number.

4.2.5.7 Effect of BA and NAA on the induction of multiple shoots

The results are presented in Table 20.

Number of shoots

After 4 weeks of culturing, the maximum number of shoots (3.00) was

Table 20. Effect of BA and combinations of BA and NAA on the induction of multiple shoots *in vitro*

Explant: Shoot node
Medium: 1/4 MS

Treatment		4 weeks			8 weeks		
NAA (ppm)	BA	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No. of roots
-	1	1.00	4.00	1.17	1.17	4.67	1.50
-	5	1.33	4.17	1.33	1.83	5.83	1.50
-	10	1.50	4.50	1.50	3.33	6.50	1.67
-	15	1.67	4.83	1.50	3.83	6.67	2.17
-	20	1.67	4.33	1.33	3.50	6.00	1.50
0.1	1	1.00	4.00	1.33	1.50	4.67	1.67
,,	5	1.67	4.17	1.50	2.17	6.00	1.67
,,	10	2.00	4.67	1.67	3.67	6.67	1.83
,,	15	1.83	5.00	1.67	4.00	5.00	2.33
,,	20	2.33	5.00	1.67	4.50	6.83	2.83
1.0	1	1.00	4.00	1.50	1.50	4.83	1.83
,,	5	2.00	4.33	1.67	2.50	6.00	2.00
,,	10	2.17	4.67	1.83	3.67	6.67	2.17
,,	15	2.33	5.17	1.83	4.33	6.83	2.67
,,	20	3.00	5.67	2.00	4.83	7.17	3.33
CD (0.05)		0.58	0.66	NS	0.93	0.91	0.75
SEm ±		0.21	0.23	0.20	0.33	0.32	0.27

Mean of 6 observations

produced in the treatment involving BA 20 ppm + NAA 1.0 ppm and was significantly superior to all the other treatments. The lowest number of shoots (1.00) was recorded at BA 1 ppm, BA 1 ppm + NAA 0.1 ppm and BA 1 ppm + NAA 1.0 ppm.

Maximum number of shoots (4.83) was observed after 8 weeks of culturing at BA 20 ppm + NAA 1.0 ppm and it was on par with BA 15 ppm + NAA 0.1 ppm, BA 20 ppm + NAA 0.1 ppm and BA 15 ppm + NAA 1.0 ppm. The lowest number (1.17) of shoots was recorded at BA 1 ppm.

Number of leaves

Maximum number of leaves (5.67) after 4 weeks of culturing was produced in the treatment combination involving BA 20 ppm + NAA 1.0 ppm and it was on par with BA 15 ppm + NAA 1.0 ppm.

After 8 weeks of culturing, the maximum number of leaves (7.17) was recorded at BA 20 ppm + NAA 1.0 ppm and the lowest number of leaves (4.67), at BA 1 ppm and BA 1 ppm + NAA 0.1 ppm.

Number of roots

After 4 weeks of culturing, no significant difference was observed among the different treatments. Maximum root number (3.33) after 8 weeks was recorded at BA 20 ppm + NAA 1.0 ppm. After 8 weeks of culturing, the minimum number (1.50) of roots was observed at BA 1 ppm, BA 5 ppm and BA 20 ppm.

4.2.5.8 Effect of BA and 2,4-D on the induction of multiple shoots

Twelve treatment combinations involving BA and 2,4-D were tried to study their effect on multiple shoot induction and the data are presented in Table 21.

Number of shoots

Maximum number of shoots (2.67) after 4 weeks of culturing was produced in the treatment combination involving BA 20 ppm + 2,4-D 2.5 ppm and it was on par with BA 10.0 ppm + 2,4-D 2.5 ppm, BA 15.0 ppm + 2,4-D 2.5 ppm, BA 15.0 ppm + 2,4-D 5.0 ppm and BA 20.0 ppm + 2,4-D 5.0 ppm. After 8 weeks of culturing, the treatment combination involving BA 20.0 ppm + 2,4-D 2.5 ppm recorded the maximum number (4.83) of shoots, which was on par with BA 15 ppm + 2,4-D 2.5 ppm and BA 15 ppm + 2,4-D 5.0 ppm.

Number of leaves

No significant difference among the treatments existed with regard to leaf number after 4 and 8 weeks of culturing.

Number of roots

Maximum number of roots (3.50 and 4.00) was recorded at BA 20 ppm + 2,4-D 5.0 ppm after 4 and 8 weeks of culturing respectively and these were on par with the treatment combination involving BA 15 ppm + 2,4-D 5.0 ppm and BA 20 ppm + 2,4-D 2.5 ppm and were significantly superior to all the other treatments.

Table 21. Effect of BA and 2,4-D on the induction of multiple shoots *in vitro*

Explant: Shoot node
Medium : 1/4 MS

Treatment		4 weeks			8 weeks		
BA (ppm)	2,4-D	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No. of roots
5	0	1.33	4.17	1.33	1.83	5.83	1.50
"	2.5	1.83	4.17	1.67	2.33	6.17	2.00
"	5.0	1.67	4.17	2.00	1.83	5.83	2.50
10	0	1.67	4.50	1.50	3.33	6.50	1.67
"	2.5	2.17	4.67	2.00	3.67	6.67	2.50
"	5.0	1.83	4.67	2.33	3.50	6.33	2.83
15	0	1.83	4.83	1.50	3.67	6.67	2.17
"	2.5	2.33	5.00	2.33	4.00	6.67	2.83
"	5.0	2.00	4.67	2.67	4.00	6.50	3.17
20	0	1.83	4.33	1.33	3.50	6.00	1.50
"	2.5	2.67	5.33	3.00	4.83	6.83	3.50
"	5.0	2.33	5.00	3.50	3.33	6.33	4.00
CD (0.05)		0.73	NS	0.86	0.95	NS	0.88
SEm±		0.26	0.29	0.30	0.33	0.38	0.31

Mean of 6 observations

4.2.5.9 Effect of BA, NAA and 2,4-D on the induction of multiple shoots

The results of the trials conducted using different combination of BA and 2,4-D in media containing NAA 2 ppm are presented in Table 22.

Number of shoots

Maximum number of shoots was produced by BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm after 4 weeks of culturing (3.33) and (5.33) after 8 weeks of culturing (Plate 6).

Number of leaves

Largest number of leaves (6.33) was recorded at BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm after 4 weeks of culturing and was significantly superior to all other treatments except BA 10 ppm + NAA 2 ppm + 2,4-D 2 ppm and BA 20 ppm + NAA 2 ppm. After 8 weeks of culturing, the maximum number of leaves (7.83) was recorded at BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm and was significantly superior to all other treatments except BA 10 ppm + NAA 2 ppm, BA 10 ppm + NAA 2 ppm + 2,4-D 2 ppm and BA 15 ppm + NAA 2 ppm.

Number of roots

After 4 weeks of culturing, no significant difference was found to exist among the treatments. At the end of 8 weeks of culturing, maximum number of roots (3.67) was recorded in the treatment, BA 15 ppm + NAA 2 ppm + 2,4-D 4 ppm. Large globular structures were found to develop from the tip of the roots in contact with the media in the treatment combination of BA 15 ppm + NAA 2 ppm + 2,4-D 2 ppm (Plate 7).

Plate 5. Effect of BA on multiple shoot induction - BA 1 ppm, 10 ppm and 20 ppm

Plate 6. Multiple shoot induction in 1/4 MS medium + BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm

Table 22. Effect of BA, NAA and 2,4-D on the induction of multiple shoots *in vitro*

Explant: Shoot node
Medium: 1/4 MS + NAA 2 ppm + sucrose 1.5%

Treatment		4 weeks			8 weeks		
BA (ppm)	2,4-D	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No. of roots
1	0	1.00	4.00	1.50	1.50	4.83	2.00
"	2	1.50	4.17	1.67	3.33	5.33	2.33
"	4	1.00	3.50	1.83	1.50	4.67	2.50
5	0	2.00	4.33	1.83	2.50	6.00	2.33
"	2	3.33	6.33	2.17	5.33	7.83	2.33
"	4	1.33	4.00	2.33	2.17	5.17	2.83
10	0	2.17	4.67	2.00	3.67	6.67	2.00
"	2	2.50	5.83	2.50	4.83	7.33	3.00
"	4	1.33	4.17	2.00	3.33	6.33	3.50
15	0	2.33	5.17	2.17	4.33	6.83	2.83
"	2	2.67	4.83	2.67	4.50	6.33	3.17
"	4	1.50	4.50	2.33	3.83	6.00	3.67
20	0	3.00	5.67	2.33	4.83	7.17	3.00
"	2	2.17	4.67	3.00	4.17	6.17	3.50
"	4	1.50	4.67	2.33	4.17	5.00	3.50
CD(0.05)		1.00	1.00	NS	1.20	1.20	1.09
SEm±		0.35	0.36	0.31	0.43	0.43	0.39

Mean of 6 observations

4.2.5.10 Effect of KIN on the induction of multiple shoots

Results of the trials conducted to study the effect of KIN in inducing shoots are presented in Table 23.

Number of shoots

No significant differences existed among the different levels of KIN, with respect to shoot number after 4 weeks of culturing.

Number of leaves

At the end of 4 weeks of culturing no significant differences existed among the different levels of KIN, with regard to leaf number.

Number of roots

After 4 weeks of culturing, no significant difference was observed among the different levels of KIN, when the root number was considered.

4.2.5.11 Effect of KIN and NAA on the induction of multiple shoots

Different levels of KIN and NAA were tried in the media, to study their effect in inducing shoots and the results are presented in Table 23.

Number of shoots

With respect to shoot number, the different treatment combinations did not show any significant difference, at the end of 4 weeks of culturing.

Table 23. Effect of KIN and combinations of KIN and NAA on the induction of multiple shoots *in vitro*

Explant : Shoot node
Medium: 1/4 MS + sucrose 1.5%
Culture period: 4 weeks

Treatment Kinetin (ppm)	No.of shoots	No.of leaves	No.f roots
0	1.00	3.33	1.00
10	1.33	4.17	1.33
20	1.67	4.33	1.50
30	1.83	4.50	1.50
40	1.83	4.33	1.33
50	1.67	4.33	1.17
CD(0.05)	NS	NS	NS
SEm±	0.25	0.27	0.18

Kinetin (ppm)	NAA	No.of shoots	No.of leaves	No.of root
20	0	1.67	4.33	1.50
,,	1	2.00	4.67	1.50
,,	2	2.33	5.00	1.50
,,	3	1.67	4.17	1.50
,,	4	1.50	3.00	1.67
,,	5	1.50	3.00	1.83
CD(0.05)		NS	0.80	NS
SEm±		0.24	0.28	0.20

Mean of 6 observations

Number of leaves

After 4 weeks of culturing, the maximum number (5.00) of leaves was recorded in KIN 20 ppm + NAA 2 ppm and it was on par with KIN 20 ppm and KIN 20 ppm + NAA 1 ppm.

Number of roots

No significant difference was observed among the different treatments with regard to root number after 4 weeks of culturing.

4.2.5.12 Effect of KIN, NAA and 2,4-D on the induction of multiple shoots

Results of the trials conducted to study the effect of KIN, NAA and 2,4-D on inducing axillary shoots are presented in Table 24.

Number of shoots

With regard to number of shoots, after 4 weeks of culturing the maximum number (2.67) was produced at KIN 20 ppm + NAA 2 ppm + 2,4-D 2 ppm and was significantly superior to all other treatments, except KIN 25 ppm + NAA 2 ppm + 2,4-D 2 ppm.

Number of leaves

After 4 weeks of culturing, maximum number of leaves (4.83) was recorded in KIN 20 ppm + NAA 2 ppm + 2,4-D 2 ppm and was on par with all the other treatments, except NAA 2 ppm + 2,4-D 2 ppm.

Table 24. Effect of KIN, BA, NAA, 2,4-D and adenine on the induction of axillary shoots *in vitro*

Medium: 1/4 MS + sucrose 1.5%
Culture period: 4 weeks

Treatment			No. of shoots	No. of leaves	No. of roots
KIN	NAA (ppm)	2,4-D			
0	2	2	1.00	3.00	1.67
5	2	2	1.67	4.00	1.83
10	2	2	1.67	4.00	1.83
15	2	2	2.00	4.50	2.00
20	2	2	2.67	4.83	2.33
25	2	2	2.33	4.33	2.33
CD(0.05)			0.54	0.97	NS
SEm±			0.18	0.33	0.20

KIN	BA (ppm)	Adenine	2,4-D	No. of shoots	No. of leaves	No. of roots
0	5	20	5	2.00	4.50	2.33
5	"	,,	"	3.00	6.00	2.50
10	"	,,	"	3.17	6.17	2.50
15	"	,,	"	3.00	6.00	2.67
20	"	,,	"	3.00	5.83	2.50
25	"	,,	"	2.67	5.33	2.50
CD(0.05)				NS	NS	NS
SEm±				0.32	0.47	0.27

Mean of 6 observations

Number of roots

No significant difference was observed among the different treatments, when the number of roots was considered.

4.2.5.13 Effect of KIN, BA, 2,4-D and adenine on the induction of multiple shoots

The results, presented in Table 24, indicated no significant difference among the treatments with respect to production of shoots, leaves and roots after 4 weeks of culturing.

4.2.6 Effect of medium supplements

In addition to growth regulators, organic additives like coconut water, tomato juice, peptone etc. when added into the medium was found to influence the growth of the cultures. Trials were conducted with these additives and the data collected are presented below.

4.2.6.1 Effect of coconut water on axillary bud development from nodal explants

Trials were conducted to know whether coconut water was having any influence on axillary bud development from inflorescence stalk nodes and the results are presented in Table 25.

With regard to number of days taken for swelling of nodes, the minimum (6.0) number of days was recorded for T_2 (BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm + CW 5%) and T_4 (BA 20.0 ppm + NAA 1.0 ppm + adenine 10.0 ppm + CW 15%) and was found to be significantly superior to all other treatments. Basal

Table 25. Effect of coconut water on axillary bud development from nodal explants

Medium : ½ MS + sucrose 3%
Culture period: 10 weeks

Treatment	Time taken for swelling of nodes (days)	Time taken for bud development (days)	Time taken for the buds to attain a size of 0.5 cm (days)
T_1 - BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm	10.0	17.0	39.8
T_2 - BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm + CW 15%	6.0	14.0	31.8
T_3 - BA 20.0 ppm + NAA 1.0 ppm + Adenine 10.0 ppm	11.0	23.2	56.2
T_4 - BA 20.0 ppm + NAA 1.0 ppm + Adenine 10.0 ppm + CW 15%	6.0	15.2	34.4
T_5 - Control (basal medium)	-	-	-
T_6 - CW 15%	-	-	-
CD(0.05)	1.46	1.63	2.38
SEm±	0.47	0.53	0.77

Mean of 5 observations
- No response

medium and medium containing CW 15 per cent alone did not show any response, during the period under study.

Minimum number (14.0) of days for bud emergence was observed for T_2 (BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm + CW 15%) and was on par with T_4 (BA 20.0 ppm + NAA 1.0 ppm + adenine 10.0 ppm + CW 15%) and were significantly superior to all the other treatments.

The time taken for the buds to attain a size of 0.5 cm was the minimum (31.8 days) for the treatment T_2 (BA 5.0 ppm + NAA 2.0 ppm + 2.4-D 2.0 ppm + CW 15%) and was significantly superior to all other treatments.

4.2.6.2 Effect of coconut water on the induction of multiple shoots

The results of the trial are presented in Table 26.

4.2.6.2.1 Apical bud

Data are presented in Table 26.

Number of shoots

During the period under study, no increase in shoot number was observed. The shoot number remained the same (1.00) for all the treatments during the 12 weeks culture period (Plate 8).

Number of leaves

After 4 weeks of culturing, no significant difference existed among the different levels of coconut water. After 8 weeks of culturing, the maximum number

Plate 7. Globular structures developing from the tip of the roots in 1/4 MS medium + BA 15 ppm + NAA 2 ppm + 2,4-D 2 ppm

Plate 8. Effect of CW on shoot production using apical bud - CW 5%, 15% and 25%

Table 26. Effect of coconut water on the induction of multiple shoots *in vitro*

Medium: 1/4MS + Sucrose 1.5%

Treatment CW (%)	4 weeks				8 weeks				12 weeks			
	a	b	c	d	a	b	c	d	a	b	c	d
Apical bud												
0	1.00	2.00	0.00	1.00	1.00	1.67	0.00	1.00	1.00	1.33	0.00	1.17
5	1.00	2.00	0.00	1.20	1.00	2.33	0.00	1.38	1.00	2.83	0.33	2.45
10	1.00	2.17	0.00	1.25	1.00	2.83	0.00	1.63	1.00	3.17	1.00	2.92
15	1.00	2.33	0.00	1.63	1.00	3.17	0.50	1.82	1.00	3.50	1.67	3.50
20	1.00	2.50	0.17	1.72	1.00	3.33	0.67	1.88	1.00	4.67	2.00	4.00
25	1.00	2.50	0.17	1.93	1.00	3.33	0.50	2.30	1.00	3.00	1.17	3.00
CD(0.05)		NS		0.23		0.83		0.21		0.92	0.92	0.47
SE±		0.17		0.08		0.28		0.07		0.31	0.31	0.16
First node												
0	1.00	2.00	0.00	0.50	1.00	2.17	0.17	0.53	1.00	1.50	0.33	0.58
5	1.50	2.67	1.00	0.73	1.50	3.67	1.00	1.75	1.83	4.67	1.17	2.75
10	2.33	3.33	1.00	1.05	2.50	5.17	1.33	1.92	3.17	7.17	1.67	2.83
15	1.83	2.83	1.00	0.98	1.83	3.83	1.50	1.92	2.17	4.83	2.17	2.50
20	1.67	2.50	1.00	0.70	1.67	3.50	1.33	1.32	2.00	4.50	1.67	1.75
25	1.50	2.50	1.00	0.67	1.50	3.50	1.00	1.23	1.83	2.33	1.33	1.43
CD(0.05)	0.58	0.71		0.23	0.52	0.67	0.27	0.49	0.43	0.78	0.36	0.52
SE±	0.20	0.25		0.08	0.18	0.23	0.01	0.17	0.15	0.27	0.12	0.18
Shoot												
0	1.00	2.00	1.00	0.50	1.00	2.33	1.33	0.53	1.00	1.50	1.00	0.60
5	1.00	2.50	1.00	0.68	1.33	2.67	1.67	1.27	1.67	3.83	2.17	1.57
10	1.67	3.33	1.33	1.05	2.33	4.83	2.67	2.30	2.83	6.33	3.50	2.92
15	1.67	3.50	1.33	1.00	2.33	4.67	2.67	2.32	3.00	6.17	3.67	2.42
20	1.50	3.17	1.33	0.93	2.00	3.83	2.00	1.88	2.67	5.83	3.17	2.15
25	1.50	3.17	1.17	0.83	2.00	3.67	2.00	1.27	2.33	6.00	2.50	2.07
CD(0.05)	0.54	0.58	NS	0.26	0.50	0.79	0.87	0.41	0.76	1.30	0.85	0.29
SE±	0.18	0.20	0.17	0.09	0.17	0.27	0.30	0.14	0.26	0.45	0.29	0.10
Basal portion												
0	1.00	1.00	0.00	0.50	1.00	1.00	0.00	0.50	1.0	1.17	0.00	0.53
5	1.17	1.33	1.00	0.65	2.17	2.17	1.17	1.18	3.17	3.17	1.67	1.48
10	1.50	1.83	1.67	1.00	2.33	2.83	2.17	1.58	3.33	3.17	3.17	2.08
15	1.50	2.00	1.33	1.07	2.33	3.00	1.67	1.75	3.50	3.17	2.00	2.25
20	1.50	2.17	1.17	1.23	2.33	3.00	1.67	1.82	3.50	3.33	1.83	2.07
25	1.83	2.50	1.17	1.47	2.67	4.33	1.50	2.20	4.33	7.17	1.83	2.70
CD(0.05)	NS	0.65	NS	0.24	0.57	0.81	NS	0.32	0.62	1.06	0.83	0.32
SE±	0.19	0.22	0.15	0.08	0.20	0.28	0.31	0.11	0.21	0.36	0.28	0.11

a - Number of shoots; b - Number of leaves; c - Number of roots
d - Length of the longest leaf (cm)

of leaves (3.33) was recorded for CW 20 per cent and 25 per cent and this was on par with CW 10 per cent and 15 per cent. At the end of 12 weeks of culturing, the maximum number of leaves (4.67) was observed for CW 20 per cent and this was found significantly superior to all other treatments. The lowest number (1.33) of leaves was observed for medium without coconut water.

Number of roots

No significant difference was noticed among the different levels of coconut water at 4 and 8 weeks after culturing. At the end of 12 weeks of culturing, the maximum number of roots (2.00) was recorded at 20 per cent level of CW, which was found to be significantly superior to all the other levels, except CW at 15 per cent and 25 per cent.

Length of the longest leaf

At the end of 4 weeks of culturing, the maximum leaf length (1.93 cm) was recorded for CW at 25 per cent and was on par with CW 20 per cent. These treatments were significantly superior to all others. The maximum leaf length (2.30 cm) was observed for CW at 25 per cent, at the end of 8 weeks of culturing and was significantly superior to all other treatments. But it was found that, after 12 weeks of culturing, CW at 20 per cent recorded the maximum leaf length (4.00 cm) and was significantly superior to all other treatments.

4.2.6.2.2 First node

Data are presented in Table 26.

Number of shoots

After 4, 8 and 12 weeks of culturing, the maximum number of shoots (2.33, 2.50 and 3.17, respectively) were observed for CW 10 per cent and it was found differing significantly from all the other levels of coconut water.

Number of leaves

Four weeks after culturing, the leaf number was found to be maximum (3.33) for CW 10 per cent and was on par with that at 5 per cent and 15 per cent. At the end of 8 and 12 weeks of culturing, the maximum number of leaves (5.17 and 7.17 respectively) were recorded for CW 10 per cent and was significantly superior to all other levels of coconut water.

Number of roots

During the 4 week culture period, no difference in root number was observed. At the end of 8 weeks of culturing, CW at 15 per cent registered the maximum (1.50) number of roots and was on par with CW 10 per cent and 20 per cent. The maximum root number (2.17) was observed after 12 weeks of culturing at CW 15 per cent and it was significantly superior to all other levels of coconut water.

Length of longest leaf

After 4 weeks of culturing, the maximum leaf length (1.05 cm) was observed for CW 10 per cent and it was significantly superior to all other levels except CW 15 per cent. The maximum leaf length (1.92 cm) after 8 weeks of culturing was recorded at CW 10 per cent and 15 per cent and was on par with CW 5 per cent. At the end of 12 weeks of culturing, the maximum leaf length (2.83 cm) was

recorded again at CW 10 per cent and this was on par with CW 5 per cent and 15 per cent.

4.2.6.2.3 Shoot node

Results are presented in Table 26.

Number of shoots

At the end of 4 and 8 weeks of culturing, maximum number of shoots (1.67 and 2.33, respectively) were recorded for CW 10 per cent and 15 per cent and this was on par with CW 20 per cent and 25 per cent. After 12 weeks of culturing, the largest number of shoots (3.00) was observed for CW 15 per cent and was on par with all the other levels except CW 5 per cent and medium containing no coconut water.

Number of leaves

Four weeks after culturing, maximum number (3.50) of leaves was recorded for CW 15 per cent and was on par with all other treatments except CW 5 per cent and media containing no coconut water. Maximum number of leaves (4.83) after 8 weeks of culturing was observed for CW 10 per cent and was significantly superior to all other levels except CW at 15 per cent. After 12 weeks of culturing, maximum number of leaves (6.33) was recorded at CW 10 per cent and was on par with all the other levels except CW 5 per cent and medium without coconut water.

Number of roots

No significant difference was observed among the different treatments at

the end of 4 weeks of culturing. After 8 weeks of culturing, the maximum root number (2.67) was recorded at CW 10 per cent and CW 15 per cent and was on par with CW 20 per cent and 25 per cent. Maximum number of roots (3.67) after 12 weeks of culturing was recorded for CW 15 per cent and was on par with CW 10 per cent and 20 per cent.

Length of longest leaf

Maximum leaf length (1.05 cm) after 4 weeks of culturing was recorded for CW 10 per cent and was on par with all other treatments except CW 5 per cent and control. After 8 weeks of culturing, maximum leaf length (2.32 cm) was observed for CW 15 per cent and was significantly superior to all other treatments except CW 10 per cent. Maximum leaf length (2.92 cm) at the end of 12 weeks of culturing was recorded at CW 10 per cent and was significantly superior to all other treatments.

4.2.6.2.4 Basal portion

Results are presented in Table 26.

Number of shoots

Four weeks after culturing, no significant difference was observed among the different levels of coconut water. After 8 weeks of culturing, maximum number of shoots (2.67) was recorded for CW 25 per cent and was on par with all other treatments except control. Maximum number of shoots (4.33), after 12 weeks of culturing was observed at CW 25 per cent and was significantly superior to all other levels.

Number of leaves

After 4 weeks of culturing, maximum number of leaves (2.50) was recorded for CW 25 per cent and was on par with CW 15 per cent and 20 per cent. Maximum number of leaves, 4.33 and 7.17, after 8 and 12 weeks of culturing, respectively was observed for CW 25 per cent and was found significantly superior to all the other treatments.

Number of roots

After 4 and 8 weeks of culturing, no significant difference was found to exist among the different treatments. Maximum number of roots (3.17) was recorded for CW 10 per cent, at the end of 12 weeks of culturing and was found to be significantly superior to all other treatments.

Length of the longest leaf

Maximum leaf length (1.47 cm, 2.20 cm and 2.70 cm) were recorded at the end of 4, 8 and 12 weeks of culturing respectively, for the treatment CW 25 per cent and was found significantly superior to all the other treatments during the period under study.

4.2.6.2.5 Effect of tender and mature coconut water

Results presented in Table 27 indicate that after 8 weeks of culturing, no significant difference was observed with regard to shoot, leaf and root production between tender and mature coconut water. The use of fresh and refrigerated coconut water in the media did not induce any significant difference on the growth of the cultures.

Table 27. Comparison of the growth of *in vitro* shoots in media containing tender and mature coconut water

Explant: Shoot node
Medium: 1/4 MS + BA 16.0 ppm + Adenine 8.0 ppm + CW 15%
Culture period: 8 weeks

Treatment (Days old)	No.of shoots	No.of leaves	No.of roots
		Tender coconut water	
0	6.50	9.17	3.17
2	6.50	9.17	2.83
4	6.33	9.00	2.67
6	6.67	8.67	2.67
		Mature coconut water	
0	6.33	9.33	3.33
2	6.50	9.50	3.33
4	6.50	9.33	3.00
6	6.67	9.17	2.67
CD(0.05)	NS	NS	NS
SEm±	0.52	0.73	0.28

Mean of 6 observations

4.2.6.3 Effect of tomato juice

Data presented in Table 28 indicate that in this case also, at the end of 4 and 8 weeks of culturing, no significant difference was observed with respect to shoot number, leaf number and root number.

4.2.6.4 Effect of adenine in combination with BA

Data are presented in Table 29.

Number of shoots

Four weeks after culturing, maximum number of shoots (3.00) was recorded for adenine 8 ppm + BA 16 ppm and was significantly superior to all other treatments except adenine 12 ppm + BA 16 ppm. After 8 weeks of culturing, maximum shoot number (5.00) was observed for adenine 8 ppm + BA 16 ppm and was on par with all the other treatments except control, BA 4 ppm and 8 ppm.

Number of leaves

After 4 weeks of culturing, maximum number of leaves (6.00) was recorded for adenine 8 ppm + BA 16 ppm and was on par with all the other treatments except control, BA 4 ppm, 8 ppm, 12 ppm and 20 ppm. Maximum number of leaves (7.67) after 8 weeks of culturing was observed for adenine 8 ppm and BA 16 ppm and was on par with all the other treatments except control, BA 4 ppm and 8 ppm.

Table 28. Effect of tomato juice on the induction of multiple shoots *in vitro*

Explant: Shoot node
Meidum: 1/4 MS + BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm

Treatment	4 weeks			8 weeks		
Tomato juice (%)	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No. of roots
0	3.33	6.33	2.17	5.83	7.83	2.33
3	3.67	6.50	2.33	6.00	8.17	2.67
6	3.83	6.67	2.50	6.33	8.50	3.00
9	4.00	7.00	2.83	6.67	8.83	3.50
CD(0.05)	NS	NS	NS	NS	NS	NS
SEm±	0.45	0.77	0.48	0.41	0.58	0.39

Mean of 6 observations

Table 29. Effect of adenine in combination with BA on the induction of multiple shoots *in vitro*

Explant: Shoot node
Medium : 1/4 MS + sucrose 1.5%

Treatment		4 weeks			8 weeks		
Adenine (ppm)	BA	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No. of roots
-	0	1.00	3.33	1.00	1.00	2.67	1.17
-	4	1.17	4.17	1.17	1.67	5.00	1.33
-	8	1.50	4.33	1.33	2.83	5.17	1.50
-	12	1.67	4.67	1.50	3.50	6.50	1.67
-	16	2.00	5.00	1.33	4.17	6.67	1.50
-	20	1.83	4.33	1.33	3.50	6.00	1.50
4	16	2.17	5.33	1.50	4.33	7.00	1.67
8	,,	3.00	6.00	1.50	5.00	7.67	2.17
12	,,	2.33	5.50	2.67	4.33	7.17	3.17
16	,,	2.17	5.50	3.00	4.33	7.17	3.50
20	,,	2.17	5.33	3.00	4.00	6.67	3.83
CD(0.05)		0.74	1.06	0.80	1.85	1.85	0.86
SEm±		0.26	0.38	0.28	0.65	0.65	0.30

Mean of 6 observations

Number of roots

Maximum number of roots (3.00 and 3.83) were recorded after 4 and 8 weeks of culturing, respectively for adenine 20 ppm + BA 16 ppm and was significantly superior to all other treatments except adenine 12 ppm + BA 16 ppm and adenine 16 ppm + BA 16 ppm.

4.2.6.5 Effect of adenine in combination with BA and NAA

Data are presented in Table 30. At the end of 4 and 8 weeks of culturing, no significant difference was observed among the levels of adenine with respect to number of shoots, leaves and roots.

4.2.6.6 Effect of peptone

Data are presented in Table 31. Peptone was found to influence the production of shoot, leaf and root (Fig.4).

Number of shoots

Maximum number of shoots (4.17, 6.67 and 7.83) were recorded after 4, 8 and 12 weeks of culturing respectively at peptone 1000 ppm, and was found significantly superior to the other levels. The lowest number of shoots (1.50, 2.50 and 0.33) were observed at peptone 2000 ppm level, at the end of 4, 8 and 12 weeks of culturing, respectively.

Number of leaves

After 4 weeks of culturing, maximum number of leaves (7.00) was observed for peptone 1000 ppm and was on par with peptone 500 ppm. Maximum

Fig.4. Effect of peptone on the induction of multiple shoots *in vitro*

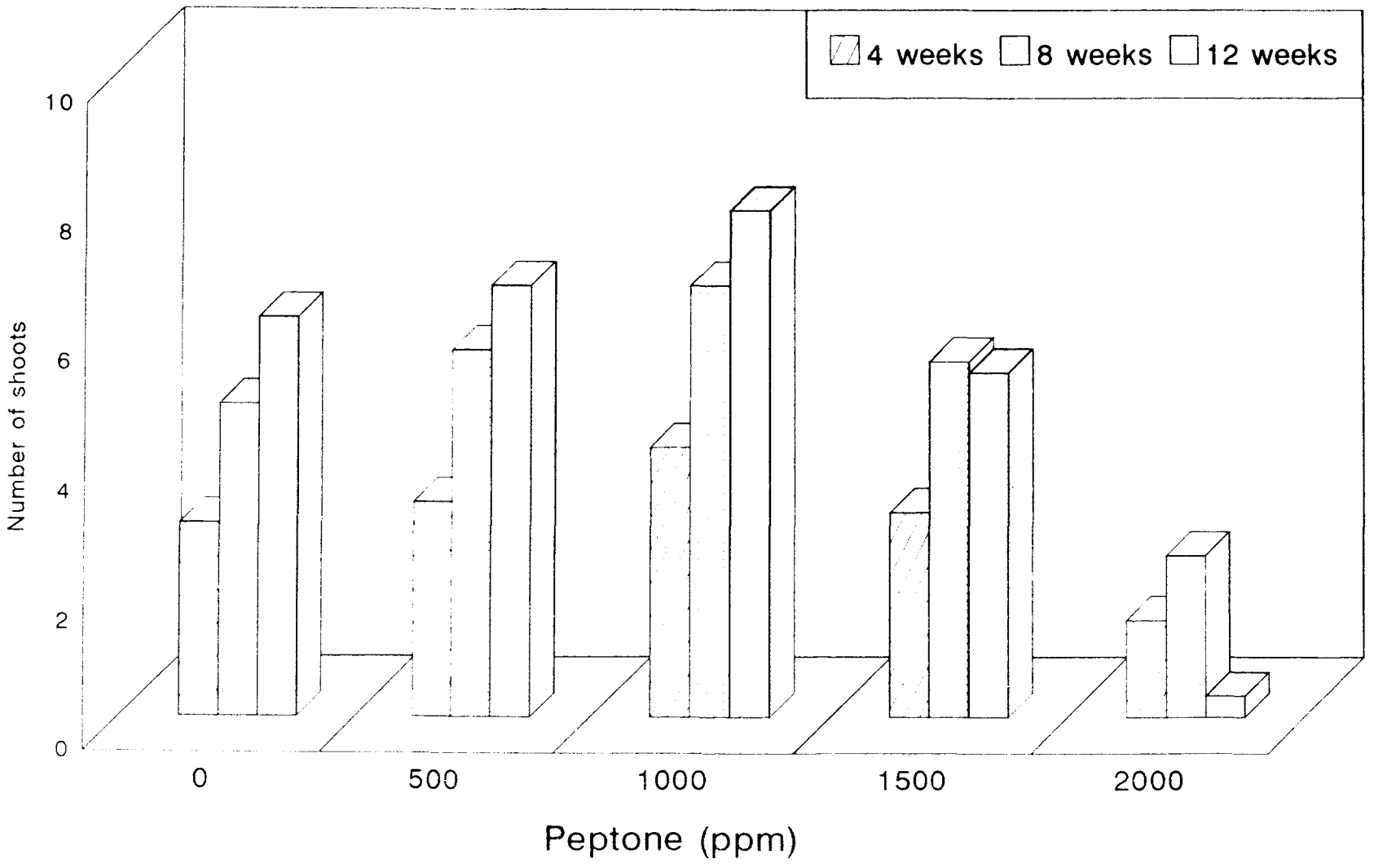

Table 30. Effect of adenine in combination with BA and NAA on the induction of multiple shoots *in vitro*

Medium : 1/4 MS + BA 10 ppm + NAA 1 ppm

Treatment	4 weeks			8 weeks		
Adenine (ppm)	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No. of roots
0	2.17	4.67	1.83	3.67	6.67	2.17
5	2.33	5.00	1.83	4.00	6.83	2.17
10	2.67	5.67	2.00	4.67	7.33	2.50
15	2.33	5.33	2.00	4.33	6.67	2.50
20	2.33	5.33	2.33	4.00	6.67	2.83
25	2.00	5.00	2.67	3.50	6.00	2.33
CD (0.05)	NS	NS	NS	NS	NS	NS
SEm±	0.28	0.52	0.31	0.54	0.64	0.39

Mean of 6 observations

Table 31. Effect of peptone on the induction of multiple shoots *in vitro*

Medium: 1/4 MS + BA 20.0 ppm + NAA 1.0 ppm

Treatment	4 weeks			8 weeks			12 weeks		
Peptone (ppm)	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No. of shoots
0	3.00	5.67	2.00	4.83	7.17	3.33	6.17	8.33	4.50
500	3.33	6.33	2.33	5.67	8.17	3.67	6.67	9.17	5.00
1000	4.17	7.00	2.83	6.67	8.67	4.33	7.83	11.00	5.67
1500	3.17	5.83	2.17	5.50	7.67	3.67	5.33	7.67	3.67
2000	1.50	3.83	1.33	2.50	5.17	2.00	0.33	1.00	0.50
CD (0.05)	0.54	1.28	0.82	0.95	1.11	1.09	1.11	1.88	1.78
SEm±	1.18	0.43	0.28	0.32	0.38	0.37	0.38	0.64	0.60

Mean of 6 observations

number of leaves (8.67) was observed for peptone 1000 ppm at the end of 8 weeks of culturing and was on par with peptone 500 ppm and 1500 ppm. At the end of 12 weeks of culturing, the maximum number of leaves (11.00) was recorded for peptone 1000 ppm, which was on par with peptone 500 ppm.

Number of roots

Maximum number of roots (2.83 and 4.33) were recorded after 4 and 8 weeks of culturing, respectively at peptone 1000 ppm and was on par with all the other levels except peptone 2000 ppm. After 12 weeks of culturing, maximum number of roots (5.67) was recorded for peptone 1000 ppm and was on par with control and peptone 500 ppm. The lowest number of roots (1.33, 2.00 and 0.50) was observed at the end of 4, 8 and 12 weeks of culturing at peptone 2000 ppm.

4.2.7 Effect of different methods in reducing phenols in nodal explants

Data are presented in Table 32. The highest percentage (40) of nodal explants was found to survive when liquid media was used for culturing and the percentage of cultures which got blackened due to phenols was the lowest (10.00) for T_4 (keeping the cultures in the dark) and T_5 (use of liquid media with filter paper bridge). The highest percentage (50) of nodes got blackened in media containing AC 0.1 per cent (T_1).

4.2.8 Effect of antioxidants on the growth of cultures

Results are presented in Table 33. Antioxidants added in the media were found to influence the growth of the cultures (Fig. 5).

Fig.5. Role of antioxidants on the induction of multiple shoots *in vitro*

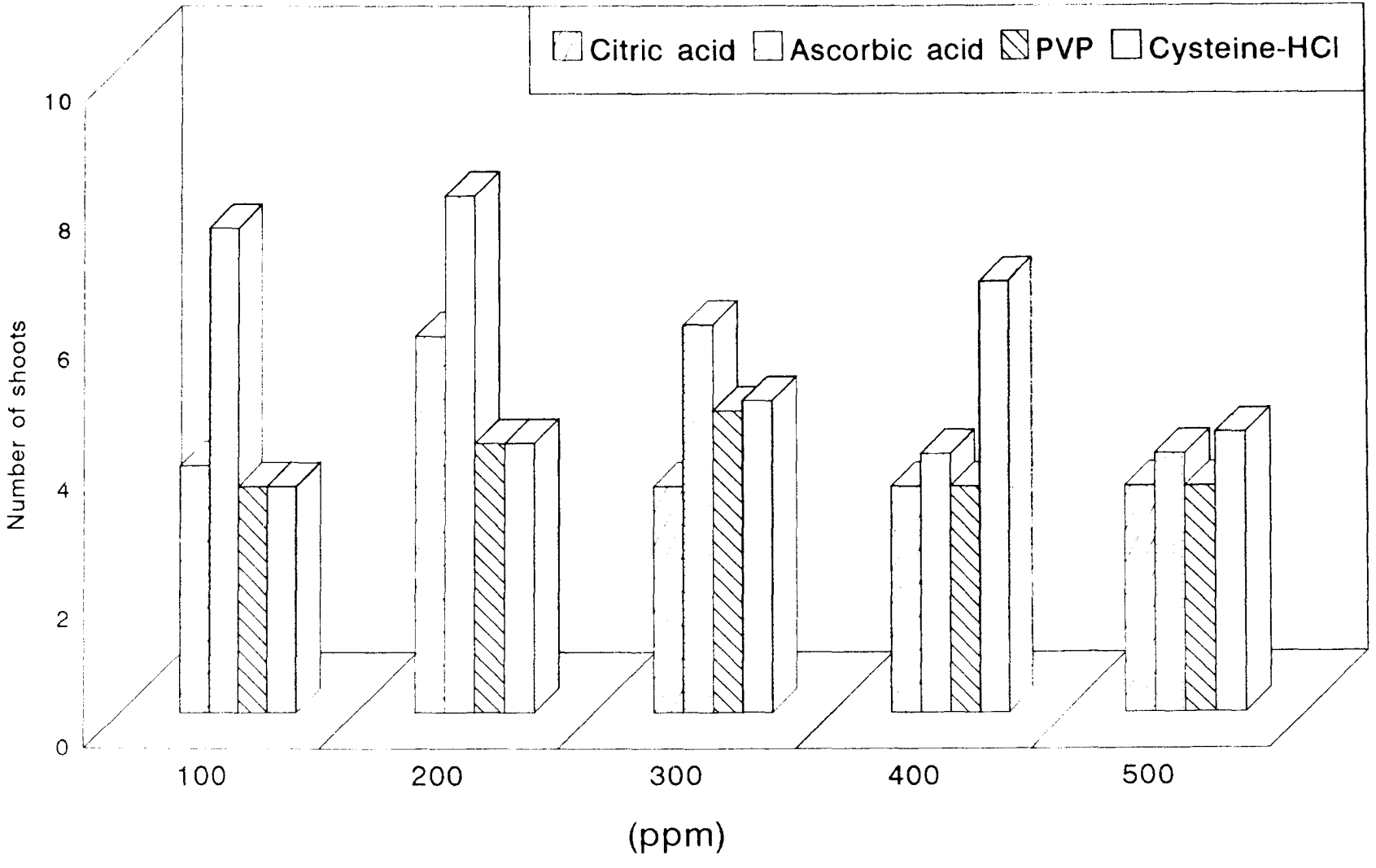

Table 32. Methods to reduce phenolic interference in nodal explants

Medium: ½ MS + BA 10.0 ppm + NAA 1.0 ppm
Culture period: 4 weeks

Treatment	Percentage culture				
	Bleached	Blackened	Fungal infected	Bacterial infected	Surviving
T_1 - Adding AC (0.1%) in the media	10	50	10	30	0
T_2 - Rinsing the explants with PVP 1000 ppm	10	20	30	40	0
T_3 - Rinsing the explants with ascorbic acid 1000 ppm	10	20	30	40	0
T_4 - Keeping the cultures in the dark	20	10	30	40	0
T_5 - Use of liquid media with filter paper bridge	15	10	10	25	40

Values taken as percentage of 20 observations

Table 33. Role of antioxidants in reducing phenolic blackening and on the induction of multiple shoots *in vitro*

Culture period: 8 weeks
Medium: 1/4 MS + BA 10.0 ppm + NAA 0.1 ppm

Treatment	Phenolic exudation			Percentage of cultures**			No. of* shoots	No. of* leaves	No. of* roots
Citric acid (ppm)	1 week	5 week	8 week	Blackened	Surviving	Dead			
100	+++	++++	++++	100	100	0	3.83	7.00	1.83
200	+	++	+++	100	100	0	5.83	7.67	1.83
300	-	+	+	60	80	20	3.50	6.67	1.83
400	-	+	+	20	80	20	3.50	6.67	1.83
500	-	-	-	0	80	20	3.50	7.00	1.67
CD(0.05)							1.38	NS	NS
SEm±							0.47	0.42	0.24
Ascorbic acid (ppm)									
100	+++	++++	+++++	100	100	0	7.50	10.00	2.17
200	++	+++	+++++	100	100	0	8.00	11.00	2.17
300	++	+++	++++	80	100	0	6.00	8.33	2.00
400	+	+	+++	80	100	0	4.00	7.50	2.00
500	-	+	+	40	100	0	4.00	7.17	2.50
CD(0.05)							1.48	1.79	NS
SEm±							0.50	0.61	0.21
PVP (ppm)									
100	+	++	+++	80	100	0	3.50	6.67	1.67
200	-	+	+++	80	100	0	4.17	7.50	1.83
300	-	-	+++	60	100	0	4.67	8.50	1.83
400	-	-	++	40	80	20	3.50	6.67	1.67
500	-	-	+	20	60	40	3.50	6.83	1.67
CD(0.05)							NS	0.93	NS
SEm±							0.32	0.32	0.20
Cysteine-HCl (ppm)									
100	+	+++	+++	60	100	0	3.50	6.67	2.00
200	+	++	++	40	100	0	4.17	8.50	1.83
300	-	+	+	20	100	0	4.83	9.17	1.83
400	-	-	+	20	100	0	6.67	11.67	1.67
500	-	-	-	0	20	80	4.33	9.00	1.67
CD(0.05)							1.02	2.05	NS
SEm±							0.35	0.70	0.20

** Mean of 6 observations

+ + + + + - Total blackening of media; + + + + - Very high phenolic blackening
+ + + - High phenolic blackening; + + - Medium phenolic blackening
+ - Low phenolic blackening

4.2.8.1 Citric acid

When the concentration of citric acid increased, phenolic blackening of media got reduced and the rate was found to increase with time. After 8 weeks of culturing, citric acid 100 and 200 ppm showed blackening of cent per cent of the cultures and at citric acid 500 ppm, there was no blackening of cultures.

With regard to the effect on shoot number, maximum number of shoots (5.83) was recorded at citric acid 200 ppm and this was highly significant than the other treatments.

When the number of leaves and roots was considered, significant difference was not found to exist among the different levels of citric acid.

4.2.8.2 Ascorbic acid

With increase in ascorbic acid content, there was reduction in phenolic blackening of media, and the rate was found to increase at a faster rate than that of citric acid, as the time advanced. Cent per cent of the cultures showed phenolic blackening at 100 and 200 ppm level and the lowest percentage (40) of cultures got blackened at 500 ppm.

After 8 weeks of culturing, maximum number of shoots (8.00) and leaves (11.00) were recorded for ascorbic acid 200 ppm and was significantly superior to other levels except ascorbic acid 100 ppm. Number of roots was found to show no significant difference among the treatments. The lowest number of shoots (4.00) was observed at 400 and 500 ppm and leaves (7.17), at 500 ppm.

4.2.8.3 Polyvinyl pyrrollidene

In this case also, with the increase in PVP concentration, phenolic blackening reduced and as time advanced there was a gradual increase in phenolic blackening. At PVP 100 and 200 ppm, the percentage of cultures blackened was the highest (80) and at 500 ppm, the lowest (20).

No significant difference was observed when the shoot and root number were considered among the different levels. But with regard to leaf number after 8 weeks of culturing, the maximum number (8.50) was observed at PVP 300 ppm and was significantly superior to all the other levels. The lowest leaf number (6.67) was observed at 100 and 400 ppm.

4.2.8.4 Cysteine-HCl

When the concentration of cysteine-HCl went on increasing, phenolic blackening showed drastic reduction, but with advance in time, blackening increased and nearly reached a stagnant point at the end of 5 weeks of culturing. The cultures which showed phenolic blackening was low (60%) when compared to citric acid, ascorbic acid and PVP, at 100 ppm level. At 500 ppm level, cent per cent of the cultures were free of blackening, but the percentage of survival was only 20.

With regard to shoot and leaf number, cysteine-HCl at 400 ppm was found to be significantly superior to all the other levels, as this recorded the maximum number of shoots (6.67) and leaves (11.67) at the end of 8 weeks of culturing. When the number of roots were considered, no significant difference existed among the different levels. The lowest number of shoots (3.50) and leaves (6.67) were observed at 100 ppm level.

4.2.9 Effect of activated charcoal on rooting of *in vitro* shoots

Data are presented in Table 34.

Number of shoots

No significant difference was observed among the different levels of activated charcoal with respect to number of shoots, at the end of 4 and 8 weeks of culturing. After 12 weeks of culturing, significant difference was noticed among the different levels and the maximum number of shoots (4.00) was observed at AC 0.40 per cent and it was on par with AC 0.10 per cent and 0.20 per cent. The lowest number (1.50) was observed for the control.

Number of leaves

In the case of number of leaves also, no significant difference was observed among the different levels of activated charcoal at the end of 4 and 8 weeks of culturing. After 12 weeks of culturing, leaf number was found to show significant difference and the maximum number of leaves (8.83) was recorded for AC 0.40 per cent and was on par with AC 0.10 per cent and 0.20 per cent. The lowest number (3.50) was observed for the control.

Number of roots

After 4 weeks of culturing, no significant difference was observed among the different levels of activated charcoal. But after 8 and 12 weeks of culturing, the largest number of roots (2.50 and 4.17 respectively) was observed at AC 0.80 per cent and was significantly superior to all the other treatments. The lowest

Table 34. Effect of activated charcoal on rooting of *in vitro* shoots

Medium: 1/4 MS + BA 5.0 ppm

Treatment	4 weeks			8 weeks			12 weeks		
AC(%)	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No. of roots	No. of shoots	No. of leaves	No. of roots
0	1.33	4.00	1.33	2.00	5.83	1.17	1.50	3.50	0.83
0.025	1.17	3.83	1.33	1.67	5.67	1.17	2.17	6.33	1.67
0.05	1.33	4.00	1.33	1.83	5.33	1.33	2.50	7.33	2.00
0.10	1.33	4.17	1.33	2.00	5.50	1.33	3.67	8.33	2.50
0.20	1.17	4.33	1.33	1.67	5.67	1.33	3.83	8.67	2.67
0.40	1.33	4.17	1.33	1.83	5.83	1.50	4.00	8.83	3.00
0.80	1.00	3.67	1.67	1.50	5.00	2.50	2.00	7.50	4.17
CD(0.05)	NS	NS	NS	NS	NS	0.48	0.58	1.24	0.69
SEm±	0.17	0.19	0.20	0.17	0.33	0.17	0.20	0.43	0.24

Mean of 6 observations

number (1.17 and 0.83) was recorded for the control at the end of 8 and 12 weeks of culturing, respectively.

4.2.10 Effect of triadimefon on rooting of *in vitro* shoots

Data are presented in Table 35.

Number of shoots

Significant difference was not observed among the different levels of triadimefon, with respect to shoot number during the period (4, 8 and 12 weeks) under study.

Number of leaves

In this case also, significant difference was not observed among the different levels of triadimefon, after 4, 8 and 12 weeks of culturing.

Number of roots

After 4 weeks of culturing, no treatment was found to differ significantly with respect to root number. But after 8 weeks of culturing, significant difference was observed and the maximum number of roots (3.00) was recorded for triadimefon 20 ppm and it was on par with triadimefon 10 ppm and 15 ppm. The lowest number (1.50) was recorded for the control. Again at the end of 12 weeks of culturing, no significant difference was observed among the different levels of triadimefon with regard to root number.

Table 35. Effect of triadimefon on rooting of *in vitro* shoots

Medium: 1/4 MS + BA 5.0 ppm + AC 0.4%

Treatment	4 weeks				8 weeks				12 weeks			
Triadimefon (ppm)	No. of shoots	No. of leaves	No. of roots	Length of the longest root (cm)	No. of shoots	No. of leaves	No. of roots	Length of the longest root (cm)	No. of shoots	No. of leaves	No. of roots	Length of the longest root (cm)
0	1.33	4.17	1.33	1.00	1.83	5.83	1.50	1.50	4.00	8.83	3.00	2.00
5	1.50	3.67	1.33	1.50	1.67	5.50	1.83	2.33	3.83	8.33	3.33	3.00
10	1.67	3.33	1.50	2.33	1.83	5.67	2.17	3.33	4.00	8.50	4.00	4.00
15	1.50	3.67	1.50	3.17	2.00	5.67	2.50	4.17	3.50	8.67	4.00	6.00
20	1.00	3.00	1.33	4.00	1.67	5.33	3.00	6.67	3.67	8.67	4.50	8.00
CD(0.05)	NS	NS	NS	1.11	NS	NS	0.93	1.10	NS	NS	NS	2.39
SE±	0.29	0.31	0.35	0.38	0.34	0.29	0.31	0.37	0.27	0.42	0.62	0.81

Mean of 6 observations

Length of the longest root

After 4 weeks of culturing, significant difference was observed among the different levels of triadimefon and the maximum root length (4.00 cm) was recorded for triadimefon 20 ppm and was significantly superior to all other treatments except triadimefon 15 ppm. The length of the root was maximum (6.67 cm) at the end of 8 weeks of culturing for triadimefon 20 ppm and it was significantly superior to all other treatments. When the root length was considered at the end of 12 weeks of culturing. The maximum length (8.00 cm) was recorded at triadimefon 20 ppm (Plate 9) and was on par with triadimefon 15 ppm and was significantly superior to all other treatments.

4.2.11 Influence of antibiotics added in the culture medium

Data are presented in Table 36. In order to achieve maximum percentage of surviving stalk nodes, the contaminated cultures were subjected to pathological examination and the organisms causing contamination were identified as *Staphylococcus*, *Streptomyces* and *Aspergillus*. The organism which was found to be the most destructive was *Staphylococcus* (Plate 10). As it was a gram positive bacteria it could be controlled using streptopencillin and based on this, streptopencillin was added in the media. With the increase in streptopenicillin level in the media, there was a proportionate increase in the survival percentage. The highest percentage of survival (80) was recorded at 160 and 200 ppm and the lowest percentage (40), for the control. When streptopenicillin was added into the media, the cultures infected by bacteria became zero. The percentage of cultures surviving in media containing coconut water was found to be higher (85) when compared to that without coconut water.

Plate 9. Effect of triadimefon 20 ppm on rooting of *in vitro* shoots

Plate 10. A bacterial contaminated inflorescence stalk node in culture

Table 36. Influence of antibiotics in reducing contamination in nodal explants

Medium: ½MS + BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm (liquid)
Culture period: 4 weeks

Treatment	I							II						
	Percentage of cultures					Days taken for		Percentage of cultures					Days taken for	
Streptopen-cillin (ppm)	Bleached	Blackened	Fungal infected	Bacterial infected	Surviving	Nodal swelling	Bud develop-ment	Bleached	Blackened	Fungal infected	Bacterial infected	Surviving	Nodal swelling	Bud develop-ment
0	15	10	10	25	40	10	17	10	10	10	30	40	6	14
40	15	5	10	0	70	10	17	10	5	10	0	75	6	14
80	15	0	10	0	75	12	19	10	0	5	0	85	7	15
120	15	0	10	0	75	11	18	15	0	0	0	85	7	16
160	20	0	0	0	80	13	18	15	0	0	0	85	8	16
200	20	0	0	0	80	12	20	20	0	0	0	80	9	17

I - Media with no CW
II - Media + CW 15%

Mean of 20 observations

When the number of days taken for nodal swelling and bud development were considered, the minimum number (10 days and 17 days, respectively) were recorded for the media containing streptopencillin at 40 ppm and control. The maximum number of days (20.00) for bud development was observed at 200 ppm. In the media containing coconut water the minimum number of days for nodal swelling (6) and bud development (14) were recorded for streptopencillin 40 ppm and the control. Here also, the maximum number (17.00 days) for bud development was observed at 200 ppm.

4.2.12 Frequency of multiple shoot formation in serial subculturing

Data are presented in Table 37. It was observed that with each subculture, the number of shoots developing was found to go on increasing. All the eight treatment combinations tried, showed a steady increase in multiple shoot formation and very high rate of production (9 to 12 shoots) was observed with combination like BA 20 ppm + NAA 1 ppm; BA 20 ppm + adenine 10 ppm + NAA 1 ppm and BA 20 ppm + adenine 15 ppm + NAA 1 ppm.

4.3 Induction of protocorm like bodies

Protocorm like bodies were found to develop from the base of shoots, tip of inflorescence stalk, leaf and roots and the results of the different trials carried out are presented below.

4.3.1 Effect of sucrose on PLB formation from *in vitro* shoots

With regard to the time taken for PLB development, minimum number of days (40.67) was recorded for sucrose at 1.5 per cent and sucrose at 3.0 per cent

Table 37. Frequency of multiple shoot bud formation during serial subculturing

Medium: 1/4 MS + NAA 1 ppm
Culture period: 4 weeks

Treatments		Subculture				Remarls
BA	Adenine (ppm)	I	II	III	IV	
10	0	+	++	+++	++++	Rate of production increased
,,	10	++	+++	++++	+++++	Very high rate of production
,,	15	+	++	+++	++++	Rate of production increased
,,	20	+	++	+++	++++	Rate of production increased
20	0	++	+++	++++	+++++	Very high rate of production
,,	10	++	+++	++++	+++++	Very high rate of production
,,	15	++	+++	++++	+++++	Very high rate of production
,,	20	+	++	+++	++++	Rate of production increased

\+ - Very low rate of multiplication (1-2 shoots)
++ - Low rate of multiplication (2-3 shoots)
+++ - Medium rate of multiplication (4-5 shoots)
++++ - High rate of multiplication (6-8 shoots)
+++++ - Very high rate of multiplication (9-12 shoots)

level took 84.83 days and other levels of sucrose did not produce PLB's during the period (14 weeks) under study. When the number of PLB's developed were considered, the maximum number (33.33) was observed at 1.5 per cent level of sucrose and a very low number (2.67) at sucrose 3.0 per cent (Table 38).

4.3.2 Effect of Thiamine-HCl on PLB formation from *in vitro* shoots

No significant difference was observed among the different levels of Thiamine-HCl with regard to the number of days taken for the formation of PLB's. When the number of PLB's developed was considered the largest number (9.67) was recorded for 25 ppm and was found to be significantly superior to all the other levels except 20 ppm, at the end of 8 weeks of culturing (Table 39).

4.3.3 Effect of BA, NAA and 2,4-D on PLB formation from *in vitro* shoots

With regard to the time taken for PLB formation, the minimum number (40.67) of days was recorded for BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm (Plates 11 and 12) and was significantly superior to all other treatments except BA 20 ppm + 2,4-D 4 ppm. The same treatment i.e., BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm recorded the largest number (4.17) of PLB's after 8 weeks of cutluring and was significantly superior to all the other treatments (Table 40).

4.3.4 Effect of BA in combination with adenine and NAA on PLB formation from *in vitro* leaf

Cent per cent of the leaf cultures developed PLB's at BA 25 ppm + adenine 10 ppm + NAA 1 ppm (Plates 13 and 14), when kept in darkness. With regard to the time taken for PLB formation, the minimum number of days (21.3) was taken at BA 25 ppm + adenine 10 ppm + NAA 1 ppm, and was on par with

Plate 11. *In vitro* shoot showing the initiation of PLB's on the margin of leaves partially submerged in the media

Plate 12. *In vitro* shoot with PLB's developed in 1/2 MS medium + BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm

Plate 13. Initiation of PLB's from leaf tip after 45 days of culturing

Plate 14. Globular PLB's developed on the leaf tip after 75 days of culturing

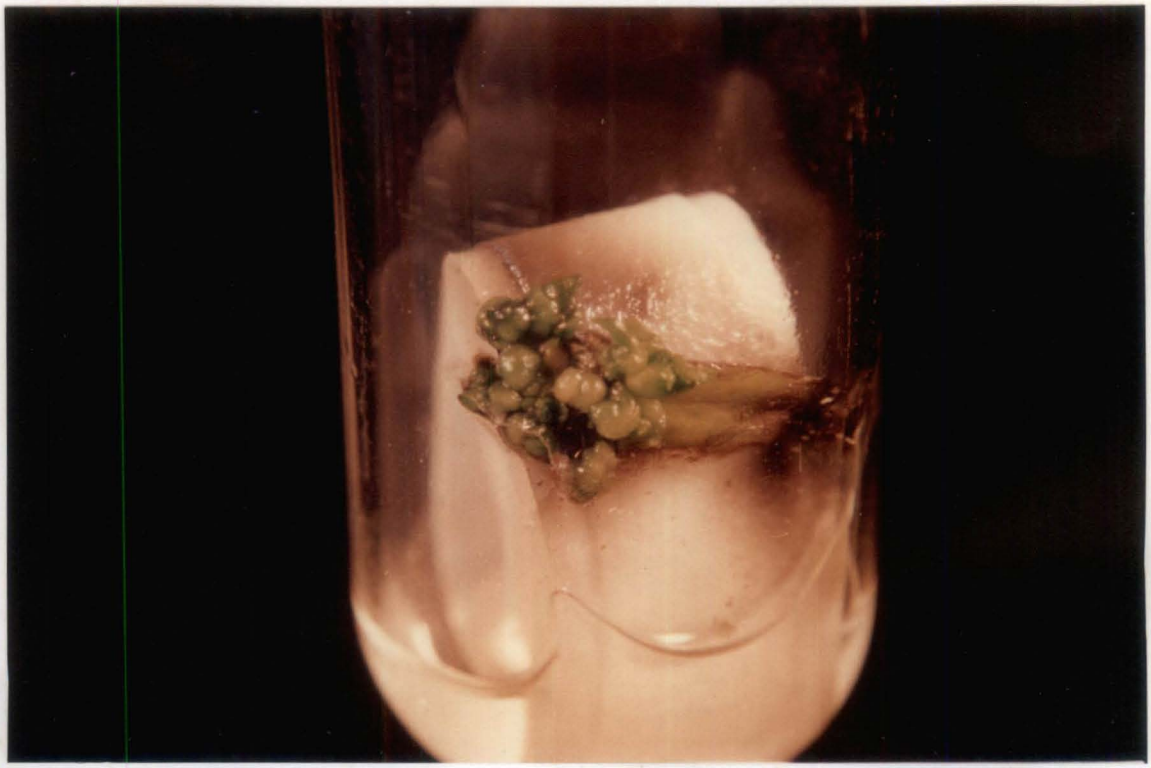

Table 38. Effect of sucrose on PLB formation from *in vitro* shoots

Medium: ½ MS + BA 5 + NAA 2 + 2,4-D 2 ppm
Culture period: 14 weeks

Treatment Sucrose (%)	Time taken for PLB formation (days)	Number of PLB's developed
0.0	-	-
1.5	40.67	33.33
3.0	84.83	2.67
4.5	-	-
6.0	-	-
7.5	-	-

Mean of 6 observations
- No response

Table 39. Effect of Thiamine-HCl on PLB formation from *in vitro* shoots

Meidum : ½MS + BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm
Culture period: 8 weeks

Treatment Thiamine-HCl (ppm)	Time taken for PLB formation (days)	Number of PLB's developed
0	40.67	4.17
5	40.00	5.17
10	38.00	5.67
15	37.30	6.50
20	33.50	9.50
25	35.67	9.67
CD(0.05)	NS	1.59
SEm ±	2.14	0.54

Mean of 6 observations

Table 40. Effect of BA, NAA and 2,4-D on PLB formation from *in vitro* shoots

Medium: ½MS
Culture period: 8 weeks

Treatment			Time taken for PLB formation (days)	Number of PLB's developed	Remarks
BA	NAA (ppm)	2,4-D			
20	-	1	-	-	No PLB formation
,,	-	2	52.50	1.33	PLB formation less
,,	-	3	51.50	1.83	PLB formation less
,,	-	4	45.33	3.00	PLB formation more
1	2	2	54.83	1.83	PLB formation less
5	"	"	40.67	4.17	PLB formation very high
10	"	"	46.00	2.50	PLB formation more
15	"	"	-	-	Large globular structure developed from the roots
CD(0.05)			5.09	1.02	
SEm ±			1.75	0.35	

Mean of 6 observations
- No response

BA 10 ppm + adenine 10 ppm + NAA 1 ppm and BA 20 ppm + adenine 10 ppm + NAA 1 ppm. Significant difference could not be observed among the different treatments with regard to number of PLB's developed and the size of the PLB's. The control (adenine 10 ppm + NAA 1 ppm) did not produce any PLB's during the 8 weeks of culture period (Table 41). Direct plantlet development was observed from the PLB's after 90-95 days of inoculation when maintained without subculturing (Plate 15).

4.3.5 Effect of BA in combination with adenine and NAA on PLB formation from *in vitro* roots

The treatment combinations BA 20 ppm + adenine 10 ppm + NAA 1 ppm and BA 25 ppm + adenine 10 ppm + NAA 1 ppm recorded PLB formation from *in vitro* roots in cent per cent of the cultures under light. With regard to the time taken for the bulging of root tips, the minimum number of days (19.80) was recorded for BA 25 ppm + adenine 10 ppm + NAA 1 ppm and was significantly superior to all other treatments. When the number of days taken for PLB formation was considered the minimum number of days (25.20) was recorded for BA 25 ppm + adenine 1 ppm + NAA 1 ppm (Plate 16) and was significantly superior to all the other treatments. No significant difference was observed among the different treatments with respect to the number of PLB's developed (Table 42).

4.3.6 Effect of BA + NAA + 2,4-D on PLB formation from *in vitro* roots

Out of the six treatments tried, only two treatments developed PLB's, at the end of 12 weeks of culturing. When the time taken for root tip bulging was considered, the minimum number of days (28.60) was recorded for BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm and was significantly superior to all the other

Plate 15. Plantlets developing directly from the leaf PLB's in 1/2 MS + BA 25 ppm + Adenine 10 ppm + NAA 1 ppm

Plate 16. Root base with globular PLB's developed in 1/4 MS + BA 25 ppm + Adenine 10 ppm + NAA 1 ppm

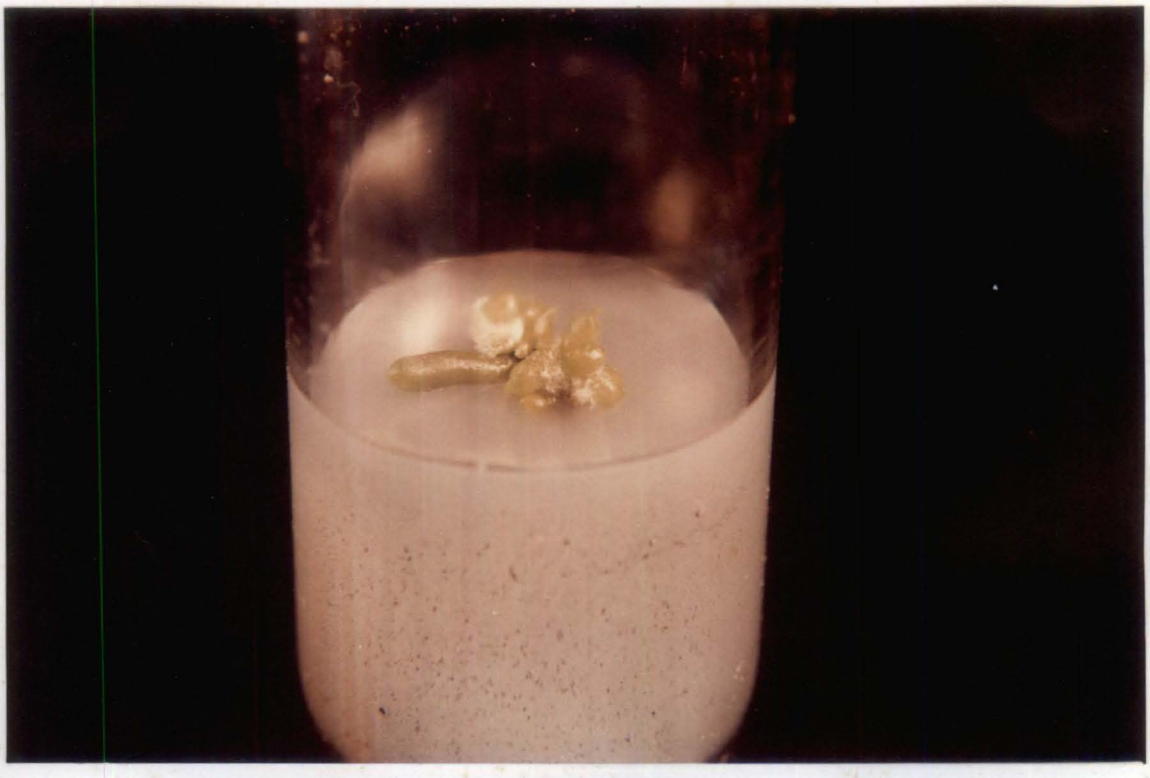

Table 41. Effect of BA in combination with adenine and NAA on PLB formation from *in vitro* leaf

Medium: ½ MS + Adenine 10 ppm + NAA 1 ppm (liquid)
Culture period: 8 weeks (under dark)

Treatment BA (ppm)	Cultures* developing PLB's (%)	Time taken for initiation of PLB's (days)	Number of PLB's developed	Size of PLB's developed (mm)	Remarks
0	--	-	-	-	Greening of leaf
5	80	43.8	3.30	1.5	Larger sized PLB's
10	80	23.4	7.90	1.5	-do-
15	80	29.5	7.20	1.4	-do-
20	80	21.9	8.10	1.2	Small sized PLB's
25	100	21.3	7.40	1.8	Very large PLB's
CD(0.05)		3.68	NS	NS	
SEm±		1.29	1.40	0.15	

*Mean of 20 osbervations
Mean of 10 observations
- No response

Table 42. Effect of BA in combination with adenine and NAA on PLB formation from *in vitro* root

Medium : 1/4 MS + Adenine 10 ppm + NAA 1 ppm (liquid)
Culture period: 8 weeks

Treatment BA (pppm)	Culture* developing PLB's (%)	Time taken for root tip bulging (days)	Time taken for PLB formation (days)	Number of PLB's developed
0	0	-	-	-
5	0	53.80	-	-
10	80	34.40	49.80	2.00
15	80	31.00	53.20	2.00
20	100	26.80	38.00	2.20
25	100	19.80	25.20	2.60
CD(0.05)		4.56	3.69	NS
SEm±		1.52	1.20	0.26

Mean of 5 observations
- No response
* Mean of 10 observations

treatments. Under this treatment the days taken for PLB formation was 37.8 and the number of PLB's developed was 2.4 (Table 43).

4.3.7 Effect of BA and combination of BA + NAA on PLB growth and plantlet development

Data are presented in Table 44.

Number of PLB's developed

At the end of 4 weeks of culturing, the maximum increase in PLB number (26.67) was observed for BA 15 ppm + NAA 1 ppm and was found significantly superior to all the other treatments except BA 10 ppm + NAA 1 ppm. After 8 weeks of culturing, the maximum increase (58.33) in PLB number was recorded for BA 15 ppm + NAA 1 ppm (Plate 17). The maximum increase in PLB number (125.00) after 12 weeks of culturing was observed for BA 15 ppm + NAA 1 ppm and was found significantly superior to all other treatments except BA 10 ppm.

It was observed that after two to three subculture cycles, the PLB's exhibited translucent and turgid appearance. And these PLB's were found to have higher capacity for differentiation into new PLB's. When these hyperhydric PLB's were cultured on media containing low concentration of BA, normal PLB's were developed.

Number of plantlets developed

With regard to plantlet development also, maximum number (5.00) was produced at BA 15 ppm + NAA 1 ppm and was significantly superior to all other treatments, after 4 weeks of culturing. At the end of 8 weeks of culturing, maximum

Table 43. Effect of BA, NAA and 2,4-D on PLB formation from *in vitro* root

Medium : 1/4 MS (liquid)
Culture period: 12 weeks

Treatment			Culture* developing PLB's (%)	Time taken for root tip bulging (days)	Time taken for PLB formation (days)	Number of PLB's developed
BA	NAA (ppm)	2,4-D				
5	-	-	0	-	-	-
5	2	-	0	70.00	-	-
5	2	2	80	28.60	37.80	2.40
20	-	-	0	-	-	-
20	2	-	0	70.80	-	-
20	2	2	60	38.60	68.80	1.80
CD (0.05)				6.78		
SEm±				2.20		

Mean of 5 observations
- No response
* Mean of 10 observations

Table 44. Effect of BA and combination of BA and NAA on PLB growth and plantlet development

Medium: 1/4 MS + sucrose 1.5%
Explant: 10 green PLB's

Treatments		4 weeks		8 weeks		12 weeks	
BA (ppm)	NAA	X	Y	X	Y	X	Y
1	-	1.00	-	2.00	1.17	4.67	3.33
5		1.67	2.33	8.50	3.83	13.83	5.50
10	-	11.17	2.50	35.00	4.33	103.33	7.17
15	-	-	-	13.33	2.33	30.00	3.17
20	-	-	-	8.67	1.33	10.00	2.00
1	1	1.83	1.00	3.67	2.33	8.00	5.00
5	"	5.67	2.50	10.50	4.00	15.83	7.83
10	"	20.83	3.00	27.50	6.00	61.67	9.33
15	"	26.67	5.00	58.33	8.67	125.00	10.83
20	"	2.17	2.33	15.33	4.67	20.00	5.83
CD(0.05)		7.25	1.74	9.93	2.99	22.84	3.19
SEm±		2.54	0.60	3.48	1.05	7.79	1.12

X - Number of PLB's developed additionally
Y - Number of plantlets developed
Mean of 6 observations
- No response

number (8.67) of plantlets was recorded for BA 15 ppm + NAA 1 ppm and was significantly superior to all other treatments except BA 10 ppm + NAA 1 ppm. After 12 weeks of culturing, the highest number (10.83) was recorded again at BA 15 ppm + NAA 1 ppm and was significantly superior, except for BA 10 ppm + NAA 1 ppm and BA 5 ppm + NAA 1 ppm.

4.3.8 Effect of coconut water on PLB formation from stalk tips

Data are presented in Table 45. When coconut water was added at 15 per cent level, PLB's were found to develop from the stalk tips in media containing growth regulators. The time taken for shoot tip bulging was minimum (8.0 days) for BA 20 ppm + NAA 1 ppm + adenine 10 ppm + CW 15 per cent and it was significantly superior to all other treatments. With regard to time taken for PLB development, the minimum number of days (49.2) was recorded for BA 20 ppm + NAA 1 ppm + adenine 20 ppm + CW 15 per cent (Plate 18) and was found significantly superior to other treatments. No significant difference could be observed among the treatments with respect to number of PLB's developed at the end of 10 weeks of culturing.

4.3.9 Effect of coconut water on PLB formation from *in vitro* shoots

Data are presented in Table 46. No significant difference was observed among the different levels of coconut water on the number of days taken for PLB formation. With regard to the number of PLB's developed, at the end of 8 weeks of culturing, the highest number (13.50) number of PLB's was produced at CW 25 per cent level (Plate 19) and was on par with CW 15 per cent and 20 per cent. The lowest number (4.17) was recorded for the control.

Plate 17. Effect of BA 15 ppm + NAA 1 ppm on PLB growth in 1/4 MS medium

Plate 18. Inflorescence stalk tip with PLB's developed in 1/2 MS medium + BA 20 ppm + NAA 1 ppm + Adenine 10 ppm + CW 15%

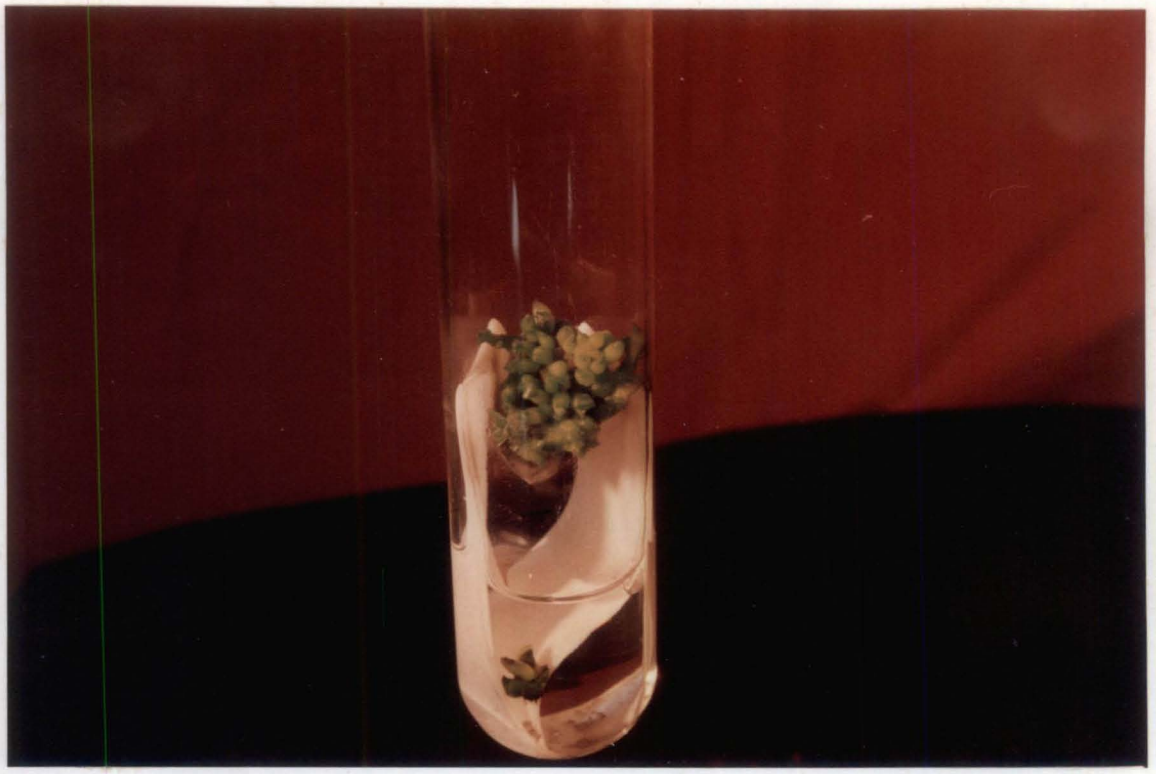

Table 45. Effect of coconut water on PLB formation from inflorescence stalk tip

Medium: ½MS + CW 15% + sucrose 1.5%
Culture period: 10 weeks

Treatment				Time taken for stalk tip bulging (days)	Time taken for PLB development (days)	Number of PLB's developed
BA	NAA (ppm)	2,4-D	Adenine			
5	2	2	--	16.8	59.0	2.8
20	1	-	10	8.0	60.8	2.8
20	1	-	20	11.0	49.2	3.6
20	5	-	--	13.4	53.2	3.2
CD (0.05)				1.84	3.26	NS
SEm±				0.60	1.06	0.54

Mean of 5 observations

Table 46. Effect of coconut water and tomato juice on PLB formation from *in vitro* shoots

Medium: ½MS + BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm
Culture period : 8 weeks

Treatment	Time taken for PLB formation (days)	Number of PLB's developed
CW (%)		
0	40.67	4.17
5	39.83	6.00
10	37.00	8.67
15	32.67	12.67
20	35.50	13.17
25	42.33	13.50
CD (0.05)	NS	2.61
SEm±	2.75	0.90
Tomato juice (%)		
0	40.67	4.17
3	33.83	8.67
6	37.50	6.33
9	40.67	4.50
CD (0.05)	NS	1.55
SEm±	2.10	0.51

Mean of 6 observations

4.3.10 Effect of coconut water on PLB growth and plantlet developmemt

Data are presented in Table 47. With the increase in the level of coconut water in the media, the number of PLB's developed also increased and the highest rate was observed for CW 15 per cent followed by CW 20 per cent and the lowest rate for CW 5 per cent and control. With regard to the number of plantlets developed, significant difference was observed among the levels of coconut water and the largest number, viz., 4.00, 8.17 and 30.00 at the end of 3, 6 and 12 weeks respectively, were recorded for CW 25 (Plate 20) per cent and was significantly superior to all other levels.

4.3.11 Effect of tomato juice on PLB formation from *in vitro* shoots

Data are presented in Table 46. No significant difference was observed among the different levels of tomato juice, with regard to the number of days taken for PLB formation. At the end of 8 weeks of culturing, the number of PLB's developed was the highest (8.67) at 3 per cent level of tomato juice and was significantly superior to other levels. The lowest number (4.17) was observed for the control and it was on par with tomato juice 9 per cent.

4.3.12 Effect of adenine in combination with BA and NAA on PLB formation from *in vitro* shoots

Data are presented in Table 48. With regard to the time taken for PLB formation, the minimum number (42.33 days) was recorded at adenine 10 ppm + BA 10 ppm + NAA 1 ppm and it was on par with adenine 12 ppm + BA 16 ppm; adenine 16 ppm + BA 16 ppm and adenine 10 ppm + BA 10 ppm + NAA 2 ppm. The maximum number (90.33 days) was observed for adenine 4 ppm + BA 16 ppm.

Plate 19. Effect of CW on PLB development from *in vitro* shoots in 1/2 MS medium + BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm

Plate 20. Effect of CW on plantlet development from PLB's on 1/4 MS medium

Table 47. Effect of coconut water on PLB growth and plantlet development

Medium: 1/4 MS

Treatment CW (%)	PLB's at the time of incubation	PLB growth			Number of plantlets developed		
		3 weeks	6 weeks	12 weeks	3 weeks	6 weeks	12 weeks
0	+	+	+	+	-	0.17	0.83
5	+	+	+	+ +	1.67	5.17	7.00
10	+	+ +	+ + +	+ + + + + +	1.67	5.17	7.50
15	+	+ + +	+ + + + +	+ + + + + + + + + + + +	2.67	6.00	7.83
20	+	+ +	+ + + +	+ + + + + + + +	2.67	6.00	13.30
25	+	+ +	+ + +	+ + + + + +	4.00	8.17	30.00
CD (0.05)					0.57	0.37	0.65
SEm±					0.19	0.13	0.22

Mean of 6 observations
+ 50 PLB's

Table 48. Effect of adenine, BA and NAA on PLB formation from *in vitro* shoots

Medium: ½MS + sucrose 1.5%
Culture period: 14 weeks

Treatment			Time taken for PLB formation (days)	No. of PLB's developed
Adenine (ppm)	BA	NAA		
4	16	-	90.33	2.83
8	,,	-	51.33	20.00
12	,,	-	47.33	23.83
16	,,	-	43.17	28.67
10	10	1	42.33	30.33
,,	,,	2	43.83	28.67
,,	,,	3	57.17	20.33
,,	,,	4	79.67	5.67
CD(0.05)			5.37	6.27
SEm±			1.88	2.15

Mean of 6 observations

When the number of PLB's developed was considered after 14 weeks of culturing, the largest number (30.33) was developed at adenine 10 ppm + BA 10 ppm + NAA 1 ppm and was significantly superior to all the other treatments except adenine 16 ppm + BA 16 ppm and adenine 10 ppm + BA 10 ppm + NAA 2 ppm. The lowest number (2.83) was recorded for adenine 4 ppm + BA 16 ppm.

4.3.13 Effect of adenine in combination with BA and NAA on PLB formation from *in vitro* leaf

Data are presented in Table 49. After 8 weeks of culturing under dark, 80 per cent of the cultures developed PLB's at adenine 5, 10 and 15 ppm in combination with BA 10 ppm + NAA 1 ppm. With regard to the time taken for development of PLB's, the minimum number of days (23.4) was recorded at adenine 10 ppm + BA 10 ppm + NAA 1 ppm and was significantly superior to all other treatments except adenine 15 ppm + BA 10 ppm + NAA 1 ppm. When the number of PLB's developed were considered, no significant difference was observed among the treatments. Regarding the size of PLB's developed, the largest size (2.1 mm) of PLB's was recorded at adenine 25 ppm + BA 10 ppm + NAA 1 ppm and it was significantly superior to all other treatments.

4.3.14 Effect of combination of adenine and BA on PLB growth and plantlet development

Data are presented in Table 50.

Number of PLB's developed

After 4 weeks of culturing, the maximum increase (10.33) in PLB number was recorded for adenine 8 ppm + BA 16 ppm and this was significantly

Table 49. Effect of adenine in combination with BA and NAA on PLB formation from *in vitro* leaf

Medium: 1/4 MS + BA 10 ppm + NAA 1 ppm (liquid)
Culture period: 8 weeks (under dark)

Treatment Adenine (ppm)	Culture* developed PLB's (%)	Time taken for initiation of PLB's (days)	Number of PLB's developed	Size of the PLB's developed (mm)
0	0	-	-	-
5	80	30.4	3.8	1.2
10	80	23.4	7.9	1.5
15	80	24.0	7.1	1.5
20	40	29.1	5.4	1.2
25	40	28.5	4.0	2.1
CD(0.05)		3.63	NS	0.41
SEm±		1.27	1.50	0.14

Mean of 10 observations
- No response
* Mean of 20 observations

Table 50. Effect of BA and combination of adenine and BA on PLB growth and plantlet development

Medium: 1/4MS + sucrose 1.5%
Explant: 10 green PLB's

Treatment		4 weeks		8 weeks		12 weeks	
Adenine (ppm)	BA	X	Y	X	Y	X	Y
--	0	-	-	-	-	-	1.50
--	4	1.17	2.17	2.83	3.17	10.17	4.50
--	8	6.67	3.00	32.50	4.33	70.00	8.00
--	12	4.33	-	21.67	2.33	46.67	3.67
--	16	-	-	9.17	1.83	20.00	2.83
--	20	-	-	8.67	1.33	10.00	2.00
4	16	1.67	1.17	4.17	2.83	8.17	4.67
8	,,	10.33	3.83	35.83	5.67	96.67	9.50
12	,,	6.67	2.17	12.17	3.67	32.33	6.50
16	,,	6.17	1.33	8.00	2.67	25.50	4.67
20	,,	5.33	0.83	5.33	1.83	11.33	3.33
CD(0.05		2.15	1.32	6.19	1.36	27.89	1.96
SEm±		0.75	0.46	2.17	0.48	9.76	0.69

X - Number of PLB's developed additionally
Y - Number of plantlets developed
- No response
Mean of 6 observations

superior to all other treatments. The maximum increase in PLB number (35.83 and 96.67) was observed again for adenine 8 ppm + BA 16 ppm at the end of 8 weeks and 12 weeks of culturing respectively, and was found significantly superior to all other treatments except BA 8 ppm.

Number of plantlets developed

It was observed that at the end of 4, 8 and 12 weeks of culturing, the largest number of plantlets (3.83, 5.67 and 9.50, respectively) was produced at adenine 8 ppm and BA 16 ppm (Plate 21) and it was significantly superior to all the other treatments except BA 8 ppm.

4.3.15 Effect of peptone on PLB formation from *in vitro* shoots

Data are presented in Table 51. With regard to the time taken for PLB formation, no significant difference was observed among the different levels of peptone. When the number of PLB's developed was considered, the highest number (10.00) was recorded for peptone at 1500 ppm (Plate 22) and was significantly superior to all the other treatments except peptone at 1000 ppm. The lowest number of PLB's (4.17) was observed for the control.

4.4. Effect of culture environment

Regarding culture environment, visual observations were recorded for light and aeration (culture vessel) maintaining humidity and temperature at constant level.

Plate 21. Effect of different levels of adenine (8, 12, 16 and 20 ppm) in combination with BA 16 ppm on plantlet development from PLB's

Plate 22. Effect of peptone (1500 ppm) on PLB formation from *in vitro* shoots in 1/2 MS medium + BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm

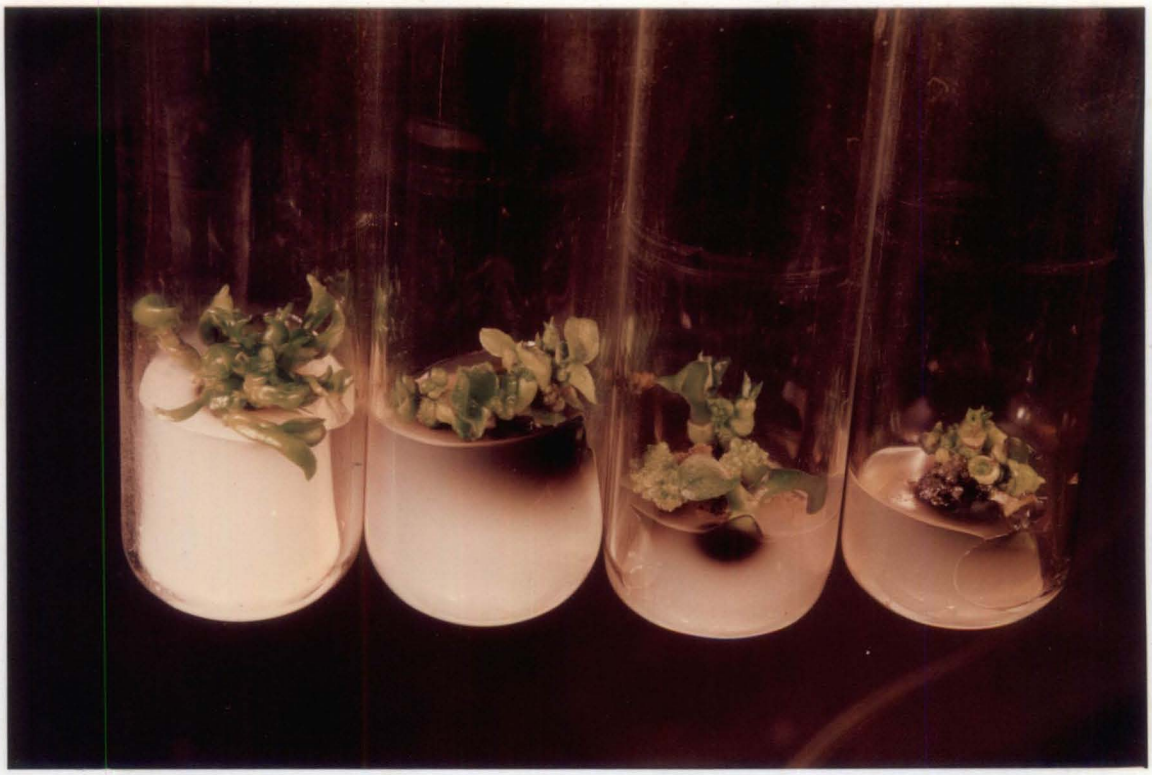

Table 51. Effect of peptone on PLB formation from *in vitro* shoots

Medium: ½ MS + BA 5.0 ppm + NAA 2.0 ppm + 2,4-D 2.0 ppm
Culture period: 8 weeks

Treatment Peptone (ppm)	Time taken for PLB formation (days)	Number of PLB's developed
0	40.67	4.17
500	38.00	6.33
1000	35.67	9.67
1500	36.83	10.00
2000	41.67	7.83
CD(0.05)	NS	1.75
SEm±	2.49	0.59

Mean of 6 observations

4.4.1 Effect of light

Data are presented in Table 52.

Leaf

Significant difference was observed with regard to time taken for PLB formation from leaf under light and dark conditions. Under dark, PLB's were found to develop earlier by one month.

Root

For roots, PLB's were found to develop earlier by one month under light than in dark. And also it was found that, PLB formation was direct under light and it was callus mediated under dark (Plate 23).

Cluster of PLB's

Under light PLB's start developing into plantlets, whereas under dark the PLB's showed callusing and further proliferated to more of PLB's with no formation of plantlets (Plate 24).

One globular PLB

When larger sized PLB's were cultured, they developed into plantlets under light, and under dark showed high callusing, proliferating to produce more of tiny PLB's.

Plate 23. Growth of roots *in vitro* under darkness

Plate 24. Growth of PLB cluster *in vitro* excluding light

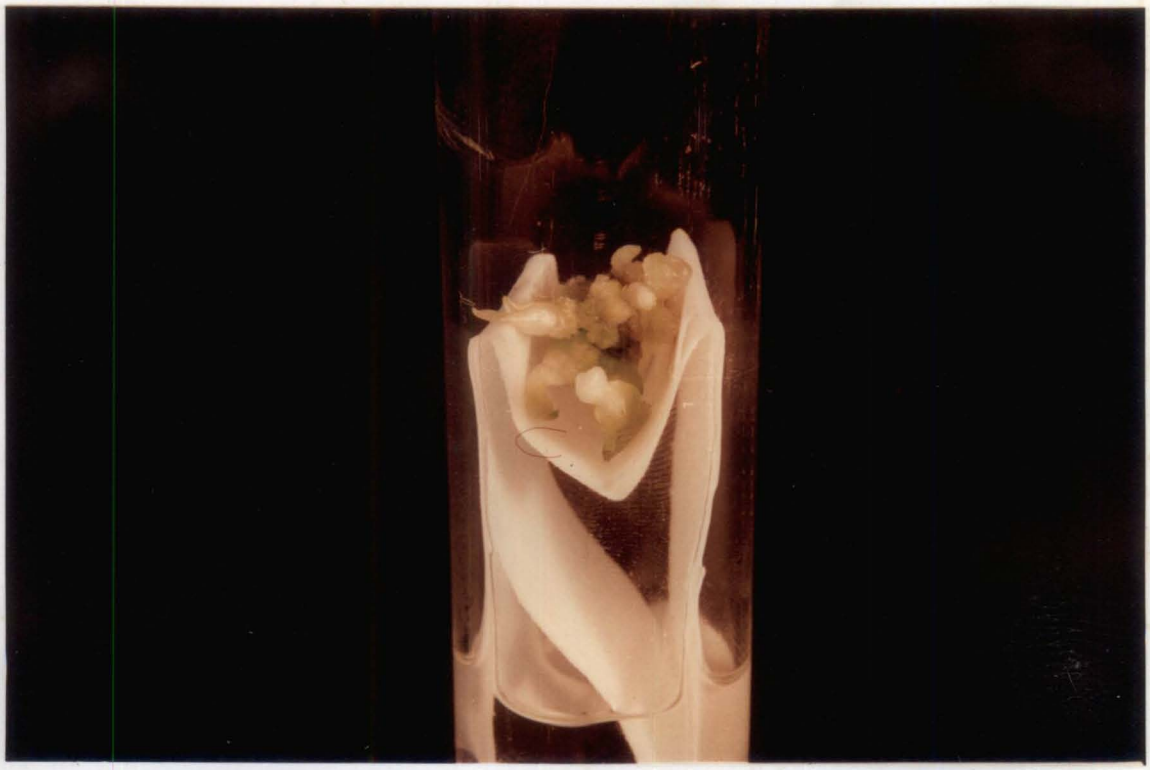

Table 52. Influence of light on the growth of the cultures

Medium: 1/4 MS + BA 10 + Adenine 10 + NAA 1 (liquid)
Period: 4 months

Explant	Light	Dark
1. Leaf	PLB development slow and takes more than 2 months	PLB development fast and develops from the side and basal part of leaf within 1 month
2. Root	Root tips bulged and developed PLB's within 1-2 months	Root tips turned white and developed callus and then PLB's within 2-3 months
3. Cluster of 10 PLB	PLB's showed high rate of multiplication and also plantlet development	PLB's turned white, showed high callusing and produced tiny PLB's on the already formed PLB and no plantlet fomation
4. One globular PLB (5 mm size)	PLB develops directly into a plantlet	PLB turned white, showed high callusing and produced tiny PLB's on the surface
5. Axillary bud (½ cm)	Elongates and develop leaves and roots and produced multiple buds	Turned white, showed high callusing and development of large number of tiny white buds
6. Shoot with 3 leaf and 2 root	Showed good development of axillary and apical buds and development of PLB's from both root and leaf within 2 months	Leaves turned white and the shoot produced elongated white and tender axillary and apical buds

Axillary bud

When newly developed bud was cultured, under light it elongated to produce shoot with leaves and roots, whereas under dark showed signs of callusing and produced large number of tiny white buds.

Axillary shoot

Under light, the shoot produced PLB's from leaf, roots and the base of the shoot and also developed axillary and apical buds. But under dark condition, no PLB formation was observed and the shoot produced elongated, white coloured very tender axillary and apical buds.

4.4.2 Effect of culture vessels

Data are presented in Table 53. With regard to the effect of culture vessels, larger and sturdy plants with very long roots were found to develop when cultured in 250 ml conical flask followed by 210 mm x 40 mm sized test tubes and 100 ml conical flask. Smaller sized plants were found to develop in 50 ml conical flask and 150 mm x 25 mm test tubes. The size of the plants were found to increase with increase in the volume of culture vessel and larger sized plants with longer roots are found to establish better in the field.

4.5 Planting out and acclimatization

Data on the influence of potting media, container and hardening treatments are presented below.

Table 53. Influence of culture vessels on the growth of plantlets

Medium: ½MS + BA 10 + NAA 1 + AC 0.4%
Explant: Shoot + 3 l + 2 r
Period: 4 months

Culture vessels	Nature of growth of the plantlets
1. 50 ml conical flask	Size of plants small and leaves and roots small in size
2. 100 ml conical flask	Size of plants big and leaves and roots longer
3. 250 ml conical flask	Robust plants, healthy, spreading, leaves large, roots very long and growing upwards. Plants very large in size.
4. 150 mm x 25 mm test tubes	Plants more elongated and compact
5. 210 mm x 40 mm test tubes	Robust plants, sturdy and large in size

4.5.1 Influence of potting media

Data are presented in Table 54. Out of the 10 treatment combinations of potting media tried, the highest survival percentage of planted out plantlets (60) was recorded for large piece of coconut husk (Plate 25), followed by coconut fibre (40) and the survival percentage was zero for T_6 [Cocoa peat + Vermiculite (1:1)], T_7 (Vermiculite) and T_8 (Sphagnum moss), at the end of 4 week period.

4.5.2 Influence of container

Data are presented in Table 55. Among the six containers tried, the polysachet method recorded the highest per cent survival (30) of planted out plantlets (Fig.6). When jam bottle was utilised, the survival percentage was zero. When clay pot was used, it recorded a survival percentage of 15, followed by black plastic pot, white plastic (ice cream) cup and egg tray (10%).

4.5.3 Influence of triadimefon added in the culture medium

Data are presented in Table 56. When the effect of different levels of triadimefon used in the culture medium for culturing the plantlets just before planting out was considered on the survival of planted out plantlets, the highest percentage (80) of survival was observed for triadimefon 10 ppm, 15 pm and 20 ppm, at the end of 4 weeks of planting out. After 8 weeks of planting out, the survival per cent decreased, and the highest percentage of survival (70) after 8 weeks of planting out was observed again at triadimefon 10 ppm, 15 ppm and 20 ppm. The survival percentage was only 40 per cent when triadimefon was not added in the medium.

Fig.6. Influence of container on the survival of planted out plantlets

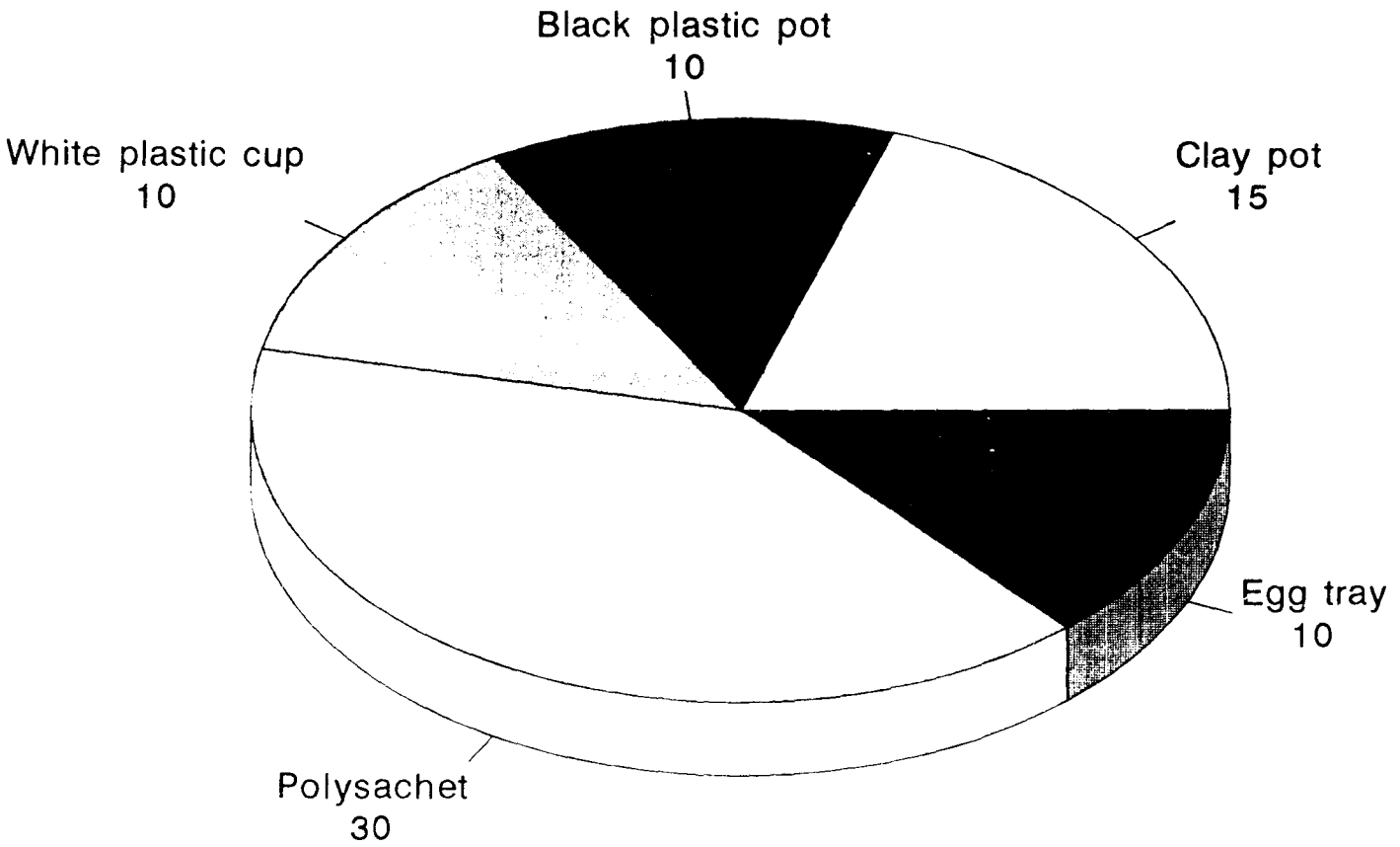

Table 54. Influence of potting media on the survival of planted out plantlets

Period : 4 weeks
Container : Egg tray

Potting media	Per cent survival
1. Charcoal	5
2. Charcoal + Brick pieces (1:1)	10
3. Charcoal + Brick pieces + Coconut fibre (1:1:1)	20
4. Charcoal + Brick pieces + Sand (1:1:1)	10
5. Charcoal + Vermiculite (1:1)	5
6. Cocoa peat + Vermiculite (1:1)	0
7. Vermiculite	0
8. Sphagnum moss	0
9. Coconut fibre (no container)	40
10. Coconut husk (no container)	60

Mean of 20 observations

Table 55. Influence of container on the survival of planted out plantlets

Period - 4 weeks
Media: Charcoal + Brick pieces (1:1)

Container	Per cent survival
1. Jam bottle	0
2. Clay pot	15
3. Black plastic pot	10
4. White plastic cup	10
5. Polysachet	30
6. Egg tray	10

Mean of 20 observations

Table 56. Influence of triadimefon added in the culture medium on the survival of palnted out plantlets

Media: Coconut husk

Triadimefon (ppm)	Period (per cent survival)	
	4 weeks	8 weeks
0	60	40
5	70	60
10	80	70
15	80	70
20	80	70

Mean of 10 observations

4.5.4 Influence of pre-transfer hardening treatments

Data are presented in Table 57. After 4 weeks of planting out, the highest percentage (90) of survival was recorded for plantlets grown in culture medium containing 20 ppm triadimefon and spread over sterile charcoal pieces for 2 weeks or those plantlets sprayed with triadimefon 20 ppm at fortnightly intervals. The lowest percentage (10) was observed for plantlets grown in liquid media and then dipped in 50 per cent glycerol before planting out and also for plantlets taken after drying up of the media and then dipped in 50 per cent glycerol.

After 8 weeks of planting out, the highest percentage (90) of survival was again noticed for plantlets which were transferred from the medium containing triadimefon 20 ppm and then spread over the sterile charcoal pieces for 2 weeks. The percentage survival was found to be zero, for those plantlets which were planted out after dipping in 50 per cent glycerol.

4.5.5 Influence of post-transfer hardening treatments

Data are presented in Table 58. At the end of 4 weeks of planting out, survival percentage was cent per cent for the plantlets grown in media containing triadimefon 20 ppm and then spread over charcoal pieces for 2 weeks and after that hung in the orchidarium with high humidity using coconut husk. All the other treatments tried recorded survival percentage ranging from 70 to 90.

When the survival percentage was considered after 8 weeks of planting out, the survival percentage was remaining the same (100) for the plantlets grown in media containing triadimefon 20 ppm and then spread over charcoal pieces for two weeks and then hung in the orchidarium with high humidity and this was followed

Table 57. Influence of pre-transfer hardening treatments on the survival of planted out plantlets

Media: Coconut husk
Post-transfer - Keeping over wire net with water beneath

Pre-transfer treatments				Per cent survival	
	In vitro		*Ex vitro*	4 week	8 week
1	Liquid media	1	Keeping in distilled water (3 h)	50	30
		2	Dipping in 50% glycerol (1 min)	10	0
		3	Smearing paraffin wax on the leaves	20	0
		4	Spraying triadimefon 20 ppm at fortnightly intervals	60	50
		5	Spreading the plantlets over sterile charcoal pieces for 2 weeks	70	60
2	Drying up of the media	1	Keeping in distilled water (3 h)	60	50
		2	Dipping in 50% glycerol (1 min)	10	0
		3	Smearing paraffin wax on the leaves	30	10
		4	Spraying triadimefon 20 ppm at fortnightly intervals	70	60
		5	Spreading the plantlets over sterile charcoal pieces for 2 weeks	80	80
3	Adding triadimefon 20 ppm into the media	1	Keeping in distilled water (3 h)	80	70
		2	Dipping in 50% glycerol (1 min)	20	0
		3	Smearing paraffin wax on the leaves	40	30
		4	Spraying triadimefon 20 ppm at fortnightly intervals	90	80
		5	Spreading the plantlets over sterile charcoal pieces for 2 weeks	90	90

Total number of observation - 10

by 90 per cent survival of the planted out plantlets kept over wire net with water beneath (Plate 26).

4.6 Post-establishment studies

Data regarding the effect of different nutrient solutions on the growth of the plantlets and their growth parameters (Fig.7) are recorded and presented below.

4.6.1 Effect of nutrient solutions

Data are presented in Table 59.

Percentage survival

Treatments with all the five nutrient solutions recorded cent per cent survival of planted out plantlets after 8 weeks of planting out. After 12 weeks of planting out the highest survival percentage (90) was observed for planted out plantlets sprayed with 30:10:10 and 17:17:17 nutrient solutions.

Plant height

After 8 and 12 weeks of planting out 17:17:17 nutrient solution recorded maximum plant height (1.12 cm and 1.24 cm, respectively), which was on par with MS (0.5%) and 30:10:10 nutrient solution (0.5%). The lowest plant height (0.76 and 0.84 cm) was observed for Hoagland (1%) solution after 8 and 12 weeks of planting out and respectively was on par with MS (0.25%).

Fig.7. Effect of nutrient solutions on the growth of planted out plantlets

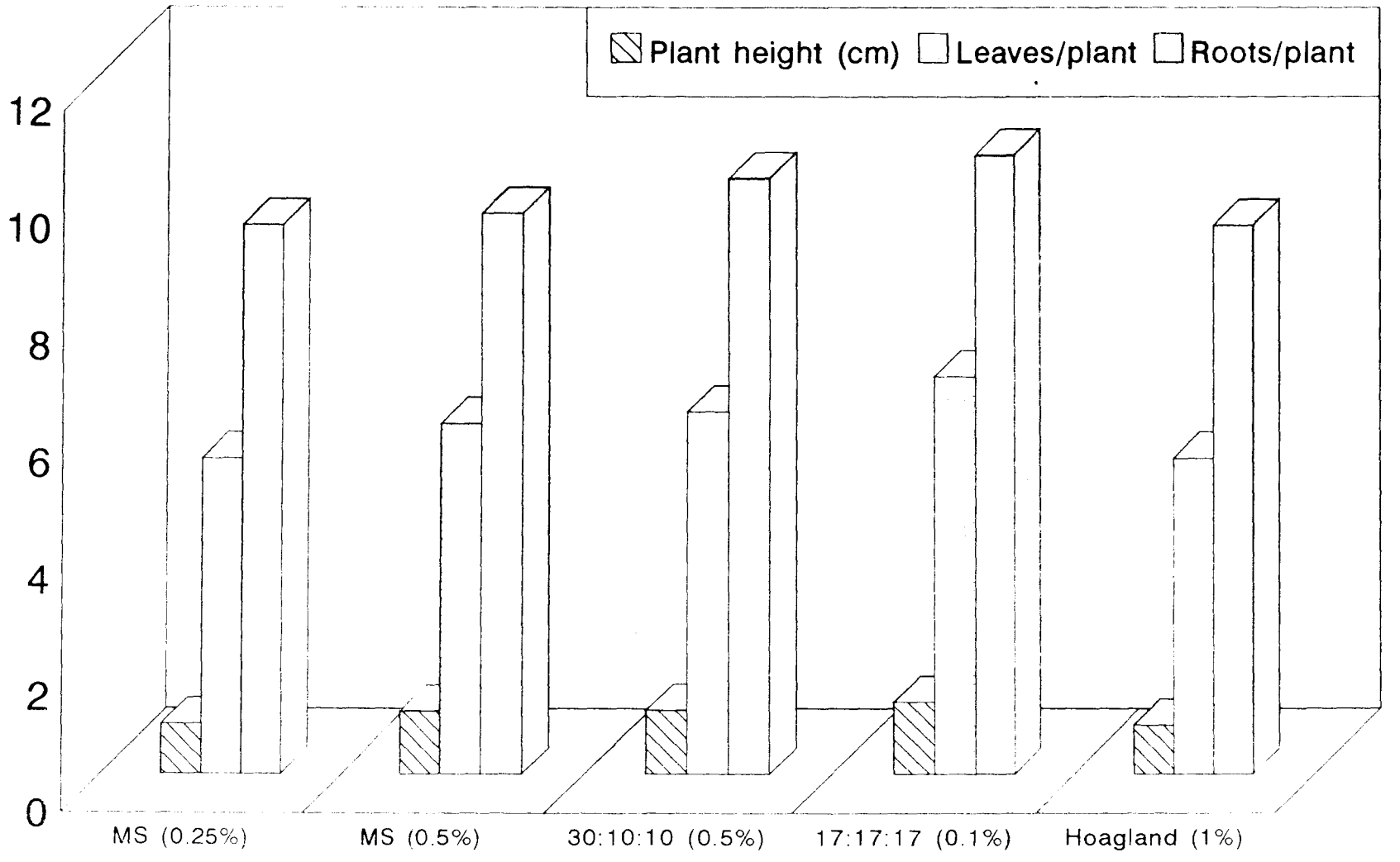

Plate 25. Plantlet grown in coconut husk

Plate 26. Plantlets kept over a basin with water for hardening

Table 59. Effect of nutrient solutions on the growth of planted out plants

Characters	Nutrient solutions						
	MS (.25%)	MS (.5%)	30:10:10 (0.5%)	17:17:17 (0.1%)	Hoagland (1%)	CD (0.05)	SEm±
After 8 weeks							
Survival (%)**	100	100	100	100	100		
Plant height (cm)	0.80	1.00	1.00	1.12	0.76	0.12	0.04
Leaves per plant (no.)	5.60	6.00	6.20	6.40	5.40	0.69	0.23
Length of longest leaf (cm)	4.60	4.90	5.20	5.40	4.50	0.66	0.22
Width of longest leaf (cm)	1.68	1.64	1.82	1.98	1.72	0.30	0.10
Roots per plant (no.)	8.80	9.00	9.20	9.60	9.00	0.43	0.38
Length of longest root (cm)	4.50	4.80	5.00	5.20	4.60	0.45	0.15
After 12 weeks							
Survival (%)	80	85	90	90	75		
Plant height	0.86	1.08	1.10	1.24	0.84	0.24	0.09
Leaves per plant	5.40	6.00	6.20	6.80	5.40	0.84	0.28
Length of longest leaf	4.90	5.40	5.60	6.00	5.00	0.60	0.20
Width of longest leaf	1.88	1.86	2.14	2.30	1.88	0.33	0.11
Roots per plant	9.40	9.60	10.20	10.60	9.40	0.81	0.27
Length of longest root	4.92*	5.22	5.36	5.80	4.94	0.63	0.21

** Mean of 20 observations
* Mean of 5 observations

Leaves per plant

After 8 and 12 weeks of planting out, 17:17:17: (0.1%) when sprayed, resulted in maximum number of leaves (6.40 and 6.80, respectively), which was on par with MS 90.5%) and 30:10:10 (0.5%). The lowest leaf number (5.40) after 8 weeks of planting out was observed for Hoagland (1%) solution and after 12 weeks of planting out, the lowest leaf number (5.40) was observed for MS (0.25%) and Hoagland (1%).

Length of longest leaf

Maximum leaf length (5.40 cm and 6.00 cm) after 8 and 12 weeks of planting out respectively, was observed for 17:17:17 (0.1%) solution, which was on par with MS (0.5%) and 30:10:10 (0.5%).

Width of longest leaf

Maximum leaf width (1.98 cm) after 8 weeks of planting out was observed for 17:17:17 (0.1%) solution, which was on par with other nutrient solutions except MS (0.5%). After 12 weeks of planting out, 17:17:17 (0.1%) solution recorded maximum leaf width (2.30 cm) which was significantly superior to other nutrient soltuions except 30:10:10 (0.5%).

Roots per plant

Eight weeks after planting out, the plantlets sprayed with 17:17:17 (0.1%) solution recorded the maximum root number (9.60) and was significantly superior to all other nutrient solutions. After 12 weeks of planting out, the maximum

root number (10.60) was observed for 17:17:17 (0.1%) solution and was significantly superior to all other nutrient solution except 30:10:10 (0.5%).

Length of longest root

Maximum root length (5.20 cm and 5.80 cm) was observed at the end of 8 and 12 weeks of planting out for the plantlets sprayed with 17:17:17 (0.1%) solution which was on par with MS (0.5%) and 30:10:10 (0.5%).

4.6.2 Growth parameters of plantlets

Data for a period of 8 month are presented in Table 60. The parameters ie., plant height, leaves and roots per plant for the period under observation is illustrated in Fig.8.

Percentage of survival

The percentage of survival was found to decrease with advancement of time. At 3 months period, the survival percentage was 90 which decreased to 80 per cent by the end of 8 month.

Plant height

Plant height was found to increase from 1.24 cm at 3 month to a maximum of 2.00 cm at the end of 8 month.

Leaves per plant

Number of leaves was found to decrease as the time advanced, as the leaf number was 6.80 at 3 month and by 8 month, the number decreased to 4.00.

Fig.8. Growth performance of planted out plantlets

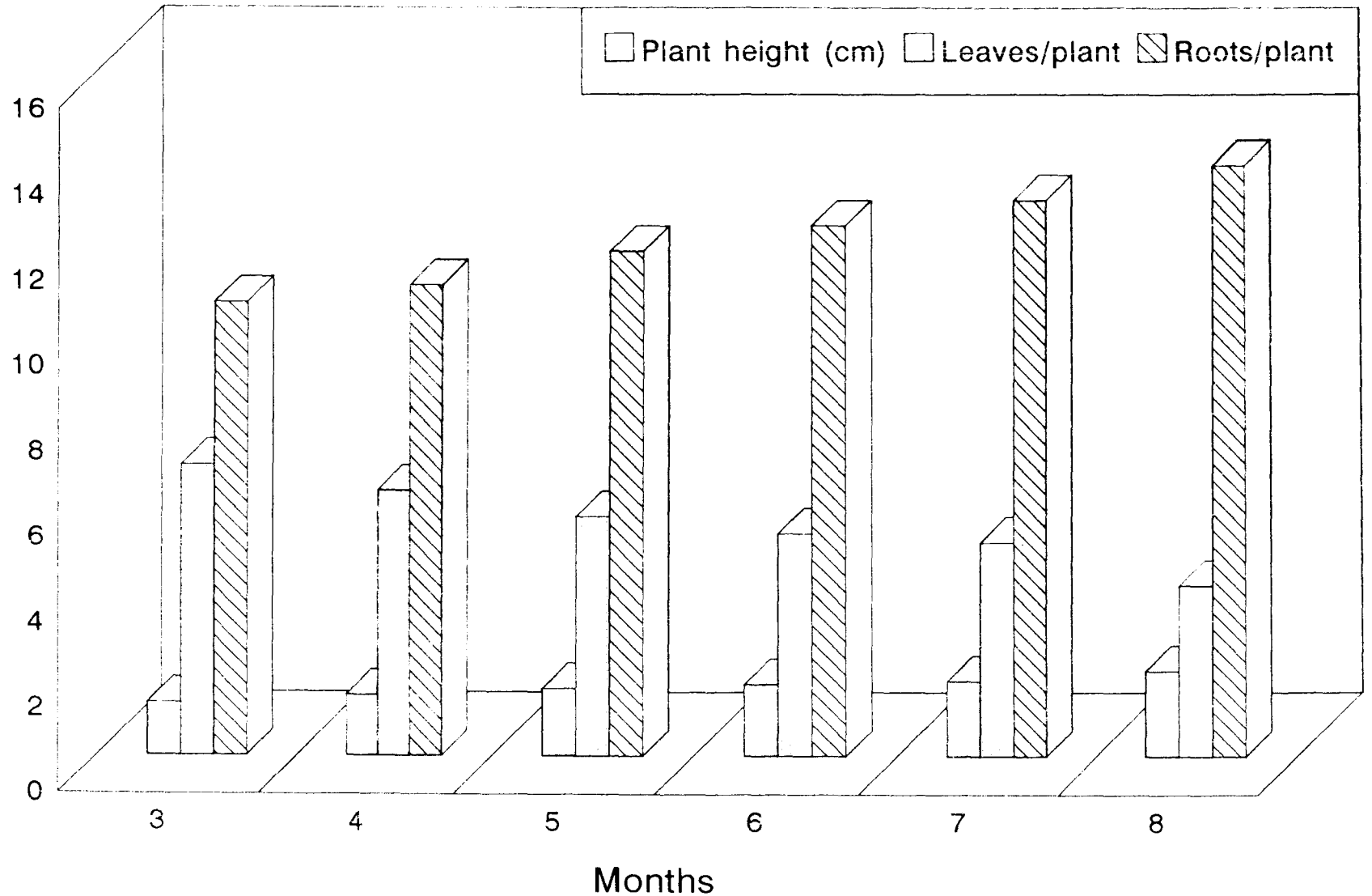

Table 60. Growth performance of planted out plants

Period	Survival (%)	Plant height (cm)	Leaves per plant	Length of longest leaf (cm)	Width of longest leaf (cm)	Roots per plant	Length of longest root (cm)
3 month	90	1.24	6.80	6.00	2.30	10.60	5.80
4 month	90	1.42	6.20	6.80	2.70	11.00	6.80
5 month	85	1.58	5.60	7.10	2.86	11.80	9.10
6 month	80	1.68	5.20	7.50	3.20	12.40	11.10
7 month	80	1.76	5.00	8.00	3.40	13.00	14.40
8 month	80	2.00	4.00	9.90	3.84	13.80	19.00
CD(0.05)		0.15	1.27	0.97	0.38	1.71	1.53
SEm±		0.05	0.43	0.33	0.13	0.58	0.52

** Mean of 20 observations
Mean of 5 observations

Length of longest leaf

Leaf length was found to increase at a faster rate i.e., from 6.00 cm to 9.90 cm as the time advanced. The leaf length (9.90 cm) observed at 8 month period was significantly superior to that of 7 month (8.00 cm).

Width of longest leaf

Width of leaf was also found to show a rapid increase (from 2.30 cm to 3.84 cm) as the time advanced by 5 month.

Roots per plant

Number of roots per plant was also found to go on increasing (from 10.60 to 13.80) with advancement in time, i.e., 3 month to 8 month.

Length of longest root

Root length showed a drastic increase with advancement of time. At 3 month the root length was only 5.80 cm, it increased to 19.00 cm by 8 month period.

Discussion

DISCUSSION

The present investigations on micropropagation of *Phalaenopsis* were carried out at the Plant Tissue Culture Laboratory attached to All India Co-ordinated Floriculture Improvement Project (AICFIP) of Department of Pomology and Floriculture, College of Horticulture, Vellanikkara, during 1993-96. The results of the study are discussed in this chapter.

Phalaenopsis belongs to the family Orchidaceae and is an important epiphytic monopodial genus of orchids, well known for its beautiful inflorescence which lasts on the plant and in the vase for a considerably longer period of time.

Propagation of *Phalaenopsis* is mainly carried out through the vegetative buds originating from the base of the main stem and those developing from the nodes on the inflorescence. This conventional method of propagation is very slow as the vegetative buds produced by the mother plant are very less in number, i.e., only 2 or 3 in an year. Because of this, the plant is highly costed and is less popular in our state.

Evolving suitable techniques of micropropagation using floral tissues and meristems of *in vitro* grown plantlets would be helpful in supplying planting materials at a reasonable rate.

Eventhough orchids represent the first floricultural crop successfully mass propagated through tissue culture technique, reports on *Phalaenopsis* is comparatively less when compared to other orchids like *Dendrobium, Cymbidium* etc. In India, works conducted on *Phalaenopsis* is very less and mainly

foreign literature has to be depended on. In our State, work on tissue culture in *Phalaenopsis* was reported only from TBGRI, Palode.

Inflorescence stalks have been reported to be the favourite explants for culture of *Phalaenopsis* (Rotor, 1949, Tse *et al.*, 1971, Intuwong *et al.*, 1972 and Intuwong and Sagawa, 1974). There is generally a decrease in the generative capacity when the buds were derived farther away from the apex (Loh *et al.*, 1978) and furthermore, the buds at the basal part of inflorescence stalk are usually highly contaminated. Among the usually highly contaminated explants used in monopodial orchids are the inflorescence stalks of *Phalaenopsis* (Intuwong *et al.*, 1972).

One major problem in *Phalaenopsis* tissue culture was explant sterilization (Goh, 1989). In the present study, mature inflorescence stalks were used and out of the sterilant combinations, none recorded total survival of nodal explants. This observation was in accordance with that of Zimmer and Pieper (1978) who observed that older tissue such as buds from basal parts of flower stalk, were especially difficult to decontaminate.

Organisms which may contaminate *Phalaenopsis* flower stalk node cultures include a wide variety of bacteria and fungi. These occur at random and their identity cannot be predicted. Because of this Johnson *et al.* (1982) used the relatively broad spectrum fungicides (Benlate and Nystatin) and two bactericides (Pencillin G and Gentamycin) in the media used for culturing *Phalaenopsis* inflorescence stalk. In the present study the organisms causing contamination were identified as *Staphylococcus, Streptomyces* and *Aspergillus*, through pathological examination. Among this, the most destructive and frequently occurring organism was identified to be *Staphylococcus*, a gram positive bacteria. By the use of

streptopencillin in the media this bacteria could be effectively controlled and the survival percentage of the nodal explants showed a good increase and the cultures were practically free of bacterial contamination. Such observations were earlier reported by Arditti *et al.*, (1981) and Tanaka *et al.* (1988).

In the present study when it was tried to induce axillary buds from mature inflorescence stalk in different growth regulator containing media, success was obtained only up to the stage of initiation and elongation of buds to a size of 0.5 cm in 1/2 MS medium and eventhough further organogenetic response was not obtained. A similar case has been reported by Tokuhara and Mii (1993) and they observed that in 1/2 MS medium further bud growth was not achieved as the explants blackened. The blackening of explants may be probably due to the toxic effect of salts present in the medium or due to the lack of some other growth factor. This problem was overcome by them by the use of New Dogashima Medium (NDM). The favourable influence of coconut water on inducing axillary buds from nodal explants was observed in the present study. The medium containing BA, NAA and 2,4-D in combination with coconut water took only minimum number of days for bud development.

Inflorescence stalk tip was found to produce PLB's in 1/2 MS medium containing sucrose at 1.5 per cent level and CW 15 per cent with combinations of BA, NAA, 2,4-D and adenine. But further growth of these PLB's were not observed. Tokuhara and Mii (1993) observed that green PLB's with high multiplication capacity can be induced from the stalk tips using New Dogashima Medium (NDM) containing 0.1 ppm NAA and 1.0 ppm BAP instead of 1/2 MS medium.

Out of the different parts of the flower bud cultured, only pollinia showed response to various treatments. Pollinia increased five times in size and showed signs of callusing in 1/2 MS medium containing BA, NAA and 2,4-D. Callus further showed no organogenetic response. Till date there is no report on callus development from pollinia in orchids.

Pod culture was attempted with very little success, as the availability of pods to carry out the trials was limited. The difficulty encountered with pod culture in *Phalaenopsis* was that the seeds showed sign of germination only when harvested at the correct stage. Immature pod did not have viable seeds and pods slightly over mature showed aborted embryos.

With regard to the type of explants, basal portion of the plantlet cultured recorded maximum number of shoots. However, shoot node was used for further studies, because during the culture it was observed that the mortality rate of basal portion was more and so to ensure consistent results shoot node was made use of. Apical bud when cultured, recorded no multiple shoot production during the period under study and this was in contrary to the opinion of most of the workers (Kunisaki *et al.*, 1972; Intuwong and Sagawa, 1974; Stewart and Button, 1976 and Karim *et al.*, 1992).

In the present study, it was observed that first node produced the longest bud followed by second, third and fourth node. Urata and Iwanaga (1965) had also reported that the position of the explant influences the growth of cultures *in vitro*. Their observation was that secondary flower stalks developed on the cuttings excised from the upper position on the flower stalk, while shoots were developed on those obtained from the lower position, in *Phalaenopsis*.

Considering the effect of media, all the media tried except MS and KC favoured the growth of the cultures. This is in contrary with the observation of Rosa *et al.* (1977) who obtained satisfactory seed germination and seedling growth on modified KC medium. Zimmer and Pieper (1978) failed to induce PLB in cultures of axillary buds from flower stalks using KC medium. In contrary, Ichihashi (1992) succeeded to induce PLB from the same explants by systematic investigation of major ions and developed a medium with optimal mineral composition for the culture. These results suggest that selection of appropriate mineral composition of the media is essential for the successful *in vitro* culture of *Phalaenopsis*.

Physical state of the medium, viz., liquid and solid did not show any significant difference with regard to shoot production. Hence the cost incurred for the production of *Phalaenopsis* plantlets can be reduced by avoiding the use of the costliest ingredient of the media upto the last stage ie. culturing the plantlets before planting out. But it was reported that liquid medium favoured the faster growth of seeds than solid medium by many workers (Singh and Prakash, 1985 and Kerbauy and Handro, 1981).

In the present study sucrose at 1.5 per cent was found to be optimum for multiple shoot development and early induction of PLB's instead of the normal concentration of 3.0 per cent used in MS media, thereby reducing the cost incurred. Hinnen *et al.* (1989) also reported the favourable effect of 1.5 per cent sucrose. In clonal propagation of *Phalaenopsis*, by shoot tip culture, yellow coloured PLB's developed on medium containing 2 per cent sucrose and upon transfer to solid medium without sucrose the PLB's turned green (Intuwong and Sagawa, 1974).

Thiamine-HCl at 20 ppm level was found to favourably influence the development of multiple shoots and PLB's. The influence was more pronounced with regard to the number of PLB's developed. Thorpe and Patel (1984), had reported that Thiamine was the most often added vitamin, followed by nicotinic acid and pyridoxine. The importance of Thiamine in orchid tissue culture was also reported by Mead and Bulard (1975); Harvais (1982) and Sanchez (1988).

In the present investigation, it was observed that different levels of NAA did not influence multiple shoot production from shoot node whereas root production increased with increased concentration of NAA. With increase in the level of BA, shoot production increased but did not influence root production. Kim and Kako (1982) too found that the addition of BA encouraged the formation of PLB's and development of shoots.

Considering the effect of combination of BA and NAA, shoot production increased when NAA at low level (1.0 ppm) was used in combination with higher level of BA (20 ppm). Vij and Pathak (1989) observed that when BA 5 ppm + NAA 1 ppm were added to the medium, 65 per cent of *Phalaenopsis* flower stalk stem segments formed adventitious buds and 17 per cent formed PLB's. Similarly, highest rate of PLB formation occurred with BA 20 ppm + NAA 0.1 ppm in *Phalaenopsis* and *Doritaenopsis* flower stalk buds (Tokuhara and Mii, 1993).

The combination of BA and 2,4-D responded similar to the combination of BA and NAA. Maximum shoot production was observed at higher level of BA and lower level of 2,4-D. With increase in level of 2,4-D, root production also registered a proportionate increase.

The most important finding of this research work was the development of an ideal growth regulator combination which favoured both multiple shoot production and PLB formation. The ideal combination identified was BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm. And this combination can be recommended as a universally accepted formulation for *Phalaenopsis* tissue culture. The same combination also favoured bud development from mature inflorescence stalk node and growth of pollinia.

Media containing KIN alone; KIN and NAA; KIN, NAA and 2,4-D and KIN, BA, 2,4-D and adenine did not show any observable difference with regard to shoot production from culturing shoot node during the course of this study.

Coconut water has been proved to promote growth and differentiation of excised tissues and organs of several crops. The beneficial effect of coconut water has been attributed to the presence of cytokinin like substances (Straus and Rodney, 1960). Coconut water also contains a number of cell division factors and free amino acids (Shantz and Steward, 1952). Addition of coconut water to the culture medium was reported to have a beneficial effect in the production of PLB's and plantlet formation in *Phalaenopsis* (Homma and Asahira, 1985; Lam *et al.*, 1991 and Ichihashi, 1992).

In the present study with regard to multiple shoot production using different levels of coconut water, the different explants recorded maximum number of shoots at different levels of coconut water, i.e., first node and second node at 15 per cent and basal portion at 25 per cent. Except apical bud, all the other three explants tried induced multiple shoots *in vitro* without the addition of plant growth

hormones. This indicates that coconut water contains all the growth hormones required for multiple shoot induction in *Phalaenopsis*. Similiar was the report of Lam *et al.* (1991) who suggested that media containing coconut water should be used to increase the number of protocorms or PLB's in cultures because hormones other than those contained in banana homogenate and coconut water are not required for the proliferation of *Phalaenopsis* protocorms and PLB's.

With regard to PLB formation, eventhough no significant difference was observed for the number of days taken for PLB formation, the number of PLB's increased with increased level of coconut water in the medium.

The effect obtained by using tender and mature coconut water in the media were similar with regard to shoot production. It was also observed that even 6 days old coconut water induced the same effect as that of fresh coconut water.

Among the undefined medium supplements used for the study, tomato juice did not register any significant influence with regard to shoot production. Loh *et al.* (1978) found that, in *Aranda* Deborah culture, both leaf and root formation was inhibited by tomato juice and only a slight increase in fresh weight was observed in cultures on 15 per cent tomato juice enriched medium. In the present study when the PLB formation from shoot node was considered, tomato juice at 3.0 per cent level recorded the highest number of PLB's.

Adenine, a nitrogenous base of DNA, have been widely used in tissue culture media for their growth regulatory effects. Davis *et al.* (1977) reported the possible role of adenine in enhancing apical dominance. Synergestic effect of adenine on cytokinins has been suggested by Nitsch *et al.* (1967). In the present

study, adenine in combination with BA recorded maximum shoot number followed by the combination of adenine, BA and NAA.

With regard to PLB formation, the combination of adenine, BA and NAA took the least number of days for PLB formation and recorded maximum number of PLB's.

Peptone added at a concentration of 1000 ppm in the media was found to be the best organic additive with respect to maximum shoot, leaf and root production in *Phalaenopsis* in this study. In *Dendrobium nobile*, Sudeep in 1994 reported the best concentration of peptone as 40 ppm in combination with BA and NAA in VW medium, whereas in *D. crepidatum* and *D. pierardi* peptone at 2 g l^{-1} favoured vegetative growth from floral buds (Vij *et al.*, 1991).

High rates of PLB formation have been obtained in cultures of flower-stalk internodal sections (Lin, 1986) and organ segments of shoots derived from flower-stalk culturers (Pieper and Zimmer, 1976; Tanaka and Sakanishi, 1978). However, the availability of flower stalks and therefore, multiplication is limited particularly in high quality breeding plants of *Phalaenopsis*, which usually exist as single plants (Reuter, 1983) and the results are often unpredictable as both vegetative and secondary flower stalks are produced in flower stalk cultures (Griesbach, 1983 and Tanaka *et al.*, 1988). Hence the large scale production of PLB's and regeneration of plantlets from the shoot node, leaf and root tissues of *in vitro* grown plantlets reported in this study are of great importance in realising quick multiplication rates.

Young plantlets appeared to be ideal material for the production of PLB's. In *Phalaenopsis*, leaves, leaf sections, stem tissues and in some cases roots

of young plantlets formed numerous PLB's, which further developed into plantlets (Zimmer and Pieper, 1978).

In the present study, PLB's were found to initiate in large number from the shoot node from its base, leaves and roots in 1/2 MS medium containing BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm + sucrose 1.5 per cent, while these organs are still attached to the shoot. Formation of PLB's was confined to the leaves and roots of the plantlet that were in contact with the nutrient medium. Neither the roots completely submerged in the medium nor the leaves freely suspended in the air developed PLB's. The PLB's were generally formed from either roots or leaves, but a majority developed PLB's from the base, leaves and roots. These reports were in confirmation with the reports of Latha and Seeni (1991), but with difference in medium and in the additives used.

When isolated leaf and root portion were used for culture in this study, PLB's were found to initiate and develop in large number in 1/2 MS medium containing BA 25 ppm + adenine 10 ppm + NAA 1 ppm, when kept under dark in the case of leaf culture and under light for root culture. The number of PLB's developed by roots was lower in number when compared to that of leaves. The initiation of PLB's near the tip region and its quick spread in a sporadic fashion all over the sides of the leaves and roots indicate that the entire surface of the organ is potentially meristematic and regenerative. Similar findings were reported in *Phalaenopsis* by Latha and Seeni (1991).

Regeneration in leaf and root tissue cultures established in a few orchid genera is restricted to the tips (Churchill *et al.*, 1973 and Loh *et al.*, 1975) and bases (Goh, 1983; Griesbach, 1983 and Mathews and Rao, 1985) of leaves and tips of

roots (Tanaka *et al.*, 1976; Kerbauy, 1984c; Phillip and Nainar, 1988 and Sanchez, 1988). Large scale development of PLB's was reported by the use of juvenile leaf segment culture (Tanaka and Sakanishi, 1978 and Vij *et al.*, 1984). Root tissues of *Phalaenopsis* were even less amenable to regeneration as only one successful report on the induction of PLB's raised from the few of the root tips after prolonged period (4-9 months) of culture is available (Tanaka *et al.*, 1976) other than the report of Latha and Seeni (1991).

When the effect of BA alone and combinations of BA and NAA on PLB growth and development were studied, BA and NAA combination (BA 15 ppm + NAA 1 ppm) was found to favour the fastest growth of PLB's and plantlet development. In the present study it was found that hyperhydric PLB's were developed after two to three subculturing cycles as reported by Su (1995). These hyperhydric PLB's (h PLB's) were in no way inferior to normal PLB's (n PLB's) as they exhibited higher capacity for proliferation, eventhough a slight delay in plantlet formation was observed. In the study conducted by Latha and Seeni (1991) the increase in the frequency and number of PLB's obtained in high concentration of BA (10 mg l^{-1}) in combination with NAA (1-2 mg l^{-1}) was consistent with the findings of others in flower stalk cultures (Tanaka and Sakanishi, 1978 and Ernst, 1985). However, a threshold level of auxin, along with high concentration of BA, was essential suggesting a possible synergistic interaction in the initiation of PLB's. This is in contrast to the published work of Lin (1986) where NAA was found to be inhibitory to PLB formation in internodal segment cultures of the flower stalk.

When the effect of adenine on PLB formation from *in vitro* leaf was considered, the combination of adenine 10 ppm + BA 10 ppm + NAA 1 ppm took

only minimum number of days for PLB formation and recorded maximum number of PLB's developed. This result was in confirmation with the report of Tanaka and Sakanishi (1985).

Regarding the effect of adenine in combination with BA on PLB growth and plantlet development, the combination of adenine 8 ppm + BA 16 ppm recorded the highest number of PLB's and plantlets developed.

Peptone when added in the medium was found to favourably influence the production of PLB's in combination with BA, NAA and 2,4-D. The addition of peptone greatly promoted the growth of PLB in *Doritaenopsis*, but more than 4 g l^{-1} increased the rate of death of PLB segments (Amaki and Higuchi, 1989). In the present study peptone at 1.0 g l^{-1} to 1.5 g l^{-1} was found to be favourable. Kukulczanka *et al.* (1989) noted best regeneration and organogenesis on MS medium provided with peptone interacting with cytokinin (BA 0.2 ppm) and auxin (NAA 0.2 ppm).

Shoot regeneration in leaf and root tissue cultures of *Phalaenopsis* was preceded by pronounced but relatively callus free formation of PLB's and the regenerated plants were morphologically uniform. Coconut water is known to promote direct formation of PLB's without callusing (Sanchez, 1988) and regeneration of typically uniform plants (Tanaka *et al.*, 1988). Latha and Seeni (1991) confirmed the cytological stability of the plantlets developed from the PLB's of leaf and root cultures and this method can be routinely used for mass propagation of identical genotypes.

To reduce phenolic blackening of nodal explants, culturing the nodal explants in liquid medium with filter paper bridge was found to be better. Keeping in the dark was also found good, but it inhibited the bud development. Activated charcoal at 0.1 per cent was found to have only very little effect in this study. The relative abundance of phenolics in *Phalaenopsis* and negative impact of the phenolic oxidates on growth and differentiation of tissues in culture as evidenced from extensive browning of the medium and loss of 20 per cent of the leaves in culture have been widely reported (Ernst, 1985 and Tanaka *et al.*, 1988). Different methods are suggested by different workers to overcome this problem. Repeated subculturing (Latha and Seeni, 1991) and use of activated charcoal (Ernst, 1974) in the media are some of the methods. Waes (1987) reported that addition of AC is beneficial for those species with a high release of phenolic compounds in the culture medium.

In the present study, using different parts of plantlets, antioxidants like citric acid, ascorbic acid, PVP and cysteine-HCl were found to be beneficial for reducing media and explant blackening.

Effect of AC depends upon the species, the stage of development and the concentration. *Phalaenopsis* seedlings generally exude phytotoxic phenolics, which diffuse into the medium. For this reason, activated charcoal has often been added to the medium (Ernst, 1975). It was shown that darkening of substrate elicited good growth and the development of positively geotropic roots. It has been reported that damaged seedlings and embryos, showing strong discolouration, occasionally recover when transplanted on charcoal agar (Werckmeister, 1970). Study conducted with AC showed that shoot number and leaf number increased with level of AC reaching a maximum value at 0.40 per cent and root number at 0.80 per cent.

The study conducted with the addition of triadimefon in the media recorded maximum healthy and lengthy roots at 20 ppm level. The plantlets grown in medium containing triadimefon 20 ppm recorded better survival percentage. It was observed in the present study that, with the increase in the level of triadimefon in the media, the length of the roots also increased. This together with the anti-transpirant activity helped in the better survival of the plants which were cultured in triadimefon containing media and planted out. The mode of action of triadimefon is that, it increased stomatal resistance, thereby contributing significantly to the maintenance of turgor in the treated plants. Transient increase in abscisic acid is triggered by triadimefon. Abscisic acid has been implicated as the initial trigger in the hardening process for various types of plant stress (Boussiba *et al.*, 1975). The effect of triadimefon on increasing the survival percentage of planted out plantlets was also reported by Hussain (1995) in gladiolus.

Light was found to favour multiple shoot production and PLB formation from shoot node, development of plantlets from PLB's and PLB formation from root; whereas darkness favoured early and good development of PLB's from leaf and callusing of buds. In Phalaenopsis, different day lengths (8, 16 or 24 h) had no effect on the production of shoots from flower stalk cuttings *in vitro*. However, shoot production was promoted when light intensity was decreased (Tanaka *et al.*, 1988).

When the plantlets cultured in different vessels were observed, the growth of plantlets was better in large sized vessel. This may be probably due to more aeration and more space for growth. Tanaka *et al.* (1988) observed that

aeration is an important factor for getting high shoot production from flower stalk cutting culture.

Large pieces of coconut husk was identified to be the best medium for planting the plants of *Phalaenopsis* in this study. Coconut husk was reported to be the best medium for planting out *Dendrobium nobile* plantlets (Sudeep, 1994). On contrary, Lakshmidevi (1992) reported that potting medium having charcoal and brick pieces was found to be better than coconut husk.

Hardening the *in vitro* raised plantlets so as to acclimatize them to the outside environment is very important. In the present study out of the different pre-transfer treatments tried, hardening the plants by just spreading over sterile charcoal pieces for two weeks was found to be the best. According to Wainwright (1988) tissue cultured plants are very poorly adapted to resist the low relative humidity, higher light levels and more variable temperature prevailing outside. Ziu (1986) reported that the success in acclimatization of *in vitro* cultured plants was dependant not only on the post transfer growth conditions, but also on the pre-transfer culture condition.

A period of humidity acclimatization was suggested for the newly transferred plantlets to make them adapted to the outside environment (Hu and Wang, 1983). Light, temperature and relative humidity are the major factors to be controlled during acclimatization. Kumar (1992) observed that *in vitro* seedlings of *Dendrobium* recorded best survival per cent in pure charcoal followed by fern roots and rubber seed husks when kept under the temperature between 29 and 35°C with RH between 70 and 90 per cent and illumination between 1000 and 1500 lux at pot level.

In the present study, the hardened plantlets after tieing on to coconut husk was hung in the orchidarium with high humidity. Even after 8 weeks of planting out, the hardened plantlets under observation recorded cent per cent survival.

Nutrient application is important for the better establishment and good growth of plantlets. In the present investigation, plantlets sprayed with 17:17:17 complex solution at 0.1 per cent level recorded, the maximum per cent of survival and other growth characters. Kumar (1992) sprayed the seedlings of *Dendrobium* hybrid with NPK nutrient solution on alternate days. Agrawal *et al.* (1992) reported that spraying the plantlets of *Vanilla* with Knudson C nutrient medium on alternate days during the hardening stage was helpful in the establishment of plants and their further growth. Seedlings of *Cymbidium kanran* fed with MS nutrient solution grew better than those fed with Knudson C solution (Kim *et al.*, 1988).

When the growth parameters of the plantlets were observed at one month interval for a period of 8 months, the percentage survival of plantlets was decreased to 80 per cent. Considering the other growth parameters an increasing trend was noticed except for leaf production. This reduction in the rate of leaf production may be attributed to the increase in leaf length and width. In a report from TBGRI, Seeni and Latha (1990) recorded cent per cent survival of the axenic seedlings of the hybrid cultivar Fire Water Ponce without any acclimatization or pretreatment of the seedlings.

Summary

SUMMARY

Investigations on micropropagation technique in *Phalaenopsis*, were carried out at the Plant Tissue Culture Laboratory attached to the All India Co-ordinated Floriculture Improvement Project (AICFIP), College of Horticulture, Vellanikkara.

Phalaenopsis commonly known as "moth orchid" is an important monopodial genus coming under the epiphytic orchids, valued for its beautiful and long lasting blooms. Besides the low number of axillary buds produced, highly recalcitrant nature make the genus less amenable to conventional micropropagation techniques. The results of the experiments conducted on various aspects of micropropagation of *Phalaenopsis* are summarised here.

Out of the different explants tried, the response was shown by inflorescence stalk node, inflorescence stalk tip and pollinia collected from the field grown plants and apical bud, shoot node, basal portion, leaf and root of plantlets grown *in vitro*.

None of the surface sterilization treatments resulted in total survival of the explants tried. However, a maximum survival per cent of 40 and 50 was recorded for nodal and internodal explants, respectively, at the sterilant combination involving mercuric chloride (0.01%) for 30 min, streptomycin and pencillin (0.01%) for 90 min followed by final sterilization using mercuric chloride (0.10%) for 10 min. For flower bud, the combination involving emisan (1.0%) for 30 min followed by alcohol (50%) for 1 min recorded the maximum survival percentage (55). Leaf explants recorded 50 per cent survival in the combination involving

emisan (1.0%) for 30 min, norfloxacin (1.0%) for 30 min followed by final sterilization using mercuric chloride (0.1%) for 10 min. Root explants, did not record survival in any of these combinations tried.

Regarding the type of explant, basal portion of the shoot was found to be the best with respect to rate of increase in shoot number (8.00) and leaf number (7.67).

Considering the position of the explant, first node cultured recorded the highest increase in the length of the buds and the rate of increase was 1.13, 2.00 and 2.83 at 20, 40 and 60 days period, respectively.

When the effect of mineral salts in the medium was considered, full strength MS and KC media were far inferior to 1/4 MS, 1/2 MS, 3/4 MS and VW media, for culturing shoot node of *Phalaenopsis*.

No significant difference existed with respect to the number of shoots in both liquid and solid media during the 4 weeks period. At the end of 8 weeks, the highest number of shoots (8.17) was recorded for 1/4 MS liquid medium containing BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm + CW 15 per cent. With regard to leaf number, at the end of 4 weeks, maximum number of leaves (8.33) was recorded for 1/4 MS solid medium containing BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm). At the end of 8 weeks, maximum leaf number (9.33) was recorded for both 1/4 MS liquid and solid media, containing BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm + CW 15 per cent. With regard to root number, the highest number (3.17) at the end of 4 weeks was recorded for 1/4 MS solid medium containing BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm + CW 15 per cent. The maximum number (4.17) of roots was

recorded for 1/4 MS semi solid medium containing BA 20 ppm + NAA 1 ppm, at the end of 8 weeks.

Sucrose at 1.5 per cent level, recorded the maximum number of shoots (3.00 and 5.00) and maximum number of leaves (6.00 and 7.33), at the end of 4 and 8 weeks of culturing, respectively. Root number was found to be maximum (2.00 and 3.00) at the end of 4 and 8 weeks respectively, for sucrose (3.0%).

As to the effect of Thiamine-HCl, maximum shoot number (4.17 and 6.50) and maximum leaf number (7.33 and 9.17) were recorded for Thiamine-HCl (20 ppm) at the end of 4 and 8 weeks, respectively. With regard to root number, no significant difference existed among the different levels of Thiamine-HCl at the end of 4 weeks. At the end of 8 weeks, the highest number (2.17) was recorded for control (media without Thiamine-HCl).

When the axillary bud development from inflorescence stalk nodes was considered, the days taken for nodal swelling was minimum (7.8) for 1/2 MS medium containing BA 20 ppm + NAA 2 ppm. With regard to the time taken for the bud emergence and for the buds to attain a size of 0.5 cm, the minimum number of days (17.0 and 39.8, respectively) were recorded at the treatment combination involving BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm in 1/2 MS medium.

As regards the effect of BA, NAA and adenine on axillary bud development from nodal explants, the minimum number of days (11.0) was observed at two treatment combination, viz., BA 5 ppm + NAA 1 ppm + adenine 40 ppm and BA 20 ppm + NAA 1 ppm + adenine 10 ppm in 1/2 MS medium. The

number of days taken for bud development and the buds to attain a size of 0.5 cm was minimum (23.2 and 56.2 respectively) at BA 20 ppm+ NAA 1 ppm + adenine 10 ppm in 1/2 MS medium.

Pollinia, when cultured, started callusing and the time taken for callusing was minimum (2.0 days) at BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm and 1/2 MS medium containing 3 per cent sucrose.

When 70, 80, 90 and 100 days old pod were used, no response was noticed for 70 days old pod. The days taken for greening of embryo was minimum in 90 days old pod (14.6) at two treatments, viz., BA 10 ppm + NAA 1 ppm and KIN 5 ppm + 2,4-D 2 ppm. No significant difference was observed between the treatments with regard to the days taken for protocorm formation.

No significant difference existed among the different levels of NAA in 1/2 MS medium, with respect to number of shoots and leaves produced at the end of 4 and 8 weeks of culturing. With regard to root number, the highest number (3.67 and 4.50) after 4 and 8 weeks respectively were recorded at the combination NAA 5 ppm + BA 10 ppm + adenine 10 ppm.

In 1/2 MS medium containing adenine 10 ppm + NAA 1 ppm, the maximum shoot number (3.33 and 5.00) were recorded at BA 25 ppm at the end of 4 and 8 weeks respectively. With regard to leaf number, maximum number (6.17) was recorded at BA 25 ppm at the end of 4 weeks and no significant difference existed among the levels of BA after 8 weeks of culturing. When the number of roots produced were considered, significant difference was not observed among the different levels of BA at the end of 4 and 8 weeks of culturing.

Maximum number of shoots (3.00 and 4.83) and leaves (5.67 and 7.17) were observed at the end of 4 and 8 weeks of culturing, respectively, at BA 20 ppm + NAA 1 ppm. The maximum number of roots (3.33) was observed at BA 20 ppm + NAA 1 ppm, after 8 weeks of culturing.

In 1/4 MS medium, shoot number was maximum (2.67 and 4.83) at BA 20 ppm + 2,4-D 2.5 ppm at the end of 4 and 8 weeks, respectively. With regard to leaf number, no significant difference was found to exist among the treatments. Maximum number of roots (3.50 and 4.00) was recorded at BA 20 ppm + 2,4-D 5.0 ppm after 4 and 8 weeks of culturing, respectively.

With regard to the effect of BA, NAA and 2,4-D, in 1/4 MS medium, the combination BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm recorded the maximum number of shoots (3.33 and 5.33) and leaves (6.33 and 7.83) after a period of 4 and 8 weeks, respectively. With regard to root production, after 8 weeks, maximum root number (3.67) was recorded at BA 15 ppm + NAA 2 ppm + 2,4-D 4 ppm.

When the effect of KIN + NAA was considered in 1/4 MS medium, for a period of 4 weeks significant difference was not found to exist among the different treatments, with regard to shoot and root production. Maximum number of leaves (5.00) was produced at KIN 20 ppm + NAA 2 ppm.

In 1/4 MS medium, the shoot number (2.67) and leaf number (4.33) were maximum (2.67) at KIN 20 ppm + NAA 2 ppm + 2,4-D 2 ppm.

Kinetin, BA, adenine and 2,4-D registered no significant difference among the treatments tried with respect to number of shoots, leaves and roots produced.

The treatment combination of BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm + CW 15 per cent in 1/2 MS medium was found to be the best, as the days taken for nodal swelling, bud development and the buds to attain a size of 0.5 cm was the minimum (6.0, 14.0 and 31.8, respectively).

Coconut water did not influence the multiple shoot production from apical bud during the 12 weeks culture period. No significant difference was observed among the different levels on the leaf production, after 4 weeks. After 8 weeks, maximum number of leaves (3.33) was recorded at CW 20 per cent and 25 per cent and that after 12 weeks (4.67) at CW 20 per cent. With regard to root production, significant difference was not observed after 4 and 8 weeks. But after 12 weeks, maximum number of roots (2.00) was recorded at CW 15 per cent. Regarding the length of the longest leaf, CW 25 per cent recorded the maximum length (1.93 cm and 2.30 cm) at 4 and 8 weeks of culturing respectively. But the maximum leaf length (4.00 cm) was recorded at CW 20 per cent after 12 weeks of cutluring.

With regard to the effect of coconut water on multiple shoot production from first node cultured, maximum number of shoots (2.33, 2.50 and 3.17) was recorded at CW 10 per cent after 4, 8 and 12 weeks of culturing respectively. After 4, 8 and 12 weeks, maximum number of leaves (3.33, 5.17 and 7.17, respectively) were observed at CW 10 per cent. With regard to root number, significant difference was not observed at the end of 4 weeks. After 8 and 12 weeks, the root number was found maximum (1.50 and 2.17, respectively) at CW 15 per cent. Leaf length was found to be maximum (1.05, 1.92 and 2.83 cm) at CW 10 per cent after 4, 8 and 12 weeks of culturing, respectively.

When the effect of coconut water on shoot node was considered, maximum number of shoots (1.67 and 2.33) were recorded at the end of 4 and 8 weeks respectively at CW 10 per cent and 15 per cent. After 12 weeks, CW 15 per cent alone recorded the maximum (3.00) number of shoots. Regarding leaf number, maximum number (3.50) was recorded at CW 15 per cent after 4 weeks of culturing. After 8 and 12 weeks, CW 10 per cent recorded maximum number (4.83 and 6.33, respectively) of leaves. With regard to root number, no significant difference was noticed among the treatments at the end of 4 weeks. After 8 weeks, the maximum number (2.67) of roots was observed at CW 10 per cent and CW 15 per cent and the number was maximum (3.67) at CW 15 per cent after 12 weeks. Length of the longest leaf was found to be maximum (1.05 cm) after 4 weeks at CW 10 per cent, after 8 weeks, the length was maximum (2.32 cm) at CW 15 per cent and after 12 weeks CW 10 per cent recorded the maximum length (2.92 cm).

Considering the effect of coconut water on the basal portion, with regard to shoot production, no significant difference was observed at the end of 4 weeks. After 8 and 12 weeks, shoot number was maximum (2.67 and 4.33 respectively) at CW 25 per cent. When leaf production was considered, maximum number (2.50, 4.33 and 7.17) were recorded at CW 25 per cent after 4, 8 and 12 weeks respectively. Coconut water 10 per cent recorded the maximum (3.17) number of roots after 12 weeks. Length of the leaf was found to be maximum (1.47, 2.20 and 2.70 cm) at CW 25 per cent after 4, 8 and 12 weeks of culturing, respectively.

Tender and mature coconut water in the culture media on the production of shoots, leaves and roots, recorded no significant difference.

Tomato juice was also ineffective on multiple shoot production, leaf and root production.

Effect of adenine when considered in combination with BA on multiple shoot production in 1/4 MS medium, the combination of adenine 8 ppm + BA 16 ppm recorded the maximum number of shoots (3.00 and 5.00) and leaves (6.00 and 7.67) after 4 and 8 weeks respectively. Maximum number of roots (3.00 and 3.83) after 4 and 8 weeks of culturing respectively were recorded for adenine 20 ppm + BA 16 ppm.

When the effect of adenine was considered in combination with BA 10 ppm + NAA 1 ppm in 1/4 MS medium, the number of shoots, leaves and roots produced during the culture period (4 and 8 weeks) did not show any significant difference among the treatments.

When the use of peptone in 1/4 MS medium was considered, maximum number of shoots (4.17, 6.67 and 7.83), leaves (7.00, 8.67 and 11.00) and roots (2.83, 4.33 and 5.67) were recorded at peptone 1000 ppm after 4, 8 and 12 weeks of culturing respectively.

Out of the different methods tried to reduce phenolic blackening of media and explant when inflorescence stalk node was used, two methods viz., keeping the cultures in the dark and use of liquid media with filter paper bridge were found better as these recorded the lowest (10) percentage of cultures which got blackened due to phenols, after a culture period of 4 weeks.

Regarding the amount of phenols exuded by the culturing of different explants of plantlets observed for a period of 2 weeks, apical bud showed the highest phenolic exudation and the PLB's produced practically no phenols.

When the role of antioxidants in reducing phenolic blackening and multiple shoot production were considered for a period of 8 weeks it was observed that with the increase in the concentration of antioxidants, there was a proportionate reduction in media discolouration. Regarding the effect of citric acid, the percentage of cultures which got blackened was zero at citric acid 500 ppm, maximum number of shoots (5.83) was recorded at 200 ppm, and significant difference was not found to exist with regard to leaf and root production.

Significant differences were not observed with regard to number of shoots and leaves after 4 and 8 weeks as influenced by activated charcoal. After 12 weeks, maximum number of shoots (4.00) and leaves (8.83) was recorded at AC 0.40 per cent. With regard to root production after 8 and 12 weeks, AC 0.80 per cent recorded the maximum number of roots (2.50 and 4.17, respectively).

In 1/4 MS medium containing BA 5 ppm, significant difference was not created by triadimefon with regard to shoot and leaf production after 4, 8 and 12 weeks of culturing. Regarding the production of roots, after 8 weeks triadimefon (20 ppm) recorded the maximum number of roots. Length of the longest root was found to be maximum (4.00, 6.67 and 8.00 cm) at triadimefon 20 ppm after 4, 8 and 12 weeks respectively.

When the influence of antibiotics added in the media was considered for a period of 4 weeks, the highest percentage (80) of survival of nodal explants was observed at streptopencillin 160 and 200 ppm in 1/2 MS liquid medium containing BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm. In the same medium containing CW 15 per cent, the percentage of survival was 85 at 80, 120 and 160 ppm. In all the treatments involving streptopencillin, the percentage of bacterial infected cultures was zero. The number of days taken for nodal swelling and bud development was minimum (6 and 14 days respectively) at streptopencillin 40 ppm and control, in medium containing CW 15 per cent.

Considering the frequency of multiple shoot production, the rate of production of multiple shoots showed a steady increase with subculturing.

As to the effect of sucrose on PLB formation, the minimum number of days taken for development of PLB's (40.67) and maximum number of PLB's (33.33) was recorded at 1.5 per cent level of sucrose in 1/2 MS medium containing BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm, when cultured for 14 weeks.

With regard to the effect of BA, NAA and 2,4-D on PLB formation from shoot node, the minimum number of days (40.67) and maximum number of PLB's (4.17) was recorded in 1/2 MS medium containing BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm with 1.5 per cent sucrose during a culture period of 8 weeks.

Cent per cent of the leaf cultures developed PLB's from the *in vitro* leaf at the combination BA 25 ppm + adenine 10 ppm + NAA 1 ppm, when BA was tried in combination with adenine and NAA in liquid 1/2 MS medium containing 1.5 per cent sucrose for a period of 8 weeks under dark condition. The time taken for

PLB formation was also minimum (21.3 days) at this combination. Significant difference was not observed with regard to number of PLB's developed and size of PLB's developed.

When the effect of BA in combination with adenine and NAA on PLB formation from *in vitro* roots was considered, in liquid 1/2 MS medium supplemented with adenine 10 ppm + NAA 1 ppm with 1.5 per cent sucrose for a period of 8 weeks, cent per cent of the root cultures developed PLB's at BA 20 ppm and 25 ppm. With regard to the time taken for bulging of root tips and PLB formation, the minimum number of days (19.80 and 25.20, respectively) were recorded at BA 25 ppm. No significant difference was observed with respect to the number of PLB's developed.

Considering the effect of BA, NAA and 2,4-D on PLB formation from roots in liquid 1/4 MS medium with 1.5 per cent sucrose during a period of 12 weeks, the combination BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm recorded 80 per cent of root culture developing PLB's, minimum number of days (28.60 and 37.80) for root tip bulging and PLB formation respectively and maximum number of PLB's (2.4) developed.

When the effect of BA and NAA on PLB growth and plantlet development was considered in 1/4 MS medium containing 1.5 per cent sucrose, the largest increase in PLB number (26.67, 58.33 and 125.00) and the maximum number of plantlets developed (5.00, 8.67 and 10.83) were recorded at the combination of BA 15 ppm + NAA 1 ppm after 4, 8 and 12 weeks respectively.

With regard to the effect of coconut water on PLB formation from stalk tip in 1/2 MS medium containing CW 15 per cent and sucrose 1.5 per cent for a period of 10 weeks, the number of days taken for stalk tip bulging was minimum (8.00) at the combination BA 20 ppm + NAA 1 ppm + adenine 10 ppm. Minimum number of days (49.2) for PLB formation was recorded at BA 20 ppm + NAA 1 ppm + adenine 20 ppm. No signfiicant difference was observed with regard to number of PLB's developed.

Addition of coconut water into 1/2 MS medium containing BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm with 1.5 per cent sucrose was found to have no significant influence on the number of days taken for PLB formation from shoot nodes.

Considering the effect of coconut water on PLB growth and development, the number of PLB's developed increased with the level of coconut water in 1/4 MS medium with 1.5 per cent sucrose and the highest rate of increase was observed at CW 15 per cent. With regard to plantlet development, the highest number (4.00, 8.17 and 30.00) after 3, 6 and 12 weeks of culturing were recorded at CW 25 per cent.

By the addition of tomato juice in 1/2 MS medium containing BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm with 1.5 per cent sucrose, no significant difference was observed with regard to number of days taken for PLB formation from shoot node. Considering the number of PLB's developed the largest number (8.67) was produced at 3 per cent level.

When the effect of adenine in combination with BA and NAA on PLB formation from shoot node in 1/2 MS medium containing sucrose 1.5 per cent was considered, during a culture period of 14 weeks, the combiantion adenine 10 ppm + BA 10 ppm + NAA 1 ppm recorded the minimum number of days (42.33) for PLB formation and largest number of PLB's (30.33).

Considering the effect of adenine on PLB formation from *in vitro* leaf in combination with BA 10 ppm + NAA 1 ppm in liquid 1/4 MS medium containing 1.5 per cent sucrose for a period of 8 weeks under dark, 80 per cent of the cultures developed PLB's at adenine 5, 10 and 15 ppm. When the number of days taken for PLB formation was considered, the minimum number (23.4 days) was recorded at adenine 10 ppm. No significant difference existed with regard to number of PLB's produced. The size of the PLB's developed was maximum (2.1 mm) at 25 ppm level of adenine.

When the effect of adenine in combination with BA on PLB growth and plantlet development in 1/4 MS medium with 1.5 per cent sucrose was considered, the largest increase in PLB number (10.33, 35.83 and 96.67) and the highest number of plantlets developed (3.83, 5.67 and 9.50) were recorded at 4, 8 and 12 weeks respectively at the combination of adenine 8 ppm + BA 16 ppm.

Addition of peptone into 1/2 MS medium containing BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm with 1.5 per cent sucrose cultured for a period of 8 weeks did not show any significant difference with regard to number of days taken for PLB formation. Regarding the number of PLB's developed, peptone at 1500 ppm recorded the largest number (10.00) of PLB's developed.

It was observed that light favoured plantlet development, multiple shoot formation and PLB formation from shoot node and *in vitro* root, whereas dark period favoured early development of PLB's from *in vitro* leaf, callusing and PLB proliferation.

When the effect of culture vessels on the growth of plantlets were compared, growing the plantlets in 250 ml conical flask was found to be best for producing healthy, large and robust plantlets with spreading leaves and very long roots.

Survival of the plantlets was the highest (60%) when plantlets were grown on large coconut husk pieces. When the effect of container was considered using charcoal + brick pieces (1:1) as the potting media, polysachet method recorded the highest percentage (30) of plantlet survival. Regarding the influence of triadimefon added in the culture medium, plantlets grown in medium containing triadimefon 10, 15 and 20 ppm recorded the highest percentage of survival (80 and 70) after 4 and 8 weeks of planting out.

Pre-transfer hardening treatment recorded the highest survival percentage (90 each) after 4 and 8 weeks of planting, using coconut husk as the medium for the plantlets cultured in medium containing triadimefon 20 ppm for 2 months before planting out, followed by spreading the plantlets over sterile charcoal pieces for 2 weeks and then planting out. The post transfer hardening treatment which recorded cent per cent survival of the plantlets after 4 and 8 weeks of planting was that of hanging the hardened plantlets in orchidarium with high humidity.

Among the nutrient solutions tried, cent per cent survival of plantlets was recorded after 8 weeks of planting at all nutrient solutions and after 12 weeks the highest survival percentage was recorded for the nutrient solutions 30:10:10 (0.5%) and 17:17:17 (0.1%). Plant height was found to be maximum (1.12 and 1.24 cm) for the plants sprayed with 17:17:17 (0.1%) solution after 8 and 12 weeks of planting respectively. Maximum leaf number (6.4 and 6.8), maximum leaf length (5.4 and 6.0 cm) maximum leaf width (1.98 and 2.3 cm), maximum number of roots (9.6 and 10.6) and maximum length of root (5.2 and 5.8 cm) was observed for plantlets sprayed with 17:17:17 nutrient solution at 0.1 per cent concentration after 8 and 12 weeks of planting, respectively.

The survival of plantlets decreased to 80 per cent at the end of 8 months of planting out. At 8 months after planting out the plants grew to a height of 2.00 cm producing 4 leaves and 13.8 roots. The length of the longest leaf was 9.9 cm and the width 3.8 cm. The length of the longest root was 19.0 cm.

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* Originals not seen

Appendices

APPENDIX-I

Composition of Murashige and Skoog (1962) medium

Constituents	Quantity	Volume made up	Volume pipetted
Solution A			
Ammonium nitrate	16.5 g	1000 ml	100 ml
Potassium nitrate	19.0 g		
Magnesium sulphate	3.7 g		
Potassium dihydrogen phosphate	1.7 g		
Solution B			
Calcium chloride	4.4 g	500 ml	50 ml
Solution C			
Boric acid	0.62 g	100 ml	1 ml
Manganese sulphate	2.23 g		
Zinc sulphate	0.86 g		
Potassium iodide	0.083 g		
Sodium molybdate	0.025 g		
Solution D			
Ferrus sulphate	2.78 g	500 ml	5 ml
Sodium EDTA	3.73 g		
Solution E			
Cobalt chloride	0.025 g	1000 ml	1 ml
Copper sulphate	0.025 g		
Solution F			
Nicotinic acid	50 mg	100 ml	1 ml
Pyridoxine HCl	50 mg		
Thiamine HCl	10 mg		
Glycine HCl	200 mg		
Sucrose	30.00 g		
Myo-inositol	100.00 mg		
Agar	7.00 g		

APPENDIX-II

Composition of Vacin and Went (1949) medium

Constituents	Quantity
Ammonium sulphate	500 mg
Magnesium sulphate	250 mg
Manganese sulphate	7.5 mg
Potassium nitrate	525 mg
Potassium dihydrogen phosphate	250 mg
Dicalcium phosphate	200 mg
Tricalcium phosphate	200 mg
Ferric tartarate	28 mg
Sucrose	20 g
Agar	8 g
pH	5.5-6.0

APPENDIX-III

Composition of Knudson's C medium (modified)

(Rowe and Richardson, 1975)

Solution	Constituents	Quantity	Volume made up	Volume pipetted
A	Calcium nitrate	10.000 g		
	Ammonium sulphate	5.000 g	1000 ml	100 ml
	Potassium dihydrogen phosphate	2.500 g		
	Magnesium sulphate	2.500 g		
	Berthelots solution			
B	Manganese sulphate	1.000 g		
	Boric acid	0.025 g		
	Potassium iodide	0.025 g		
	Cobaltous chloride	0.025 g	500 ml	0.5 ml
	Zinc sulphate	0.050 g		
	Copper sulphate	0.025 g		
	Sulphuric acid	0.5 ml		
	Iron solution			
C	Ferrous sulphate	2.785 g	500 ml	5 ml
	Sodium EDTA	3.725 g		
	Thiamine solution			
D	Thiamine-HCl	0.250 g	250 ml	1 ml
	Sucrose	40.000 g		
	Agar	5.000 g		
	pH	5.6-5.8		

APPENDIX-IV

Abstract of analysis of variance for the effect of different treatments

Sl. No.	Character	Treatment SS	Error SS	Treatment DF	Error DF	Treatment MSS	Error MSS
1	2	3	4	5	6	7	8
1. Response of explants on multiple shoot development							
a) Apical bud							
i)	Number of leaves	51.533	10.067	4	20	12.883	0.503*
ii)	Number of roots	5.356	4.493	3	15	1.785	0.300*
b) Shoot node							
i)	Number of shoots	168.133	21.067	4	20	42.033	1.053*
ii)	Number of leaves	168.533	30.667	4	20	42.133	1.533*
iii)	Number of roots	183.867	37.333	4	20	45.967	1.867*
c) Basal portion							
i)	Number of shoots	250.133	43.467	4	20	62.533	2.173*
ii)	Number of leaves	268.133	29.467	4	20	67.033	1.473*
iii)	Number of roots	90.200	13.800	4	20	22.550	0.690*
2. Effect of position of nodes on elongation of buds							
i)	Length of the bud in 20 days	2.00	0.289	3	15	0.669	0.019*
ii)	Length of the bud in 40 days	5.729	0.615	3	15	1.910	0.041*
iii)	Length of the bud in 60 days	5.729	0.708	3	15	1.910	0.047*
3. Effect of media on the induction of multiple shoots							
a) After 4 weeks							
i)	Number of shoots	29.889	9.111	5	25	5.978	0.364*
ii)	Number of leaves	57.222	26.111	5	25	11.444	1.044*
iii)	Number of roots	3.250	4.917	5	25	0.650	0.197*

Contd.

Appendix-IV. Continued

1	2	3	4	5	6	7	8
44. Effect of nutrient solutions on the growth of planted out plantlets							
a) After 8 weeks of planting out							
i)	Plant height	0.494	0.150	4	16	0.124	0.009*
ii)	Leaves per plant	3.440	4.160	4	16	0.860	0.260*
iii)	Length of longest leaf	2.940	3.960	4	16	0.735	0.247**
iv)	Width of longest leaf	0.370	0.858	4	16	0.093	0.054NS
v)	Roots per plant	1.840	11.760	4	16	0.460	0.735*
vi)	Length of longest leaf	1.640	1.860	4	16	0.410	0.116*
b) After 12 weeks of planting out							
i)	Plant height	0.774	0.602	4	16	0.193	0.038*
ii)	Leaves per plant	6.960	6.240	4	16	1.740	0.390*
iii)	Length of longest leaf	4.040	3.260	4	16	1.010	0.204*
iv)	Width of longest leaf	0.786	0.902	4	16	0.197	0.056*
v)	Roots per plant	5.760	5.840	4	16	1.440	0.365*
vi)	Length of longest leaf	2.602	3.654	4	16	0.651	0.228*
45. Growth performance of planted out plantlets							
i)	Plant height	1.767	0.243	5	20	0.353	0.012*
ii)	Leaves per plant	23.867	18.133	5	20	4.773	0.907*
iii)	Length of longest leaf	44.475	10.733	5	20	8.895	0.537*
iv)	Width of longest leaf	7.451	1.721	5	20	1.490	0.086*
v)	Roots per plant	36.700	33.800	5	20	7.340	1.690*
vi)	Length of longest root	619.267	26.733	5	20	123.853	1.337*

* Significant at 1 per cent level of probability
** Significant at 5 per cent level of probability

MICROPROPAGATION OF *Phalaenopsis*

By

JYOTHI BHASKAR

ABSTRACT OF A THESIS

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Kerala Agricultural University

DEPARTMENT OF POMOLOGY AND FLORICULTURE
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR - 680 654

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ABSTRACT

Investigations were carried out at the Plant Tissue Culture Laboratory attached to the All India Co-ordinated Floriculture Improvement Project (AICFIP), College of Horticulture, Vellanikkara, during 1993-1996 to standardise the micropropagation technique in *Phalaenopsis*.

Out of the different explants tried, response was shown by inflorescence stalk node, inflorescence stalk tip and pollinia collected from the field grown plants and apical bud, shoot node, basal portion, leaf and root of plantlets grown *in vitro*.

Maximum survival (40%) of nodal explants was recorded at the sterilant combination involving mercuric chloride (0.01%) for 30 min., streptomycin + pencillin (0.01%) for 90 min., followed by final sterilization using mercuric chloride (0.10%) for 10 min. For flower bud, the combination involving emisan (1.00%) for 30 min. followed by alcohol (50%) for 1 min. recorded the maximum survival percentage (55).

The 1/2 MS liquid medium containing BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm + CW 15 per cent recorded the minimum number of days for nodal swelling and bud development (6 and 14 days, respectively). Basal portion was found to be the best explant with respect to rate of increase in shoot number (8.00) and leaf number (7.67), followed by shoot node. The latter recorded the highest root number (7.67) at the end of 12 weeks of culturing. As to the position of explant, highest increase in the length of the buds was recorded for first node, followed by second, third and fourth.

Full strength MS and KC media were far inferior to 1/4 MS, 1/2 MS, 3/4 MS and VW media, for culturing. Maximum number of shoots (5.00) and leaves (7.67) were recorded for 1/4 MS and 1/2 MS media after 8 weeks of culturing.

Physical state of the medium, viz., liquid and semi-solid did not show any significant difference.

Sucrose at 1.5 per cent level recorded the maximum number of shoots and leaves after 8 weeks of culturing. Thiamine-HCl increased the shoot and leaf number at 20 ppm level at the end of 8 weeks, whereas the presence did not favour the production of roots.

The time taken for callusing in pollinia was minimum (2.0 days) at BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm in 1/2 MS medium containing 3 per cent sucrose. When 90 day old pod was used, protocorm formation was observed in 1/2 MS medium containing BA 10 ppm + NAA 1 ppm and KIN 5 ppm + 2,4-D 2 ppm.

When the effect of NAA was considered root production was increased at the combination NAA 5 ppm + BA 10 ppm + adenine 10 ppm.

With regard to the effect of BA, that at 25 ppm in combination with adenine 10 ppm + NAA 1 ppm recorded maximum number of shoots and leaves.

Shoot and leaf number was maximum at 1/4 MS medium containing BA 20 ppm + 2,4-D 2.5 ppm, whereas root production was maximum at BA 20 ppm + 2,4-D 5 ppm. As to the combined effect of BA, NAA and 2,4-D in 1/4 MS

medium, the combination BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm recorded the maximum number of shoots and leaves.

Different levels of KIN, was found to have no significant influence on the production of shoots, leaves and roots. When the combined effect of KIN, NAA and 2,4-D was considered 1/4 MS medium containing KIN 20 ppm + NAA 2 ppm + 2,4-D 2 ppm recorded maximum number of shoots and leaves.

The apical bud did not show any multiple shoot production during the 12 weeks culture period as influenced by coconut water, whereas first node at CW 10 per cent, shoot node at CW 15 per cent, and basal portion at CW 25 per cent recorded the highest number of shoots. Maximum number of leaves was produced by apical bud at CW 20 per cent, first node and shoot node at CW 10 per cent, and basal portion at CW 25 per cent.

Both tender and mature coconut water were equally effective. Fresh and upto 6 days old coconut water could also be used with similar effect.

Peptone at 1000 ppm was found to influence favourably the induction of multiple shoots from *in vitro* shoots.

Culturing the nodal explants in liquid media with filter paper bridge or keeping in the dark were found to reduce phenolic blackening. With the increase in the concentration of antioxidants, there was a proportionate reduction in media discolouration.

Activated charcoal and triadimefon added in the media were found to influence the root production from shoots. Length of the root was maximum at triadimefon 20 ppm.

Sucrose at 1.5 per cent level recorded the minimum number of days for PLB development and the maximum number of PLB's developed. Thiamine-HCl, coconut water, tomato juice and peptone did not significantly influence the time taken for PLB formation, but favoured the number of PLB's developed.

With regard to PLB formation from shoot node, 1/2 MS medium containing BA 5 ppm + NAA 2 ppm + 2,4-D 2 ppm recorded the minimum number of days for PLB development and maximum number of PLB's. When PLB formation from *in vitro* leaf was considered, cent per cent of the leaf cultures developed PLB's at the combination BA 25 ppm + adenine 10 ppm + NAA 1 ppm and the time taken for PLB formation was minimum.

Cent per cent of the cultures developed PLB's from *in vitro* roots at BA 25 ppm + adenine 10 ppm + NAA 1 ppm and the time taken for PLB formation was minimum. Further growth of PLB's and plantlet development was the best in 1/4 MS medium containing BA 15 ppm + NAA 1 ppm followed by adenine 8 ppm + BA 16 ppm. Regarding the effect of coconut water, PLB growth at CW 15 per cent and plantlet development at CW 25 per cent recorded the best results.

Light favoured plantlet development, multiple shoot formation and PLB formation from shoot node and *in vitro* root, whereas dark period favoured early development of PLB's from *in vitro* leaf, callusing and PLB proliferation.

Healthy, large and robust plants were produced when plantlets were grown in 250 ml conical flask, followed by large test tubes.

When the plantlets grown previously in medium containing triadimefon 20 ppm were hardened by spreading over sterile charcoal pieces for two weeks, planted in coconut husk and were hung in the orchidarium with high humidity, cent per cent survival was recorded even after 8 weeks of planting out.

The nutrient solution 30:10:10 (0.50%) and 17:17:17 (0.10%) recorded the highest survival percentage after 12 weeks of planting out. The growth characters of the plants, viz., plant height, leaf number, leaf length and width, root number and root length were found to be maximum for the plants sprayed with 17:17:17 at 0.10 per cent level.

The survival percentage of plants showed a slight decrease with time and all the plant characters increased with time except the number of leaves.