CLONAL MULTIPLICATION AND ESTABLISHMENT OF *Dendrobium wardianum Warner* : SOME ASPECTS OF PHYSICO-CHEMICAL REQUIREMENTS, NITROGEN METABOLISM AND PRESERVATION

A Thesis Submitted for the Degree of

DOCTOR OF PHILOSOPHY

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CERTIFICATE

I certify that the thesis entitled "Clonal multiplication and establishment of Dendrobium wardianum Warner: Some aspects of physico-chemical requirements, nitrogen metabolism and preservation", submitted by Ms. Abha Sharma for the degree of Doctor of Philosophy in Botany of the North-Eastern Hill University, Shillong embodies the record of original investigation carried out by her under my supervision. She has been duly registered and the thesis presented is worthy of being considered for the award of the Ph.D. degree. This work has not been submitted for any degree of any other university.

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Dated December 22 , 1993
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Chapter 1

General Introduction

Orchidaceae, a highly sophisticated family of distinction is one of the largest and includes diverse groups of flowering plants. Constituting a group of prized ornamentals, orchids culminate in one of the evolutionary lines of monocots and are still in the process of active speciation. These are botanically very interesting for their floral complexities, free gene flow across the specific barriers, minute seeds with undifferentiated embryos, suppressed endosperm formation and dependence on a suitable mycorrhizal association for germination. The uniqueness of the family is also reflected in its peculiar pollination contrivances and wide natural hybridization. Acclaimed all over the world for the bewitching beauty of its flowers, the family orchidaceae comprises of 20,000 - 23,000 species spread over 725 genera (Atwood, 1986). Besides, the exertions of man over the past 120 years have produced a parallel population of more than an equal number of artificial hybrids.

Orchids enjoy wide distribution, found in areas from sea-

level to snowline and are reported from all the continents except Antarctica. In India, the estimates of the number of orchid species vary from about 800-1300, but a fairly critical appraisal (Jain and Mehrotra, 1984) shows the presence of about 925 species. North-East India, including North-East Himalayas and Khasi and Jaintia Hills, forms the richest geographical region for orchids. About 700 species are reported to occur in terrestrial and/or epiphytic forms in the region (Hegde, 1984).

Phytogeographical studies have revealed that North-East region harbours about 50% of the total Indian flora (about 10,000 species). However, it has been recently observed that increasing biotic influences including socio-economic development and unrestrained commercial exploitation of forest wealth have threatened the survival of the genetic resources amounting to a great loss of natural heritage. The area of cultivation, distributional range/spread of such plants is shrinking in native habitats. On the other hand, for a number of taxa of this region, potentialities and desirable attributes are as yet not fully known and exploited. Therefore, the preservation of plant genetic resources of unknown promise as well as threatened types for posterity do need priority.

Orchids are perennial plants which grow either as epiphytes (growing on trees) , lithophytes (growing on rocks), terrestrials (growing on the ground) or as saprophytes (leaf-less forms growing on decaying organic matter) and bloom annually. Depending on their mode of growth, orchids could be monopodial where a

single stem continues to grow from its apex year after year, producing new leaves continuously; sympodial, where each new shoot springing from the rhizomes of the previous growth is complete in itself and terminates in a potential inflorescence, or diapodials, where growths are built up in a similar way to the sympodials, but lack the characteristic feature, the pseudobulbs (swollen stems, providing the plant with a means of water storage).

In natural conditions, majority of the orchid flowers are not pollinated and their ovules not fertilized. As a consequence, capsules are rarely formed. Orchid seeds are extremely minute (0.3-2.0 mm, Stoutamire, 1964), and usually undifferentiated with the endosperm underdeveloped or completely lacking (Henrich *et al.*, 1981). This insufficient nutrition results in the inefficiency of the orchid seeds to attain the autotrophic stage of development which could be provided under natural conditions by a mycorrhizal association. As such, less than 5% of orchid seeds are able to germinate in nature (Rao, 1977).

The pioneering work of Bernard (1909) laid the foundation of *in vitro* symbiotic cultures of orchids. Later Knudson (1922) showed that germination of orchid seeds was possible *in vitro* without fungal association by providing balanced organic and inorganic nutrition for the developing embryos. However, propagation of plants by seeds has a number of limitations *viz.*:

a) viability of orchid seeds is remarkably less,

b) seeds are available only for a limited period and

c) the regenerants are heterozygous.

While applying existing tissue culture techniques (White, 1951) to the study of virus transmission in Cymbidium, Morel (1960) noted that protocorm-like-bodies (plbs) developed around shoot tips cultured in vitro which eventually produced roots and shoots. Such plbs when cut into sections and transferred to new medium, multiplied in number. This process of protocorm multiplication could be repeated indefinitely and large tissue stocks of any one clone could be obtained within a relatively short period.

Clonal propagation, now a fairly common practice in orchid culture is particularly important as orchid genotypes are heterozygous. Furthermore, asexual propagation is essential for plants which may be completely sterile. Normally, members of a clone have identical genomes thereby exhibiting true-to-type characteristics. Since the initial publication (Morel, 1960), this technique and its modifications (Wimber, 1963; Kim et al., 1970; Intuwong and Sagawa, 1973; Lay, 1978; Sagawa and Kunisaki, 1982; Kukulczanka and Wojciechowska, 1983; Homma and Asahira, 1985; Sanchez, 1988; Kraus and Monteiro, 1989; Goh and Wong, 1990; Vij and Pathak, 1990; Shimasaki and Uemoto, 1991) have become important in the mass propagation of desirable varieties at rates which were undreamt of earlier.

Unlike other plant families, differences in requirements for propagation in vitro exist among the diverse genera, species and hybrids of orchidaceae. Nutrient formulations and steps that are

satisfactory for one may not be applicable to another (Huang, 1988). Growth as well as differentiation can be controlled by various media components including mineral nutrition. Several plant tissue and cell culture media are in use including formulations devised by Murashige and Skoog (1962), White (1963) and Gamborg *et al*. (1968) besides the more commonly used orchid culture medium of Vacin and Went (1949). Composition and components of culture media have been investigated and reviewed by various workers (Gamborg *et al*., 1976; Huang and Murashige, 1977). In the last three decades, many reports on the growth and development of various orchids as affected by different defined and undefined media have been made (Rao and Avadhani, 1963; Raghavan, 1964; Raghavan and Torrey, 1964; Arditti, 1966; Zeigler *et al*., 1967; Fonnesbech, 1972a, b; Harvais, 1973; Ernst, 1975; Mead and Bulard, 1979; Henrich *et al*., 1981; Krishna Mohan and Jorapur, 1984; Amaki and Higuchi, 1989). Pierik *et al*. (1988) have brought out a detailed report on the germination and further growth of *Paphiopedilum*, a genera considered to be difficult to propagate *in vitro*.

Besides nutritional requirements, other physico-chemical factors have been reported to influence the physiology and development of plants in culture. Orchids vary considerably in their requirements for light. Though seed germination can take place in light or total darkness, depending on the taxa involved, further development of seedling does require illumination (Stoutamire, 1974; Arditti, 1979). The effect of light, both

quantitative and qualitative including photoperiod on orchid seed germination and growth has been studied (Withner, 1959; Zeigler et al., 1967; Ueda and Torikata, 1972; Ernst, 1976; Hasegawa et al., 1978; Arditti et al., 1982; Zeigler et al., 1985; Pierik et al., 1988). Considerable differences exist in the light intensities used for orchid tissue culture. Light intensity has been shown to affect the type of growth in culture and higher light intensities have been reported to improve the survival of ornamental plants transferred to soil (Hughes, 1981). Photoperiod requirement of orchids has been found to vary from none at all (i.e. complete darkness, Morel, 1971) to continuous illumination (Wimber, 1963) ranging from 400 - 5000 lux (Werckmeister, 1970a,b,; 1971).

Although little information is available about the influence of pH of a culture medium on in vitro morphogenesis, the growth promoting properties and the selectivity of the culture medium are pH dependent (Sarma et al., 1990). Most tissue culture media are poorly buffered, and pH fluctuations that occur during culture may be detrimental to the growth and development. The majority of efficient nutrient solutions have pH values between 5.0-6.0 and these limits are associated with healthy growth of many plants. Though several studies have been conducted to investigate the effect of different physico-chemical factors on orchid seedling growth and development (Arditti et al., 1981), not much is known about the acidity effect on their asymbiotic cultures. The pH lower than 4.0 and higher than 8.0 has been

found to be inhibitory for seedling growth of orchids (Arditti et al., 1982).

Early studies on the growth of plant cells in cultures indicated that the optimum temperature range was between 26 and 28°C, but that species requirements differed considerably (Carew and Staba, 1965; Puchan and Martin, 1971). In practice, plant cultures are grown around 25°C and few studies investigating specific temperature requirements for various plant species are available (Hughes, 1981). Temperature between 20-25°C has been found to be suitable for the growth and development of most of the orchid species (Harvais, 1973; Stoutamire, 1974; Tanaka and Sakanishi, 1978; Arditti, 1982; van Waes and Debergh, 1986).

Results of experiments with auxins, cytokinins and gibberellins are inconsistent and consequently inconclusive with the responses on the germination and seedling growth of orchids differing from species to species (Wither, 1959, 1974; Arditti, 1977, 1982; Hadley and Harvais, 1968; Ueda and Torikata, 1969; Goh, 1970; Hadley, 1970; Fonnesbech, 1972a, b; Pierik and Steegmans, 1972; Harvais, 1973; Strauss and Reisinger, 1976; Tamanaha et al., 1979; Kusumoto, 1980; Nakamura, 1982; Sharma and Tandon, 1986; van Waes and Debergh, 1986; Huang, 1988). In most cases, auxins, mostly indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α-naphthaleneacetic acid (NAA) enhanced germination and/or seedling growth. Inhibition was reported in some cases (Arditti and Ernst, 1984). Auxin : cytokinin ratio may be important for growth in some instances (Hadley and

Harvais, 1968; Harvais, 1972; Fonnesbech, 1972a, b) and inhibitory (Kano, 1965) in others. High concentrations of 6-banzylaminopurine (BAP) resulted in formation of numerous plbs in Cattleya (Pierik and Steegmans, 1972), lily (Kawarabayashi and Asahira, 1988) and Phalaenopsis (Wang, 1989). Gibberellic acid (GA_3) has been found to inhibit seedling growth in Bletilla striata, Dendrobium (Kano, 1965) and Galeola (Nakamura, 1982). On the other hand, seedling growth in Cattleya and Cymbidium (Blowers, 1958; Hirsh, 1959; Harvais, 1982) was stimulated with GA_3 application. The suitability of some plant growth regulators over others and their inhibitory action in some thus suggest specific requirements of a particular system (Liu et al., 1988).

Nitrogen assimilation, both ammonium (NH_4) and nitrate (NO_3) ions, has an important role in plant growth and differentiation. Nitrogen is required for growth, production of proteins and formation of chlorophyll and cytochrome. The demand for nitrogen is closely related to the amount of growth and differentiation (Kramer and Kozlowski, 1979) and nitrogen deficiency is the most common limitation on growth after water stress. Of all the mineral nutrients, the form of nitrogen used is shown to affect the chemical composition of plant tissue as well as pattern of plant growth and differentiation (Ozias-Akins and Vasil, 1985). Number of studies have been conducted on the effect of different inorganic and organic nitrogen sources on orchid seed germination, growth and development (Withner, 1959; Raghavan, 1964, 1976; Raghavan and Torrey, 1964; Mitra, 1971; Mead and

Bulard, 1975, 1979; Ichihashi and Yamashita, 1977; Nakamura, 1982; van Waes and Debergh, 1986). Most of the orchid species are reported to be incapable of utilizing nitrate during the early stages of the growth and development and their ability to utilize nitrate parallels the appearence of nitrate reductase in plants (Raghavan and Torrey, 1964). Several workers have reported ammonium nitrate to be the most suitable form of nitrogen for the development of orchids in culture (Arditti and Ernst, 1984). Results with urea are contradictory even with species in the same genus. A culture medium for orchids formulated by Thompson (1977), however, contains only urea and ammonia nitrogen. Individual amino acids or mixtures of amino acids do not stimulate growth in orchids when added to a medium already supplying inorganic nitrogen. Some of the amino acids were observed to replace ammonium nitrate in orchid culture (Raghavan and Torrey, 1964; Mead and Bulard, 1975, 1979; Raghavan, 1976; van Waes and Debergh, 1986). However, results obtained from experiments with amino acids have been extremely variable (Arditti, 1967a, b, 1979, 1982; Nakamura, 1982).

Several enzymes are secreted by orchid seedlings into the culture medium and many additional enzymes probably are produced though very few have been studied (Arditti and Ernst, 1984). Alvarez (1968) and Alvarez and King (1969) found the peroxidase activity to be the exact reciprocal of IAA production by Vanda seedlings. Kumaria et al. (1990) studied the activities of some oxidative enzymes in Cymbidium giganteum Wall. as influenced by

different growth regulators. They have reported the specific activity of both peroxidase and polyphenoloxidase in the auxin and cytokinin treated protocorms to be slightly suppressed in comparision to the untreated controls. Varner and Ho (1977) dealt with the physiological and biochemical aspects by treating the best known response of plant hormones with regard to control of enzyme activity. It is accepted that changes in enzyme levels cause developmental changes and growth regulators bring about a transient change in the enzyme activity (Varner and Ho, 1977; Letham et al., 1978; Moore, 1980; Kumaria et al., 1990). Relations between growth regulators and nitrogen metabolism have been reported to be reciprocal. Not only do these hormones control certain phases of protein synthesis and degradation, but two of the three main classes of hormones, the auxins and cytokinins are themselves N-containing compounds whose production is invariably linked with the nitrogen metabolism of the plant (Luckwill, 1968). Though nitrogen assimilation in plants has been studied to a wide extent (Jackson et al., 1986), there is very little information on uptake, transport and storage of nitrogen in orchids (Hew et al., 1993).

Indiscriminate exploitation of natural resources, destruction of natural habitats and other unwarranted human activities have resulted in the deterioration of natural ecosystem. North-East India with its diverse topography, altitude, climate and rainfall plays a vital role in the occurrence of diverse orchid species, however, majority of

orchids of this region are faced with threats of extinction not only due to habitat destruction through deforestation and shifting agriculture (locally termed as 'jhum') which involves clearing up of vegetation, but also due to overexploitation for their ornamental value (Hore and Sharma, 1988). A large number of orchid species (e.g. Paphiopedilum wardii, Dendrobium wardianum, Pleione lagevieria, Coelogyne assamica etc.) have reached such a critical level that there is an urgent need for their preservation. D. wardianum Warner, a deciduous long-stemmed epiphyte, is found in isolated pockets of North-East India and Burma. A dendrobe of delicate, fragrant beauty with an exceptionally long period of blooming inflorescence of 29 days, it is in great demand in the South-East Asian cut flower industry. Once found in reasonable numbers, this splendid orchid has become endangered and now, is scarcely found in the wild (Kataki et al., 1984). Preservation of such endangered/rare plant species in vitro can be carried out by their rapid mass multiplication using tissue culture techniques for reintroduction into the wild and by preserving the germplasm using slow growth approaches (limiting the multiplication rate) or by cryo-exposure (arresting the metabolic activities). In vitro conservation refers to maintenance of germplasm in a relatively stable form under more or less defined nutrient conditions in an artificial environment (Withers, 1987). The major aim in developing in vitro storage methods is to reduce the frequent demands of subculturing and preserving the unique genetic constitution of the germplasm.

Freezing at liquid nitrogen (LN_2) temperature tends to suppress cell division, arrests growth and retains the cells in metabolically inactive state. The suspended animation prevents the cells from ageing and provides indefinite life-span with no genetic change. However, the technique is not yet applicable to many plant species. Hence, shoot cultures of many plant species have been stored under conditions in which growth is slowed down by use of a reduced culture temperature or by the application of osmotica or growth retardants (Mix, 1982, 1985; Monette, 1986; Staritsky et al., 1986; Love et al., 1987; Roca, 1990; Schoofs, 1990). Besides, mineral oil overlay (Crane and Hughes, 1990), reduced oxygen tension (Bridgen and Staby, 1981; Engelmann, 1990) and defoliation of shoots (Withers, 1987) have also been used for slow growth storage. In recent years, artificial seeds, consisting of somatic embryos enclosed in a protective coating, have been proposed as a low-cost, high volume propagation system (Redenbaugh, 1990). The inherent advantages of artificial seeds are the production of many somatic embryos and the use of conventional seed handling techniques for embryo delivery. Besides, artificial seeds can be stored for a considerable period at low temperature or by treating them with growth retardants. Storage of alginate-encapsulated loblolly pine and Norway spruce somatic embryos have been reported by Gupta and Durzan (1986, 1987). Also, inhibited germination of alginate-encapsulated alfa-alfa somatic embryos for one week at $4^{o}C$ was reported by Redenbaugh et al. (1986). Fujii et al. (1989) arrested the

germination of encapsulated alfa-alfa somatic embryos by treating them with abscissic acid (ABA), thus attaining maturation of the plants before transferring them to greenhouse conditions thereby enhancing the survival rate. Research on artificial seeds has increased significantly and various reports have been made (Kitto and Janick, 1985; Bapat et al., 1987; Mathur et al., 1989; Senaratna et al., 1990; Fernandes et al., 1992), however, the germplasm conservation reports in orchids remain scanty.

Plantlets cultured in vitro wilt rapidly on transfer to normal green house or field conditions. Poor water uptake and excessive water loss (Grout and Aston, 1977) may lead to high rates of mortality unless plantlets are acclimatized by gradual stages to reduced humidity and increased light intensity (George and Sherrington, 1984). The problems of poor water relations are compounded by damage to shoots and roots during transplantation (Debergh and Maene, 1981). Thus, the establishment and healthy growth of in vitro cultured plants in the glass house require suitable conditions. Different potting mixtures, containers and composts influence the growth of orchids extentively and differ from genera to genera (Bose and Bhattacharjee, 1980; Stewart, 1988; Talukdar et al., 1988; Yadav et al., 1988; Cribb, 1990; Robbins and Bell, 1990). Water retaining capacity of sphagnum and osmunda moss makes them suitable for the initial establishment of the orchid plantlets. Addition of manure and fertilizers is considered beneficial and the amount as well as the type varies from one species to the other.

The present work on *D. wardianum* Warner was undertaken with the following main objectives :

1) development of a feasible protocol for the mass propagation and preservation,
2) study of enzymatic activities related to nitrogen assimilation and,
3) hardening and establishment of regenerants.

Chapter 2

Effect of nutrient media on protocorm-like-body differentiation, growth and development of regenerants

New vistas in propagation of orchids through tissue culture techniques were opened after Morel's observation of green, globular plb formation around the shoot tips of Cymbidium cultured in vitro (Morel, 1960, 1964). These plbs when cut into smaller sections and transferred to fresh nutrient medium, multiplied and gave rise to secondary and tertiary plbs. However, on leaving undisturbed, these developed into complete plantlets with roots and shoots. This technique of meristem culture has been used since, by many workers for virus elimination and production of asexual plantlets on a large scale (Arditti, 1977). Of the numerous factors affecting the successful induction of morphogenesis in plant tissue cultures, medium composition is one of the most important. Following Knudson's discovery (1922) that orchids could be grown on a medium supplemented with organic and inorganic nutrients, a number of media for tissue culture of orchids have been used viz., Vacin and Went (1949), Murashige and

Skoog (1962), White (1963), Nitsch and Nitsch (1969) and Mitra *et al*. (1976) etc. In general, plant tissue culture media consist of mineral salts, a carbon and energy source, vitamins and growth regulators, however, other organic compounds have often been included (Ozias-Akins and Vasil, 1985). The disaccharide sucrose at a concentration of 2-3% is the most commonly used carbohydrate in plant tissue culture media. Homes and Vansveren-Van Espen (1973) while working on *Cymbidium* reported an enhancement in plb proliferation by supraoptimal levels of sucrose, and an increased organogenesis at suboptimal concentrations. A wide variety of complex natural extracts like yeast extract, coconut water, peptone, casein hydrolysate, fruit and vegetable homogenates, honey, fish emulsion, beef extracts and even silkworm pupae and Malayasian beer have been used to supplement the orchid culture media (Arditti and Ernst, 1984). While the beneficial effects of complex natural extracts and liquid endosperms are more pronounced in low salt media due to the contribution of inorganic as well as organic constituents, their primary role in the high salt media is the supplementation of carbohydrates, plant growth regulators, vitamins and amino acids (Ozias-Akins and Vasil, 1985).

Growth and development of plants *in vitro* is largely due to the uptake of nutrients, including carbohydrates from the medium. Since plants are likely to differ in their responses to nutrients when grown *in vitro*, there is a clear need to understand the

optimal nutrient requirements which would lead to increased growth rates and would also enable plant morphogenesis to be directed more efficiently. A number of reports on the nutritional requirements for *in vitro* growth and development of orchids have been made and effects of media studied (Prasad and Mitra, 1975; Stimart and Ascher, 1981; Arditti, 1982; Kukulczanka *et al.*, 1987; Pierik *et al.*, 1988; Soedjono, 1988; Tay *et al.*, 1988; Hew and Lim, 1989; Oyamada, 1989). However, nutrient and culture conditions found suitable for one orchid species may not be applicable to others (Arditti *et al.*, 1981).

D. wardianum, a deciduous, long stemmed epiphyte, is found growing wild on trees and rocks at 1200-1500 m in the hills bordering Assam and Burma (Mukherjee, 1983). A splendid orchid, it has a pendulous stem, about 60 cm long and somewhat thickened at the nodes. Flowers are produced from the nodes in twos or threes and are waxy glistening white with magenta or purple spots on the lips, on the sepals and petals. Lip is white with a golden disc and has two dark maroon blotches at the base and pale magenta tips. A dendrobe of delicate, fragrant beauty with an exceptionally long period of blooming inflorescence of 29 days, it is in great demand in the South-East Asian cut flower industry. Ruthless commercial exploitation and unplanned human activities have endangered the existence of this orchid and it is now scarcely found in the wild (Kataki *et al.*, 1984). The following chapter deals with the plb initiation and effect of different nutrient media on their morphogenetic responses.

Materials and Methods

Plb initiation

Shoot apices, excised from *D. wardianum* Warner plants growing *in vivo* in the Botanical Garden of North-Eastern Hill University (Fig. 1) were used for regeneration studies. The explants were first washed carefully under running tap water and then treated with 1% (v/v) cetavlon, [20% (w/v) cetrimide, an antiseptic and detergent] for 8-10 min. These were then washed with distilled water 4-5 times. Under aseptic conditions, the explants were surface-sterilized with 5% (v/v) NaOCl solution (0.2-0.3% available chlorine) for 20 min and finally washed 5 times with sterilized distilled water. The explants were cultured on Murashige and Skoog (MS, 1962) medium after trimming the exposed ends with a scalpel blade. The medium contained 0.8% (w/v) bacteriological agar (Qualigens, Glaxo), 3% (w/v) sucrose and was supplemented with different growth regulators viz., IAA, IBA, NAA, 2,4-dichlorophenoxyacetic acid (2,4-D), BAP and kinetin (KN) at a concentration range of 0-5 mg/l. The pH of the medium was adjusted to 5.8 before autoclaving at 121^{o}C for 20 min. The cultures were incubated at 24$\pm$2^{o}C at 16 h illumination (cool, flourescent light) of 2,000 lux. Ten replicates of each treatment were taken and the experiment was repeated twice.

Shoot regeneration

Plbs of uniform size were subcultured for 3 passages on MS hormone-free medium and then transferred to six different basal

media viz., Knudson C (KC, 1946), modified Vacin and Went (mod. VW + 15% coconut water, 1949), MS, Gamborg *et al*. (B_5, 1968), Nitsch and Nitsch (N & N, 1969) and Mitra *et al*. (1976) (Table 1) and the effects studied on the growth and development of the plantlets. The cultures were transferred to fresh medium every 4 weeks. The growth and development of the plbs were studied in terms of fresh and dry weight, number and length of shoot and root. Data were collected every 30 days for 3 months. Five replicates of each treatment were taken and the experiment was repeated twice.

Results

The effect of growth regulators on morphogenetic responses of shoot apices cultured on MS medium is presented in Table 2. Initiation of plbs was noticed in certain treatments about 3 weeks after inoculation. Green protuberances developed all over the shoot tip surface by the 6th week and in about 8 weeks, the whole explant turned into a mass of small, round, green plbs (Fig. 2). The highest plb formation (8-14 plbs per culture) was obtained in 60% cultures with BAP (2.5 mg/l) alone. An increase in BAP concentration, however, reduced the percentage response as well as number of plbs formed per culture. When cultured in medium with IAA and BAP in combination, shoot apices showed a lower morphogenetic response than BAP alone. Using different concentrations of IAA and BAP in combination, the maximum response was 40 with the shoot apices producing 7-10 plbs. A

Table 1 Inorganic and organic components of the media used (mg/l)

	N & N	KC	Mitra et al	B_5	VW	MS
Inorganic						
NH_4NO_3	720	-	-	-	-	1650
KNO_3	950	180	180	2500	52500	1900
$Ca(NO_3)_2.4H_2O$	-	200	200	-	-	-
$(NH_4)_2SO_4$	-	100	100	134	-	-
$MgSO_4.7H2O$	185	250	250	250	25000	370
$CaCl_2$	166	-	-	-	-	-
$CaCl_2.H_2O$	-	-	-	150	-	440
$Ca_3(PO_4)_2$	-	-	-	-	20000	-
KH_2PO_4	68	150	-	-	25000	170
$NaH_2PO_4.H_2O$	-	-	150	150	-	-
$FeSO_4.H_2O$	27.8	25	55.7	-	2100	27.8
$Na_2EDTA.2H_2O$	37.3	74.6	74.5	-	1570	37.3
Sesquestrene 330 Fe	-	-	-	28	-	-
$MnSO_4.H_2O$	-	0.075	-	10	6.8	-
$MnSO_4.4H_2O$	25	-	-	3	-	22.3
$MnCl_2.4H_2O$	-	-	0.4	-	-	-
H_3BO_3	25	6.2	0.6	3	-	6.2
$ZnSO_4.7H_2O$	10	-	0.05	2	-	8.6
$ZnCl_2$	-	3.9	3.9	-	-	-
$NaMoO_4.2H_2O$	0.25	0.25	0.05	0.25	-	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.05	0.025	-	0.025
KI	-	0.8	0.03	0.75	-	0.83
$CoCl_2.2H_2O$	-	0.025	-	-	-	-
$CoCl_2.6H_2O$	-	-	-	0.025	-	0.025
$Co(NO_3)_2.6H_2O$	-	-	0.05	-	-	-
Organic						
M-inositol	100	-	-	100	-	100
Nicotinic acid	5	-	1.25	1.0	-	0.5
Pyridoxine HCl	0.5	0.3	0.3	1.0	-	0.5
Thiamine HCl	0.5	0.3	0.3	1.0	-	0.1
Glycine	2.0	-	-	-	-	2.0
Folic acid	0.5	-	0.3	-	-	-
Biotin	0.05	-	0.05	-	-	-
Riboflavin	-	0.3	0.05	-	-	-
pH	5.5	5.2	5.5	5.5	5.2	5.8

Table 2 Effect of different concentrations of NAA+BAP and IAA+BAP on morphogenetic responses of cultured shoot apices*.

Growth regulator	Conc. (mg/l)	Morphogenetic responses (%)	Remarks
MS	0.0	-	-
MS+BAP	0.5	20	Pale green 4-7 plbs
	2.5	60	Green, globular 8-14 plbs
	5.0	40	Green, globular 7-10 plbs
MS+ IAA+BAP	0.5+0.0	-	-
	0.5+0.5	20	Single shoot formation
	0.5+2.5	30	Green, globular 7-10 plbs
	0.5+5.0	40	Green, globular 7-10 plbs
	2.5+0.0	-	-
	2.5+0.5	-	-
	2.5+2.5	-	-
	2.5+5.0	20	Explant remains green
	5.0+0.0	-	-
	5.0+0.5	-	-
	5.0+2.5	-	-
	5.0+5.0	-	-
MS + NAA+BAP	0.5+0.0	-	-
	0.5+0.5	40	Single shoot formation
	0.5+2.5	30	Green, globular 6-10 plbs
	0.5+5.0	40	Green, globular 6-8 plbs
	2.5+0.0	-	-
	2.5+0.5	20	Swelling of the explant
	2.5+2.5	-	-
	2.5+5.0	10	Pale green 3-4 plbs
	5.0+0.0	-	-
	5.0+0.5	-	-
	5.0+2.5	-	-
	5.0+5.0	-	-

* Data based on 10 replicates per treatment, collected after 8 weeks

\- No response

Fig. 1 Dendrobium wardianum Warner in bloom.

Fig. 2 Proliferation of plbs from shoot apices on MS + 2.5 mg/l BAP (after 8 weeks).

Fig.1

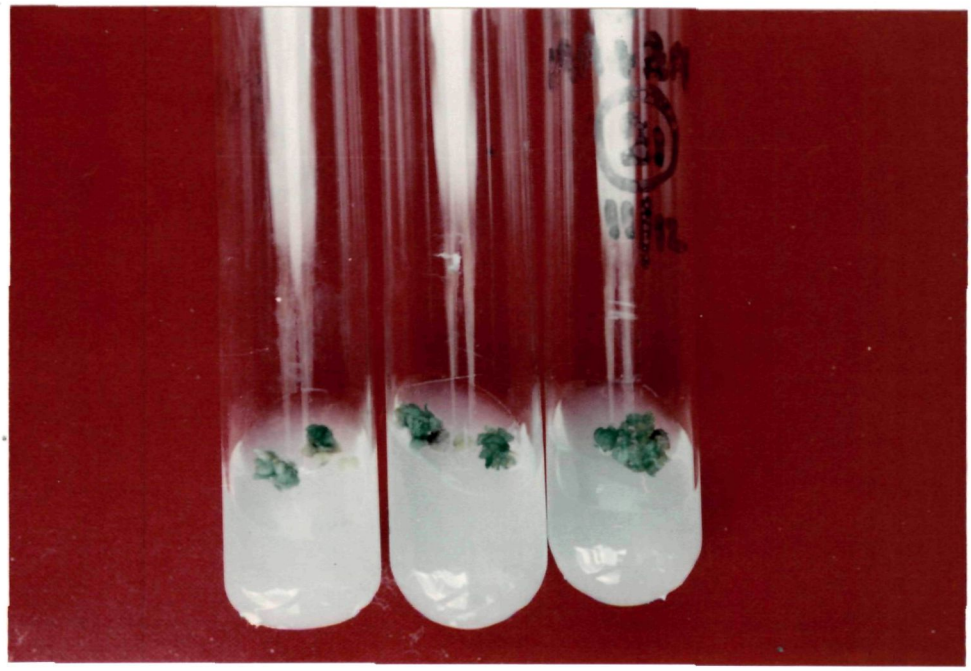


Fig.2

Fig. 4 Effect of different nutrient media [Nitsch & Nitsch (a), Knudson C (b), Mitra et al. (c), Gamborg et al.(d), mod. Vacin and Went (e), and Murashige and Skoog (f)] on growth and development of regenerants (after 90 days).

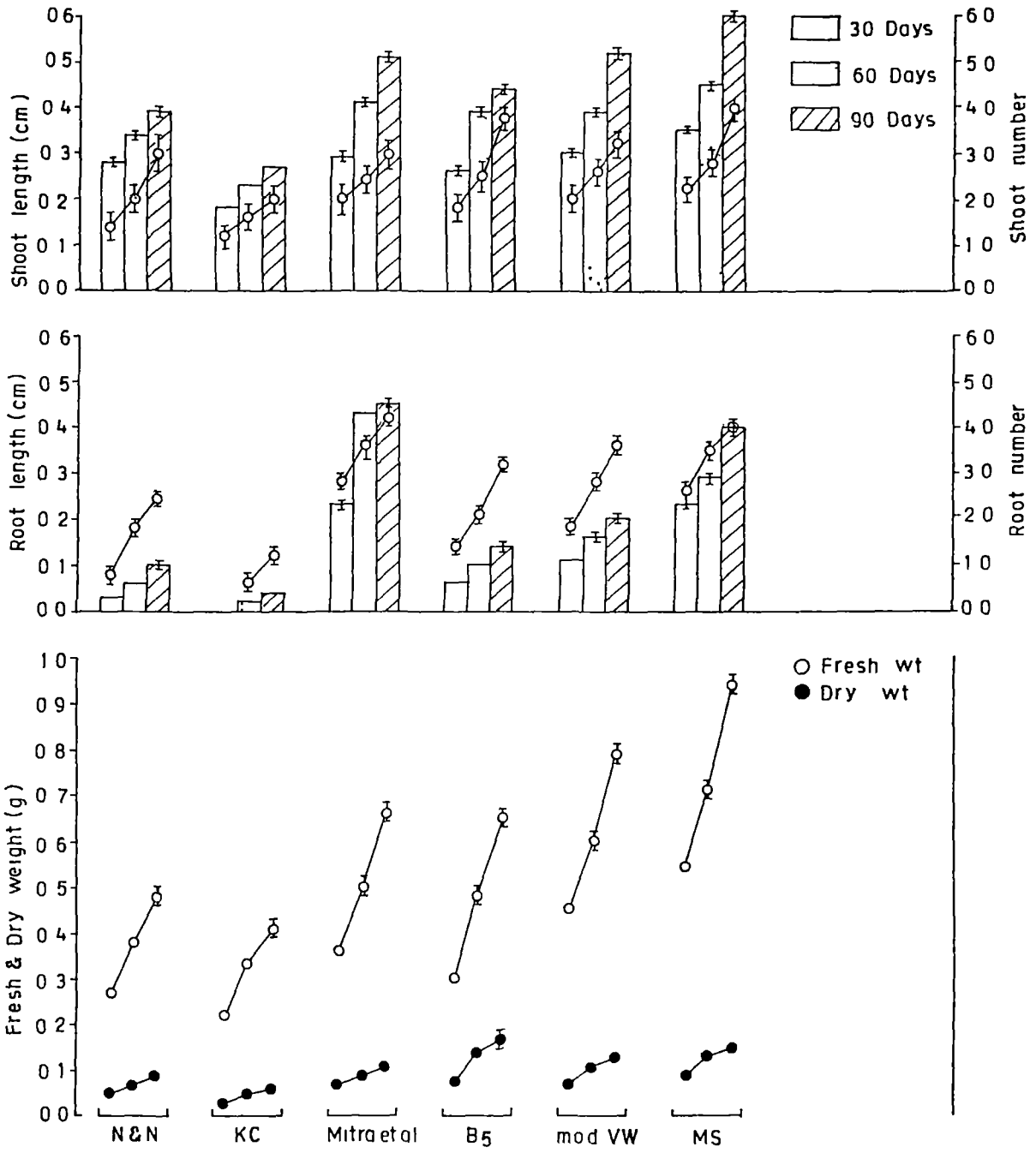

Shoot length (cm)
Shoot number
30 Days
60 Days
90 Days
Root length (cm)
Root number
Fresh & Dry weight (g)
Fresh wt
Dry wt
N&N
KC
Mitra et al
B5
mod VW
MS

Fig.3

Fig. 3 Effect of different nutrient media [Nitsch & Nitsch, Knudson C , Mitra et al., Gamborg et al., mod. Vacin and Went and Murashige and Skoog] on growth and development of regenerants. Bar and line represent shoot / root length and number respectively.

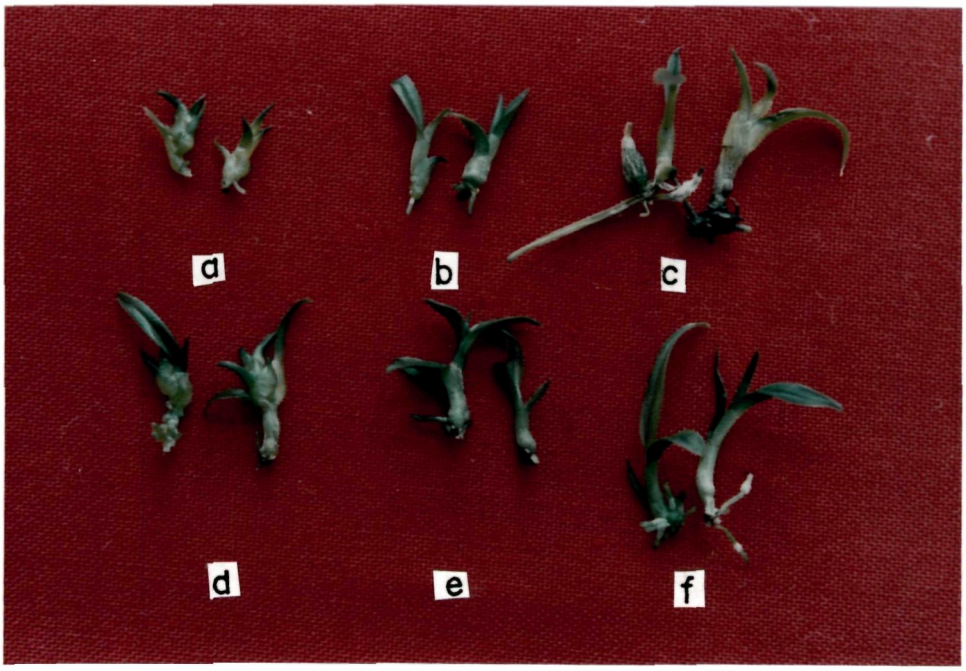


Fig.4

decline in plb formation was also observed using NAA and BAP in combination. With an increase in auxin (IAA/NAA) concentrations, there was a decrease either in percentage response or in number of plbs formed per culture. There was no morphogenetic response at 5.0 mg/l auxin in the medium.

Of the six nutrient media tested, optimum growth and development of D. wardianum plantlets resulted in MS medium followed by Mitra et al., mod. VW and B_5 (Fig. 3). Besides resulting in highest shoot number, shoot length and fresh weight, long, dark green leaves were also produced in MS medium (Fig. 4). Highest root number and length were, however, observed in plantlets growing in Mitra et al. medium. Plantlets performed poorly on N & N and KC media.

Discussion

Rapid propagation, production of phenotypically and genetically identical (Murashige, 1974) and virus-free (Boxus and Druart, 1986) plants has resulted in the wide application of meristem culture technique in orchidaceae (Arditti, 1977; Kawarabayashi and Asahira, 1988; Wang et al., 1989). Reports have been made reflecting morphogenetic responses of combinations of IAA+BAP in Vanilla (Duan and Hu, 1989), NAA+BAP in Phalaenopsis (Zimmer and Pieper, 1977), Nervilia tubers (Chow, 1986) and lily (Kawarabayashi and Asahira, 1989) and BAP alone in Cattleya (Pierik and Steegmans, 1972), Serapias (Pouchet et al., 1985) and Phalaenopsis (Wang, 1989). On the other hand, Widiastoety et al.

(1986) have reported an inhibitory effect of either NAA or BAP on plb formation from shoot tips in case of Aranda cv. Christine. Parallel to our report, BAP alone was found promotory for plb proliferation by Goh and Wong (1990) in their studies with Aranda 'Deborah'. Moreover, the need of BAP concentration to be equivalent or higher to that of auxin for development of plbs from Phalaenopsis inflorescence tips has been reported (Yoneda, 1986). The results in present study show a promotory effect with BAP alone. This morphogenetic potential of BAP does not rule out auxin involvement as there might be some endogenous auxin formation at the organ forming loci. Combinations with higher auxin concentrations found unfavourable for plb differentiation could be attributed to high endogenous auxin level in the explants manifesting apical dominance.

The successful establishment and growth of plant cells in vitro generally is determined by the nature of the explant and the composition of the nutrient medium (White, 1951). The development of the orchid plantlets in vitro is reported to require a balanced supply of organic and inorganic nutrients, growth regulators and other physical conditions (Arditti and Ernst, 1984; Zeigler et al., 1985). Moreover, mixture of vitamins in the medium is found to influence growth and development of certain orchid species positively (Arditti and Harrison, 1977). It has been reported that orchids are unable to absorb and utilize nitrates during the initial phase of germination and development (Raghavan, 1964). Form of nitrogen has been shown to

affect the chemical composition of plant tissues as well as plant growth and development and demand for nitrogen is reported to be closely related to the amount of growth (Kramer and Kozlowski, 1979). In the present studies, MS medium was found to favour optimum growth and development of plbs. Presence of high amount of ammonium nitrate in MS medium might have promoted the growth with NH_4^+ and NO_3^+ ions being readily assimilated during the initial and later stages of development respectively. Yam and Weatherhead (1988) also reported high contents of nutrient concentrations especially nitrogen and potassium in MS medium responsible for promotion of the plantlet regeneration in some Hong Kong orchids. Low concentration of the NH_4^+ ions in B_5, VW, N & N and KC media could have influenced growth otherwise. Potassium nitrate, present in very high concentrations in B_5 medium did not support growth which could be accounted for by inability of orchid protocorms to utilize NO_3^- in earlier stages (Raghavan and Torrey, 1964).

Chapter 3

Effect of physical factors on growth and development of regenerants

Physical factors contribute greatly to the plant growth *in vitro* and much progress has been made toward a better understanding of their roles in recent years (Read, 1992). However, the available details on their influence on the physiology and development of orchid plantlets are few and far between (Arditti, 1982). Light requirements which may be subdivided into photoperiod and light intensity, vary considerably in orchids (Arditti and Ernst, 1984; Chow, 1986). Morphogenetic requirements for light in an *in vitro* system may be satisfied by one or both of these factors. However, only a few studies are available where these factors are considered separately (Hughes, 1981). It is well known that light exerts a marked influence on plant growth independently of photosynthesis (Economou and Read, 1986). Besides, it is important for regulating photomorphogenetic processes in tissue cultures in terms of duration, intensity and spectral quality (Murashige, 1978). Orchids appear to require light for induction or improvement of shoot and/or root formation (Ueda and Torikata,

1972; Stoutamire, 1974; Pierik et al., 1988). Werckmeister (1971) working on Cymbidium observed that high light intensities were important for the development of shoots alone, but that darkness was necessary around the roots for the best results.

Early studies on the growth of plant tissues in culture indicated that the optimum temperature range was between 26 and 28°C in a number of cases but the requirements were found to differ considerably (Carew and Staba, 1965; Puchan and Martin, 1971). Though, a range of 32-35°C was found to be optimum for certain plants (Hughes, 1981), a temperature as low as 12°C was reported suitable for Streptocarpus (Appelgren and Hiede, 1972). The optimal temperature for growth and development of most orchid species is 20-25°C with the range extending from 6-40°C for seed germination (Arditti, 1967a,b; Mukherjee et al., 1974; Thompson, 1977; van Waes and Debergh, 1986; Lee et al., 1988). Tanaka and Sakanashi (1978) have reported influence of temperature on the morphogenic events in case of Phalaenopsis, with the flower stalk buds cultured at 25°C exhibiting position effects i.e., development of reproductive shoots from the upper nodes and vegetative shoots from the lower ones. At 28°C, however, all the buds were reported to develop into vegetative shoots irrespective of their positions.

Although little information is available about the influence of pH of a culture medium on in vitro morphogenesis, it is one of the important factors, as the growth promoting properties and the selectivity of the culture media are pH dependent. Changes in pH

in plant tissue culture systems have been reported by various workers (Eriksson, 1965; Butenko *et al.*, 1984; Skirvin *et al.*, 1986). Butenko *et al.* (1984) have attributed the media pH changes to the release of compounds from the plant material or uptake of particular nutrients such as ammonium, nitrate or phosphate ions. The pH values lower than 4.0 or higher than 8.0 have been found to be inhibitory for orchid growth *in vitro* (Arditti *et al.*, 1982). No generalizations regarding different physical factors, however, can be made as different orchids show different requirements for their optimal growth and development.

Materials and Methods

The plbs subcultured on MS hormone-free medium for 3 passages as described in Chapter II were transferred to fresh MS medium supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar without growth regulators. The cultures were then incubated in white, flourescent light at a range of 1,000 - 4,000 lux (16 hr photoperiod) and at 8 - 24 hr photoperiod (2,000 lux) at $24\pm2^{o}C$ to study the growth and development of *D. wardianum* regenerants *in vitro*. Besides, plbs were also maintained in complete darkness. The effect of temperature at light intensity 2,000 lux (16 hr photoperiod) was studied by incubating the cultures at a range from 15 - $35^{o}C$. The pH of the MS medium for the above experiment was adjusted to 5.8. To standardize the acidity level for optimum growth and development, the plbs were transferred to fresh MS media (as described above) having pH range of 4.5-7.5.

The cultures were incubated at 24±2°C under 16 hr photoperiod of 2,000 lux light intensity. Growth parameters studied were number and length of shoot and root and their fresh and dry weights. Data were collected every 30 days for 3 months. Five replicates were taken for each treatment and the experiments were repeated twice.

Results

Effect of light

i) Light intensity - Light was promotory in conversion of plbs to plantlets (Fig. 5). Growth and development of plantlets enhanced from 0 to 2,000 lux light intensity, declining thereafter (Fig. 7). Highest fresh and dry weight were recorded at 2,000 lux light intensity, however, extremes of 0 and 4,000 lux were observed to inhibit the growth of the regenerants.

ii) Photoperiod - The increase in photoperiods from 0 to 16 hr resulted in an increase in growth of regenerants with highest shoot, root number, length, fresh and dry weights observed in regenerants formed at 16 hr photoperiod (Fig. 6). Total darkness and continuous illumination were, however, found to be inhibitory (Fig. 8).

Effect of temperature

Growth of regenerants was observed to increase from 15 to 25°C

Fig. 5 Effect of different light intensities (0 - 4,000 lux) on growth and development of regenerants. Bar and line represent shoot/root length and number respectively.

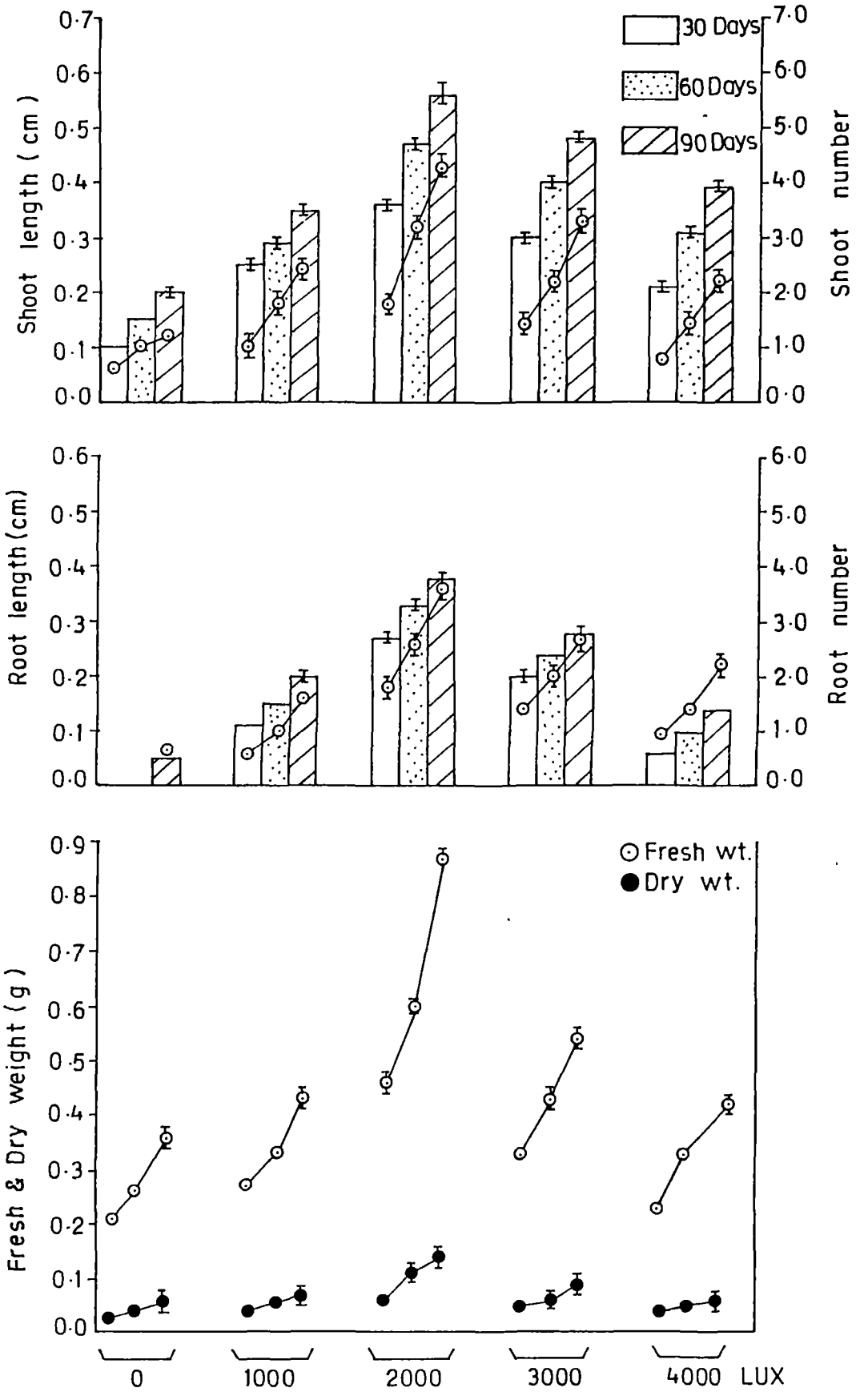

Shoot length (cm)
Shoot number
30 Days
60 Days
90 Days
Root length (cm)
Root number
Fresh & Dry weight (g)
Fresh wt.
Dry wt.
0
1000
2000
3000
4000
LUX

Fig.5

Fig. 6 Effect of different photoperiods (0 - 24 hr) on growth and development of regenerants. Bar and line represent shoot/root length and number respectively.

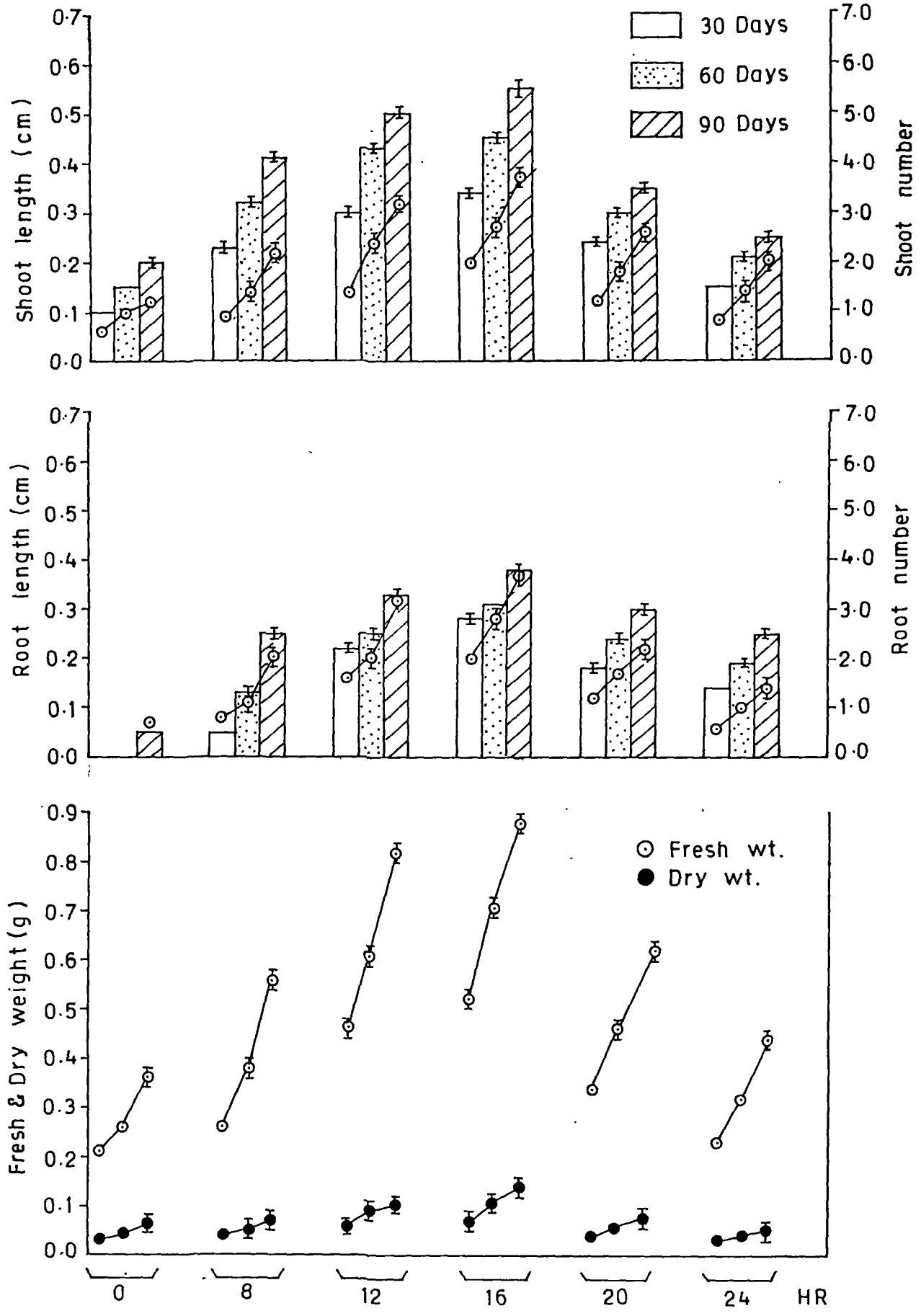
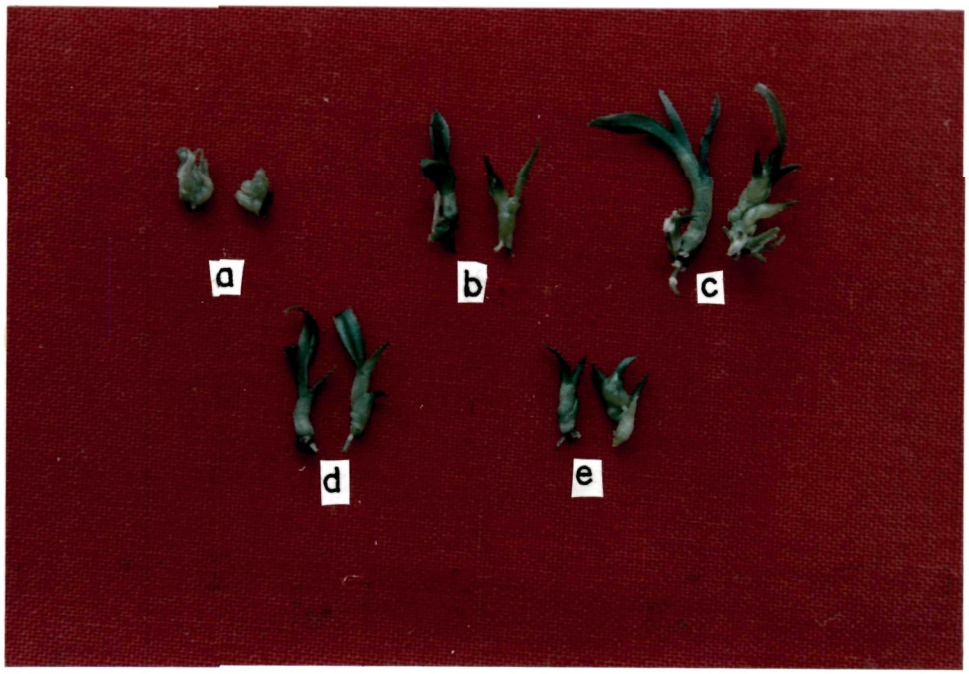

30 Days
60 Days
90 Days
Shoot length (cm)
Shoot number
Root length (cm)
Root number
Fresh wt.
Dry wt.
Fresh & Dry weight (g)
0
8
12
16
20
24
HR

Fig.6

Fig. 7 Regenerants developed at different light intensities [0 (a), 1,000 (b), 2,000 (c), 3,000 (d) and 4,000 (e) lux] (after 90 days).

Fig. 8 Regenerants developed at different photoperiods [0 (a), 8 (b), 12 (c), 16 (d), 20 (e) and 24 (f) hr.] (after 90 days).

Fig.7



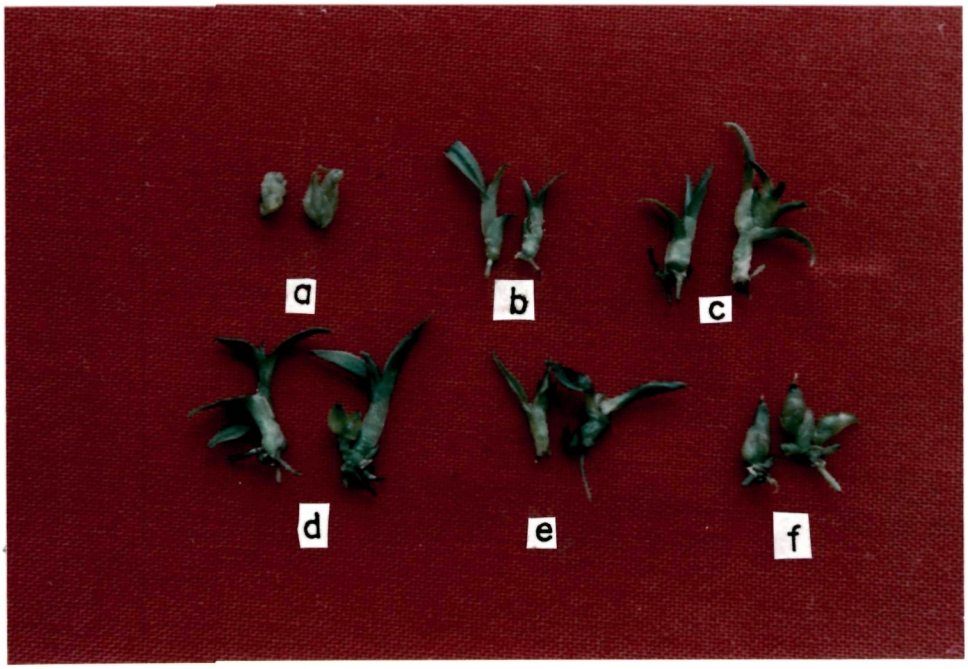


Fig.8

after which a decline in growth was recorded (Fig. 9). Shoot and root number and length, fresh and dry weights were recorded to be highest at 25°C. Poor growth and development of regenerants, however, was observed at higher temperatures (Fig. 11).

Effect of pH

Growth and development of regenerants varied markedly due to different pH levels of the medium (Fig. 10). Optimum growth was observed at pH 6.0 (Fig. 12). Fresh and dry weights were observed to be at their maximum at pH 6.0 with the development of green and healthy plantlets after 90 days of culture. Both pH 5.5 and 6.5 were also found suitable for the growth and development of the regenerants. Low pH levels (>5.0) were inhibitory. At pH 4.5, thick, stout, stunted bodies were developed. A pH range higher than 6.5 was also inhibitory for the growth and development of the plantlets .

Discussion

Environment exerts an important effect on the physiology and development of orchids (Arditti and Ernst, 1984). The influence of light intensity seems to be related to species, with some benefitting from high intensities, others responding to intermediate levels, while still others best cultured under low light or darkness (Thorpe and Murashige, 1970; Miller and Murashige, 1976; Papachatzi et al., 1981). Murashige (1974) reported that optimum light intensities for plant tissues in

Fig. 9 Effect of different temperatures (15 - 35^{o}C) on growth and development of regenerants. Bar and line represent shoot/root length and number respectively.

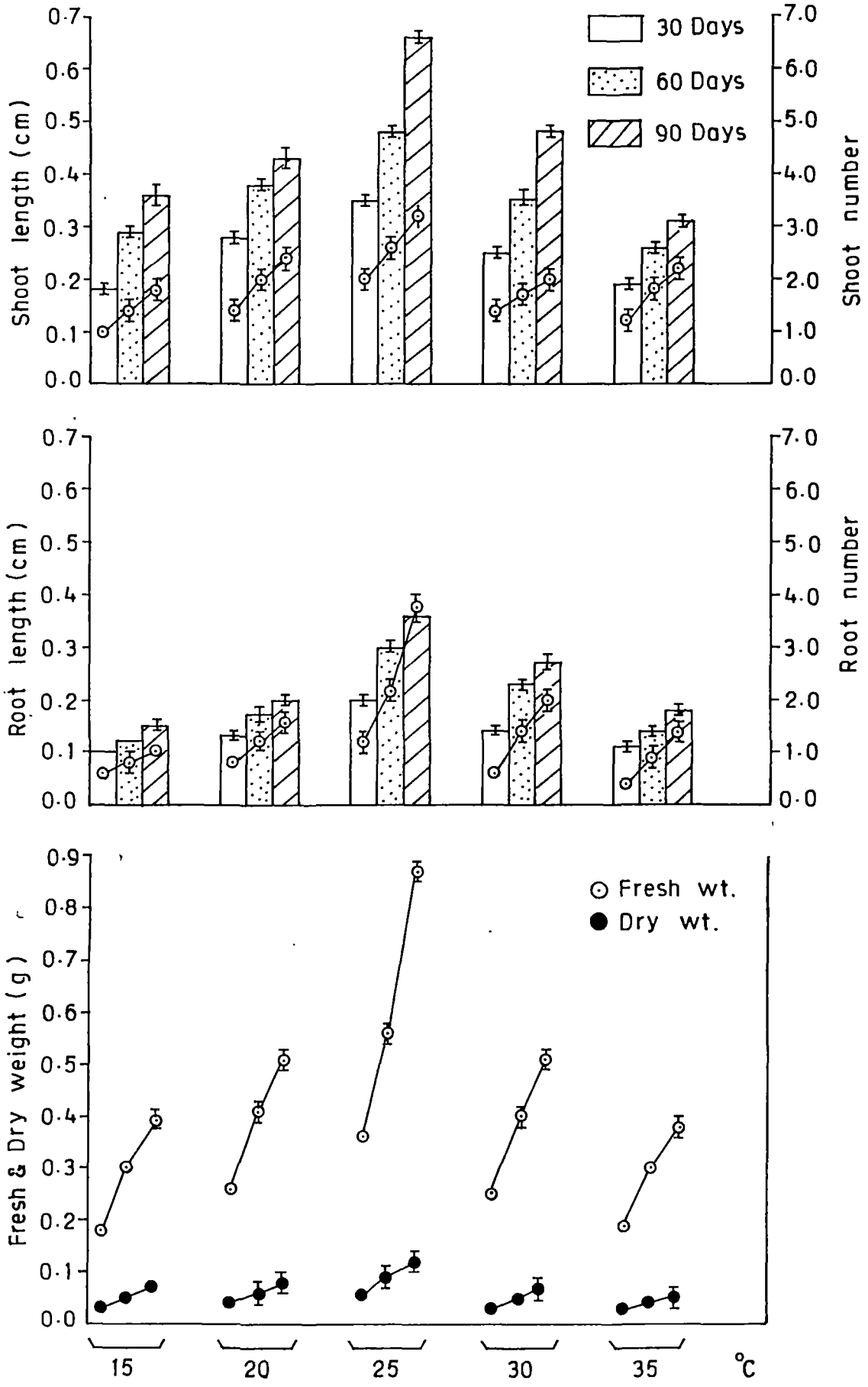

30 Days
60 Days
90 Days
Shoot length (cm)
Shoot number
Root length (cm)
Root number
Fresh & Dry weight (g)
Fresh wt.
Dry wt.
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
2.0
3.0
4.0
5.0
6.0
7.0
15
20
25
30
35
°C

Fig.9

Fig. 10 Effect of different pH values (4.5 - 7.5) on growth and development of regenerants. Bar and line represent shoot/root length and number respectively.

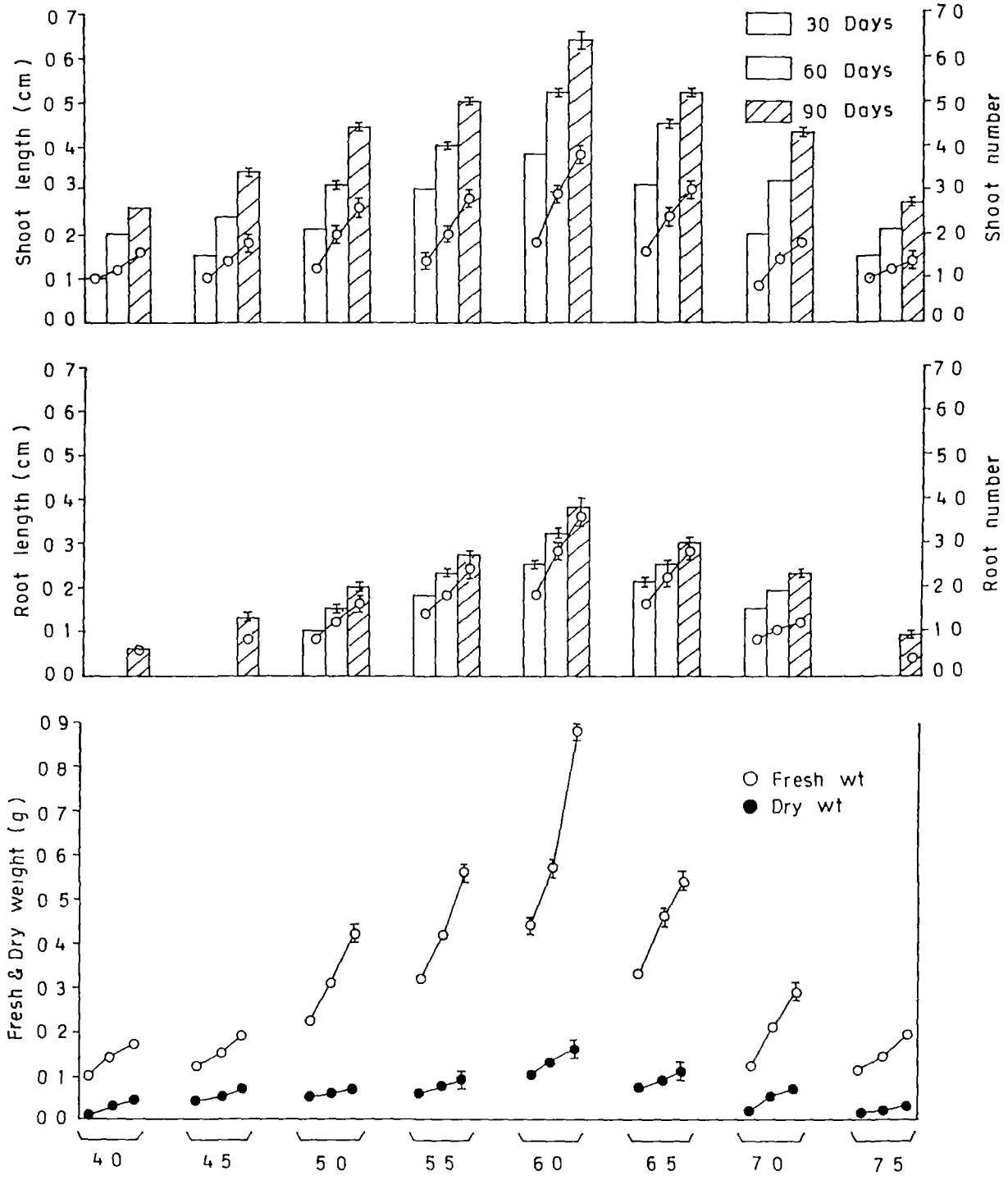
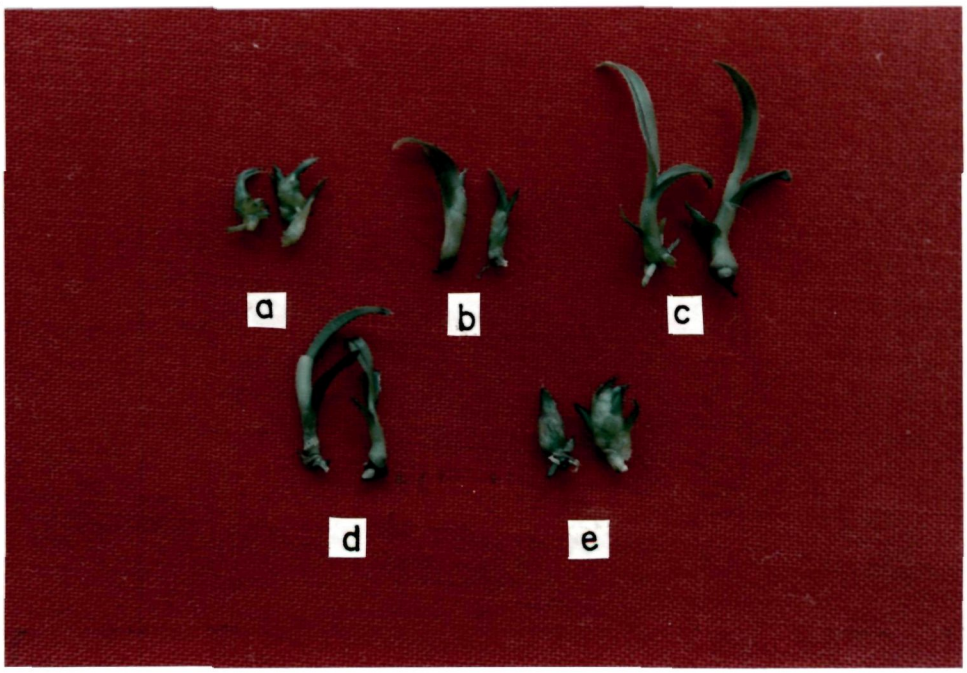

30 Days
60 Days
90 Days
Shoot length (cm)
Shoot number
Root length (cm)
Root number
Fresh & Dry weight (g)
Fresh wt
Dry wt
40
45
50
55
60
65
70
75

Fig.10

Fig. 11 Regenerants developed at different temperatures [15 (a), 20 (b), 25 (c), 30 (d) and 35 (e)°C] (after 90 days).

Fig. 12 Regenerants developed at different pH [4.0 (a), 4.5 (b), 5.0 (c), 5.5 (d), 6.0 (e), 6.5 (f), 7.0 (g) and 7.5 (h)] (after 90 days).

Fig.11



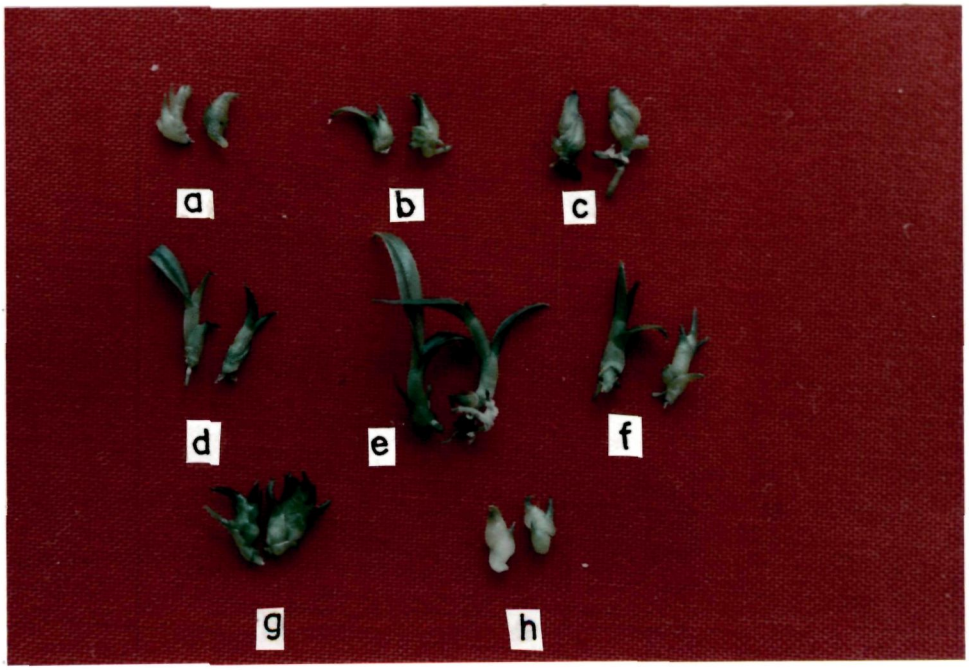


Fig.12

culture may differ from the requirements of the plants themselves growing in nature. Though light intensity of about 400-500 lux was reported favourable for the growth and development of Calanthe discolor (Hasegawa et al., 1978), in case of Arundina bambusifolia it was 3000 lux (Mitra, 1971). Werckmeister (1970a,b, 1971) has indicated a range of 400 - 5,000 lux light intensity suitable for the growth and development of Cymbidiums. In our study, 2,000 lux was found to be optimum for the morphogenesis followed by 3,000 lux light intensity.

The effective photoperiod for morphogenesis varies between taxa (Hughes, 1981). In the case of orchids, these have been found to vary from none at all (i.e. complete darkness, Morel, 1971) to continuous illumination (Wimber, 1963). Homes et al., (1971a,b) have reported a photoperiod of as much as 23.5 hrs for the development of Cymbidium protocorms. A range of 12 - 16 hr photoperiod has been observed to be beneficial for the optimum growth and development of a number of orchid species (Mitra, 1971; Hasegawa et al., 1978). Parallel to their study, we have found 16 hr photoperiod to result in optimum growth in D. wardianum. Light-triggered induction of morphogenesis may be related either directly or indirectly to the increased accumulation of starch in specific cells through the photosynthetic processes which may serve as a readily available source of energy for shoot production (Thorpe and Meier, 1972). A concomitant increase in growth with photoperiod could be due to improved metabolic processes besides higher photosynthetic

activity (Ziegler *et al.*, 1985). Also, an alteration in endogenous levels of growth inhibitors and promoters by different light levels are reported to influence growth and development (Economou and Read, 1986).

Optimum temperatures for the growth of plant tissue cultures have been investigated by several researchers and a range of 20-27^{o}C has been employed most often. Influence of temperature on basic physiological processes such as respiration, and on cell and organ formation is well known (Read, 1992). For the growth and development of orchids, a range of 20-25^{o} C is reported to be most suitable (Harvais, 1973; Stoutamire, 1974; Arditti *et al.*, 1982; van Waes and Debergh, 1986). The present study also shows a temperature of 25^{o}C to be optimum for the development of *D. wardianum* plantlets. An increase in the growth rate in the range 20-30^{o}C could be due to an increase in their metabolic rate (Muire, 1982; Ziegler *et al.*, 1985). Poor performance of the plantlets at extreme temperatures could however, be attributed to the loss of water from the medium as well as the living system. Bazzaz *et al.* (1970) have reported photosynthesis to be affected negatively above 30^{o}C which may result in poor growth and development.

Plant tissue cultures are known to tolerate a wide range of pH and a value between 5.2 and 5.8 is most often used (Kruse and Patterson, 1973). Murashige and Skoog (1962) reported that a pH value of 5.7-5.8 is suitable for maintaining all the salts in soluble form, even with relatively high phosphate levels, and is

low enough to permit rapid growth and differentiation of the tissue. From the data obtained on the growth of Ipomoea suspension cultures under controlled pH regimes, Martin and Rose (1975) suggested that the influence of pH was through its effect on the utilization of ammonia and nitrate rather than from any general effect on cell physiology. Some orchid plantlets can tolerate acidity and grow well even at a pH of 3.3 to 3.7 (Ernst, 1967a,b; Miyazaki and Nagamatsu, 1965). The present study shows optimum growth of D. wardianum plantlets at pH 6.0. This could be attributed to better uptake of nutrients and water from the medium (Knudson, 1946; Ito, 1955). Conversely, injury of root cells, depression in the uptake of potassium and calcium at pH 4.5 and precipitation and/or non-utilization of iron compounds at higher pH values could be the reason for the detrimental effect of the extreme pH values on the growth and development of in vitro growing plantlets. Evidently, pH of the medium surrounding the roots can affect the growth of the plants by controlling the availability and rate of uptake of nutrients by plants.

Chapter 4

Morphogenetic responses of cultured shoot apices to some nitrogenous adjuvants

A major part of nitrogen is required for the production of proteins used in the formation of protoplasm for new cells. Of all the mineral nutrients, the form of nitrogen (oxidized or reduced, organic or inorganic) probably is responsible for the most pronounced effects on growth and differentiation of cultured tissues (Ozias-Akins and Vasil, 1985). A number of studies on the suitability of different nitrogen sources for different orchids have been carried out with ammonia, nitrate and urea, all reported to be suitable for most orchid seedlings (Arditti and Ernst, 1984). A number of species, however, seem unable to utilize nitrate during the early stages of development (de Bruijne and Debergh, 1974). Cattleya seedlings were reported to grow on nitrate only after a 60-day growing period (Raghavan and Torrey, 1964). Reports with urea are contradictory even with species in the same genus. There are reports that the growth of Dendrobium and Phalaenopsis was inhibited, but development of Vanilla planifolia, Laeliocattleya, Cattleya, and Vanda was

enhanced (Arditti and Ernst, 1984). Amino acids and related substances have also been used for orchid growth and development *in vitro*. Arginine, ornithine and urea were able to replace ammonium nitrate in *Cattleya* cultures. Proline and α - aminobutyric acid served as moderately good sources of nitrogen, however; glycine, valine and leucine were found to be inhibitory (Raghavan, 1964, 1976; Raghavan and Torrey, 1964). Thus, results obtained from experiments with amino acids have been extremely variable and the conclusions drawn inconsistent and inconclusive (Sanford, 1974; Withner, 1959, 1974; Arditti and Ernst, 1984).

Materials and Methods

Shoot apices excised from *D. wardianum* plantlets growing under *in vitro* conditions were inoculated on MS medium containing 3% (w/v) sucrose, 0.8% (w/v) agar and 2.5 mg/l BAP (optimal conditions for plb formation) and supplemented with different inorganic and organic nitrogen sources viz., calcium nitrate, ammonium sulphate, urea and amino acids (alanine, glutamine, glycine, leucine and serine) at a range of concentrations (0.5-5.0 mg/l). Explants cultured on MS medium supplemented with 2.5 mg/l BAP served as control. The pH of the medium was adjusted to 5.8 prior to addition of agar and autoclaving carried out at 121^{o}C for 20 min. The cultures were incubated at 24$\pm$2^{o}C at 16 hr illumination of 2,000 lux from cool, white fluorescent light. Data were collected every 30 days for 3 months. Twenty replicates for each

treatment were taken and the experiments repeated thrice.

Results

Globular and green plbs were formed in explants cultured in the MS medium with 2.5 mg/l BAP (Table 3; Fig. 13a). Shoot apices cultured on MS medium supplemented with 0.5 and 2.5 mg/l calcium nitrate resulted in direct multiple buds in 8 weeks in 55 and 60% cultures respectively (Fig. 13b,c). Higher concentration of calcium nitrate, however, produced plbs alone in 50% cultures (Table 3). Green and healthy shoot buds (4-5 in number) were produced in the medium containing 2.5 mg/l calcium nitrate (Fig. 13d). If transferred to MS nutrient salt medium and left undisturbed, the shoot buds grew into plantlets (Fig. 13e) and developed healthy, green roots in 12 - 14 weeks (Fig. 13f). Using urea (2.5 and 5.0 mg/l) shoot buds were produced, however lower concentration resulted in green, globular plbs. Explants cultured on ammonium sulphate supplemented MS medium developed plbs at all concentrations. With an increase in the ammonium sulphate concentration, a decrease in the percentage response of plb formation was observed. Addition of individual amino acids did not show any significant morphogenetic effect. Maximum plb proliferation was observed at 2.5 mg/l of glycine and 0.5 mg/l of serine. Besides producing plbs, some of the amino acid treatments resulted in a small percentage of single shoot formation.

Table 3 Effect of different organic and inorganic nitrogen sources on the morphogenetic potential of shoot apices cultured on MS1 medium (MS+2.5 mg/l BAP)*

Nitrogen source	Conc (mg/l)	Morphogenetic response (%)	Nature of response Plb	ms	ss	Remarks
MS medium (control)	-	60	+	-	-	Green, globular plb formation
Inorganic N-source						
MS1+ Calcium nitrate	0.5	55	+	+	-	50% cultures form 2-3 ms
	2.5	60	-	+	-	Formation of 4-5 ms
	5.0	50	+	-	-	Green, globular plb formation
MS1+ Ammonium sulphate	0.5	45	+	-	-	Green, globular plb formation
	2.5	35	+	-	-	Green, globular formation
	5.0	20	+	-	-	Pale green plbs
Organic N-source						
MS1+ Urea	0.5	50	+	-	-	Green, globular plb formation
	2.5	55	+	+	-	45% cultures form 2-3 ms
	5.0	55	+	+	-	45% cultures form 3-4 ms
MS1+ Alanine	0.5	50	+	-	+	40% cultures form green plbs
	2.5	55	+	-	+	50% cultures form green plbs
	5.0	50	+	-	+	40% cultures form green plbs
MS1+ Glutamine	0.5	45	+	-	+	30% cultures form green plbs
	2.5	50	+	-	+	40% cultures form green plbs
	5.0	40	+	-	-	Green, globular plb formation

...contd.

MS1+ Glycine	0.5	50	+	-	-	Green, globular plb formation
	2.5	55	+	-	-	Green, globular plb formation
	5.0	40	+	-	+	30% cultures form green plbs
MS1+ Leucine	0.5	45	+	-	-	Green, globular plb formation
	2.5	40	+	-	+	35% cultures form green plbs
	5.0	40	+	-	+	35% cultures form green plbs
MS1+ Serine	0.5	55	+	-	-	Green, globular plb formation
	2.5	50	+	-	+	40% cultures form green plbs
	5.0	50	+	-	+	40% cultures form green plbs

* Data based on 20 replicates per treatment, collected after 8 weeks.
ms Multiple shoots
ss Single shoot

Fig. 13(a-f) Morphogenetic responses of shoot apices

a) proliferation of plbs from shoot apices cultured on MS + 2.5 mg/l BAP (control)

b,c) multiple shoot bud formation in shoot apices cultured on MS + 2.5 mg/l BAP + 2.5 mg/l calcium nitrate (after 8 weeks)

d) multiple shoot development from shoot buds transferred to MS (hormone-free) medium (after 12 weeks

e) regeneration of many complete plantlets (after 16 weeks)

f) healthy rooted shoot, ready for transfer to pot.

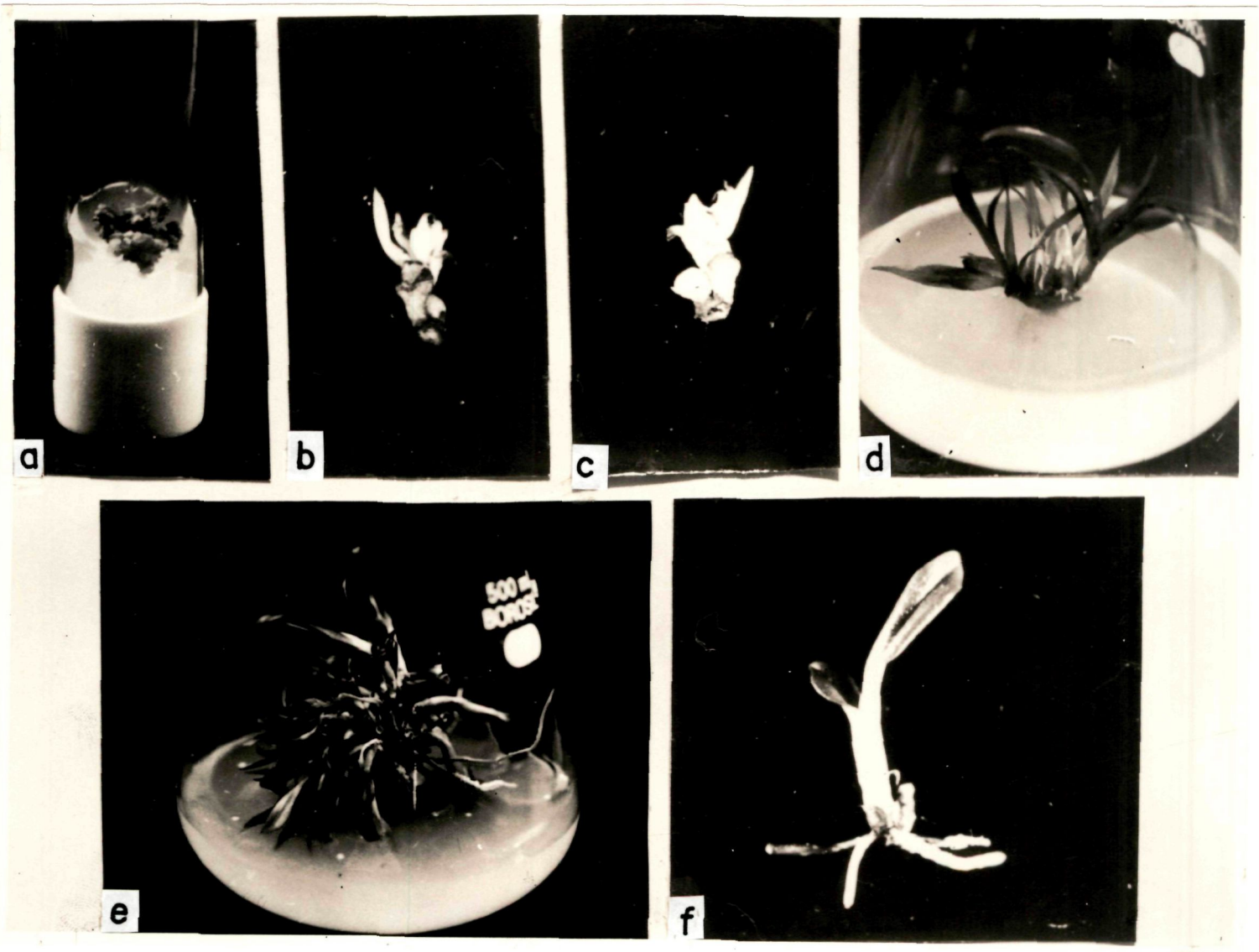


Fig. 13

Discussion

Extra/intra cellular ionic environment has been, besides other factors, found to affect cell elongation, enlargement, division and morphogenesis (Tran Thanh Van and Mutaftschiev, 1990). Also, it affects the chemical composition of plant tissues, thus influencing the plant growth and development (Alan, 1989). Nitrogen generally is supplied in the form of ammonium along with nitrate. It is believed that reduced forms of nitrogen have value for certain plant tissue cultures (Skirvin, 1981). However, in orchids, no such generalizations can be made as different orchids have been found to have different nitrogen-form requirements (Arditti and Ernst, 1984). Form of nitrogen supplied to plants has a great influence on the absorption of nutrients. Alan (1989) reported that nitrate generally increased all mineral contents except for nitrogen in the tissues whereas ammonium decreased them. The decrease of potassium, calcium and magnesium contents of the plant tissues with ammonium have been well documented in other plants also (Maynard et al., 1968; Edwards and Horton, 1982; Hartman et al., 1986). Mitra et al. (1976) have recognised the promotory influence of nitrates on plb proliferation and differentiation in D. fimbriatum. Additions of calcium nitrate tetrahydrate and potassium nitrate in combinations were shown to result in very high rates of differentiation of protocorms. Urea has been reported to serve as an effective nitrogen source for Cattleya embryos (Mariat, 1948). On the other hand, it has also been reported to cause necrosis at higher concentrations as it

significantly increases the nitrogen content and decreases calcium content. Although ammonium is rarely adequate as the sole nitrogen source, small amount of it may be essential for good growth. In certain cases, however, it has been reported to be detrimental to survival and growth of plant cells (Ozias-Akins and Vasil, 1985). Kirkby and Mengel (1967) observed that ammonium-N as a nitrogen source resulted in lower levels of cations. The unsuitability of ammonium as sole nitrogen source can be attributed to the tendency of the pH of the medium to fall below 5 under such conditions (Gamborg et al., 1968; Ohira et al., 1973; Wetherell and Dougall, 1976). Gamborg and Shyluk (1970) reported that ammonium can serve as the sole nitrogen source when the medium is provided with an organic acid such as malate, succinate, citrate or fumarate. The function of organic acids though, has not been elucidated. Poor performance of shoot apices subjected to ammonium sulphate in the medium at higher concentrations can thus be attributed to ammonium toxicity which is caused by higher nitrogen accumulation when ammonium is used (Barker et al., 1967; Barker and Maynard, 1972). Requirement for specific organic nitrogen compounds has been reported including arginine, glutamine and asparagine (Linsmaier-Bedman and Skoog, 1967; Dougall, 1972). However, growth inhibitions have also been observed very often following the addition of a combination of amino acids, a phenomenon that has been attributed to competitive interactions between the amino acids (Street, 1969). In the present study, amino acids did not result in any significant

morphogenetic response. Withner (1959), working with orchids reported that individual amino acids or mixtures of amino acids did not give any growth stimulation when added to a medium already supplying inorganic nitrogen. No particular increase in growth by addition of amino acids to a medium supplemented with ammonium was also reported by Nitsch *et al*. (1970).

Chapter 5

Activities of nitrogen metabolism enzymes as influenced by growth regulators during formation of regenerants from protocorm-like-bodies

Nitrogen assimilation, both ammonium (NH_4^+) and nitrate (NO_3^-) ions, has an important role in the growth and differentiation. Though, nitrogen metabolism enzymes have been studied extensively in plants (Jackson *et al.*, 1986), very little information is available in the case of orchids (Hew *et al.*, 1993). Physiological and biochemical studies have been carried out on different aspects during *in vitro* organogenesis (Kavi Kishore and Mehta, 1988; Auer *et al.*, 1992; Gopalan *et al.*, 1992). It is well established now that energy cycles like Kreb's, glycolytic and pentose phosphate pathway are enhanced during organogenesis which may be attributed to the need for energy regeneration (Kavi Kishore, 1988).

Compounds containing nitrogen constitute only a small proportion of the total dry weight of plants but they are extremely important physiologically. Nitrogen is required for growth and nitrogen deficiency is the most common limitation on plant growth and development after water stress. Required for

production of proteins and formation of new cells, chlorophyll and cytochrome, the demand for nitrogen is closely related to the amount of growth and differentiation (Kramer and Kozlowski, 1979). Growth regulators play an important role in differentiation (Ozias-Akins and Vasil, 1985), and regulation of enzyme activity by growth regulators has been reported by various researchers (Varner and Ho, 1977; Letham *et al.*, 1978; Moore, 1980). Developmental changes during growth are associated with changes in enzyme levels. Ashton (1976) reported that differentiation processes are controlled by the interactions between several hormones. Hence, presence of a hormone in different tissues or in the same tissue during different stages of its development may influence enzyme activity/synthesis through different mechanisms. Studies on enzymatic activities during *in vitro* differentiation of orchids as influenced by various growth regulators are, however, scanty (Kumaria *et al.*, 1990). Activities of the main enzymes of nitrogen metabolism during *in vitro* differentiation of *D. wardianum* as influenced by different growth regulators are presented here.

Materials and Methods

Plbs (derived from shoot apices) and multiplied in MS liquid hormone-free medium as described in chapter VI, were used for this study. Two week old plbs were transferred to MS semi-solid medium containing 0.8% (w/v) agar, 3% (w/v) sucrose and

supplemented with IAA, IBA, NAA and BAP at a concentration range of 0-5 mg/l. Cultures were incubated at 24±2°C at 2,000 lux light intensity (16 h photoperiod) from cool, white, fluorescent light. Samples collected every 0, 15, 30 and 45 days of growth were used for assay of enzyme activities.

Enzyme Extraction

500 mg plbs were homogenized in 0.1M phosphate buffer (pH 7.5) containing 1mM disodiumethylene diaminetetraacetate, 1% polyvinylpyrrolidone, 1mM dithiothreitol and 1% bovine serum albumin, and centrifuged at 10,000 x g for 30 min. The supernatant was collected and used for measurement of nitrate reductase (NR) and nitrite reductase (NiR) activity. For glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutamate synthase (GOGAT), Tris-HCl buffer (pH 7.6) with 5mM cysteine was used instead of phosphate buffer.

Enzyme Assay

NR activity was assayed using the method of Jaworski (1971). The 5 ml reaction mixture contained 1.0 ml of 0.1 M phosphate buffer (pH 7.5), 2.5 ml of 0.2 M potassium nitrate, 0.25 ml of 5% n-propanol, 1.0 ml of distilled water and 0.25 ml of enzyme extract. Incubation was carried out at room temperature (25°C) for 30 min. Reaction was terminated by adding 1.0 ml of 1% sulphanilamide in 3 N HCl and 1.0 ml of 0.02% N- (1 naphthyl) ethylenediamine dihydrochloride. The absorbance of the solution was read at 540 nm and activity is expressed as μM

nitrite produced per min per mg protein. Standard curve was made by taking sodium nitrite at a range of concentration and making up the volume to 5 ml with distilled water. Reaction was terminated by the addition of sulphailamide followed by naphthyl ethylenediamine reagent. Absorbance was read at 540 nm.

NiR assay was done following the method of Hucklesby et al. (1972). The reaction mixture contained 0.25 ml of 0.1 M phosphate buffer (pH 7.5), 0.08 ml of 1 mM $NaNO_2$, 0.08 ml of 0.155 mM methylviologen, 0.39 ml of distilled water and 0.1 ml of enzyme extract. The reaction was started by addition of 0.1 ml of 5 mM sodium dithionite buffered in $NaHCO_3$. After 15 min of incubation at room temperature, the reaction was stopped by vigorous agitation in order to oxidize methylviologen. Nitrites were determined by addition of 1.0 ml of 1% sulphanilamide and 1.0 ml of 0.02% N - (1- naphthyl) ethylenediamine dihydrochloride as described above. The enzyme activity is expressed as the amount of nitrite (μM) reduced per min per mg protein.

GDH activity was assayed by using the amination reaction based on glutamate formation (Thalouarn, 1988). The 2.5 ml reaction mixture contained 1.0 ml of 50 mM Tris-HCl (pH 8.0)., 0.4 ml of 180 mM ammonium acetate, 0.3 ml of 16 mM α - ketoglutarate, 0.12 ml of 0.12 mM NADH and 0.1 ml of enzymatic extract. Incubation of the reaction mixture was carried out at room temperature for 30 min followed by recording of change in absorbance at 340 nm. The amount of NADH oxidised is calculated from the molar extinction coefficient. Activity is expressed as

μM NADH oxidised per min per mg protein.

GS was assayed by using the biosynthetic reaction based on glutamyl hydroxamate formation as described by Sadashivam and Manikam (1992). The 4 ml reaction mixture contained 2.0 ml of 0.2 M glutamine, 0.5 ml of 20 mM sodium arsenate, 0.3 ml of 3 mM manganese chloride, 0.5 ml of 50 mM hydroxylamine, 0.5 ml of 1 mM adenosine-di-phosphate and 0.2 ml enzyme extract. Incubation of the reaction mixture was carried out at room temperature for 30 min. Reaction was stopped by addition of 1.0 ml of ferric chloride reagent [10g trichloro-acetic acid (TCA) and 8g ferric chloride in 250 ml of 0.5 N HCl] and absorbance was read at 540 nm. Enzyme activity is expressed as μM - glutamyl hydroxamate formed per min per mg protein.

GOGAT activity was determined by following NADH oxidation (Sadasivam and Manikam, 1992). The 5 ml reaction mixture contained 1.8 ml of 50 mM Tris-HCl buffer (pH 7.6), 1.0 ml of 5 mM glutamine, 1.0 ml of 5 mM 2-oxoglutarate, 1.0 ml of 0.25 mM NADH and 0.2 ml enzyme extract. Reaction mixture was incubated at room temperature for 30 min and change in absorbance recorded at 340 nm. Activity is expressed as μM NADH oxidised per min per mg protein.

Soluble proteins were also determined in the extract (Lowry et al., 1951). 500 mg of each of the treated tissue was macerated in alcohol using chilled mortar and pestle. The volume was made up to 5 ml with 80% alcohol and centrifuged at 5,000 rpm for 20 min. The supernatant was discarded and the residue suspended in

5% TCA for 15 min after which 2 ml aliquot was taken and resuspended in 10% TCA in the ratio 1:1 (v/v) for 15 min. The mixture was centrifuged at 5,000 rpm for 10 min. The supernatant was discarded and the residue was washed twice with distilled water and dissolved in 1.0 ml of 0.5 N NaOH and diluted to desired volume. Suitable volume of this solution was mixed with 5.0 ml freshly prepared alkaline solution (1.0 ml of 0.3% copper sulphate in 1% sodium-potassium tartarate mixed with 50 ml of 2.0% sodium carbonate solution). The reaction mixture was allowed to stand for 10 min. at room temperature. After incubation, 0.5 ml Folin-Ciocalteau reagent (equally diluted with water) was added with immediate shaking and held at room temperature for 30 min. The colour was read at 750 nm. Protein is expressed as µg per 100 mg fresh wt. of tissue using standard curve prepared by bovine serum albumin.

Besides, the tissue extract was subjected to polyacrylamide gel electrophoresis (PAGE) for localizing isozymes of soluble proteins according to the method of Ornstein (1964). Following solutions were used for electrophoresis :

Solution A :	1 N HCl	- 48 ml
	Tris(hydroxymethyl) amino methane	- 36.6 g
	N,N,N',N'- tetramethylethylenediamine	- 0.46 ml

Final volume was made up to 100 ml with double distilled water.

Solution B :	Acrylamide	- 30.0 g
	Methylene-bis-acrylamide	- 0.8 g

Final volume was made up to 100 ml with double distilled water.

Solution C : Ammonium per sulphate - 0.14 g

Final volume was made up to 100 ml with double distilled water.

All the stock solutions except solution C (to be prepared fresh at the time of use) were kept in thoroughly washed amber coloured bottles at $4^{o}C$.

Tank buffer : Tris-glycine (25 mM, pH 8.3) was made by dissolving 0.6 g Tris (hydroxymethyl) amino methane and 2.88 glycine in 1 litre of double distilled water.

Preparation of gel

Stock solutions were brought to room temperature to avoid bubble formation in the gels. Solutions A and B were mixed gently in 1:1 ratio followed by 2 parts of solution C. The mixed gel solution was immediately loaded in gel tubes (0.5 cm x 7.0 cm) with one end sealed with parafilm. A layer of water was loaded at the top of the gel to prevent contact with atmospheric oxygen, and to straighten the meniscus. Gels were allowed to polymerise for 30 min at room temperature.

Preparation of tissue

A suitable weight of freshly chopped and chilled tissue was ground in chilled pestle and mortar in a medium containing Tris glycine buffer, 40% sucrose and 5% polyvinyl pyrrolidone. The extract was strained through four folds of muslin.

Electrophoresis

Electrophoresis was done at 4°C. Bromophenol blue (500 mg/10ml distilled water) was used as the marker dye by adding 1 or 2 drops of it to the extract. In each gel tube, suitable extract (containing 200-300 ug protein) was loaded on top of the gel. A current of 2 mA per tube was used for the first 15-20 min which was susequently increased to 4 mA per tube. The power was turned off when the marker dye reached 5 mm above the bottom. The gels were removed from the tubes by squirting water from the syringe between the gel and the glass wall.

Staining of gels

Proteins were localized by staining the gels with amidoblack (0.1%) in acetic acid (7%) for 30 min followed by destaining in 7% acetic acid. Proteins appeared as dark blue bands against a clear background.

Results

Effects of growth regulators on the nitrogen metabolism enzyme activity were appraised.

Nitrate Reductase

The activity of NR along the time course of the experiments in control and treated plbs at different concentrations of growth regulators showed different profile plots (Fig. 14a-d). An increase in the activity on 30th day was observed in plbs treated

Fig. 14(a-d) NR activity of differentiating plbs as influenced by different growth regulators (1, 2 and 3 represent 0.5, 1.0 and 2.5 mg/l concentrations).

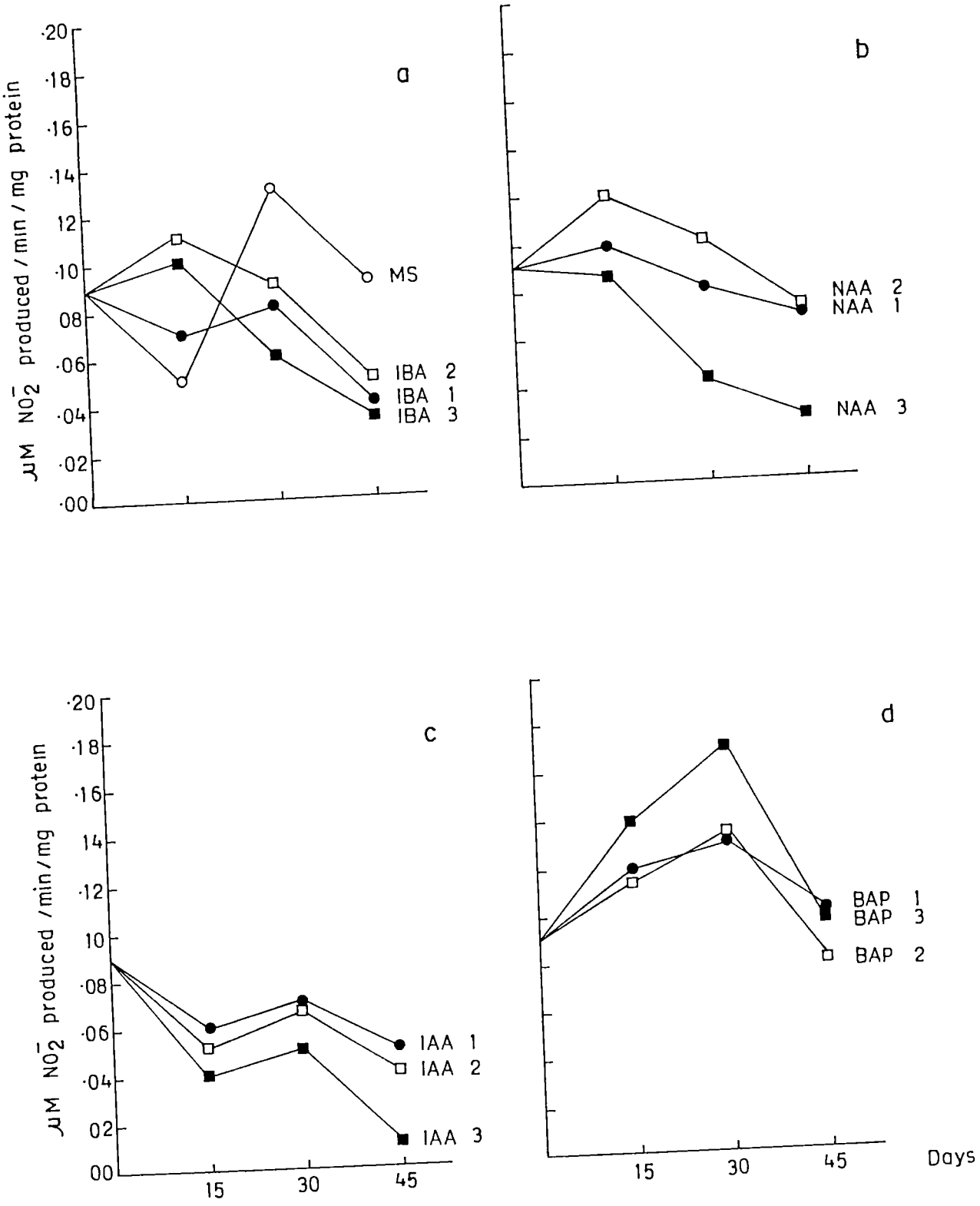

a
b
c
d
µM NO2- produced / min / mg protein
·20
·18
·16
·14
12
·10
08
·06
·04
·02
·00
MS
IBA 2
IBA 1
IBA 3
NAA 2
NAA 1
NAA 3
14
·12
10
·08
02
00
IAA 1
IAA 2
IAA 3
BAP 1
BAP 3
BAP 2
15
30
45
Days

Fig.14

with BAP followed by a decline thereafter. Highest level of enzymatic activity was recorded on the 30th day in plbs treated with 1.0 mg/l BAP. In plbs treated with IBA, a steady increase in the activity on day 15 followed by a decrease was observed at higher concentrations, however, in plbs treated with 0.5 mg/l IBA a slight decrease in the activity from zero to 15 day followed by an increase till day 30 and a decline thereafter was observed. An increase in the NR activity from day zero to day 15 was also noted in plbs treated with 0.5 and 1.0 mg/l NAA which then declined till the end of the study. IAA treated plbs, at all concentrations showed a decline from zero to 15 day followed by a slight increase on the 30th day and a decline after that. Enzymatic activity in control declined on 15th day and showed a peak value on 30th day and declined later.

Nitrite Reductase

NiR profiles observed for different growth regulators understudy were more or less similar to those of NR (Fig. 15a-d). An increase in NiR activity in plbs treated with IBA and NAA was observed from zero to 15th day which then declined till the end of the study. IAA treated plbs, however, showed a decline in the NiR activity from zero to 15th day that increased in the case of 0.5 and 1.0 mg/l treated plbs on day 30 and decreased thereafter. BAP treated plbs showed an increase in activity on the 30th day which then decreased till the end of the study. The pattern of NiR activity in control plbs was similar to NR.

Fig. 15(a-d) NiR activity of differentiating plbs as influenced by different growth regulators (1, 2 and 3 represent 0.5, 1.0 and 2.5 mg/l concentrations).

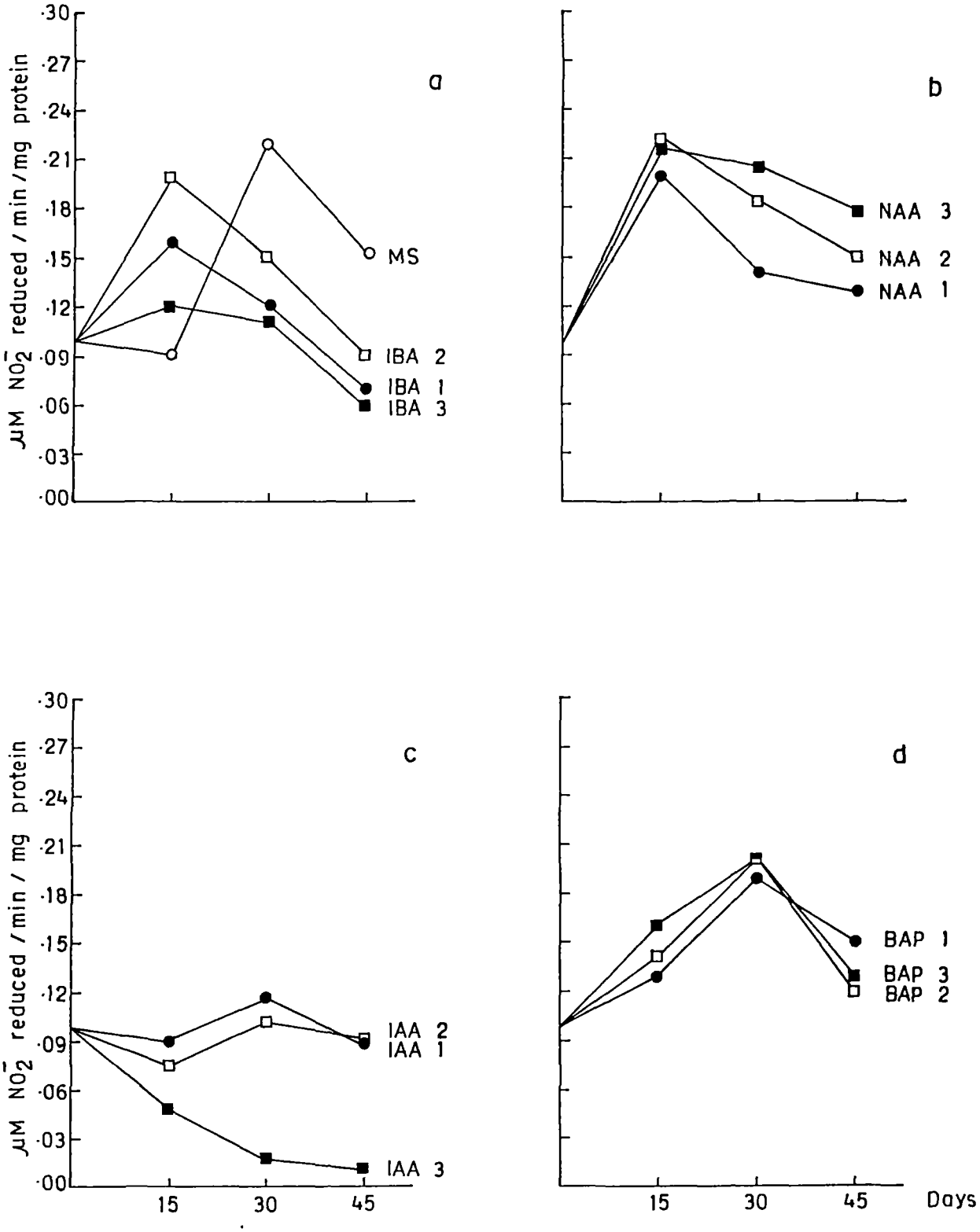

·30
·27
·24
·21
·18
·15
·12
·09
·06
·03
·00
µM NO2 reduced / min / mg protein
a
MS
IBA 2
IBA 1
IBA 3
b
NAA 3
NAA 2
NAA 1
c
IAA 2
IAA 1
IAA 3
d
BAP 1
BAP 3
BAP 2
15
30
45
Days

Fig.15

Glutamate dehydrogenase

Profiles of GDH activity were observed to be different from those of NR and NiR (Fig. 16a-d). In IBA, NAA, IAA and BAP treated plbs, a decline in the activity was observed from day zero onwards till the end of the study except for 1.0 mg/l IBA and 2.5 mg/l BAP treatments where an increase in the activity was recorded on 15th day followed by a decline. A decline in GDH activity from day zero onwards was also observed in control which continued till the end of the study.

Glutamine synthetase

Significant differences in GS activities were observed amongst different concentrations of growth regulators (Figs. 17a-d). The highest enzymatic activity was recorded in plbs treated with 2.5 mg/l BAP on the 15th day. A slight increase on the 15th day followed by a decrease till the end of the study was, however, observed in plbs treated with lower concentrations of BAP. A marked increase in GS activity on day 15 was also observed in plbs treated with 1.0 mg/l IBA. IAA treated plbs, at all concentrations, showed a decline in activity from zero day till the end of the study. A similar decline in activity was also observed in the case of control.

Glutamate synthase

Highest GOGAT activity was recorded on day 15 in plbs treated with 1.0 mg/l BAP followed closely by 1.0 mg/l IBA.

Fig. 16(a-d) GDH activity of differentiating plbs as influenced by different growth regulators (1, 2 and 3 represent 0.5, 1.0 and 2.5 mg/l concentrations).

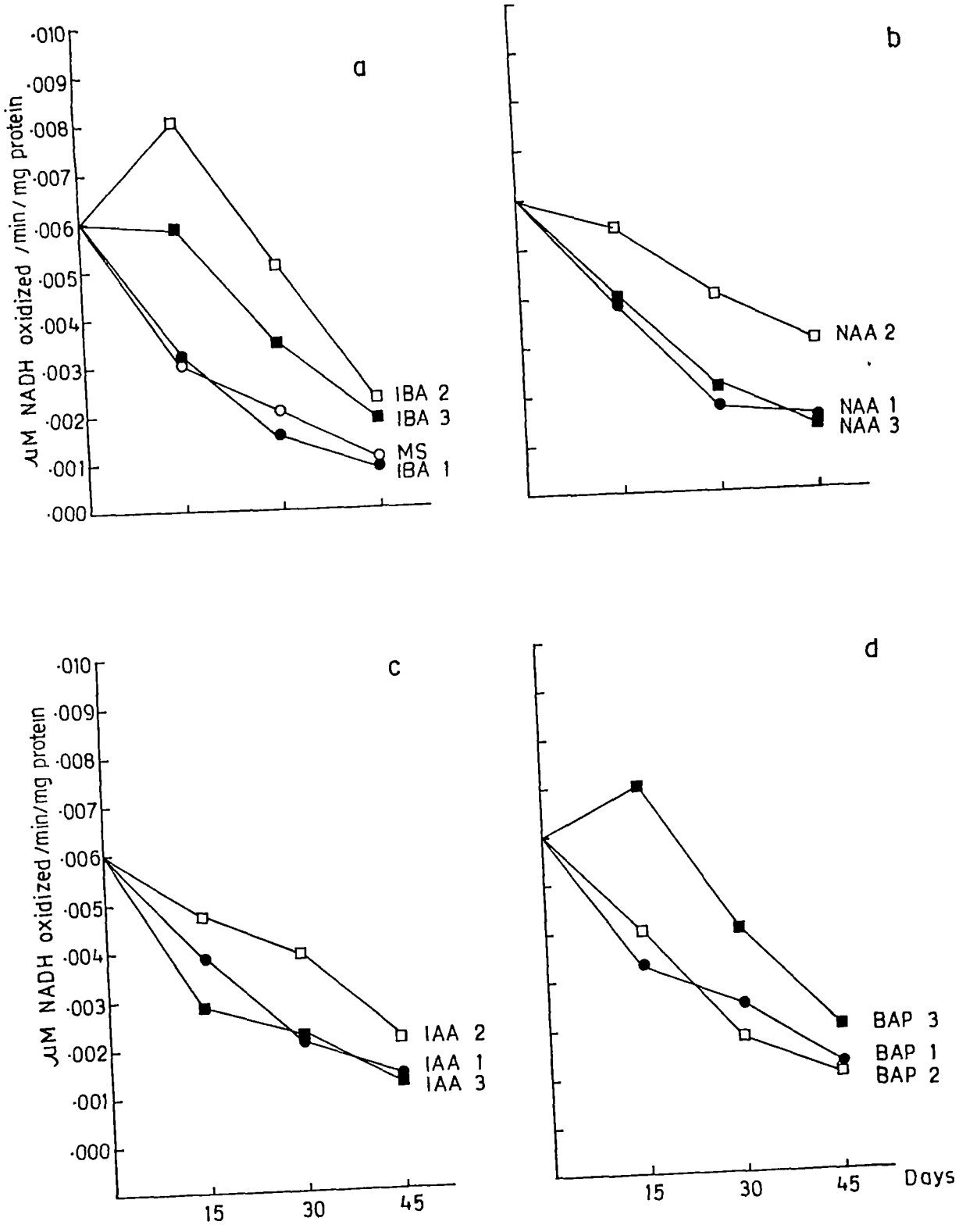

a
b
c
d
µM NADH oxidized /min/mg protein
·010
·009
·008
·007
·006
·005
·004
·003
·002
·001
·000
IBA 2
IBA 3
MS
IBA 1
NAA 2
NAA 1
NAA 3
IAA 2
IAA 1
IAA 3
BAP 3
BAP 1
BAP 2
15
30
45
Days

Fig.16

Fig. 17(a-d) GS activity of differentiating plbs as influenced by different growth regulators (1, 2 and 3 represent 0.5, 1.0 and 2.5 mg/l concentrations).

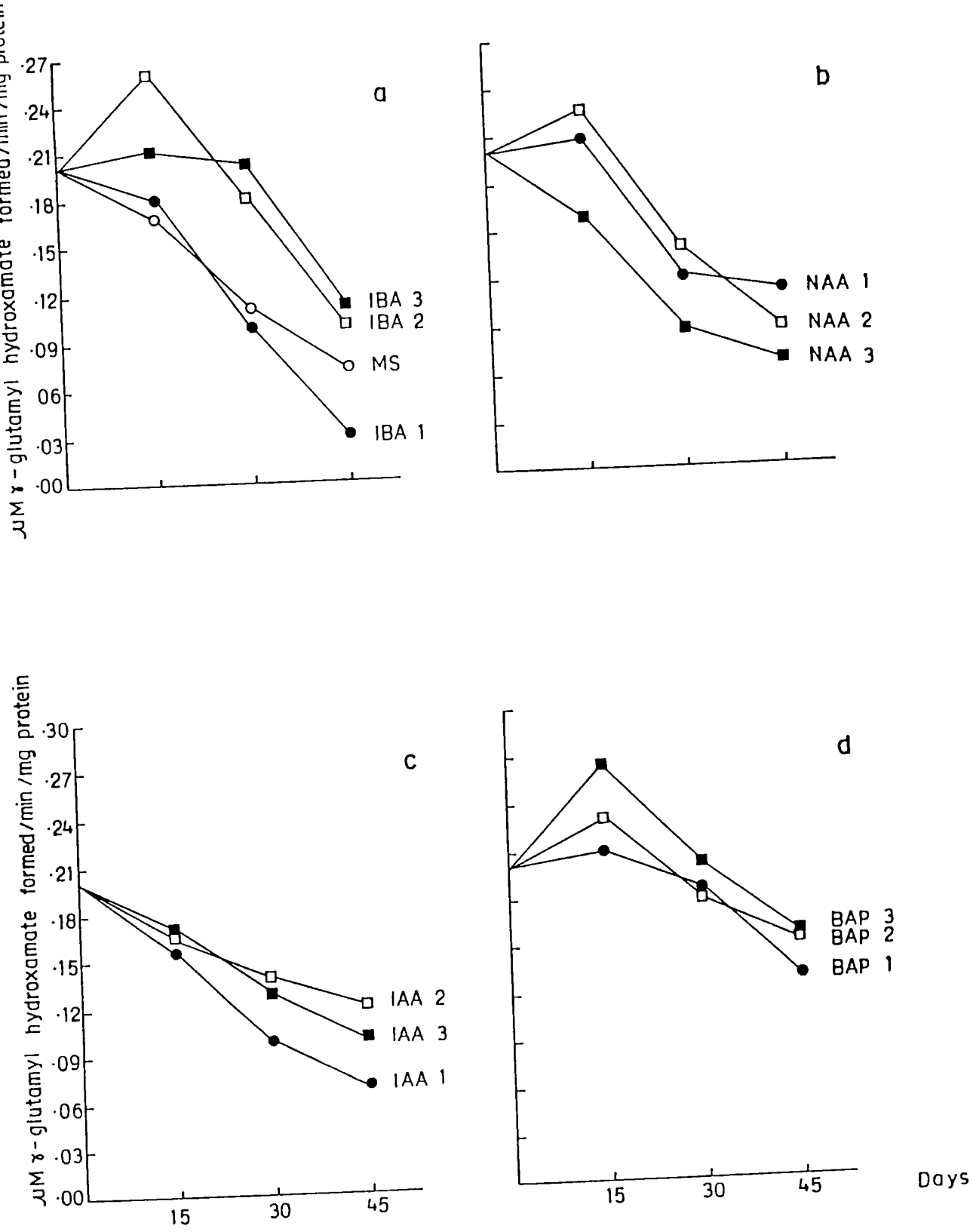

µM γ-glutamyl hydroxamate formed/min/mg protein
·27
·24
·21
·18
·15
·12
·09
06
·03
·00
a
IBA 3
IBA 2
MS
IBA 1
b
NAA 1
NAA 2
NAA 3
µM γ-glutamyl hydroxamate formed/min/mg protein
·30
·27
·24
·21
·18
·15
·12
·09
·06
·03
·00
c
IAA 2
IAA 3
IAA 1
15
30
45
d
BAP 3
BAP 2
BAP 1
15
30
45
Days

Fig.17

Fig. 18(a-d) GOGAT activity of differentiating plbs as influenced by different growth regulators (1, 2 and 3 represent 0.5, 1.0 and 2.5 mg/l concentrations).

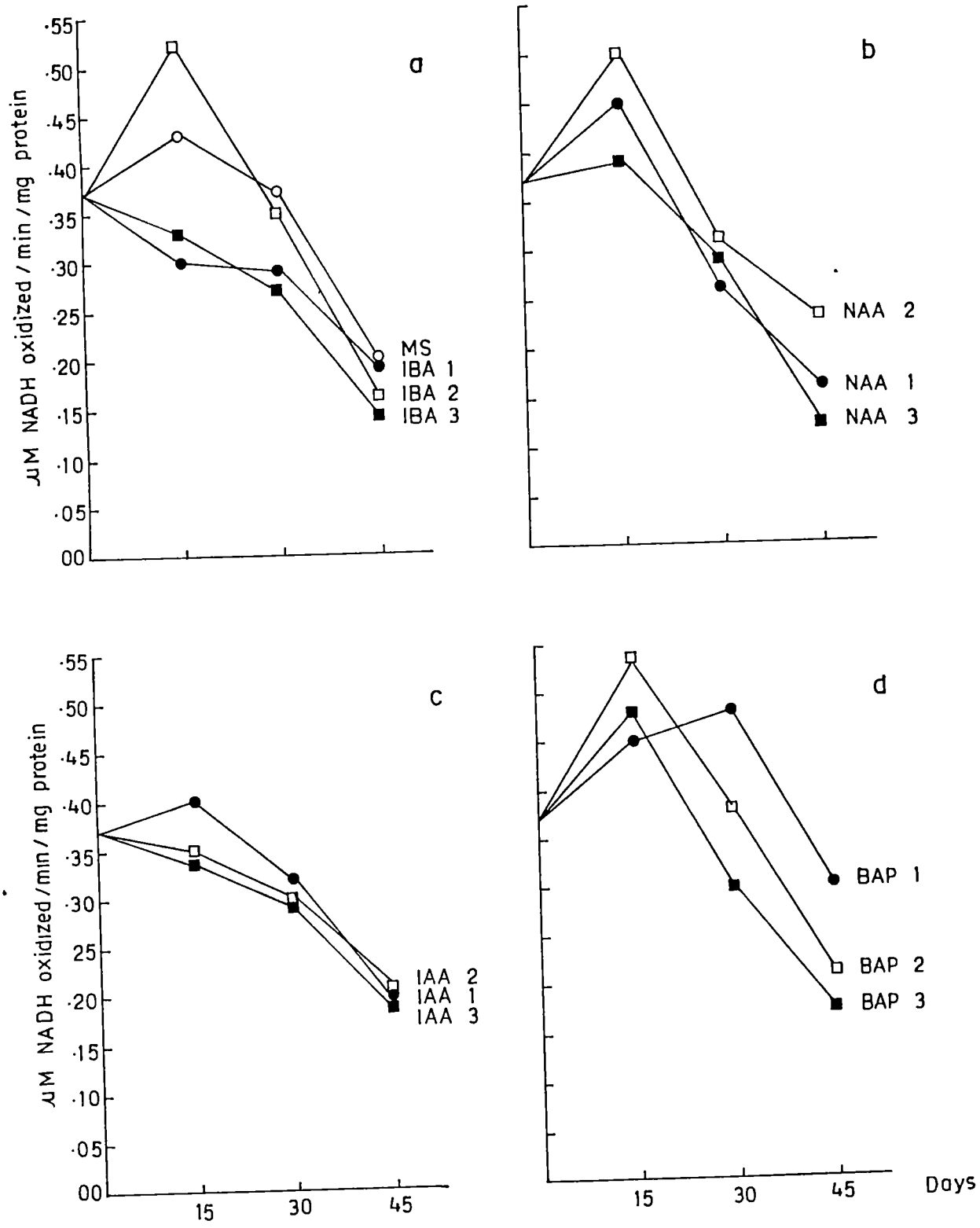

a
b
c
d
µM NADH oxidized / min / mg protein
µM NADH oxidized / min / mg protein
·55
·50
·45
·40
·35
·30
·25
·20
·15
·10
·05
00
MS
IBA 1
IBA 2
IBA 3
NAA 2
NAA 1
NAA 3
IAA 2
IAA 1
IAA 3
BAP 1
BAP 2
BAP 3
15
30
45
Days

Fig.18

Increase in activity was also observed in all NAA treated plbs and in plbs treated with 0.5 mg/l IAA, on day 15. A decline in activity was observed which continued till the end of the study in plbs treated with higher concentrations of IBA and IAA. Control plbs showed an increase in the activity on 15th day, declining thereafter.

Proteins

The protein profile of control and treated plbs is depicted in Fig. 19a-d. An increase in soluble protein content was observed from zero day onwards which continued up to the end of the study in all the treatments. Maximum level of soluble proteins was observed in plbs treated with 1 mg/l IBA which was slightly higher than that in 2.5 mg/l BAP treated plbs. On 45th day, control had higher soluble protein levels than IAA and NAA treated plbs of the same age.

Determination of the electrophoretic profile for proteins revealed the presence of five major bands in control and all the growth regulator treatments. The bands, however, differed in their Rm, colour intensity and width (Fig. 20a-e).

Discussion

Relations between nitrogen metabolism and endogenous hormones are reciprocal. Not only do these hormones control certain phases of protein synthesis and degradation but two of the three main classes of hormones, the auxins and the cytokinins are themselves

Fig. 19(a-d) Soluble protein content of differentiating plbs as influenced by different growth regulators (1, 2 and 3 represent 0.5, 1.0 and 2.5 mg/l concentrations).

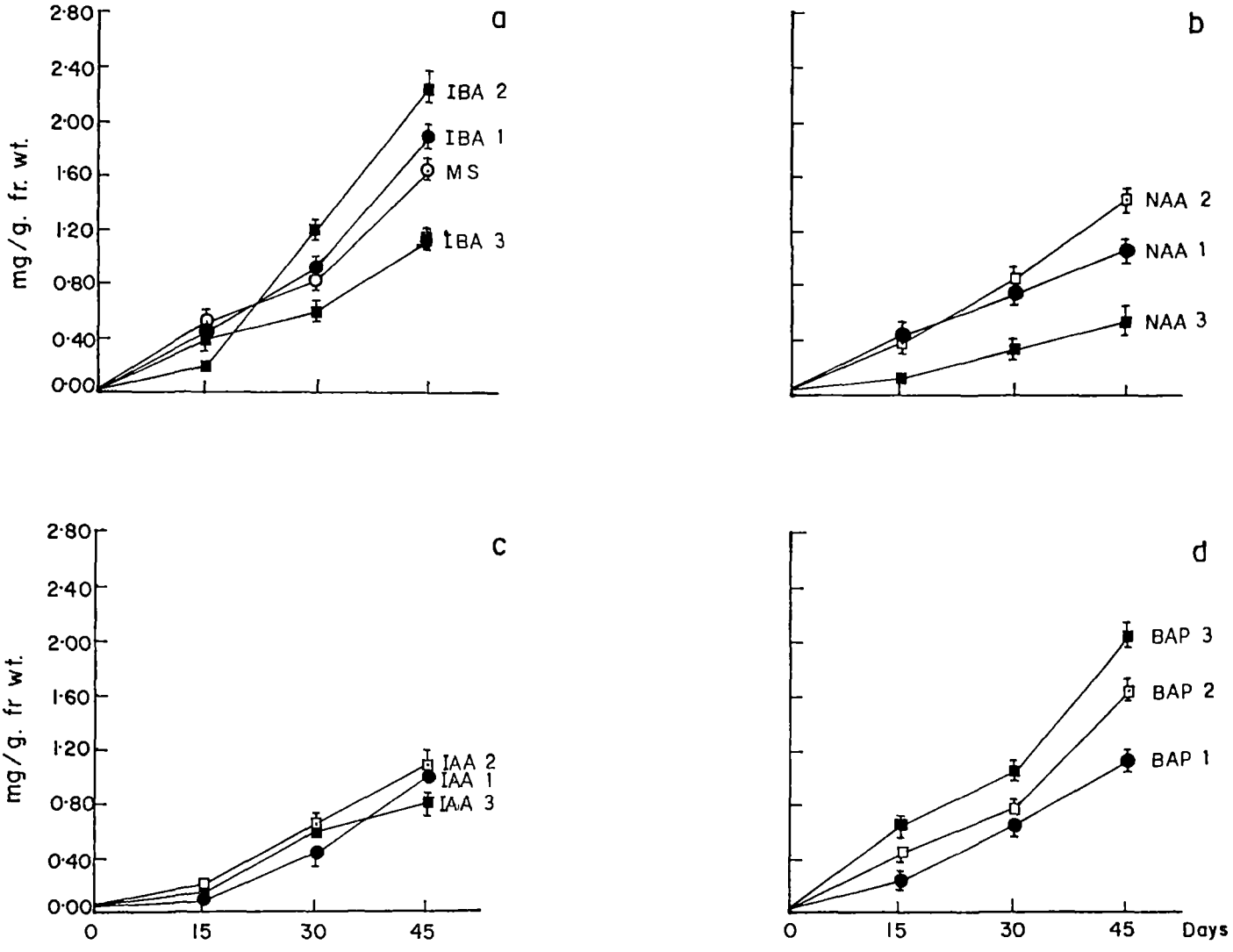

a
2·80
2·40
2·00
1·60
1·20
0·80
0·40
0·00
mg/g. fr. wt.
IBA 2
IBA 1
MS
IBA 3
b
NAA 2
NAA 1
NAA 3
c
2·80
2·40
2·00
1·60
1·20
0·80
0·40
0·00
mg/g. fr wt.
IAA 2
IAA 1
IAA 3
0
15
30
45
d
BAP 3
BAP 2
BAP 1
0
15
30
45
Days

Fig.19

Fig. 20(a-e) Electrophoretic profiles of protein isozymes of plbs cultured in MS (a) and medium supplemented with different growth regulators [IAA (b), IBA (c), NAA (d) and BAP (e)]. 1, 2 and 3 represent 0.5, 1.0 and 2.5 mg/l concentrations.

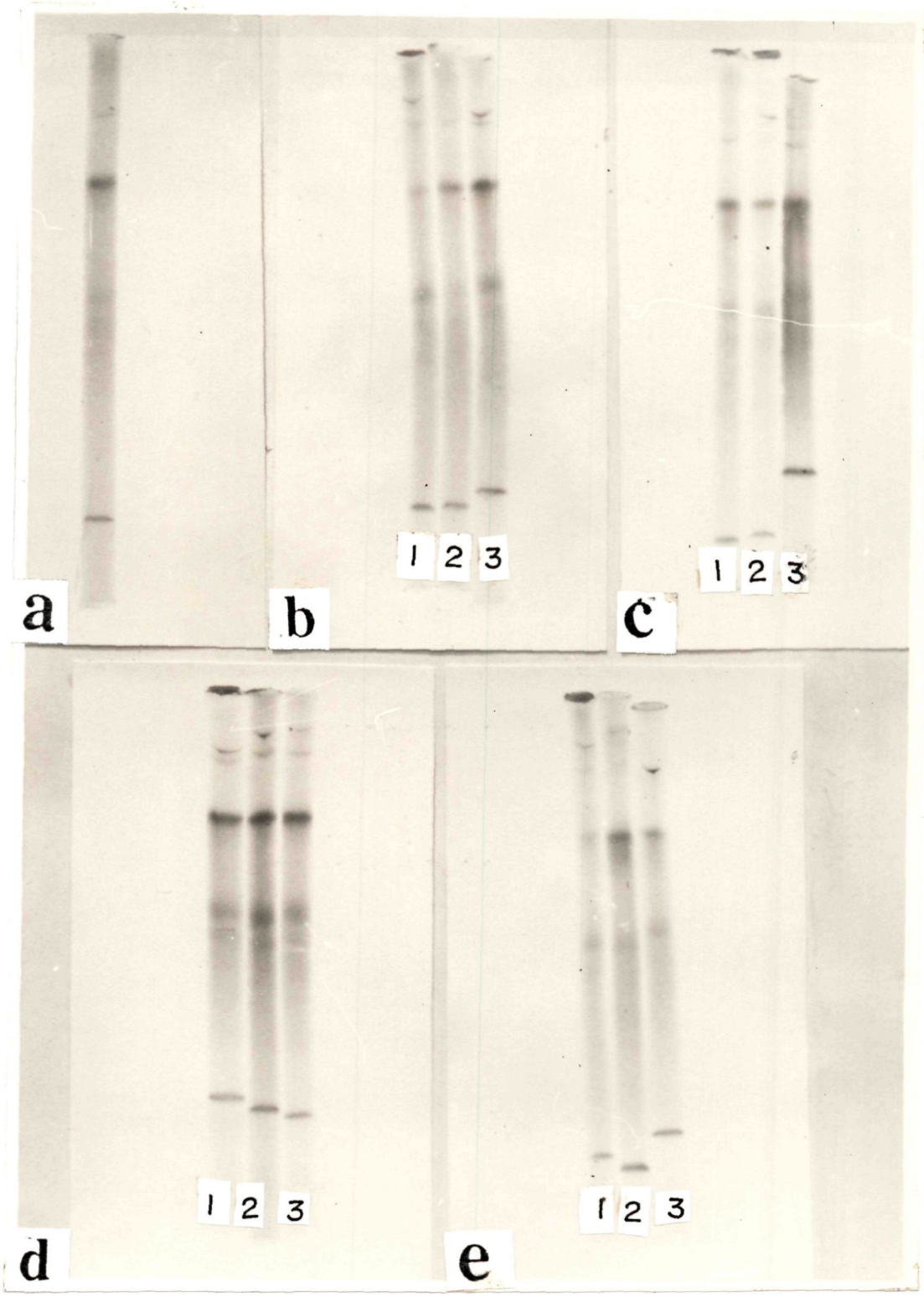


Fig. 20

Fig. 21(a-c) Regeneration of multiple shoots from nodal explants
a) shoot formation from nodal bud cultured on MS + 2.5 mg/l BAP (after 8 weeks)
b) multiple shoots transferred to MS hormone-free medium (after 10 weeks)
c) well developed rooted shoots.

Table 4 Effect of different concentrations of NAA+BAP and IAA+BAP on morphogenetic responses of cultured nodal buds in MS medium*.

Growth regulators	Conc. (mg/l)	Morphogenetic responses (%)	Remarks
MS medium (control)	-	40	Single shoot formation
MS+BAP	0.5	30	3-5 ms formation
	2.5	50	7-10 ms formation
	5.0	20	2-4 ms formation
MS + IAA+BAP	0.5+0.0	10	Single shoot formation
	0.5+0.5	10	1-2 shoot formation
	0.5+2.5	20	2-4 ms formation
	0.5+5.0	30	2-4 ms formation
	2.5+0.0	-	-
	2.5+0.5	10	Single shoot formation
	2.5+2.5	10	Single shoot formation
	2.5+5.0	20	1-2 shoot formation
	5.0+0.0	-	-
	5.0+0.5	-	-
	5.0+2.5	-	-
	5.0+5.0	-	-
MS + NAA+BAP	0.5+0.0	20	Single shoot formation
	0.5+0.5	20	2-3 ms formation
	0.5+2.5	40	3-5 ms formation
	0.5+5.0	30	2-4 ms formation
	2.5+0.0	-	-
	2.5+0.5	10	Single shoot formation
	2.5+2.5	10	Single shoot formation
	2.5+5.0	20	1-2 shoot formation
	5.0+0.0	-	-
	5.0+0.5	-	-
	5.0+2.5	-	-
	5.0+5.0	-	-

* Data based on 10 replicates per treatment, collected after 8 weeks
ms Multiple shoots
\- No response

nitrogen containing compounds whose production is inevitably linked with the nitrogen metabolism of the plant (Luckwill, 1968). In plants, the rate at which nitrate can be assimilated into simple organic forms and eventually into protein, depends largely on the activity of the NR and NiR systems in the plant and under certain conditions, these may well become limiting factors. During the course of a growth passage in tissue culture, the initially rich nutrient culture medium is progressively depleted by the tissue growth and development until one or more nutrient factors becomes limiting, and growth ceases on entry into stationary phase (Robinson et al., 1992). NR, a substrate-inducible enzyme, mediates the reduction of nitrate to nitrite. In the present study, increase in NR activity was recorded in NAA and IBA treatments on 15th day which is possibly due to progressive uptake of NO_3^- from the medium and consequent stimulation of nitrogen metabolism enzyme activities. NR activity in BAP treated plbs increased up to day 30. Also, highest NR activity was observed in 1.0 mg/l BAP treated plbs which could be due to its stimulatory effect as cytokinins are reported to be capable of inducing NR synthesis (Dwivedi et al., 1984). Stimulation of NR synthesis due to the presence of BAP in the medium has also been reported by Kende et al. (1971). Decline in the NR activity in the later stages could be due to a decrease in the NO_3^- concentration in the medium, in agreement to references that both induction and steady state level of NR activity are affected by nitrate concentration (Knypal, 1973; Chantarotwong et

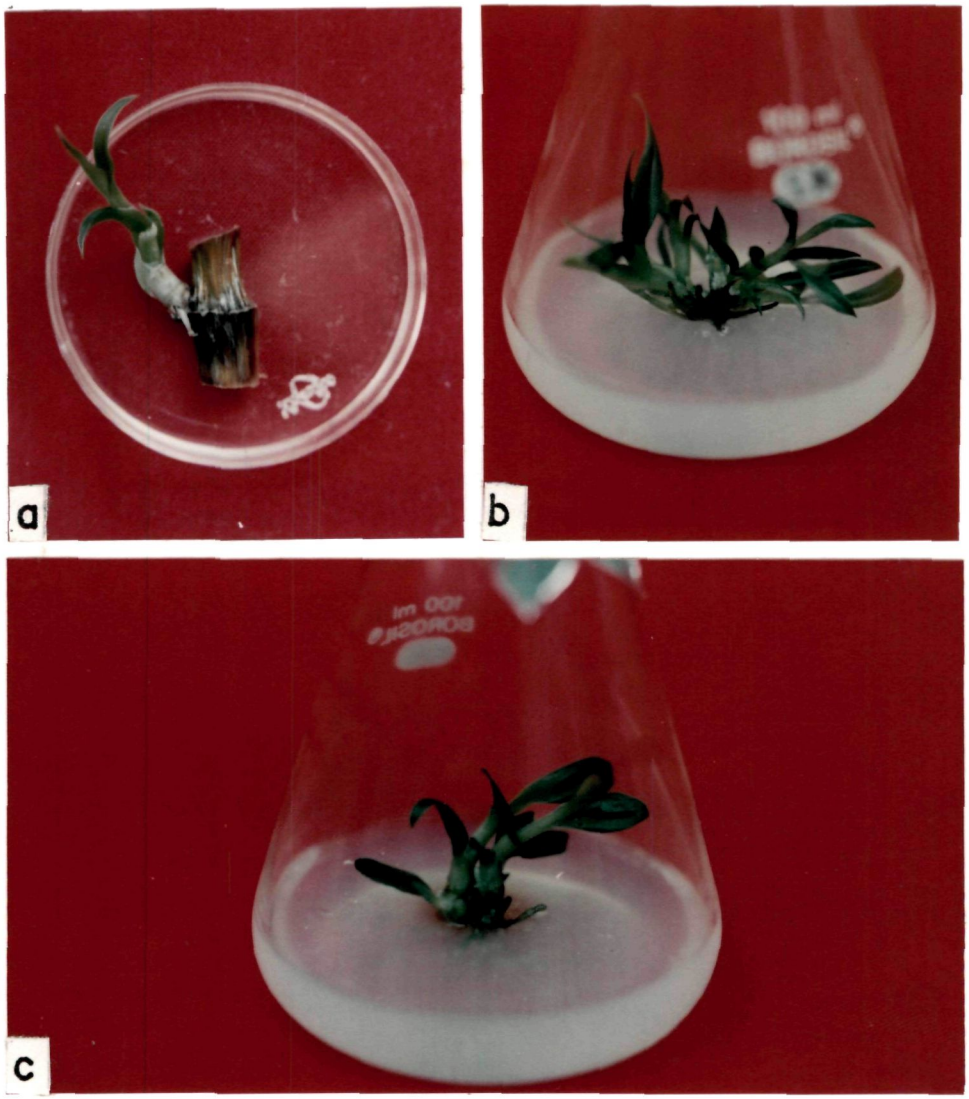


Fig.21

al., 1976). Decrease might also be due to NR-inactivating factors as suggested by Shaner and Boyer (1976). An initial decline in activity from zero day to 15th day in control could be due to the readjustment to the new conditions, required by the plbs when subcultured to a fresh medium as suggested by Santos and Salema (1989).

NiR activity was always higher than that of NR. Thus, a nitrite accumulation, toxic for the plbs, is avoided. A steady availability of the substrate to act upon could be the possible reason for the higher nitrite activity which in its turn provides substrate for GDH. A decrease in the activity in the later stages of the study might be due to a parallel decrease in the NR activity.

GDH activity in control and treated plbs was found to be low in comparision to other nitrogen metabolism enzyme activities studied. No significant differences in GDH activity were observed among plbs treated with different growth regulators at different concentrations. Except for plbs treated with 1.0 mg/l IBA and 2.5 mg/l BAP concentrations which showed an increase in activity on 15th day, all other growth regulator treated plbs showed a decline in the GDH activity from day zero which continued till the end of the study suggesting thereby that ammonium assimilation might not be taking place via this pathway in the case of D. wardianum. The route of NH_4^+ assimilation, thus, fades away in favour of GS-GOGAT pathway, an assumption supported by high GS and GOGAT activities (Figs. 17a-d, 18a-d). Low GDH

activity can also be attributed to its low affinity for ammonium as suggested by Sprent (1979). Until recently, controversy existed regarding the metabolic role of GDH. A number of researchers assigned GDH a function in ammonium assimilation (Yamaya et al., 1986; Srivastava et al., 1987; Rhodes et al., 1989) whereas others suggested its role in catalyzing the oxidation of glutamate, thus providing carbon-skeletons to the TCA cycle (Furuhashi and Takahashi, 1982; Robinson et al., 1990). Robinson et al. (1992) attribute increased GDH activity in their study during the stationary phase of carrot cell cultures to carbon limitation and protein catabolism leading to the release of glutamate which is oxidised by GDH to 2-oxoglutarate with the concomitant release of ammonia. The primary role of GDH, thus, according to Robinson et al.(1992) is to provide carbon skeletons for TCA cycle under conditions of carbon limitation, the enzyme thereby playing an important role in linking the carbon and nitrogen metabolism.

GS activity in plbs, treated with higher concentrations of IBA and BAP was more on 15th day decreasing thereafter. Also, an increase in activity was observed in plbs treated with NAA at 0.5 and 1.0 mg/l concentrations on 15th day, declining in the later stages. A much greater affinity of GS for ammonium than GDH has been reported by Sprent (1979). This additional benefit of GS, of rapid assimilation of ammonium from pools of even low concentration prevents ammonium accumulation in sufficient quantities thus checking toxicity. Hew et al. (1993) while

studying the nitrogen uptake by tropical orchids have reported a much higher activity of GS than GDH in the case of Cymbidium, Dendrobium and Bromheadia and have suggested that GS in orchids might be playing a major role in nitrogen assimilation. In the present study too, an increased GS-GOGAT activity indicates greater involvement of GS in the nitrogen metabolism.

GOGAT activity reaches its highest level with BAP treated plbs followed by IBA and NAA respectively. The activity depends on glutamine synthesis which acts as its substrate, thus GS activity decides the follow up activity of GOGAT. However, some diversion of glutamine towards other pathways might take place as this metabolite is essential for nucleic acid and protein synthesis. Low activities of the nitrogen metabolism enzymes in the later stages of the study could be accounted for by a depletion in the nutrients of the medium. Many tissue culture experiments have attempted to examine the relationship between exogenous plant hormones in the medium and subsequent plant growth and development (Fosket, 1980; Skivirsky et al., 1982). The developmental response of explants to exogenous hormones is the result of a variety of biochemical processes including hormone uptake, transport and metabolism (Horgan, 1987). Thus, although explant growth is typically described in relation to the hormone concentration in the medium, hormone concentration does not necessarily reflect the level of active endogenous hormone in the explant (Auer et al., 1992) which might account for the results with different auxins in the present study. Also,

suitability of some growth regulators over others in controlling the morphogenetic responses and their inhibitory action in some has been well documented in orchids (Liu et al., 1988). Lower activities of the enzymes in control when compared to treated plbs might be due to the absence of the growth regulators. Moreover, a higher soluble protein content could be the result of the active state of other enzymatic pathways in the present case. The present study on nitrogen metabolism enzyme activities with D. wardianum in vitro cultures, thus, suggest that GS plays an important role in nitrogen assimilation and also that growth regulators modify the activities of the nitrogen metabolism key enzymes.

The importance and utility of natural plant resources has led to the need for conservation of plants. Wherever possible, species are being protected *in situ* failing which *ex situ* conservation is being resorted to. In recent years, intensive efforts have been made for the development of totipotent tissue culture systems for a large number of plants. *In vitro* techniques have become increasingly popular for the conservation of endangered/threatened plants by rapid mass multiplication of the species with reproductive problems and/or having extremely reduced populations (McComb, 1985; Powers and Backhaus, 1989; Iriondo and Perez, 1990; Agarwal *et al.*, 1991; Bunn and Dixon, 1992). In highly heterozygous crops such as orchids, tissue culture can also serve as an efficient means for rapid clonal propagation of a large number of selected progenies for commercial use. Since Morel's successful experiment with *Cymbidium* (Morel, 1960), propagation by *in vitro* induction of adventitious buds on explants of shoot tips has become a widely

accepted method for clonal propagation of orchids. However, for recalcitrant genera, species, hybrids or progenies, other approaches are needed (Sagawa et al. 1990). Besides shoot apices, a number of alternative explants such as leaf tips, leaf bases, inflorescence stalks, floral buds and root tips have been used by various researchers (Champagnat et al., 1970; Churchill et al., 1971; Intuwong and Sagawa, 1973; Reisinger et al., 1976; Tanaka et al., 1976; Stewart and Button, 1978; Arditti, 1982; Goh and Tan, 1982; Yoneda et al., 1983; Griesbach, 1983; Vij et al., 1984, 1986; Chaturvedi and Sharma, 1986; Lim-Ho et al., 1986; Yoneda, 1986; Tanaka, 1987; Huang, 1988; Sanchez, 1988; Tanaka et al., 1988; Wang et al., 1988; Yoneda and Momose, 1988; Kraus and Monteiro, 1989; Goh and Wong, 1990; Holters and Zimmer, 1990; Vij and Pathak, 1990; Sagawa et al., 1990; Shimasaki and Uemoto, 1991). Almost all the orchid genera have responded to the tissue culture techniques except Paphiopedilum. Huang (1988) has reported propagation by proliferation of lateral (axillary) shoots in some Paphiopedilum hybrids. A number of reports on rapid propagation of Dendrobiums under aseptic conditions have been made using different techniques (Sagawa and Shoji, 1967; Kim et al., 1970; Arditti et al., 1973; Intuwong and Sagawa, 1975; Mitra et al., 1976; Arekal and Karnath, 1980; Sagawa and Kunisaki, 1982; Kukulezanka and Wojciechowska, 1983; Liu et al., 1988; Devi et al., 1990). However, the explant source, age, physiological state and nutritional environment are all found to influence the responses (Vij et al., 1983, 1984; Read, 1992).

Unlike other plant families, differences in requirements for propagation *in vitro* exist among the diverse genera, species and hybrids of Orchidaceae and the nutrient formulations and steps that are satisfactory for one may not be applicable to another (Huang, 1988).

Numerous factors affect the successful induction of morphogenesis in plant cell and tissue culture. Besides various physico-chemical factors, growth regulators markedly influence the growth and development of *in vitro* growing plantlets. Skoog and Miller (1957) indicated that auxin to cytokinin ratios determined the type and extent of organogenesis. While no universal ratio for root or shoot induction exists, both an auxin and a cytokinin are usually added to the medium in order to obtain morphogenesis. Orchids have responded differentially to various auxins, cytokinins and gibberellins and the results have been inconsistent and consequently inconclusive (Arditti, 1967a, b, 1979; Withner, 1959, 1974; Arditti and Ernst, 1984). Auxins, mostly IAA, IBA and NAA have been reported to enhance the growth. Israel (1963) reported death of the excised *Dendrobium* ovaries in the absence of auxin. Enhanced growth and development in *Bletilla* was reported by Strauss and Reisinger (1976) when treated with NAA. In addition, Kusumoto (1979a, b) observed a stimulatory effect of 2,4-D on shoot formation and NAA on shoot proliferation in *Cattleya*. An inhibitory effect of 2,4-D on *Cymbidium* cultures was, however, reported with the development of

abnormal plbs (Kusumoto, 1978; Harvais, 1982; Sharma and Tandon, 1986). A similar inhibition of Cymbidium seeds to NAA concentrations above 1 ppm was indicated by Torikata et al. (1965). In vitro growth of seedlings of several species is generally enhanced by cytokinins (Arditti and Ernst, 1984). A promotory influence of BAP on protocorm proliferation was suggested by Pierik and Steegmans (1972). Also, Kusumoto (1979a, b) reported an increased shoot formation with BAP in the case of Cattleya. Developmental retardation and root/root hair inhibition were, however, observed by Rucker (1974) in Cymbidium when treated with BAP. Kinetin in medium is reported to stimulate as well as inhibit the growth and development in different orchid species (Hadley, 1970; Harvais, 1973, 1982; Hadley and Harvais, 1968; Chennaveeraiah and Patil, 1973; Uesato, 1978; Nakamura, 1982). Sharma and Tandon (1986) observed promotion in both seed germination and seedling growth at low kinetin concentrations in Coelogyne. Effects of exogenous gibberellins on the growth of orchid seedlings have generally been negetive (Nakamura, 1982; Arditti and Ernst, 1984; van Waes and Debergh, 1986).

Clonal propagation, now a fairly common practice in orchid culture, is important as orchid genotypes are heterozygous. The present chapter deals with morphogenetic responses of the regenerants to various growth regulator treatments and large scale clonal propagation and establishment of D. wardianum.

Material and Methods

Nodal buds, leaves and root tips were excised from *D. wardianum* Warner plants growing in Botanical Garden, Department of Botany, North-Eastern Hill University and were used for regeneration experiments. Besides, leaves and root tips from *in vitro* growing plants were also used. Sterilization of the explants, except those obtained from *in vitro* plants, was carried out as described in Chapter II. Leaves excised from *in vitro* growing plantlets were cut into 4 sections (leaf base, leaf tip and the remaining portion cut in halves perpendicularly to the mid rib). All the explants except nodal buds were cultured on both, MS and modified VW media under aseptic conditions. MS and modified VW media contained 3 and 2% (w/v) sucrose respectively and 0.8% (w/v) agar. Both the media were supplemented with various growth regulators at a concentration range from 0-5 mg/l, for plb initiation.

To study the effect of different growth regulators on differentiation, growth and development of regenerants, plbs of uniform size, obtained from shoot apices as described in Chapter II, were transferred to fresh MS medium supplemented with different auxins, cytokinins and gibberellin at a concentration range from 0-5 mg/l.

The pH of MS medium was adjusted to 5.8 and that of modified VW to 5.2 before autoclaving at 121^{o}C for 20 min. The plbs cultured in liquid medium were multiplied by cutting into smaller

pieces and subcultured into fresh medium every week. Liquid cultures were maintained at 75 rpm on gyratory shaker All the cultures were maintained at 2,000 lux light intensity (16h photoperiod) from cool, white fluorescent tubes, at 24+2^{o}C. Ten replicates per treatment were taken and the experiments repeated twice.

Results

Nodal buds started developing about 3 weeks after inoculation on MS medium containing BAP alone or BAP with IAA/NAA (Table 4). Buds cultured in the medium containing 2.5 mg/l BAP alone developed multiple shoots (7-10 shoots/culture) in 50% cultures. With an increase in the concentration, however, a decline in the response was observed. Multiple shoots were also observed on buds cultured in the medium containing combinations of IAA + BAP and NAA + BAP. However, the number of multiple shoots developed and the percentage response were less compared to the response obtained by using BAP alone. Single shoots emerged out of every 4 of 10 nodes when no growth regulators or low auxins were added to the medium. Multiple shoots were obtained from nodal buds in 8 weeks (Fig. 21a). Further growth and development was accomplished in MS hormone-free medium (Fig. 21b). Isolated shoots developed viable roots (Fig. 21c). The leaf and root explants from *in vivo* plants did not show any morphogenetic response in the present experiment.

Morphogenetic responses from in vitro leaves were observed only from the leaf bases in modified VW medium containing NAA+BAP (Table 5). Initiation of leaf base differentiation started by the second week (Fig. 22a) and a rosette of bright green plbs was formed (Fig. 22b) after 6 weeks of culture. The plbs once formed, severed their contact with the mother explant which later dried. It was observed that NAA and BAP if used at 0.5 - 1 mg/l and 1 - 2 mg/l respectively induced plb formation. An equal concentration of auxin and cytokinin, however produced 3-4 plbs and a poor percentage response. The optimum plb formation resulted in the medium containing 0.5 mg/l NAA and 2.0 mg/l BAP. Other growth regulators tried were found unsuitable for plb formation or multiple shoot development. Swelling of root tips (from in vitro grown plantlets) was observed on modified VW medium with no growth regulators added to it but no further development was observed.

Plbs obtained from shoot apices (as described in Chapter II) and leaf bases were multiplied by cutting into smaller pieces and culturing on MS medium devoid of growth regulators. Each segment got converted into smaller clumps of plbs and the process could be repeated to get a large number of protocorm cultures. Faster proliferation resulted in liquid medium of the same composition. Plbs thus obtained, when subcultured on MS nutrient salt medium without growth regulators and left undisturbed, differentiated (Fig. 22c). Complete plantlets with well developed green, healthy roots were formed within 12-16 weeks (Fig. 22d).

Table 5 Effect of different concentrations of NAA+BAP on morphogenetic responses of cultured leaf explants*.

Growth regulators	Conc. (mg/l)	Morphogenetic responses (%)	Remarks
modified VW	-	-	-
modified VW + NAA+BAP	0.5+0.0	-	-
	0.5+0.5	20	Swelling of explant
	0.5+1.0	40	4-6 green plbs
	0.5+2.0	60	8-10 green plbs
	0.5+4.0	30	3-5 green plbs
	0.5+5.0	-	-
	1.0+0.0	10	Swelling of explant
	1.0+0.5	10	2-3 green plbs
	1.0+1.0	20	2-3 green plbs
	1.0+2.0	50	6-8 green plbs
	1.0+4.0	10	2-3 green plbs
	1.0+5.0	-	-
	2.0+0.0	-	-
	2.0+0.5	-	-
	2.0+1.0	-	-
	2.0+2.0	10	Swelling of explant
	2.0+4.0	20	3-4 green plbs
	2.0+5.0	20	2-3 green plbs
	5.0+0.0	-	-
	5.0+0.5	-	-
	5.0+1.0	-	-
	5.0+2.0	-	-
	5.0+4.0	-	-
	5.0+5.0	-	-

* Data based on 10 replicates per treatment, collected after 8 weeks
- No response

Fig. 22(a-e) Clonal propagation using leaf bases and shoot tips
a) proliferation of plbs from léaf base cultured on modified VW + 1.0 mg/l NAA + 2.0 mg/l BAP (after 2 weeks)
b) rossette of bright green plbs formed (after 6 weeks)
c) differentiating plbs (after 8 weeks of culture)
d) proliferating plbs obtained from shoot apices
e) well developed plantlets (after 16 weeks of culture).

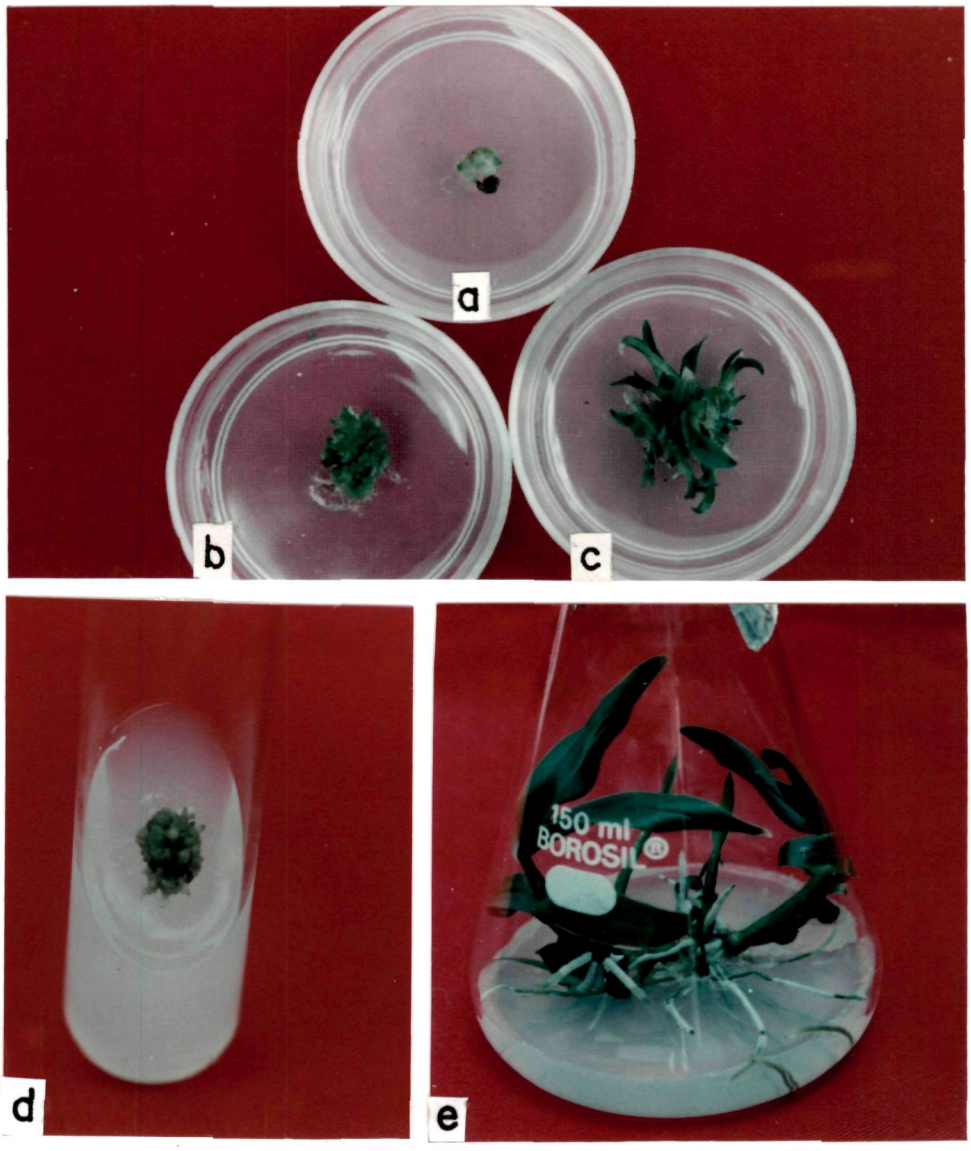


Fig.22

Of the different auxins used to study their effect on the growth and development of the regenerants, NAA (1.0 mg/l) resulted in morphogenetic responses in 70% cultures with healthy, green multiple shoot formation (Table 6, Fig. 23a). Higher concentrations, however, resulted in a decrease in the percentage response. IBA and IAA resulted in plb multiplication as well as multiple shoot formation with optimum response at 1 mg/l each (Fig. 23b,c), though the percentage response with IAA was less. Plbs treated with low concentrations of 2,4-D resulted in poor growth and development (Fig. 23d). However, higher concentrations did not support the growth and the cultures dried. Of the two cytokinins used, BAP was observed to stimulate the multiple shoot formation (Fig. 23e). Eighty percent of the plbs developed into multiple shoots at 2.5 mg/l BAP. Kinetin proved inhibitory at low concentrations with only 10% plbs developing into plantlets (Fig. 23f). At higher concentrations, cultures turned brown and died eventually. A negative influence of GA_3 at all concentrations was observed with a percentage response of 30 at 1 mg/l. Further, the plantlets developed showed poor growth (Fig. 23g).

Discussion

Tissue culture of orchids has increased remarkably in recent years and several reports have been made using different explants (Sanchez, 1988). An important technique for rapid mass multiplication of desired genotypes, shoot-meristem culture

Table 6 Effect of growth regulators on the growth and development of regenerants*.

Growth regulator	Conc. (mg/l)	Morphogenetic responses (%)	Nature plb	ms	ss	Remarks
MS	-	80	+	+	-	Green, globular plbs; Stout, green ms
MS + IBA	0.5	50	+	+	-	Green, globular plbs; Stout and short ms
	1.0	70	+	+	-	Green, globular plbs; Stout and short ms
	2.5	30	+	+	-	Stout, short ms
	5.0	20	+	-	-	Green, globular plbs
MS + IAA	0.5	40	+	+	-	Green, globular plbs; Stout and short ms
	1.0	50	+	+	-	Green, globular plbs; Stout and short ms
	2.5	30	-	+	-	Stout, short ms
	5.0	20	-	+	-	Stout, short ms
MS + NAA	0.5	40	+	+	-	Green, globular plbs; Stout, dark green ms
	1.0	70	-	+	-	Dark green, stout ms
	2.5	30	-	+	-	Dark green, stout ms
	5.0	10	-	+	-	Green, stout ms
MS + 2,4-D	0.5	20	+	+	-	Pale green plbs and ms
	1.0	10	+	-	-	Pale green plbs
	2.5	-	-	-	-	-
	5.0	-	-	-	-	-

...contd.

MS + KN	0.5	10	+	+	-	Small, pale green plbs and ms
	1.0	10	+	+	-	Small, pale green plbs and ms
	2.5	-	-	-	-	-
	5.0	-	-	-	-	-
MS + BAP	0.5	30	+	+	-	Green, globular plbs; Dark green, stout ms
	1.0	60	+	+	-	Green, globular plbs; Dark green, stout ms
	2.5	70	+	+	-	Green, globular plbs, Dark green, stout ms
	5.0	30	+	+	-	Green, globular plbs, Dark green, stout ms
MS + GA_3	0.5	20	-	-	+	Elongated, pale green ss
	1.0	30	-	+	-	Pale green ms
	2.5	10	-	-	+	Elongated, pale green ss
	5.0	-	-	-	-	-

* Data based on 10 replicates per treatment, collected after 45 days
\- No response
ms Multiple shoots
ss Single shoot

Fig. 23(a-g) Effect of different growth regulators [NAA (a), IBA (b), IAA (c), 2,4-D (d), BAP (e), KN (f) and GA_3 (g) (each at 1.0 mg/l concentration)] on growth and development of regenerants.

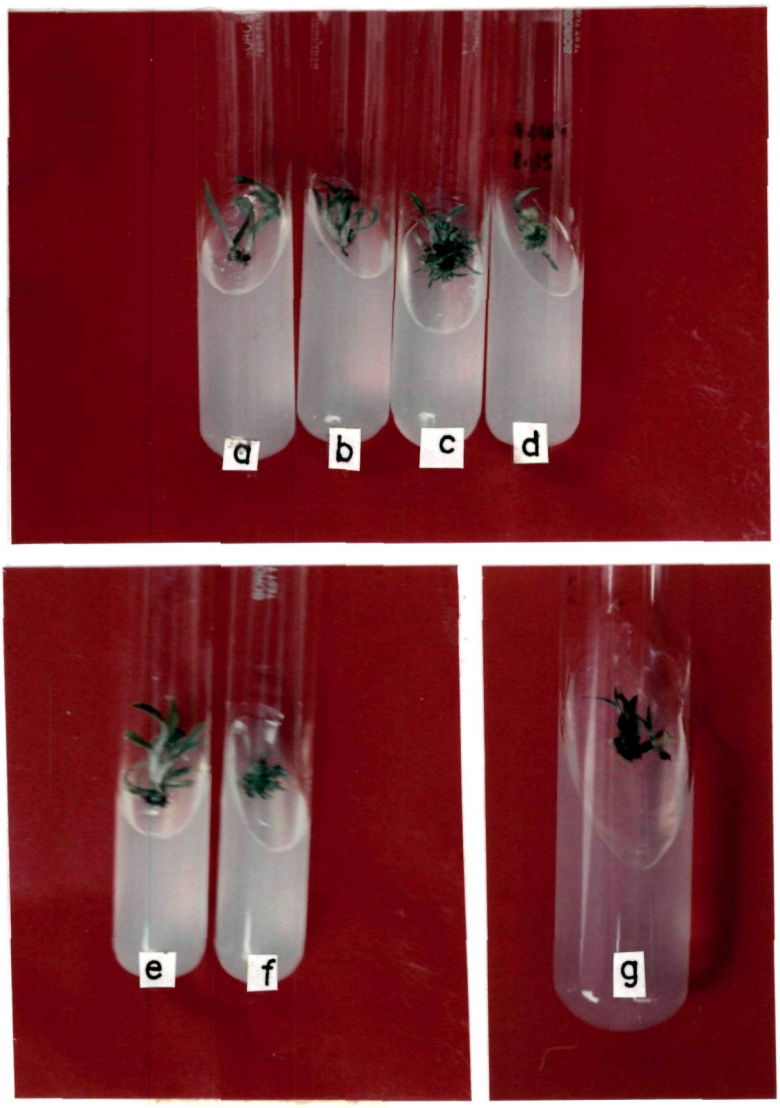


Fig. 23

requires the sacrifice of the growing point thus having a limited utility in monopodial taxa. Hence, there has been an emphasis on explants other than the shoot apices for *in vitro* orchid propagation (Vij and Pathak, 1990). Plant growth regulators elicit different morphogenetic responses in orchid culture. In the present study, BAP alone and BAP + NAA/IAA enhanced multiple shoot formation from the cultured nodal buds. High concentration of the auxins, however, did not prove beneficial which might be due to the presence of high endogenous auxin level in the nodes resulting in supraoptimal concentrations. As mentioned in the case of cultured shoot apices (Chapter II), a benefactory high cytokinin : auxin ratio for development of multiple shoots concurrent to the report of Yoneda (1986) was also observed with nodal explants.

Wimber (1965) opened up new vistas in orchid regeneration by producing plbs from leaf tissues. The regeneration ability of the orchid leaves, traced to the dermal layers (Loh *et al.*, 1975; Manorama *et al.*, 1984; Mathews and Rao, 1985), has been reported from orchid genera representing diverse habits and habitats (Churchill *et al.*, 1973; Loh *et al.*, 1975; Tanaka and Sakanishi, 1977; Chaturvedi and Sharma, 1986; Tanaka *et al.*, 1988; Wang, 1989). Restriction of morphogenetic potential to the leaf bases only, in the present study, is in accord with the earlier suggestions of Zimmer and Peiper (1975) that the leaf base is, generally, meristematic in monocots and upon isolation and culture it differentiates plants. Also, Abdul Karim and Hairani

(1990) have reported a greater proliferative potential of leaf base than the leaf tip in the case of Dendrobiums. Vij et al. (1984) and Vij and Pathak (1990) have, however, observed a positive response in both the apical and the basal leaf explants in different orchid genera. A direct correlation of the regeneration potential with physiological age and source of the leaf explants has also been suggested (Vij et al., 1986). Auxins and cytokinins, in combination, activate meristematic loci and/or multiplication of the resultant plbs depending upon their nature and the species employed. Zou and Qian (1987) have observed the promotory influence of 2,4-D and NAA for callus proliferation and differentiation in Orychophragmus violaceus L. An enhanced response in the seedling leaves of Rhyncostylis retusa was correlated with a synergistic action of KN and IAA/NAA in peptone enriched medium (Vij et al., 1984). A similar action of auxins was apparent during morphogenesis in Oncidium cultures (Abdul Karim and Hairani, 1990). NAA has also been effectively used with BAP in Vanda cultures (Mathews and Rao, 1985; Vij et al., 1986). Tanaka and Sakanishi (1977) recommended the utility of the combination of adenine, NAA and BAP, but, Wang (1989) indicated that adenine can be eliminated from the combination if NAA and BAP are used at lower concentrations.

Results with different growth regulators have been species specific. Enhanced germination and/or seedling growth with auxin treatment has been reported in most instances, with the inhibition cited in few cases. Though NAA has been found to

stimulate plb proliferation (Kusumoto, 1979a), shoot formation (Kusumoto, 1979b) and bud formation (Kusumoto, 1978), IAA most often has been reported to inhibit germination (Goh, 1971). Rao and Avadhani (1963) observed IAA to be ineffective for Vanda seedling formation. Also, Hadley and Harvais (1968) attributed impeded germination and protocorm elongation in Orchis purpurella to the application of IAA in the medium. Hayes (1969), however, reported an increased protocorm differentiation in Odontoglossum grande, O. schilieperianum and Miltonia spectabilis. In addition, promotion of seedling growth with IAA treatments is reported by Arditti and Ernst (1984) and Sharma and Tandon (1986). Pages (1971) observed a growth enhancement in Dendrobium protocorms when treated with IBA. On the other hand, a reduced percentage of plantlet development with IAA and NAA was indicated in Dendrobium by Miyazaki and Nagamatsu (1965). In the present study, however, promotory influences of both NAA and IBA and to a lesser extent of IAA on shoot multiplication were observed. In concurrence with the present results with 2,4-D, an inhibitory effect has been elucidated earlier by Goh (1971) in the case of Vanda cv. Miss Joaquim. Also, Kusumoto (1978) has reported development of abnormal plbs in Cymbidium when subjected to different concentrations of 2,4-D. Moreover, growth and development in Galeola septentrionalis (Nakamura, 1982), Cymbidium reginae (Harvais, 1982) and Cymbidium elegans (Sharma and Tandon, 1986) has also been influenced negatively by 2,4-D. Inconsistent reports regarding auxin effects indicate that requirements of

plants vary from species to species. Differential responses with cytokinins are well documented. BAP influenced the formation and proliferation of Cattleya aurantiaca plbs at low and high concentrations respectively (Pierik and Steegmans, 1972). However, retarded protocorm development was observed by Rucker (1974). Hadley and Harvais (1968) have reported a pronounced effect of kinetin on growth and development of O. purpurella. A stimulatory effect of the same has also been reported by Harvais (1982) and Nakamura (1982). Increased number of protocorms and shoots were indicated by Hadley (1970) and Uesato (1978) in Platanthera bifolia and Phalenopsis respectively when treated with KN. In the present study, though BAP had pronounced effects on multiple shoot development, KN proved to be inhibitory with higher concentrations resulting in browning of the cultures. Teratogenic and toxic effects of KN at high concentrations have also been suggested by Rucker (1974). Effects of exogenous GA_3 in orchid culture media vary with the species and growth stage (Arditti, 1967a,b, 1979; Arditti and Ernst, 1984; Sharma and Tandon, 1986). In general, however, the effects of GA_3 are mostly negative. In the present study too, an inhibitory effect of the same was observed with poor developoment of the regenerants.

Arditti and Ernst (1984) have given several possible reasons for the inconsistencies found in results obtained by various workers in their experiments with plant growth regulators:

1) interactions may have occurred between various combinations of hormones and the culture conditions,

2) physiological responses and requirements of species and genera may vary,
3) different forms and analogues of each hormone were used,
4) culture conditions were different in each case,
5) a wide range of dosage concentrations was used and
6) the age of the plantlets used for experimentation may have varied.

Chapter 7

In vitro preservation of protocorm-like-bodies using different slow growth approaches

Threats to biodiversity posed by the destruction of natural habitat notably by urbanization and global warming has led to an extinction of a large number of plant species (Ford-Lloyd and Jackson, 1986; Pearce, 1989; Wilkins, 1991). Preservation of any such endangered/rare plant species *in vitro* can be carried out in two steps:

1) by use of micropropagation technique for rapid mass propagation and,

2) by preservation of germplasm either using slow growth approaches (limiting the multiplication rate) or by cryo-exposure (arresting the metabolic activities).

Plant tissues cultured *in vitro* are generally maintained by periodically transferring them to fresh medium. However, genetic, physiological or biochemical variations are often observed during serial subculture (Withers, 1991). To diminish such variations and to save expense and time for routine culture maintenance, preservation of germplasm in the inactive state has been

emphasized (Henshaw, 1975; Withers, 1991). Though freeze-preservation has been widely studied, it is not yet applicable to many plant species. Hence, extensive investigation has been carried out over the years into increasing or decreasing culture medium components and modifying the physical environment to reduce growth rates (Withers, 1992). A common approach is to reduce the temperature at which cultures are maintained. Banerjee and Langhe (1985) reported a temperature of 15°C to be optimal for the storage of Musa species. On the other hand, a temperature of 9°C was reported optimal to store Colocasia esculenta shoot cultures (Zandvoort and Staritsky, 1986). Slow growth storage has also been reported in Dioscorea species (Ng and Hahn, 1985), Eucalyptus species (Aitken-Christie and Singh, 1987) and Pyrus species (Wanas et al., 1986; Wilkins et al., 1988) by a reduction in incubation temperature. Besides, limitation of growth has been achieved by using osmotic inhibitors (Espinoza et al., 1984; Ng and Hahn, 1985; Staritsky et al., 1986; Love et al., 1987), natural or synthetic hormonal inhibitors (Mix, 1982, 1985; Ng and Ng, 1991; Wilkins, 1991), mineral oil overlay (Druart, 1985; Moriguchi et al., 1988; Crane and Hughes, 1990; Mathur et al., 1991), reduced oxygen tension (Bridgen and Staby, 1981; Engelmann, 1990) and defoliation of shoots (Withers, 1987). Singh (1988) used organic solvents for the preservation of orchid seeds and reported petroleum ether to store Spathoglottis plicata 'Alba' seeds effectively for a considerable period. Recent technique of artificial seed production has opened up new

possibilities for preservation by storing the encapsulated tissues at low temperature (Bapat and Rao, 1988). Artificial seeds, consisting of somatic embryos enclosed in a protective coating have been proposed as a low-cost, high-volume propagation system (Redenbaugh et al., 1986). These provide a potential method to deliver plant material produced from tissue culture directly to the greenhouse or field. The direct planting of the somatic embryos into soil, thus, bypasses in vitro plant production steps that require high labour effort and expense (Fujii et al., 1989). Insufficient, and inconsistent information for different slow growth approaches, however, has made it difficult to recommend any one method suitable for a group of plants. In an attempt to preserve the germplasm of threatened and endangered D. wardianum, different approaches to slow growth storage viz., reduced temperature, osmotic inhibitor, and natural hormone inhibitor were used.

Materials and Methods

Effect of reduced temperature

Plbs obtained from excised shoot apices (as described in Chapter II) were inoculated on MS medium and the cultures incubated at -10, 0 and 4^{o}C (in the dark). Control was maintained at 24$\pm$2^{o}C (in the light). Cultures stored at low temperatures were transferred to room temperature (RT, 24$\pm$2^{o}C every 7 days for 3

weeks and the data collected on the plb survival, growth and development. Ten replicates per treatment were taken and the experiment were repeated twice.

For artificial seed production, sodium alginate (Sigma, 2-5% w/v) and calcium chloride (50-150 mM) solutions were prepared separately in growth regulator free - MS liquid medium containing 3% (w/v) sucrose. Plbs, mixed with sodium alginate solution and dropped singly in calcium chloride using a wide mouth pipette, were left in the solution for 40 min. on gyratory shaker (75 rpm) to form round, firm beads (a result of ion exchange reaction between Na^{+} and Ca^{+2} ions). The beads were then, recovered by decanting the calcium chloride solution and washed thrice with MS liquid medium. These were plated on MS medium devoid of growth regulators for further growth and developmental studies. Besides, both encapsulated and non-encapsulated plbs were stored at $4^{o}C$ in the dark. After storage, the 'synthetic seeds' were tested for their regeneration potential at 15 day interval up to day 180. Twenty replicates per treatment were taken and the experiment repeated twice.

Effect of mannitol and abscissic acid

Plbs were inoculated on MS medium supplemented with mannitol (MOH), an osmotic inhibitor and abscissic acid (ABA), hormonal growth retardant at a concentration range of 0-15 % (w/v) and 0-5 mg/l respectively to study their potentialities for slow growth storage. Data were collected every 30 days for 3 months. Ten

replicates per treatment were taken and the experiment repeated twice.

The pH of the MS medium and other solutions was adjusted to 5.8 prior to autoclaving at 121^{o}C for 20 min. and the cultures incubated at 24±2^{o}C with a 16 h photoperiod at an illumination of 2000 lux from cool, white, fluorescent light.

Results

Effect of reduced temperature

Cultures incubated at different low temperatures showed different responses (Table 7). At temperatures 4^{o}C and below, the plbs inoculated were found to turn pale green in comparision to the bright green plbs at 24±2^{o}C.

Observation after 7 days- Cultures incubated at 0 and 4^{o}C turned green with a percentage survival of 20 and 30 respectively. At RT, a 20% plb differentiation was recorded, however, plbs maintained at -10 ^{o}C lost colour and eventually died.

Observation after 14 days - Out of the 10 plbs incubated at 0^{o}C initially, only one survived while all others lost their green colour. Twenty percent of the initial 30% surviving plbs incubated at 4^{o}C, showed slight differentiation with 10% remaining pale green. Initiation of differentiation was also observed in 70% cultures incubated at RT.

Observation after 21 days - Ninety percent of the plbs

Table 7 Percentage survival and/or differentiation of plbs after storage at low temperature in the dark

Day	7	14	21
Temp.(°C)			
24+2 (control)	100(20)	100(70)	100(90)
-10	0	0	0
0	20	10	10
4	30	30(20)	(30)

Figures in parentheses indicate differentiating plbs.

maintained at RT and 30% of those initially incubated at 4°C were in different stages of differentiation. Thus, of all the temperature treatments, plbs could be preserved for a week at 4°C with a percentage survival of 30.

Plbs stored for 14 and 21 days at reduced temperature in the dark did not survive.

Different concentrations of sodium alginate (2-5%) and calcium chloride (50-150 mM) had profound effect on bead quality (Table 8). A three percent sodium alginate solution on complexation with 100 and 150 mM of calcium chloride for 40 min. produced firm, round beads. Lower (2%) and higher (5%) concentrations of sodium alginate resulted in fragile, difficult to handle and too viscous beads respectively. Lower concentrations of calcium chloride prolonged the complexation time and also affected firmness of the beads. Hence, encapsulation of plbs for all subsequent investigations was done using 3% sodium alginate and 100 mM calcium chloride.

Encapsulated plbs (synthetic seeds) could be germinated in vitro and the conversion frequency was 100% on MS medium after 45 days (Fig. 24), however, a 60% conversion was recorded in case of non-encapsulated plbs. Ninety percent of the 'synthetic seeds', incubated at 4°C for 15 days in the dark were capable of germinating compared to non-encapsulated plbs which did not germinate. The synthetic seeds remained viable and germinated successfully up to day 120 though germination percentage was reduced to 30 which further declined to 10% by day 180 (Table 9).

Table 8 Effect of different concentrations of calcium chloride and sodium alginate on bead quality*

Sodium alginate (%)	Calcium chloride (mM) 50	100	150
	Bead quality (shape, firmness)		
2	Fragile, difficult to handle	Fragile, of irregular shape	Fragile, difficult to handle and of irregular shape
3	Fragile, of irregular shape	Firm, round beads	Firm, round beads
5	Viscous, irregular shape	Very viscous with irregular shape	Very viscous and of irregular shape

* Complexation allowed to take place for 40 mins.

Table 9 Conversion frequency of the encapsulated plbs stored at $4^{o}C$*

No.of days	Percentage conversion	Time required to germinate** (days)
15	90	20
30	80	20
45	80	25
60	50	30
90	30	30
120	30	40
180	10	60

* Each batch consisted of 20 plbs stored on sterile moist cotton pads in petridishes.
** Conversion on MS + BAP (2.5 mg/l).
Stored non-encapsulated plbs did not germinate.

Fig. 24(a-c) Regeneration of complete plantlets from 'synthetic seeds'

a) germinating 'synthetic seeds' (after 4 weeks)
b) development of regenerants from 'synthetic seeds' (after 8 weeks)
c) well developed rooted shoots (after 12 weeks).

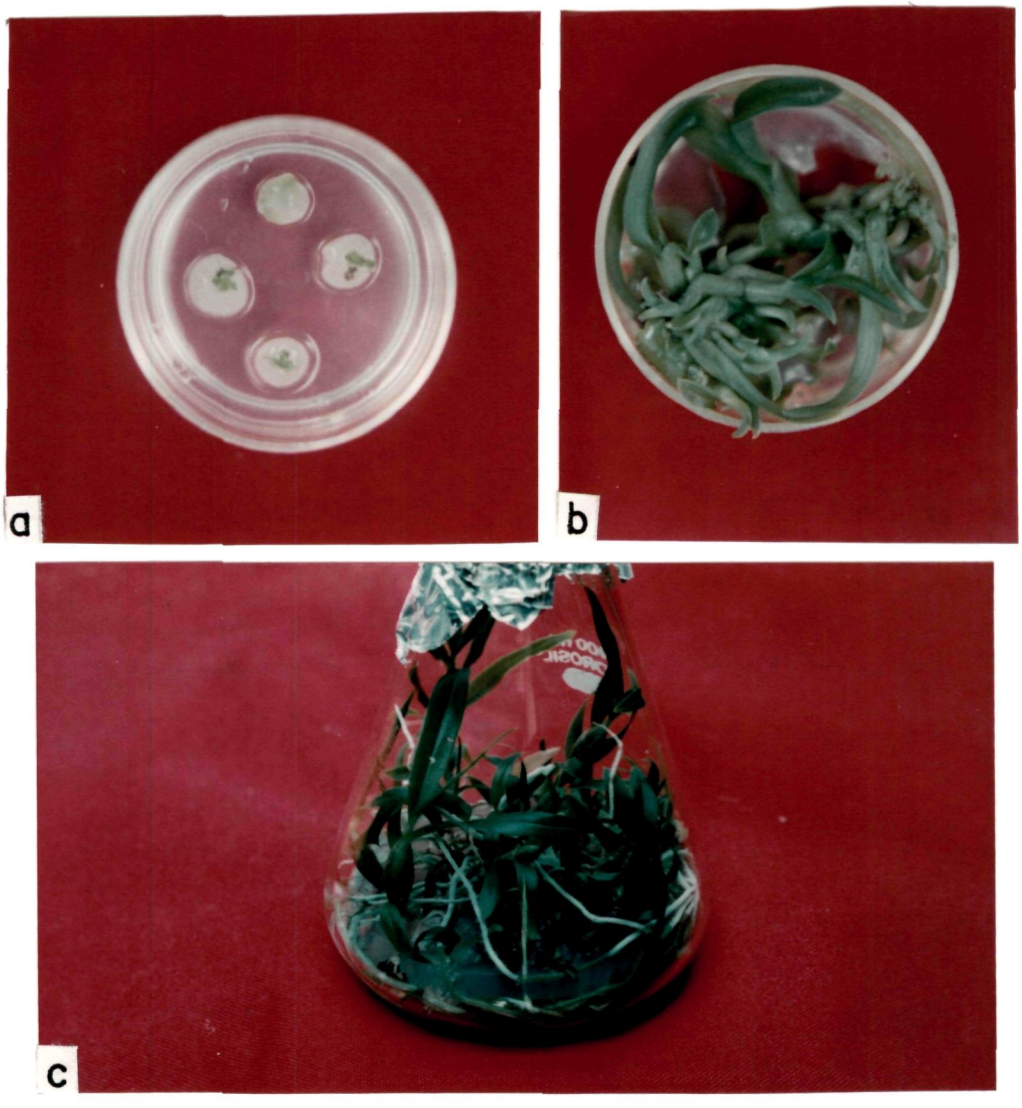


Fig. 24

Effect of mannitol and abscissic acid

Mannitol was observed to suppress the plb growth effectively (Fig. 25, 26a-e). Percentage growth suspension of plbs treated with 10% MOH was 44 after 90 days. Using 7.5% MOH, 38% of the plbs showed growth suppression whereas 25% plbs were in the proliferating stage and 12% were fully differentiated. Lower MOH concentrations (2.5 and 5%) could suspend the growth of plbs in 19% cultures after 90 days of incubation. Control (plbs inoculated on MS free of MOH) showed a 100% plb conversion to well developed rooted shoots at the end of 90 days. However, a steady increase in the drying up of the explants was observed with an increase in MOH concentration.

Varied responses were observed when plbs were subjected to ABA (Figs. 27, 28a-e). Plbs treated with ABA (2 mg/l) showed maximum growth reduction in 35% cultures with no visible differentiation even after 90 days of incubation period. Lower ABA concentrations (0.1 and 0.5 mg/l), however, were not found to be effective in suppressing the plb growth and differentiation (Fig. 28a-e). Higher ABA concentration (5 mg/l), on the other hand, could suppress the growth in 9% of the cultures only with 91% of the cultures turning brown.

Regeneration after slow growth treatment

The plbs treated with growth suppressing concentrations of both MOH and ABA, when subcultured on MS medium devoid of either

Fig. 25 Effect of different concentrations of mannitol (0 - 12.5%) in the medium on growth suppression of plbs (after 90 days).

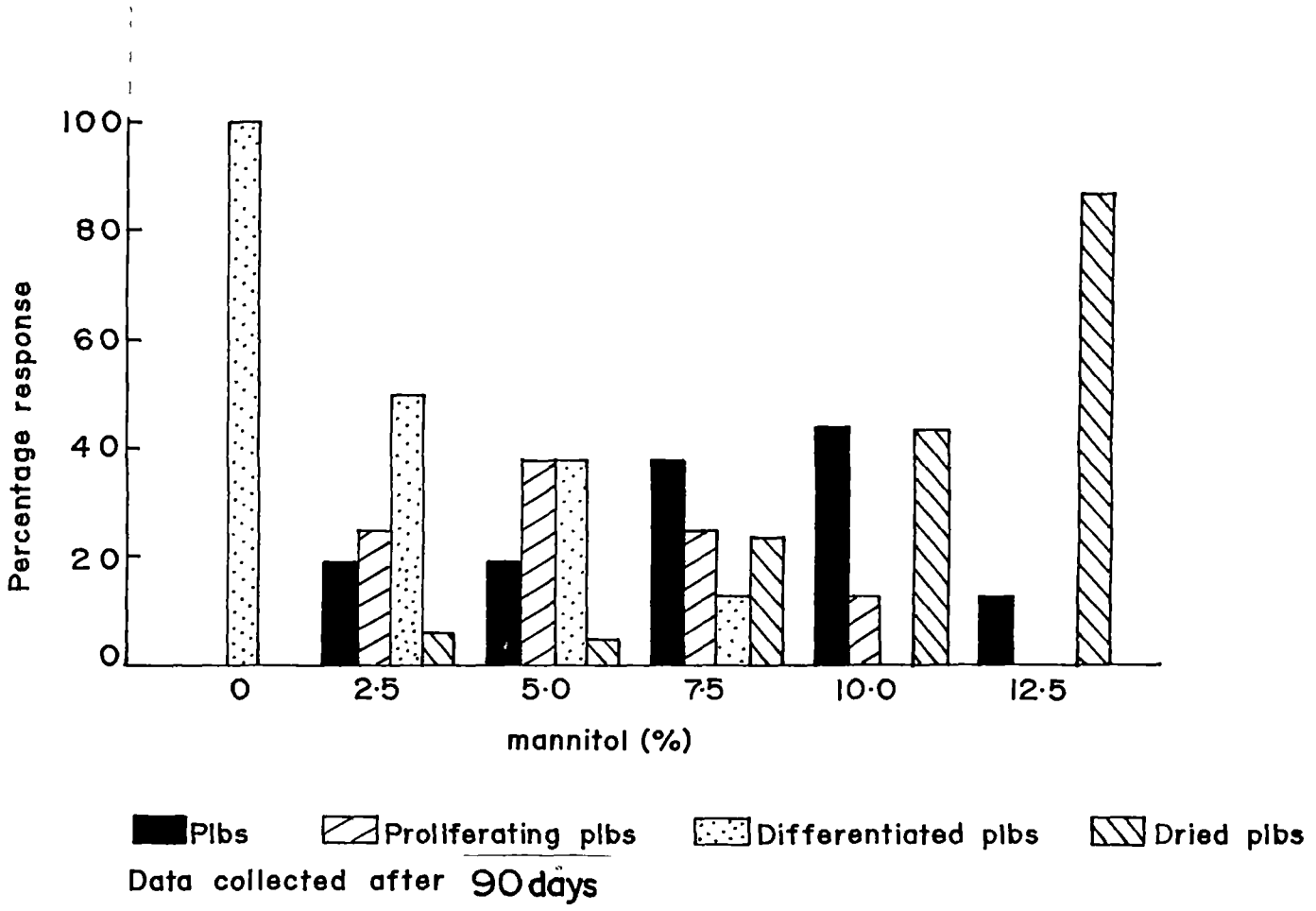

Percentage response
100
80
60
40
20
0
0
2·5
5·0
7·5
10·0
12·5
mannitol (%)
Plbs
Proliferating plbs
Differentiated plbs
Dried plbs
Data collected after 90 days

Fig.25

Fig. 26(a-e) Plb growth suppression after 90 days of culture in MS medium containing different concentrations of mannitol, a) control, b) 2.5%, c) 5.0%, d) 7.5%, and e) 10%.

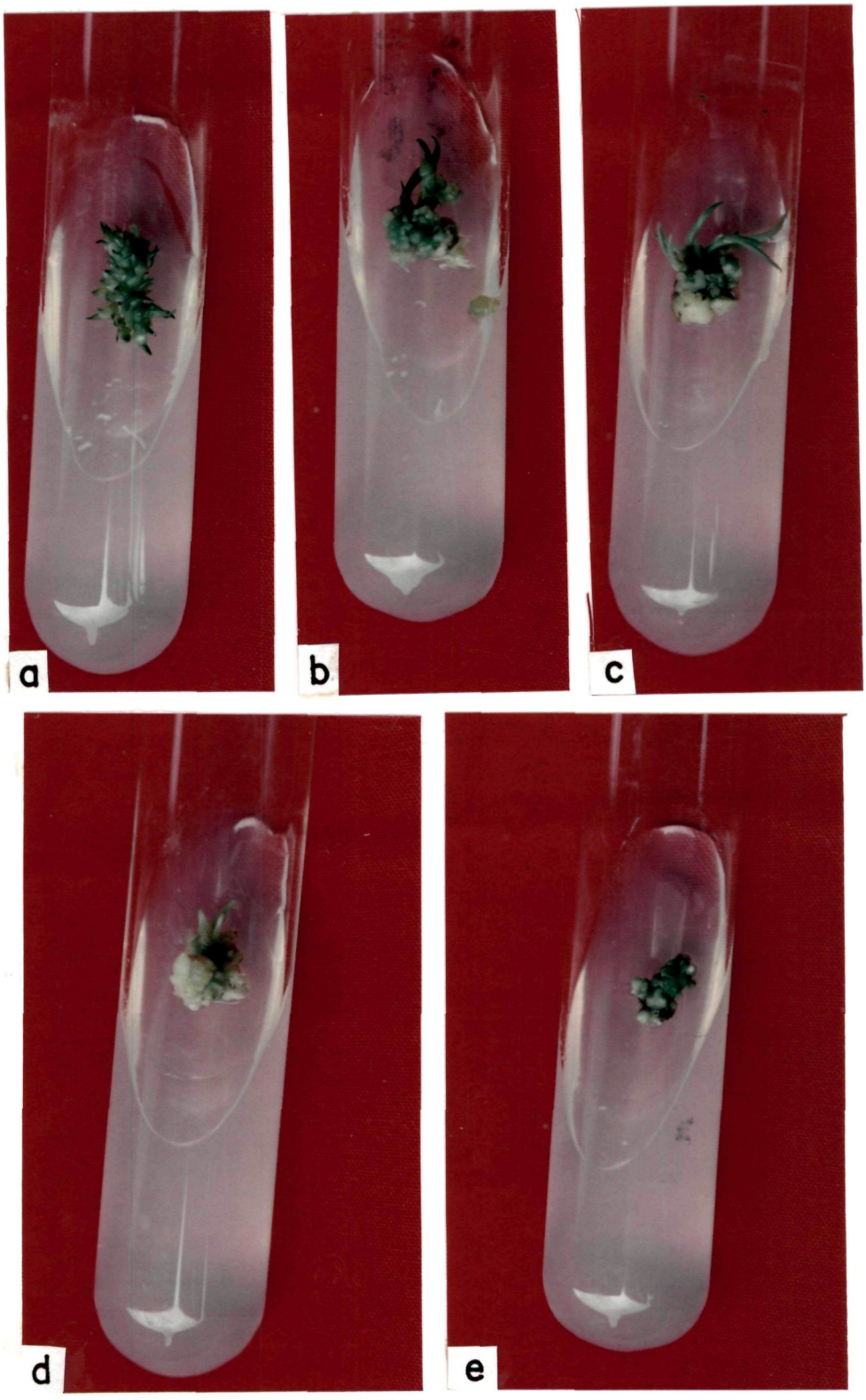


Fig. 26

Fig. 28(a-e) Growth suppression of plbs in MS medium containing different concentrations of ABA after 90 days of culture, a)control, b) 0.1 mg/l, c) 0.5 mg/l, d) 1.0 mg/l and, e) 2.0 mg/l.

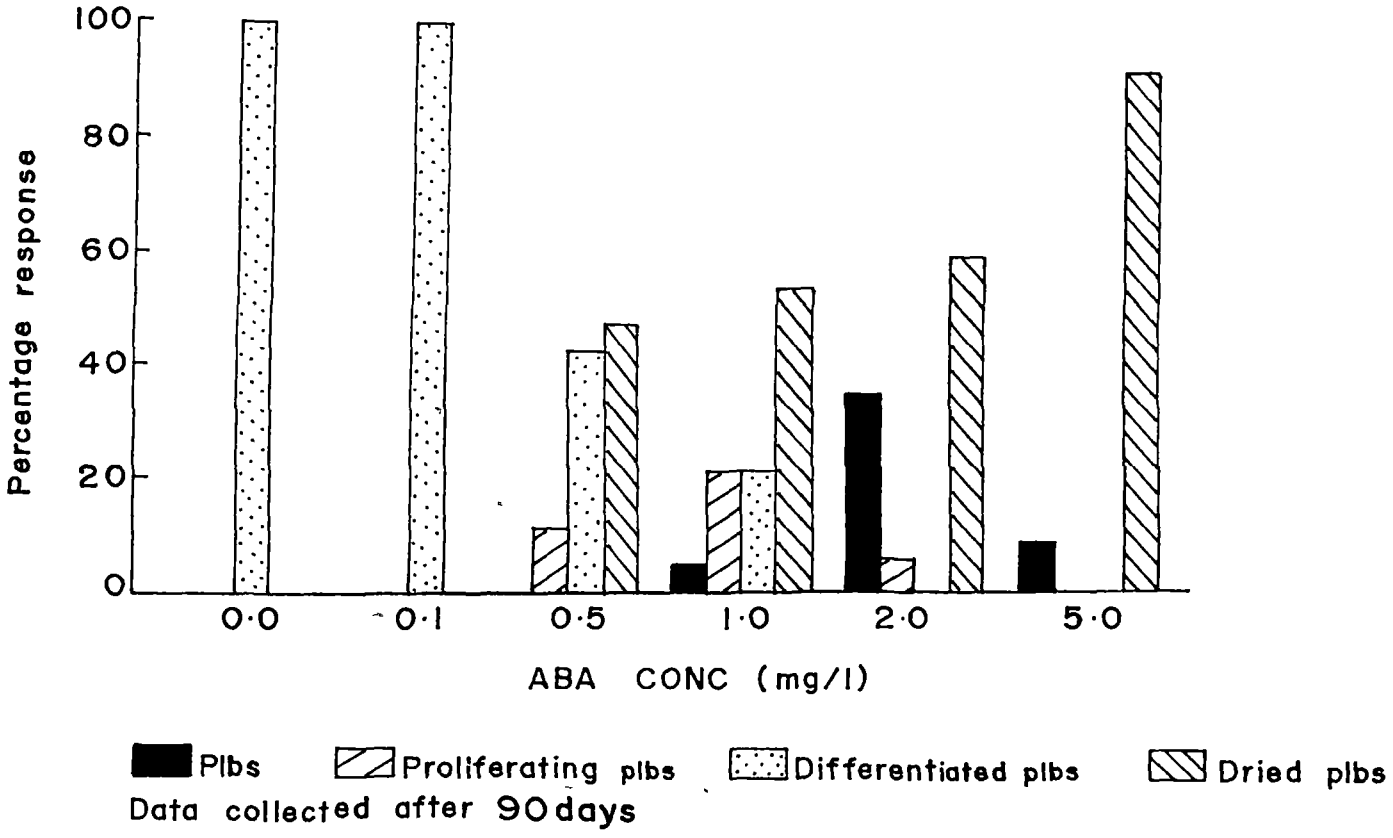

Percentage response
100
80
60
40
20
0
0·0
0·1
0·5
1·0
2·0
5·0
ABA CONC (mg/l)
Plbs
Proliferating plbs
Differentiated plbs
Dried plbs
Data collected after 90 days

Fig.27

Fig. 27 Effect of different concentrations of ABA (0 - 5 mg/l) in the medium on growth suppression of plbs (after 90 days).

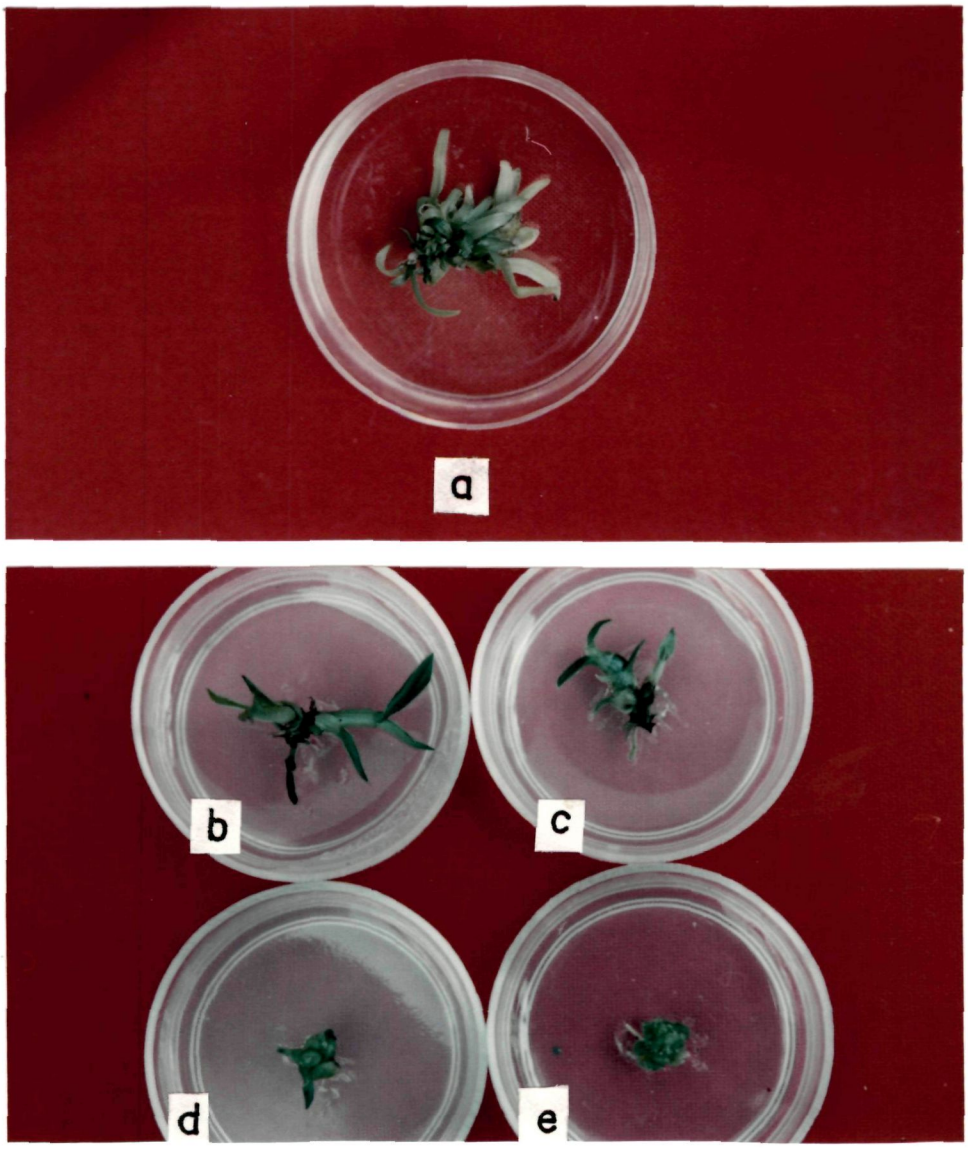


Fig. 28

of the two growth retardants, developed into complete plantlets in 12 - 16 weeks (Fig. 29a,b). The plants look healthy and similar to the mother plants with no observable phenotypic change.

Discussion

In vitro conservation refers to maintenance of germplasm in a relatively stable form under more or less defined nutrient conditions in an artificial environment. The major aim in developing in vitro storage method is to reduce the frequent demands of subculturing and preserve the unique genetic constitution of germplasm. The most successful and widely applied approach to reduce the rate of growth and thereby extend the interval between transfer to fresh medium for convenient storage involves reducing the growth temperature (Smith et al., 1982; Monette, 1986; Aitken-Christie and Singh, 1987; Evers et al., 1988; Wilkins et al., 1988; Arora and Bhojwani, 1989; Withers, 1991). Moriguchi et al. (1988) reported that moderate depression of growth, which is important for minimum growth during the storage period could be obtained by reduction of the temperature. Cultures have been stored at reduced temperatures in the dark (Cheyne and Dale, 1980; Marino et al., 1985; Arora and Bhojwani, 1989) as well as under reduced light conditions (Dale, 1980; Miedema, 1982; Banerjee and De Langhe, 1985; Evers et al., 1988; Roca, 1990; Schoofs, 1990). In the present study, however,

Fig. 29(a,b) Plantlet development from plbs treated with growth retardants

a) after 12 weeks of transfer to MS hormone-free medium

b) complete rooted shoots (after 16 weeks).

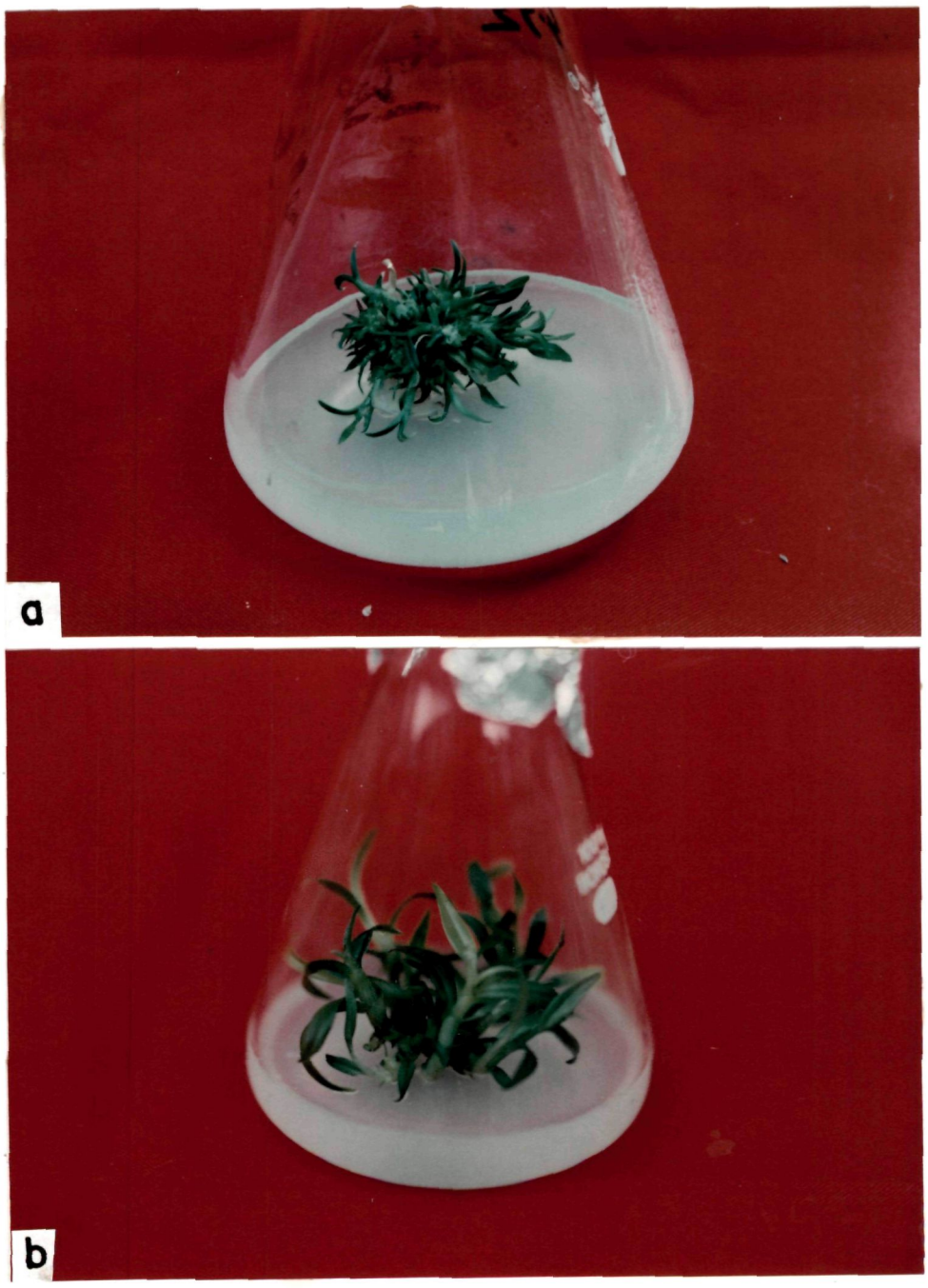


Fig. 29

plbs could be preserved only for a week in the dark at $4^{o}C$ with lower temperatures resulting in the death of the cultures. Poor performance in terms of growth rate and secondary product synthesis in certain cases after low temperature treatment has been reported earlier by Hiraoka and Kodama (1984) and Withers (1986).

Production of 'synthetic seeds' by encapsulating regenerative tissues has been reported in a large number of plant species (Kitto and Janick, 1985; Redenbaugh et al., 1986; Bapat et al., 1987; Bapat and Rao, 1988; Mathur et al., 1989; Senaratna et al., 1990; Fernandez et al., 1992). However, very few reports on 'synthetic seed' production in the case of orchids have been made (Sharma and Tandon, 1992; Corrie and Tandon, 1993) which produce plbs in culture, thus offering most potential method of clonal propagation. Highly heterozygous nature of orchids and low seed viability under natural conditions emphasize the need for development of the technique for storing of the germplasm for a considerable period. In the present study, high germination percentage in case of encapsulated plbs than non-encapsulated plbs could be due to the matrix that not only facilitates regular nutrient supply but also protects the dispersed, delicate tissue from mechanical injury during handling and from dessication. The ability of encapsulated plbs to develop into complete plantlets was directly proportional to the quality of the plb. Tissue culture techniques for clonal propagation of plants that have a high per unit value, like ornamentals and fruits are of great

significance. High volume propagation potential combined with formation of 'synthetic seeds' for low cost delivery is expected to open new vistas. Efforts have been intensified in this area by encapsulating somatic embryos (Redenbaugh, 1990) and axillary buds (Bapat et al., 1987; Bapat and Rao, 1988) in various matrices and growing them on different substrata. A significant outcome of the present study was the observation that the synthetic seeds retained their viability even after 120 days at 4°C and the plants recovered also maintained their clonal identity as there were no morphological differences observed among the regenerants.

The sugar alcohol, mannitol is most widely employed as pre-growth media additive for preservation studies (Withers and King, 1980; Espinoza et al., 1986; Ng and Hahn, 1985; Pritchard et al., 1986; Love et al., 1987). However, variations exist between plants in their physiological and structural responses to osmotically active compounds. Pritchard et al. (1986) reported growth rate reductions and cell wall thinning in Sycamore and Soyabean cells following osmotic stress but emphasized that cells display a different capacity for osmotic adjustment and alteration in their cytoplasmic complement. Mannitol (10%) was found to be optimum, in the present study, for the suppression of plb growth followed by a concentration of 7.5%. Dehydration of the plbs at these concentrations seem to be at a critical level, thus resulting in the suspension of growth. Espinoza et al. (1986) have also reported that mannitol in the medium exerts an

osmotic stress that leads to reduction in the growth rate at 25°C. For successful freeze-hardening of maize embryos, a high concentration of osmoticum (mannitol) was reported to give an increased survival rate (Delvallee et al., 1989). Further, it was observed that the culture of excised embryos at high osmoticum induced water stress thus achieving freezing tolerance by low water content. However, a very high osmoticum resulted in a decrease in embryo survival thereby emphasizing that the water stress, apart from the protective action also had a detrimental effect.

The inhibitory effect of ABA on the growth of roots, shoots and leaves has been reported in many plant systems (Bornmann, 1983; Davies et al., 1990). Wakabayashi et al. (1991) suggested that suppression of growth by ABA application could be the result of inhibition of cell elongation as well as cell wall synthesis (in particular, the synthesis of cellulose and hemi-cellulose polysaccharhides) and that ABA-mediated inhibition of cell wall synthesis occurs prior to the suppression of growth of the explants. ABA concentration, in our study, seems to affect the plb responses greatly at 1.0 and 2.0 mg/l suppressing the growth from 9 to 35% cultures. Higher ABA concentration increases the cytotoxicity possibly, with maximum cultures turning brown by the end of 90 days. Fujii et al. (1989) have shown ABA-induced arrest in the germination of alfa alfa somatic embryos. They have reported that an arrest in the germination of the somatic embryos using ABA leads to their maturation which is beneficial for

direct germination of the same under non-sterile soil conditions. Further, they suggest that the increased conversion frequency of somatic embryos in soil might be due to ABA promoted storage of carbohydrates, proteins, and other nutrients within the embryo. Slow growth storage methods that are reproducible and are widely applicable among different crops and among genotypes of a crop could be used for the conservation of the germplasm especially the rare and endangered species (Withers, 1991). However, germplasm storage techniques developed should reduce the detrimental effects of growth inhibiting treatment while retaining the desired retardation of growth. Very few reports on slow growth storage of orchids in vitro have been made so far. Dubus (1980a,b) reported preservation of Cymbidium protocorms by increasing the sucrose concentration and maintaining the cultures at low temperature. The present studies suggest effective suppressio plb growth by mannitol, ABA and also by storage of encapsulated plbs at low temperature.

Chapter 8

Hardening, transfer and establishment of clonally propagated plantlets

Plantlets which are cultured *in vitro* on agar-based media, in a water-saturated atmosphere, wilt rapidly on transfer to normal greenhouse or field conditions. Poor water uptake and excessive water loss (Grout and Aston, 1977) may lead to high rates of mortality unless plantlets are acclimatized by gradual stages to reduced humidity and increased light intensity (George and Sherrington, 1984). The problems of poor water relations are compounded by damage to the fragile shoots and roots during transplantation (Debergh and Maene, 1981). Thus, the transfer of *in vitro* raised plantlets to community pots or field conditions need a careful, step-wise procedure for better survival. General principles for orchid transfer and maintenance are more or less same, but each genus and often each species in a genus is reported to have its special requirements (Mukherjee, 1983). Bhojwani and Razdan (1983) have stressed high humidity conditions during the initial days for successful transplantation. Light requirements of early growth of orchid

plantlets are reported to vary, though, under natural conditions they usually grow in semi-shade. Besides, temperature is a major determining factor for the in vivo growth and development of the orchid transplants. In general, a range of 18.3 to 29.4°C is reported to be optimum for orchid growth (Mukherjee, 1983). Robbins (1989) suggested a minimum (17°C) and maximum (30°C) temperature for better establishment of Phragmipedium hesseae. On the other hand, 18°C and 25°C were found suitable for Dendrobium alexandrae (Cribb, 1990).

Type of composts and pots play an important role in the survival rate of transplants of orchids. Clay pots are better than the glazed ones as there is sufficient aeration of roots and compost in the former. Moreover, good drainage is essential for the luxuriant growth of the plants which can easily be achieved in the clay pots. Besides pots, baskets or cradles, wooden logs and tree-fern blocks have also been used as orchid containers (Hegde, 1984).

Orchids are grown on a variety of media depending on their terrestrial or epiphytic nature. Osmunda fibre consisting of the intricate root system of two types of ferns of the genus Osmunda is generally used for epiphytes, however, majority of orchid plantlets can be conveniently grown on a mixed compost of osmunda and sphagnum moss. Mosses can retain sufficient amount of water for a long period, thus being an ideal medium for plants that require a constant supply of moisture. A number of different combinations of containers and composts have been used by various

workers (Rao *et al.*, 1979; Rao and Mohanan, 1983). Meyer (1951) used tree fern fibres as the compost. Also, bark preparations obtained from the shredded and/or chopped bark of a variety of conifers like Douglas fir, white fir and several types of pines and cedars have been used (Stewart, 1988; Cribb, 1990; Robbins and Bell, 1990). More watering is required for composts of bark preparation due to increased drainage. A disadvantage of bark preparation is its rapid deterioration necessitating a frequent change. Right stage of transplants, suitable compost, containers and other physical factors affect the survival rate of plants. In the present chapter, successful hardening of the *D. wardianum* regenerants, their transfer and establishment under glasshouse conditions has been accomplished.

Material and Methods

Plantlets regenerated *in vitro* using the experimental protocol as described in Chapter VI were used for the transplantation studies. Plastic (diameter 5 cm) and clay (diameter 10 cm) pots were used for the present study. Minute holes were pierced into plastic pots using a hot needle. Both pots and crocks were thoroughly washed before use. They were then filled 3/4th with different compost combinations and watered for planting convenience. Potting mixtures used for the present study were -

i) charcoal chunks and brick pieces (1:1)

ii) charcoal chunks, brick pieces and coconut fibres (1:1:1/2)

iii) charcoal chunks, brick pieces and a layer of moss at the surface (1:1:1/2)

iv) sand covered with a layer of moss

v) vermiculite covered with a layer of moss

Well developed rooted shoots measuring 3-5 cm in height were carefully removed from the culture vessels by means of a spatula. Agar adhering to the roots was removed to the last trace and care taken to avoid any damage. Plantlets were now washed, picked up with the help of forceps and the roots immersed in 0.1% diethane solution to minimise the fungal infection. Plants were then carefully placed into the crevices of the compost and covered with the moss if required, in the plastic pots. Plants were fed with 1/10 MS nutrient salt liquid medium every alternate day for the first two weeks, every third day during the third week and once a week thereafter. For the second experiment, plantlets transferred to a potting mix of charcoal chunks, brick pieces and moss were fed with water or liquid MS nutrient salt medium (strength ranging from 1/2 to 1/10th) in a pattern similar to that in previous experiment. After 16 weeks of growth in the plastic pots, the plantlets were carefully transferred to clay pots. The average minimum and maximum temperatures of the glasshouse at the time of transplantation were 17^{o}C and 23^{o}C. The relative humidity was around 70 -80%. In order to maintain high humidity in the glasshouse, mist was produced by using humidifier.

Results

Of the various compost combinations used, charcoal pieces, brick bats and moss was found to be the best substratum for survival and healthy growth of the plantlets (Table 10 ; Figs. 30 a-c). Though use of charcoal and brickbats alone was found satisfactory, the presence of moss indeed made a difference. Vermiculite and moss supported good growth in the initial days, however, only 10% plants survived at the end of the 4th week. Compost comprising charcoal chunks, brick bats and coconut fibres or sand and moss could not support the healthy growth of the regenerants. Transplants growing in both, plastic as well as earthen pots showed healthy growth.

The diluted liquid MS medium supported better growth of the transplants (Table 11). Optimum survival of 70% was observed when plants growing on a compost of charcoal chunks, brick bats and moss were fed with MS 1/10th strength liquid nutrient salt medium. The plantlets developed were dark green and new leaves were formed by thesecond week. Higher strength of MS liquid medium did not prove beneficial.

Discussion

The deflasked regenerants are often plunged directly into a fungicide solution before being planted in community pots and the practice is believed to prevent damping off (Sessler, 1978). At times, however, fungicide solutions are harmful and the treated

Table 10 Responses of *in vitro* grown plantlets to different potting substrates*

Substrate used	% Survival	Growth
i) Charcoal chunks + brick pieces	50	+
ii) Charcoal chunks + brick pieces + coconut fibres	30	-
iii) Charcoal chunks + brick pieces + moss	70	++
iv) Charcoal chunks + moss + vermiculite	10	-
v) Charcoal chunks + moss + soil	10	-

- Poor growth; + fair growth; ++ good growth

* Data collected after 4 weeks of transfer, based on 20 plantlets transferred per substratum.

Table 11 Growth response of potted plants fed with different strengths of MS liquid medium*

Nutrient feed	% Survival	Growth
i) MS	5	-
ii) MS 1/2 strength	10	-
iii) MS 1/4 strength	30	-
iv) MS 1/8 strength	50	+
v) MS 1/10 strength	70	++
vi) MS 1/12 strength	60	++

\- Poor growth; + fair growth; ++ good growth
* Data collected after 4 weeks of transfer, based on 20 plantlets per treatment.

Fig. 30(a-c) Plantlets transferred to pots containing charcoal pieces + brick bats + moss after

a) 12 weeks
b) 16 weeks and
c) 20 weeks.

Fig. 30

a

b

c

plantlets remain permanently stunted (Kang, 1979). Successful transplantation also depends on suitable size of the plants and their state of growth *in vitro*. Hence, regenerants showing vigorous *in vitro* growth were selected for the present study. Easy transplantation and less susceptibility to diseases and mechanical injury of hardy plantlets is a well established fact. The culture of plantlets *in vitro* at reduced humidity has been shown to improve the ability of stomata to respond to stimuli that induce closure in *Chrysanthemum* (Wardle *et al.*, 1983 ; Short *et al.*, 1987), and carnation (Ziv *et al.*, 1983), increase the deposition of epicuticular wax on the leaves of *Iris germanica* (Maene and Debergh, 1986), and cauliflower (Wardle *et al.*, 1983; Short *et al.*, 1987) and reduce wilting after transfer to soil in *Calathea ornata* (Maene and Debergh, 1986), chrysanthemum and cauliflower (Short *et al.*, 1987) and carnation (Ziv *et al.*, 1983). Methods that have been used to reduce humidity in the culture vessel include the use of desiccants (Wardle *et al.*, 1983; Ziv *et al.*, 1983; Short *et al.*, 1987), increasing the concentration of agar in the culture medium (Ziv *et al.*, 1983), the use of culture vessels with porous closures (Short *et al.*, 1987) and cooling the bottom of the culture vessel (Maene and Debergh, 1986). Smith *et al.* (1990) reported that inclusion of paclobutrazol in the culture medium gave effects similar to those obtained by reduction of humidity (though the mechanisms may differ) thus influencing the rate of survival. Plant growth retardants generally induce a shortening of the internodes of

higher plants *in vivo* and some have additional effects such as a reduction in leaf size, an intensification of the green colouration of leaves and a thickening of roots (Graebe, 1987). A number of workers, thus, using different techniques have tried for increased survival of transplants.

Among the most commonly used materials for potting orchid plantlets are fir and redwood bark, pieces of cork bark, tree fern, osmunda fern root, sphagnum moss, hardwood charcoal, coarse perlite, gravel and coconut fibre (Fitch, 1981). In the present study, charcoal chunks, brick pieces and moss formed the best substratum for the plantlet growth which could be due to proper drainage and aeration for root respiration. Layer of moss at the surface might have facilitated high retention of moisture content. Feeding the plantlets with 1/10th MS nutrient salt solution gave highest (70%) survivability. The promotory effect of low nutrient salt solutions on the growth and development of orchid plantlets has been reported by various researchers (Sheehan, 1960; Sander, 1969, 1979; Mukherjee, 1983). Low survival rates of transplants fed with high nutrient salt media, in the present study, show low nutrient requirements of *D. wardianum*. Besides, the temperature and humidity conditions of the glasshouse were most suitable for vigorous growth and development of orchid plantlets.

Summary

SUMMARY

Plbs obtained from culturing shoot apices excised from *in vivo* growing *Dendrobium wardianum* Warner plants on Murashige and Skoog (MS) medium supplemented with 0.3% (w/v) sucrose, 0.8% (w/v) agar and 2.5 mg/l BAP were transferred to different media viz., Nitsch & Nitsch (N & N), Knudson C (KC), Mitra et al, Gamborg et al. (B_5), modified Vacin and Went (VW + 15% CW) and MS to study their effects on the growth and development of the regenerants. For assessing the growth, shoot number-length, root number-length, fresh and dry weights were measured at 30 day intervals for 90 days. Plantlets showed optimum growth with respect to shoot number-length and fresh weight on MS medium followed by VW. Highest dry weight was observed in plantlets growing on B_5 and highest root number-length of the regenerants were observed to be in Mitra *et al*. medium. Plantlets growing on KC medium performed poorly.

Besides the nutritional factors, effects of physical factors like light (light intensity and photoperiod), temperature and pH

were also studied on the growth and development of regenerants. Optimum growth was recorded at 2000 lux light intensity followed by 3000 lux. Higher light intensity, however, was detrimental. Photoperiod from 8 to 16 hr showed promotory effect on shoot/root growth and fresh and dry weights. On the other hand, total darkness and continued illumination were inhibitory. At 25^{o}C, growth of the regenerants was optimum. Medium with pH 6.0 was suitable for plantlet growth and development.

Morphogenetic effects of some nitrogenous adjuvants were studied by culturing shoot apices excised from *in vitro* grown plantlets on MS medium supplemented with inorganic and organic nitrogenous sources, *viz.*, calcium nitrate, ammonium sulphate, urea and amino acids (alanine, glycine, glutamine, serine and leucine). Calcium nitrate (0.5, 2.5 mg/l) and urea (2.5 mg/l) resulted in direct multiple shooting besides forming plbs. Explants inoculated on ammonium sulphate supplemented MS medium resulted in poor percentage of plb formation at higher concentrations. Addition of individual amino acids did not show any significant effect.

Nitrogen metabolism enzyme activities as influenced by various growth regulators during plb growth and differentiation were carried out. Soluble proteins were also determined in the tissue extract. Besides, the tissues were subjected to PAGE to localize the isozymes of soluble proteins. The activity of nitrate reductase in plbs treated with IBA and NAA increased from zero day to day 15, however, an increase in the activity till day

30 was observed in BAP treated plbs at all concentrations. IAA treated plbs, on the other hand, showed a decline in the nitrate reductase activity from day zero to 15th day, a slight increase till day 30 and a decrease thereafter. Control showed a trend parallel to IAA treated plbs. Nitrite reductase activity was found to be higher to that of nitrate reductase activity in plbs treated with different growth regulators at all concentrations. Profiles of glutamate dehydrogenase activity were different from those of nitrate and nitrite reductase activities. Of the four growth regulators studied, plbs treated with IBA and BAP at concentrations of 1.0 and 2.5 mg/l respectively showed an increase in activity from zero day to the 15th day and declined thereafter till the end of the study. However, a decline from zero day onwards that continued up to the 45th day was observed in plbs treated with IAA, NAA and the rest of the IBA and BAP concentrations. An increase in the glutamine synthetase activity was recorded in plbs treated with all BAP concentrations till day 15 followed by a decline. Activity increase was also observed in plbs treated with IBA and NAA at 1.0 mg/l each. IAA treated plbs showed a decline in activity from zero day up to the end of the study. The activity of glutamate synthase was recorded to be highest on 15th day in plbs treated with 1.0 mg/l IBA followed by 2.5 mg/l BAP treated plbs. An increase in activity was also recorded from day zero till day 15 in NAA treated plbs and in 0.5 mg/l IAA treated plbs. The protein profile of plbs grown in control or treated conditions showed an increase from zero day to

the end of the study. Determination of the electrophoretic profile for proteins revealed the presence of five major bands in all the growth regulator treated and control plbs.

Clonal propagation of *D. wardianum* was carried out using different explants *viz.*, shoot apices, axillary buds, leaf tips and bases and root tips, both from *in vivo* and *in vitro* plants. Plbs were produced from shoot tips (in vivo) and leaf bases (*in vitro*) on MS and modified VW medium respectively under the influence of different concentrations of IAA/NAA and BAP. A response of 60% was observed in shoot tips cultured in medium with 2.5 mg/l BAP alone. NAA (1 mg/l) and BAP (2 mg/l) resulted in profuse plb proliferation from leaf bases. Nodal buds from *in vivo* plants, cultured in MS medium supplemented with 2.5 mg/l BAP produced 7-10 multiple shoots. The plbs were multiplied by cutting into smaller pieces and agitating in hormone-free MS liquid medium. If left undisturbed, plbs developed into complete plantlets with healthy, viable roots in 12-16 weeks.

Morphogenetic responses of plbs as influenced by different growth regulators *viz.*, IAA, IBA, NAA, 2,4-D, BAP, KN and GA_3 were studied. Of the different auxins used, NAA (1.0 mg/l) resulted in morphogenetic responses in 70% cultures with healthy, green multiple shoot formation. Plantlets treated with 2,4-D, at all concentrations, performed poorly. Of the two cytokinins used, BAP stimulated the multiple shoot formation. Kinetin proved inhibitory as very small, light green, folded leaves were observed at low concentrations. A negative influence of GA_3 was

observed on growth of plantlets.

In vitro storage of plbs was attempted using reduced temperature, mannitol and abscissic acid. Besides, plbs were successfully encapsulated in calcium alginate beads to get 'synthetic seeds' which were then stored and the conversion frequencies studied. Optimum storage of the plbs for 90 days in 44% cultures was achieved using 10% mannitol. ABA at 2 mg/l concentration could suppress the growth in 35% cases. These plbs, when transferred to re-growth medium, showed 100% conversion to plantlets. Poor performance was observed when plbs were subjected to low temperature storage in the dark. Thirty percent plbs could survive a temperature of $4^{o}C$ for a week, the rest facing a loss of colour and eventual death. All the 'synthetic seeds' developed into plantlets *in vitro*. Those stored at $4^{o}C$ could germinate even after 180 days, though poorly.

Plantlets (4-6 cm) with well developed root system and 3-4 healthy leaves were transferred to pots containing different potting mixtures. A potting mix of charcoal: brick bats: moss (1:1:1/2) was found to be the most suitable substratum. Plantlets fed with 1/10th liquid MS nutrient salt medium showed a survival rate of 70% and healthy growth.

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Standardizing acidity level for growth and development of Dendrobium wardianum

Abha Sharma and Pramod Tandon

Sharma Abha, Tandon Pramod 1991 Standardizing acidity level for growth and development of *Dendrobium wardianum* IBC 8 : 27–29

Differentiating protocorms of *Dendrobium wardianum* were cultured on MS medium with varying pH (4–7.5) to assess the optimal acidity level for its growth. The acidity levels below pH 5.0 and above 6.5 were not favourable for healthy growth of plantlets. The best growth of plantlets was observed at pH 6.

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Dendrobium wardianum Warner belongs to Orchidaceae, a unique family with huge amount of diversity (both vegetative and floral) coupled with peculiar pollination contrivances and wide natural hybridization. It is a splendid dendrobe of rare occurrence, getting threatened in its natural habitat due to ruthless exploitation of forest resources and its poor natural regeneration. *In vitro* technique provides better understanding of different physiological and biochemical aspects of regeneration at various levels (Kavi Kishore, Mehta 1984). Various factors, physical and nutritional both, affect growth and behaviour of orchid species differently and a species may even respond differetly at various stages of its growth (Arditti 1967). Though several studies have been conducted to investigate the effect of different media on seed germination and seedling development (Arditti et al 1981), not much is known about the acidity effect on their asymbiotic cultures (Curtis, Spoerl 1948; Ito 1955). Studies however, in other plant groups, on the influence of pH on growth (Hewitt 1966), have revealed that healthy seedings can be obtained at a particular pH (Norstog 1973).

The present investigation was carried out to study the effect of pH on growth and development of *Dendrobium wardianum* Warner seedlings.

Materials and Methods

Dendrobium wardianum Warner protocorms of one–leaf stage (size ca 2–3 mm) grown on Murashige and Skoog's (1962) basal medium under sterilized conditions were transferred to fresh MS medium of varying acidity and alkalinity, pH ranging from 4.0 to 7.5, obtained by adding 1N HCl or 1N NaOH as per requirement. The cultures were incubated at 24±2°C under 12 h photoperiod of 2500 lux illumination and observations were made at every 30–day interval for 3 months. Each treatment

consisted of 10 replicates and the experiment was repeated thrice. Cultures were subcultured at every 10-day interval to maintain the stability of the pH as it tends to shift after a certain period owing to the absorbance of ions by the inoculum of the media (Scragg, Fowler 1985).

Results

Growth and development of *Dendrobium wardianum* seedling varied markedly due to different pH levels of the medium. Seedling growth was observed to be satisfactory at pH 6.0 which is also manifested by fresh and dry weight of the seedlings (Table 1). However, pH ranging below 5 and above 6.5 was not found to be favourable for seedling growth and development. Lower pH inhibited growth of the protocorms and resulted in thick, stout, stunted bodies. The absorbing hair/-roots and leaf primordia were found to be the highest in number at pH 6.0.

Table 1. Effect of pH on growth and development of *Dendrobium wardianum*

pH	*Seedling growth after 90 days of treatment*			
	Average shoot length ± SD (cm)	*Average fresh weight ± SD (g)*	*Average dry weight ± SD (g)*	*Remarks*
4.0	0.46 ± 0.04	0.429 ± 0.051	0.091 ± 0.130	+
4.5	0.63 ± 0.06	0.521 ± 0.062	0.100 ± 0.014	+
5.0	0.97 ± 0.09	0.612 ± 0.073	0.115 ± 0.017	+ +
5.5	1.04 ± 0.10	0.707 ± 0.085	0.117 ± 0.017	+ +
6.0	1.33 ± 0.12	0.890 ± 0.107	0.131 ± 0.019	+ + +
6.5	0.92 ± 0.08	0.600 ± 0.072	0.105 ± 0.015	+ +
7.0	0.85 ± 0.08	0.581 ± 0.069	0.100 ± 0.014	+
7.5	0.62 ± 0.05	0.512 ± 0.061	0.110 ± 0.015	+

+, poor; + +, moderate; + + +, best

Discussion

Acidity level of the media has pronounced effect on the growth of cell, tissue and organ (Smith, Krikorian 1990). Acidity, lower than 4.0 or higher than 8.0 has been found to be inhibitory for seedling growth in case of orchids (Arditti et al 1982). In the present investigation, seedling development was found to be the best in medium with pH 6.0, and minimum below pH 5.0 and above 6.5. Better seedling growth at pH 6.0 in the present study may be attributed to better uptake of nutrients and water from the medium (Knudson 1946; Ito 1955). Besides injury of

root cells, depression in the uptake of potassium and calcium at pH 4.0 and precipitation and/or non-utilization of iron compounds at higher pH values could be behind the detrimental effect of the extreme pHs on seedling growth and development (Hewitt 1966). Evidently, pH of the medium surrounding the roots can affect the growth of the plants by controlling the availability of nutrients, thus deciding the rate of uptake by plants. Our findings suggest pH 6.0 to be the most suitable for growth and development of *Dendrobium wardianum*, and the results can be exploited in rearing nurseries and also in *in vitro* technique for mass multiplication of the plant.

Acknowledgement

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Nutritional studies on in vitro raised Protocorms of endangered dendrobe : Dendrobium wardianum Warner

Abha Sharma and Pramod Tandon

Sharma Abha, Tandon Pramod 1991 Nutritional studies on *in vitro* raised protocorms of endangered dendrobe : *Dendrobium wardianum* Warner – IBC 8 : 75–78

Protocorm–like bodies obtained by culturing shoot apices of *Dendrobium wardianum* were subjected to various nutrient media with different salt levels for proliferation and plantlet–regeneration. Remarkable differences were observed in growth and development of regenerant in response to various nutrient levels. This study can be of immense importance to nursery industry.

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Studies on the nutrition of plant embryos *in vitro* are of considerable importance in understanding the physiological and biochemical aspects of growth and development in plants. Besides activation of enzymes and hormonal factors, the harmonious development of embryo and functional differentiation within it may result from induction by gradients of nutritional substances (Raghavan, Torrey 1964). Although repeatable regeneration of plants has now been made possible, understanding of the nutritional and hormonal balance is still incomplete. Small variations in conditions for sterilization and culture media can result into qualitative and quantitative variations.

Taxonomically the orchids represent the most highly evolved family among monocotyledons. Unfortunately a large number of orchid species are underway of extinction due to the increasing devastation of forest lands and their overexploitation to cater a great demand in the medical and cut–flower industries. Besides, less than 5% of orchid seeds germinate in nature (Rao 1977) owing to their extremely small size, underdeveloped or lack of endosperm and the requisition of a specific fungi to enter a symbiotic association with. Mass clonal propagation of a number of orchids has been developed in recent years and various protocols developed (Arditti et al 1982). However, only a few studies are carried out for the selection of specific culture media for the maximum regeneration and multiple shooting, for use in nursery. *Dendrobium wardianum* Warner – a beautiful epiphytic orchid – is fast becoming an endangered species. It is one of the commercially important dendrobes. The present communication describes experiments to establish the optimum culture conditions for shoot multiplication, and to maximize the overall nutrient efficiency of *in vitro* regeneration of *D wardianum* Warner.

Materials and Methods

Establishment of initial cultures :

shoot tips (ca 2-3 mm) were excised from *Dendrobium wardianum* Warner plants and sterilized with 5% (v/v) NaOCl solution (with 1-3% available chlorine) for 5 minutes, and were inoculated on Murashige and Skoog's (1962) basal medium containing 3% sucrose, 0.8% agar and 2.5 mg l^{-1} 6-benzylaminopurine (BAP) (under report). The pH of the medium was adjusted to 5.8 prior to addition of agar and autoclaved for 15 min at 120°C. The cultures were incubated at 24± 2°C with 16 h illumination of 2000 lux. Protocorm-like bodies (plbs) analogous to embryoids were produced after 6 weeks of culture.

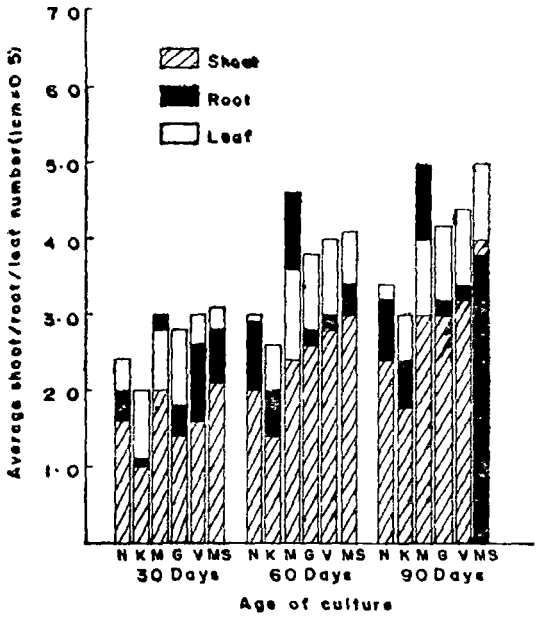

Fig 1. Effect of various nutrient media on shoot, root and leaf number of regenerants. (*N*, Nitsch; *K*, Kundson C; *M*, Mitra et al, *G*, Gamborg; *V*, Vacin & Went; *MS*, Murashige & Skoog)

Shoot multiplication:

Protocorm-like bodies (plbs) so developd were transferred to fresh medium containing six different basal media : Vacin and Went (1949), Mitra et al (1976), Gamborg et al (1968), Nitsch and Nitsch (1969), Knudson–C (1946), and Murashige and Skoog (MS), and the effects of six macroelement formulae were studied on growth and development of seedlings. To avoid the possibility of a carry over effect of growth regulators from the original mother explants, all the results given in this paper were obtained during the third set of experiment. Each experiment was repeated thrice. The cultures were transferred to a fresh medium every four

weeks. The growth and development of the plbs were studied in terms of fresh weight; number of shoot, root and leaf; and length of shoot and root.

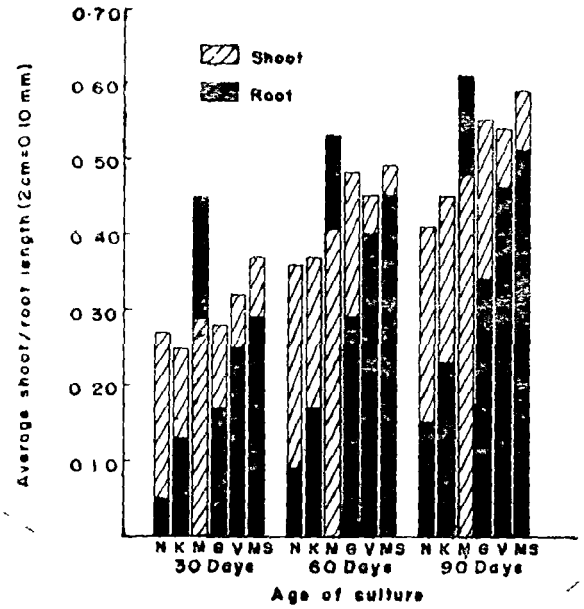

Fig 2. Effect of various nutrient media on shoot and root length. (*N*, Nitsch; *K*, Knudson C; *M*, Mitra et al; *G*, Gamborg; *V*, Vacin & Went; *MS*, Murashige & Skoog)

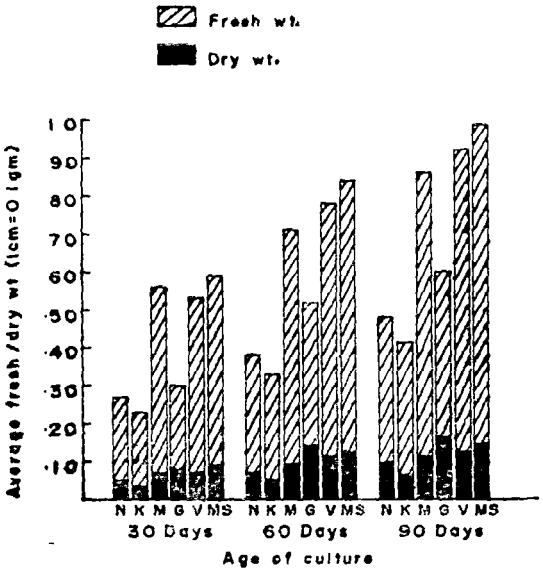

Fig 3. Effect of various nutrient media on fresh and dry weight of regenerants. (*N*, Nitsch; *K*, Knudson C; *M*, Mitra et al; *G*, Gamborg; *V*, Vacin & Went; *MS*, Murashige & Skoog)

Results

Of the six mineral media tested, best results were obtained with MS medium. Besides resulting into the highest shoot and leaf number, shoot length and fresh weight (0.984 g), it produced dark green stems and long leaves. However, the

highest avarage root number and root length were observed with Mitra et al medium. Gamborg's medium yielded the highest dry weight; and average shoot, root and leaf number were found to be the lowest on Knudson–C. Growth on both, Knudson–C and Nitsch media was stunted. The cultures grown on Vacin and Went medium were the second best in their growth.

Discussion

The successful establishment and growth of plant cells *in vitro* generally is determined by the nature of the explant and the composition of the nutrient medium (White 1951). Growth and differentiation are controlled by various components of media and mineral nutrients. It has been reported that seeds/protocorms of orchid are unable to absorb and utilize nitrates during the initial phase of germination and development. However, appearance of nitrate reductase activity has been reported to be correlated with the absorption of nitrates in the later stage (Raghavan 1964). Presence of high amount of ammonium nitrate in MS might favour the growth as NH_4^+ ions are readily assimilated during the initial phase and NO_3^- at later stage of development. Moreover, most tissue culture media are poorly buffered and pH fluctuations that occur may be detrimental to long term survival and to growth of cells at either low density (Caboche 1980) or as single cells (Koop et al 1983).

We derive that the nutrient medium containing high concentration of ammonium nitrate favours luxuriant growth of *D wardianum*, which can be exploited for nursery and orchard developments.

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Financial assistance from Department of Environment, Government of India is gratefully acknwoledged.

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IN VITRO CULTURE OF *DENDROBIUM WARDIANUM* WARNER: MORPHOGENETIC EFFECTS OF SOME NITROGENOUS ADJUVANTS

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SUMMARY

Excised shoot apices of *Dendrobium wardianum*, cultured on MS medium supplemented with inorganic and organic nitrogenous sources, viz., calcium nitrate, ammonium sulphate, urea and amino acids (alanine, glutamine, glycine, leucine and serine) showed varied responses. Calcium nitrate and urea gave rise to direct multiple shooting besides forming protocorm—like bodies. Regenerants obtained by rooting isolated shoots and germinating protocorm—like bodies were successfully transplanted to a substratum containing charcoal pieces, brick bats and coconut fibres. The rate of survival was recorded to be 65% under glass house conditions.

INTRODUCTION

Orchids are slow growing plants which could be accounted for by their sluggish nitrogen metabolism (Poddubnaya-Arnold, 1967). A major part of nitrogen is required for the production of proteins and formation of chlorophyll and cytochrome. Nitrogen deficiency, after water stress, is the most common limitation on growth. In their initial stage of growth and development, orchids are unable to utilize nitrate but the ability to absorb nitrate ions indicates the appearance of nitrate reductase activity (Raghavan and Torrey, 1964). Dendrobes are splendid orchids important both commercially and/or medicinally. An experiment was designed to investigate the effect of different inorganic nitrogen sources on the morphogenetic potential of cultured shoot apices of an epiphytic, commercially favoured ornamental, *Dendrobium wardianum* Warner.

MATERIALS AND METHODS

Excised shoot apices (ca. 2-3 mm) from *Dendrobium wardianum* Warner plants were sterilized with 5% (v/v) NaOCI solution (with 1-3% available chlorine) for 5 minutes. These were then inoculated on Murashige and Skoog (MS, 1962) basal

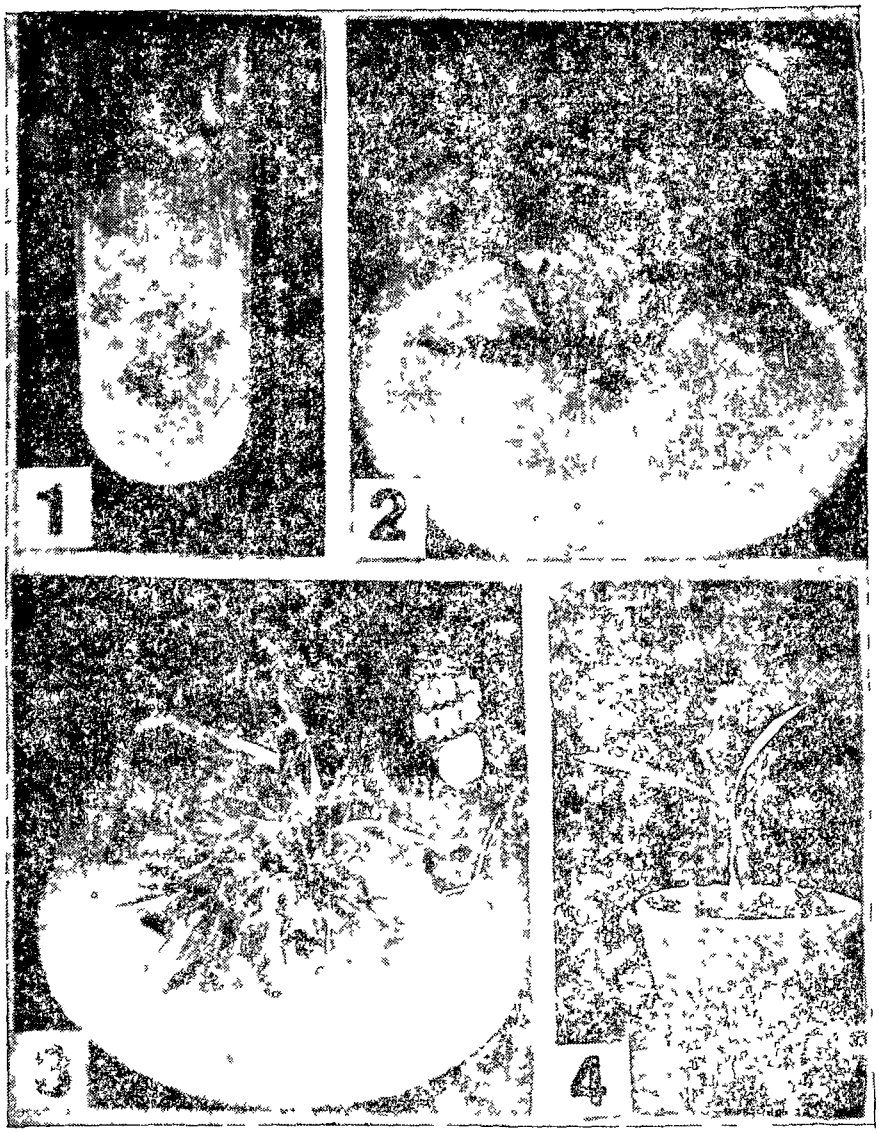

Fig. 1. Proliferation of protocorm—like (plb) from cultured shoot apices.
Fig 2. Direct multiple shooting from the cultured shoot apex under influence of calcium nitrate (2.5 mg/1).
Fig. 3. Regeneration of plantlets by transferring protocorm—like bodies on MS basal medium.
Fig. 4. Potted plantlet ready for hardening.

medium containing 3% sucrose, 0.8% agar and 2.5 mg/l 6-benzylaminopurine (BAP) for protocorm-like body formation (Sharma and Tandon, 1991), supplemented with different inorganic and organic nitrogen sources, viz. calcium nitrate, ammonium sulphate, urea and aminoacids (alanine, glutamine, glycine, leucine and serine) at a range of concentrations (0.5-5.0 mg/l). Explants cultured on MS medium supplemented with 2.5 mg/l BAP served as control. The pH of the medium was adjusted to 5 8 prior to addition of agar and the cultures incubated from cool fluorescent white lamp at 24 ∓ 2°C at 16 h illumination of 2000 lux. Data were collected every 30 day up to 3 months and the experiment repeated thrice.

RESULTS AND DISCUSSION

All the cultures more or less gave rise to globular, green protocorm-like bodies with 0.5 as expected (Fig. 1). Shoot apices cultured on MS medium supplemented and 2.5 mg/l calcium nitrate gave rise to direct multiple shooting thus bypassing the usual intervening protocorm-like body (plb) formation phase (Fig. 2). However, higher concentrations of calcium nitrate resulted in drying of the explants. Response with urea was parallel to that of calcium nitrate at 2.5 and 5 mg/l, (Table 1). Lower concentration of the urea gave rise to plb's which on transfer to basal MS medium regenerated into complete plantlets. Explants inoculated on ammonium sulphate supplemented MS medium gave a poor percentage of plb formation at higher concentrations. Most cultures turned brown within a week and dried ultimately. Addition of individual aminoacids did not show any significant effect.

Ionic extra/intra cellular environment has been, besides other factors, found to affect cell elongation, enlargement, division and morphogensis (Tran Thanh Van and Mutaftschiev, 1990). It is generally believed that reduced forms of nitrogen have value for certain plant tissue cultures. Although ammonium (NH^+_4) is rarely adequate as the sole nitrogen source, small amount of it may be essential for good growth; however, NH^+_4, in certain cases may be detrimental to survival and growth of plant cells (Ozias-Akins and Vasil, 1985). The browning and drying of explants treated with ammonium sulphate can be attributed to ammonium toxicity which is caused by higher N-accumulation when NH^+_4 is used in higher concentrations (Barker *et al*, 1967; Barker and Maynard, 1972). The form of N supplied to plants has a great influence on the absorption of nutrients. Alan (1989) reported that nitrate generally increased all mineral contents except for N in the tissue whereas ammonium decreased them. Decrease of K, Ca and Mg content of the plant tissues with NH^+_4 have been well documented in other plants (Hartman *et al*, 1986). Urea has been reported to serve as an effective N source for *Cattleya* embryos (Mariat, 1948) but has also been reported to cause necrosis at higher concentrations as it significantly

Table 1 : Effect of different organic and inorganic cnitrogen sources on the morphogenetic potential of shoot apices cultured on MS_1 (MS+2.5 mg/l BAP)*

Nitrogen source	Conc. (mg/l)	% Morpho-genetic	Nature of response			Remarks
			Plb	MS**	SS***	
MS+No N—Source	—	60	√	—	—	Green, globular plb
Inorganic N-Source						
MS_1+Calcium Nitrate	0.5	55	√	√	—	50% cultures formed 2-3 thick MS
	2.5	60	—	√	—	4-5 healthy MS
	5.0	50	√	—	—	Green, globular plb
MS_1+Ammonium	0.5	45	√	—	—	-do-
sulphate	2.5	35	√	—	—	-do-
	5.0	20	√	—	—	Pale green plbs
Organic N-Source						
MS_1+Urea	0.5	50	√	—	—	Green, globular plb
	2.5	55	√	√	—	45% cultures formed MS
	5.0	55	√	√	—	45% cultures formed 3-4 MS
Amino Acids						
MS_1+Alanine	0.5	50	√	—	√	40% cultures formed globular plbs
	2.5	55	√	—	√	50% cultures gave rise to plbs
	5.0	50	√	—	√	40% cultures formed plbs
MS_1+Glutamine	0.5	45	√	—	√	Green, globular plbs
	2.5	50	√	—	√	-do-
	5.0	40	√	—	—	Green, globular plbs
MS_1+Glycine	0.5	50	√	—	—	-do-
	2.5	55	√	—	—	-do-
	5.0	40	√	—	√	30% cultures formed green plbs
MS_1+Leucine	0.5	45	√	—	—	Green plbs
	2.5	40	√	—	√	35% cultures gave rise to plbs
	5.0	40	√	—	√	-do-
MS_1+Serine	0.5	55	√	—	—	Green, globular plbs
	2.5	50	√	—	√	40% cultures gave rise to healthy plb
	5.0	50	√	—	√	-do-

* Data collected every 30 day for 3 months. Each experiment repeated thrice.

** Multiple shoots

*** Single shoot.

increases the N content and decreases Ca content (Alan, 1989) Individual amino acids or mixtures of aminoacids have been found to give no growth stimulation in orchids when added to a medium already supplying inorganic N (Withner, 1959). No specific growth enhancement following the addition of amino acids could be attributed to competitive interactions between the amino acids (Street, 1989).

Isolated shoots, obtained directly from calcium nitrate and urea treated shoot apices (Fig. 2), were transferred to basal MS medium where they grew well developed, stout roots in 2 weeks. Protocorm-like bodies when sub-cultured to the same basal MS media regenerated into complete plantlets (Fig. 3). The regenerated plantlets after attaining a height of 4-5 cms. were transferred to small, earthen pots (Fig. 4) containing charcoal pieces, brick bats and cocount fibres with a layer of moss at the surface and kept under glasshouse conditions. Survival rate was found to be 65%. The results obtained can be immensely important to and exploited by the nursery growers, orchard developers and cut flower industries for rearing the species on mass scale.

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Regeneration of *Dendrobium wardianum* Warner (Orchidaceae) from synthetic seeds

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Protocorm-like-bodies (plbs) of *D. wardianum* were successfully encapsulated in calcium alginate beads to get synthetic seeds and 100% conversion frequency was recorded after 45 days of culture when germinated on MS medium. The beads could be stored at 4°C up to 180 days. However, the germination declined with increase in duration of storage. The regenerants were phenotypically similar to controls.

Mericloning has opened new vistas in orchid tissue culture[1-3]. It becomes particularly important for orchids as their genotypes are highly heterozygous and vegetative propagation by division is slow[4]. Natural regeneration of orchids through seeds is very poor due to their minute size, lack of embryo and/or presence of a reduced endosperm[5]. Plant regeneration *in vitro* can occur via adventitious bud development, somatic embryogenesis or axillary/apical meristem proliferation. Recently, production of synthetic seeds by encapsulating somatic embryos (SE) has been reported in a few plants[6,7]. However, this technique has not been amenable for use in case of orchids which do not produce SE in true sense rather give rise to protocorm-like-bodies (plbs) in culture offering the most potential method for clonal propagation. *Dendrobium wardianum* Warner, a beautiful, epiphytic, commercially favoured orchid has become endangered in its natural habitat due to large scale denudation of lands and ruthless exploitation to cater its demand in the cut flower industry. In the present study an attempt has been made to produce synthetic clonal seeds by encapsulating plbs of *D. wardianum*, preserve them under cold condition over a considerable period and study the conversion frequency.

Sodium alginate (Sigma) was mixed with Murashige and Skoog[8] (MS) liquid medium with 3% sucrose without growth regulator and the solution *p*H was adjusted to 5.8. For formation of insoluble calcium alginate, 100 m*M* calcium chloride was prepared in liquid MS medium and the autoclaving carried out at 1.05 kg/cm^2 for 15 mins.

Plbs were obtained by culturing shoot apices of *D. wardianum* on MS medium with 3% sucrose, 0.8% agar and 2.5 mg/l 6-benzylaminopurine (BAP) as reported[9]. These plbs were separated, blot dried, mixed with sodium alginate solution, and dropped into $CaCl_2.2H_2O$ solution using a wide mouth dropping pipette. The drops, each containing a single plb, when left in $CaCl_2.2H_2O$ solution for 40 min on gyratory shaker (75 rpm) formed round, firm beads as a result of the ion exchange reaction between Na^+ and Ca^{2+} ions. Later these beads were recovered by recycling and decanting $CaCl_2.2H_2O$ solution and washed thrice with autoclaved MS liquid medium. The beads were then plated on basal MS medium for further growth and development studies.

The cultures were maintained at 24° ± 1°C under 16 hr photoperiod (fluorescent light 30 $Em^{-2}S^{-1}$ Encapsulated plbs (10 sets of 20 beads each) were stored at 4°C. Non-encapsulated plbs stored at 4°C served as control. The regeneration potential was recorded at every 15 day interval up to 180 days and the complete experiment was repeated twice.

Encapsulated plbs (without storage) when germinated directly on medium (Fig. 1a) with or without BAP showed the conversion frequency of 100% after 45 days of culture. On the other hand, a conversion frequency of 60% was recorded in case of non-encapsulated plbs (without storage) under the same cultural conditions. The synthetic seeds stored at 4°C remained viable and germinated up to day 120. However, a poor germination of 30% was recorded which further declined to 10% by day 180. Higher germination percentage in case of synthetic seeds (without storage) could be due to the matrix which not only facilitates regular nutrient supply but also protects the dispersed, delicate tissue from any mechanical injury during handling and from desiccation. Synthetic seeds plated on MS medium free of growth regulators eventually developed into multiple plantlets (Fig. 1b) whereas the same when cultured on medium containing BAP 5 mg/l) resulted in their multiplication only. The multiple plantlets obtained from synthetic seeds were cultured in flasks containing MS medium without growth regulators. Within 16 weeks of transfer, plantlets of 6-8 cm height with 3-4 nodes and well developed root system were obtained. These were potted in earthen pots containing charcoal pieces, brickbats, coconut fibres and a layer of moss at the surface and hardened under glass house conditions

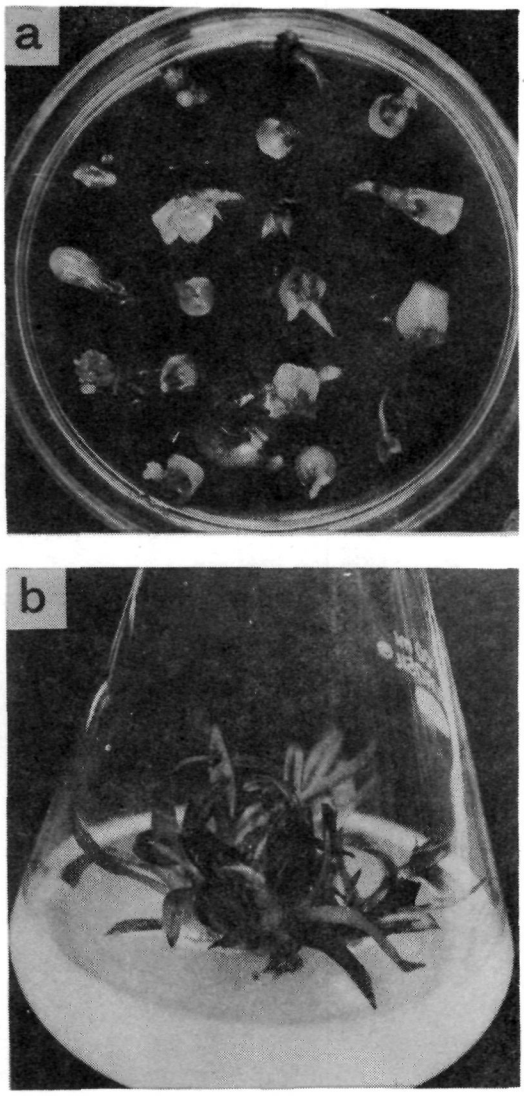

Fig.1—(a) Germinating synthetic seeds on MS basal medium; and (b) multiple plantlets obtained from synthetic seeds

(temperature 20°-25°C, RH 80-90%). About 65% of the regenerants survived.

Tissue culture techniques for clonal propagation of plants that have a high per unit value, like ornamentals and fruits are of great significance. High volume propagation potential combined with formation of synthetic seeds for low cost delivery is expected to open new vistas. Recently, efforts have been intensified in this area by encapsulating SE[6] and shoot bud explants[10,11] in various matrices and growing them in different substrata. A significant outcome of the study was the observation that the synthetic seeds retained their viability even after 120 days at 4°C and the plants recovered also maintained their clonal identity as there were no morphological differences observed among the regenerants. Attempts are underway to directly transfer the synthetic seeds to natural substrata by adding antifungal and antibacterial compounds in the matrices.

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Morphogenetic responses of cultured cells of cambial origin of a mature tree – *Dalbergia sissoo* Roxb.

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ABSTRACT

Regeneration of plantlets was achieved from cell suspension derived calli of cambial origin from mature 'elite' trees of Dalbergia sissoo. Callus proliferation occurred on the cambial tissue pieces cultured on MS medium supplemented with 2,4-dichlorophenoxyacetic acid (2.0 mg/l) and benzylaminopurine (0.1 mg/l). Suspension cultures were obtained by transferring and agitating callus lumps in liquid medium composed as above. Aggregates of about 30 cells were plated on semi solid medium, which developed into calli. Shoot bud differentiation was observed in the calli transferred to medium devoid of auxin but containing 0.5-2.0 mg/l benzylaminopurine. The isolated microshoots were rooted on modified MS medium containing low organic salts and auxins.

ABBREVIATIONS

BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; IAA - indole-3-acetic acid; IPA - indole-3-propionic acid; KN - kinetin; MS - Murashige and Skoog (1962); NAA - 1-naphthaleneacetic acid; IBA - indole-3-butyric acid.

INTRODUCTION

In vitro propagation of mature trees has progressed significantly during the last decade (von Arnold, 1988). Organogenesis has been difficult to achieve in woody legume cultures (Datta and Datta, 1985); however, limited success has been reported in recent years (Gresshoff and Mohapatra, 1982; Mohan Ram et al., 1982; Datta and Datta, 1985; Kapoor and Gupta, 1986; Lakshmana Rao and De, 1987; Kumar, 1987; Ravishankar Rai and Jagadish Chandra, 1988; Varghese and Kaur, 1988).

Dalbergia sissoo (Leguminosae) is an important timber yielding tree. It ranks among the finest woods in durability, adaptability and working qualities. Besides, its heartwood contains compunds having the potential for leprosy treatment (Chopra et al., 1958). There are reports on the micropropaga-tion of D. sissoo by culturing root segments and axillary buds (Mukhopadhyay and Mohan Ram, 1981; Datta et al., 1982). Adventitious regeneration directly from organs generally gives a limited number of propagules. This number may be increased several folds by an intervening callus phase that regenerates an increased supply of adventitious shoots (Hussey, 1983). Moreover, cell culture offers many advantages for isolation of mutants in higher plants. As the cells are grown in uniform cultural conditions, reproducible selection schemes can be employed. However, isolation of auxotrophic cell lines and recovery of mutants in higher plants has lagged due to the lack of stable haploid cell lines and suitable selection systems. Therefore, the present investigation was aimed at developing a suitable methodology for rapid propagation of mature 'elite' Dalbergia sissoo lines using cell suspension derived calli which can be exploited in future for isolation of mutants.

MATERIALS AND METHODS

Wood blocks with intact bark were cut from young branches of 'elite' Dalbergia sissoo Roxb. trees. The blocks were soaked in 'Teepol' detergent solution and washed thoroughly under running tap water. These were then surface sterilized with 0.5% aqueous mercuric chloride for 8-10 minutes and washed several times with sterile distilled water. After removing the bark, the cambial layer was scraped off with the help of a sterilized scalpel and cultured aseptically on MS medium (Murashige and Skoog, 1962) containing 3% sucrose, 0.8% agar and various concentrations of auxins and cytokinins either individually or in combinations. The cultures were maintained at 25±2°C under 12 h daily illumination with fluorescent cool light at 45 $Em^{-2}s^{-1}$ intensity and 60-70%

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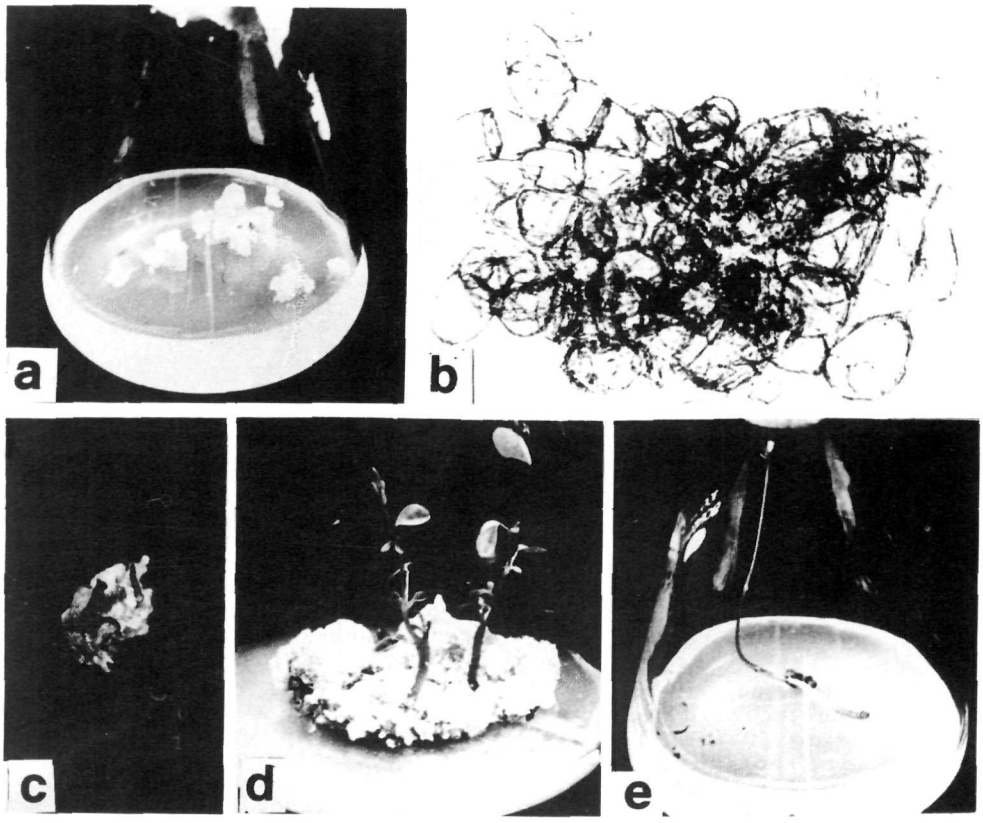

Fig.a Formation of callus lumps on MS+2,4-D (2.0 mg/l)+BAP(0.1 mg/l) from cambial tissues of Dalbergia sissoo

Fig.b Cell aggregates obtained from suspension culture of D. sissoo

Fig.c Differentiation of shoot buds on MS+BAP (1.0 mg/l) in the suspension derived callus of D. sissoo

Fig.d Regeneration of shoots from the callus of D. sissoo

Fig.e Rooted shoots obtained by culturing excised microshoots on MS+IAA+IBA+NAA (1.0 mg/l each)

relative humidity. Suspension cultures were obtained by transferring friable callus lumps (20 d old) to liquid medium of the same composition at 100 rpm with an orbital motion stroke of 2-3 cm. At the first subculture on fresh medium, after 15 d of transfer, large clumps of initial inoculum were removed by passing the suspension through stainless steel mesh of 60 um. Cell aggregates with upto 30 cells were plated on semi-solid medium of the same composition for colony growth. Larger cell colonies were isolated and subcultured on fresh medium to generate calli. Calli (60 d old), were subjected to various growth regulator treatments for organogenesis. For rooting of excised microshoots, half-strength MS medium supplemented with various auxins was used. Experiments were repeated twice.

RESULTS AND DISCUSSION

Callus proliferation occurred on the cambial tissues cultured on MS+2,4-D (2.0 mg/l) + BAP (0.1 mg/l), and the entire explant generated a lump of greenish-white, friable callus within 15 d (Fig.a). Filtered cell suspensions, free of larger clumps, with cell aggregates of ca. 30 cells (Fig.b) when plated on semi-solid medium of same composition formed larger cell colonies after 30 d. However, smaller cell aggregates, i.e. less than 30 cells, failed to grow. On fresh medium, these larger cell colonies grew vigorously and formed calli in 30 d. Organogenesis was observed in the calli subcultured on MS+IAA and/or BAP within 45-60 d. Callus cultures subjected to IAA (0.05-2.0 mg/l) exhibited rhizogenesis after 30 d of subculture. Percentage of culture showing rooting was maximum (45.5±2.8) on MS+IAA (1.0 mg/l) (Table 1). Calli subcultured on MS+BAP (0.05-2.0 mg/l) showed rapid growth and produced 2-6 green nodular structures (i.e. shoot buds) (Fig.c) after 45-60 d, which eventually developed into shoots (Fig.d).

Table 1. Morphogenetic effects of BAP and IAA on the regeneration frequency in callus derived from cell suspension of Dalbergia sissoo[a]

MS+growth regulators (mg/l)		Percentage of cultures showing organogenesis ± S.D.	
BAP	IAA	Shoots	Roots
0	0	-	-
0.05	0	29.1±3.6	-
0.5	0	37.4±4.5	-
1.0	0	60.0±5.8	-
2.0	0	24.6±3.0	-
0	0.05	-	25.0±2.8
0	0.5	-	18.4±2.6
0	1.0	-	45.4±2.8
0	2.0	-	22.2±3.1
0.5	0.5	25.0±3.2	-
1.0	0.5	41.6±6.7	-
1.0	1.0	27.0±3.5	7.6±1.8
0.05	0.5	13.5±1.6	26.4±3.5
0.05	1.0	-	38.1±4.7
2.0	2.0	-	-

[a]Each treatment consisted of 20 replicates
- no response

Table 2. Root formation in excised microshoots of D. sissoo cultured on modified MS[a] medium containing different auxins[b]

Growth regulator (mg/l)		Percentage rooting 20 d (± S.D.)	30 d
IAA	0.5	20±2.8	40±2.1
	1.0	30±2.0	50±1.6
	2.0	20±2.0	30±1.6
IBA	0.5	10±0.9	40±1.5
	1.0	30±1.4	60±1.6
	2.0	20±2.0	30±1.6
IPA	0.5	10±1.2	40±0.9
	1.0	40±0.8	70±1.6
	2.0	10±0.8	30±1.2
NAA	0.5	40±0.9	70±1.2
	1.0	60±1.2	80±0.9
	2.0	50±0.4	70±1.2
2,4-D	0.5	-	-
	1.0	-	-
	2.0	-	-
NAA+IBA	1.0+1.0	30±1.4	60±1.2
NAA+IPA	1.0+1.0	30±2.8	60±2.4
NAA+IAA	1.0+1.0	40±1.8	60±1.6
IAA+IBA+IPA	1.0 mg/l each	50±0.8	80±1.2
IAA+IBA+NAA	1.0 mg/l each	60±1.2	90±1.0

[a]MS medium with 1/2 strength inorganic salts
[b]Each treatment consisted of 20 replicates
- no response

Percentage of shoot-forming cultures was maximum (60±5.8) with 1.0 mg/l BAP in the medium (Table 1), and a higher BAP concentration (2.0 mg/l) did not favour regeneration of shoots. Caulogenetic potential using BAP in the present study is in agreement with that of Phillips and Collins (1979) in red clover. The presence of IAA along with BAP always suppressed shoot-formation. A similar antagonistic auxin-cytokinin effect has been observed in callus cultures of Aegel marmelos (Arya et al., 1981). The regenerative callus could be maintained by subculturing on basal MS medium containing 2,4-D (2.0 mg/l) and BAP (0.1 mg/l) for almost 2 years.

Isolated shoots were cultured on half-strength MS medium supplemented with NAA, IAA, IBA, IPA and 2,4-D individually and in combinations over a range of concentrations (0.5-2.0 mg/l) for rooting. Eighty percent of the cultured shoots produced fibrous roots in MS+IAA+IBA+IPA (each at 1.0 mg/l concentration) (Table 2). Combinations of IAA+IBA+NAA (1.0 mg/l each) produced stout tap roots in 90 percent of the cultured shoots (Fig.e). Plantlets were transferred to pots containing a soil:sand (1:1) mixture under glasshouse conditions. Percentage survival in the pots was about 45. Attempts are underway to improve the survival rate of transplants.

Plantlet regeneration from calli of adult trees has been very limited (Jones, 1983). Despite some drawbacks like genetic aberrations (Thomas, 1981) and loss of regeneration potential with the age of the cultures (Murashige, 1974; Halperin, 1986), this approach is considered to be most potential for cloning plant species (Murashige, 1978). The regeneration of plantlets from cambial cultures of mature Dalbergia sissoo trees is similar to the previous report in Ulmus compestris (Jacquiot, 1966). Variant cell lines are usually isolated from protoplasts or more commonly from cell suspension cultures either by plating cell aggregates on semi-solid agar medium (Flick et al., 1981) or by direct selection in cell suspensions. The regeneration protocol described in this experiment offers many advantages for the future isolation of mutants in D. sissoo.

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