IN VITRO PROPAGATION OF DENDROBIUM FIMBRIATUM VAR. OCULATUM HK. F. : SOME FUNCTIONAL AND BIOCHEMICAL ASPECTS OF ITS GROWTH

A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Forwarded
17.12.91
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CERTIFICATE

I certify that the thesis entitled ***In vitro* propagation of *Dendrobium fimbriatum* var. *oculatum* Hk. f. : Some functional and biochemical aspects of its growth** submitted by Ms. Suman Kumaria 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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ACKNOWLEDGEMENTS

I offer my heartiest gratitude to my honourable guide and supervisor, Dr. Pramod Tandon, Professor of Plant Physiology, Department of Botany, North-Eastern Hill University, for his able and expert guidance, intellectually acute ideas and thoughts, and continued keen interest during the course of the present investigation.

I am grateful to Professor Y.S. Chauhan, Head, Department of Botany, North-Eastern Hill University, for providing me the facilities.

I also offer my sincere thanks to my lab. mates -Drs A.L.S. Rajee, A. Kumar, Mr. J.C. Dang, Ms A. Sharma, Mrs S. Coorie, Miss Virginia Kalita and Mrs S. Purkayastha who always gave me their whole-hearted co-operation by making themselves available for consultations/assistance whenever I looked forward to them.

Words are inadequate to express my sincerest thanks to my friends Prathima, Seema, Debashis, Dhruva and Maitreyee for their generous help in innumerable ways throughout my research period and during the preparation of the manuscript.

I do not have words to express my gratitude and indebtedness to my parents for their blessings and inspiration and to my sister and brother for their encouragement and support.

Award of Junior and Senior Research Fellowships during the course of this study under the North-Eastern Council, Ministry of Home Affairs, Government of India, sponsored research project is gratefully acknowledged.

Shillong,
December 17, 1991

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CONTENTS

I. GENERAL INTRODUCTION

Orchids are unique plants having a distinct mode of growth and reproduction which make them incredible and fascinating. They belong to Orchidaceae, one of the largest families of the flowering plants, and are economically important for their horticultural and floricultural appeal. The orchids can be found in almost all the parts of the world except the Antarctica. There are about 17,000 species in about 750 genera distributed all over the world (Hegde, 1984), but Atwood (1986) has estimated the total number of orchid species to be 19,218. He also suggests that ultimately there may be 20,000-23,000 species with an

improbable maximum of 25,000. India is one of the richest reservoirs of orchids in tropical Asia and has about 1076 species (including varieties) (Rao, 1986). Jain and Mehrotra (1984) in their preliminary inventory estimated 925 species out of which 284 species are endemic to India. North-Eastern India and Sikkim are reported to harbour 650 orchid species (Kataki *et al.*, 1984).

Orchids have been attracting floriculturists since time immemorial due to their fads, fancies and fashions, and this has led to "Orchid Mania" throughout the world. Orchids are considered as the luxury flowers because of their high price and difficulty in cultivation. These beautiful and wondrous plants were thought to be parasites growing on trees but now it has been proved beyond doubt that the orchids are autotrophs which use their hosts merely for anchorage. They can withstand many abnormalities and endure drought and frost. Because of their wide range of tolerance, one can experiment with the orchids and learn about them through trial and error.

Orchids are considered to be important in medicines, food, perfumes etc. besides their value in commercial market. In nature, orchids are generally epiphytes growing on trees. However, lithophytes, terrestrials and saprophytes growing on rocks, ground and organic matter are also found. Orchids are perennial plants blooming annually under favourable

conditions of light, temperature and humidity. The flowers are produced either singly or in a spray or branched spike. These flowers are pollinated by different means, followed by fertilization which results in the formation of seed capsules containing extravagantly thousands of minute seeds. Nazarov (1988) has established a close relationship between the external volume of capsules and the amount of seeds produced in two species of *Dactylorhiza*. The orchid seeds are difficult to germinate in nature because they lack endosperm and possess immature embryo (Zeigler *et al.*, 1967). Even the presence of cotyledon in orchids is not clear. Batygina and Adronova (1988) have reported the absence of cotyledons in seven of the eight orchid species studied by them. The orchids depend on the specific mycorrhizal association for germination. Less than 5% of the seeds are reported to germinate in nature (Rao, 1977) as the frequency of the seeds to come into direct contact with its own mycorrhiza is very low.

A large number of orchids are propagated from seeds rather than vegetative means. Based on seed germination, the orchids can be divided into three categories: i) Tropical epiphytes and lithophytes (*Cattleya* from tropical America, *Phaius, Dendrobium and Cymbidium* , from Asia and Pacific) which germinate readily under asymbiotic conditions, ii) Tropical terrestrial and lithophytes (*Paphiopedium*) are

difficult to germinate asymbiotically and many require special media, and iii) Temperate climate terrestrials (north and south), which do not germinate under asymbiotic conditions and are solely dependent on their symbionts. The North American genus, *Spiranthes* is an exception to the above defined groups. It readily germinates under asymbiotic conditions (Yam and Weatherhead, 1988).

It was Bernard (1909) who for the first time isolated the root infecting fungus which helped orchid seed germination and paved the way for the development of *in vitro* asymbiotic germination of orchid seeds. Comparative germination studies were made of infected and non-infected seeds. The percentage of germination improved considerably when the sterilised seeds were grown in association with the fungus. Masuhara and Katsuya (1989) have reported the promotion of germination and stimulation of protocorm growth in *Spiranthes sinensis* var.*amoena* when the seeds are grown in association with mycorrhizal fungi. However, the work of Knudson (1922, 1924, 1925) suggested that the seed germination of orchids *in vitro* could be accomplished without fungal association by providing a nutrient rich medium having balanced organic and inorganic nutrients for the developing embryos. Different workers suggested a number of media and their modification for orchid seed germination (Vacin and Went, 1949; Zeigler *et al.*, 1967; Hadley and Harvais, 1968;

Rao, 1977; Reyburn, 1978; Henrich *et al.*, 1981; Harvais, 1982; Nakamura, 1982; Krishan Mohan and Jorapur, 1984; Oliva and Arditti, 1984; Pierik *et al.*, 1988; Yam and Weatherhead, 1988; Yam *et al.*, 1989). The orchid multiplication has been standardized and the major orchids tackled belong to the species of *Vanda*, *Dendrobium*, *Cymbidium* and few others (Goh, 1990; Sagawa, 1990). Besides the selection of media, other physico-chemical factors for orchid seed germination and seedling growth have been investigated. Many scientists have reported the effect of light, both qualitative and quantitative including photoperiod, on orchid seed germination and growth (Zeigler *et al.*, 1967; Mitra, 1971; Ueda and Torikata, 1972; Ernst, 1976; Hasegawa *et al.*, 1978). The optimal temperature for seed germination of most species is reported to be between 20°C to 25°C (Grillo Mensa *et al.*, 1985) with a wide range from 6°C to 40°C (Withner, 1959; Arditti, 1967a; Mukherjee *et al.*, 1974; Thompson, 1977). Stoutamire (1974) reported the requirement of chilling in several species. The specific pH value between 5.0 to 6.0 has been recommended for orchid seed germination (Withner, 1942; Knudson, 1946; Israel, 1963; Goh, 1971; Raghuwanshi *et al.*, 1986). The range of pH for asymbiont seed germination of orchids extends from 3.6 to 7.6 (Arditti, 1967a, 1979).

The responses of orchid seeds to the growth factors supplemented in the media differ from one species to another

(Arditti, 1982). The positive response of the germinating seeds to indole-3-acetic acid (IAA) incorporation in the medium has been reported in many instances (Arditti, 1967a, 1979; Withner, 1974; Mathews and Rao, 1980; Vij *et al.*, 1981). Tamanaha *et al.* (1979) suggested that the germinating orchid seeds and seedlings do not require exogenous auxins in most cases. However, 2,4-dichlorophenoxyacetic acid (2,4-D) has been shown to either inhibit germination or stimulate callusing of seeds (Mitra, 1986). Among cytokinins, kinetin (KN) has been shown to promote greening of protocorms and formation of plantlets which eventually lead to greater survival (Fonnesbech, 1972a; Harvais, 1972; Pierik and Steegmans, 1972). Benzylaminopurine (BAP) is reported to retard development and differentiation of cells and tissues of *Cymbidium* protocorms (Gailhofer and Thaler, 1975). The effects of exogenous gibberellins on growth of orchid seedlings in most cases is negative and vary with the species and growth stage (Arditti and Ernst, 1984). According to Mukherjee *et al.* (1974), incorporation of gibberellic acid (GA) in the medium induced callus formation; reduced percentage of normal seedlings; increased length and number of leaves in *Dendrobium* seedlings. Interactions between auxins (∝-naphthaleneacetic acid, NAA or 2,4-D) and cytokinins (KN and BAP) may result in enhanced growth, but the effects of these combinations vary with the hormones

used, their concentration, and ratios and the orchid (Kusumoto, 1978, 1979a, 1979b; Uesato, 1978). Several growth regulators have been incorporated in the media to promote orchid seed germination and seedling growth in different orchid species (Pierik and Steegmans, 1972; Strauss and Reisinger, 1976; Arditti, 1982; Nakamura, 1982; Sharma and Tandon, 1986; Van Waes and Debergh, 1986).

The tissue culture studies in orchids is gaining wide importance (Charanasri, 1989). The application of tissue culture techniques to the production of quality orchids in large quantities by clonal multiplication, establishment of hybrid plants, improvement of orchid trade and industry are unlimited. *In vitro* multiplication of orchids is also an effective method of saving many species from extinction (Clements and Ellyard, 1979; Clements *et al.*, 1986). Morel (1960) observed that the shoot tips of *Cymbidium* cultured on the suitable medium formed a spherule-like body with rhizoids at the base. These structures resembled morphologically the protocorms developed from the embryos and were hence called protocorm-like bodies (plbs). Regular chopping of these plbs and culturing them on fresh medium resulted in their multiplication, but when left undisturbed developed into complete plantlets without addition of any growth adjuvants. Most of the economically important orchids, except *Paphiopedilum* are clonable *in vitro* (Murashige, 1978). Shoot

tips measuring less than 1 mm can develop into a large number of plbs (Morel, 1960, 1972) and hence give rise to many plantlets. Different explants from orchid plants have been used for multiplication of orchids *in vitro*. Many studies have been conducted using shoot tips (Intuwong and Sagawa, 1974; Kusumoto, 1979a,b), flower stalk nodes (Reisinger *et al.*, 1976; Lay, 1978), internodal sections of flower stalk (Homma and Asahira, 1985), leaf segments (Tanaka *et al.*,1975, 1989; Vij *et al.*, 1984), root tips and root meristems (Chaturvedi and Sharma, 1981; Sood and Vij, 1986; Sanchez, 1988; Yoneda and Momose, 1988; Kraus and Monteiro, 1989). Meristem culture of orchids has also gained importance (Kunisaki *et al.*, 1972). The success of a particular species through tissue culture of explants largely depends on the medium and the explant source used and it differs from species to species. The incorporation of certain additives and growth factors into the media proves to be beneficial for tissue culture of many orchids (Kusumoto, 1979a,b; Yoneda and Momose, 1988).

The perusal of literature reveals the influence of plant hormones on enzyme synthesis or activity. The exogenous supply of plant growth regulators brings changes in the metabolic activities of the plant. The range of different enzymes affected is large and covers a wide spectrum of metabolic activities in the plant (Barendse,

1983). The changes brought about by the growth regulators in the enzyme activity are rather transient, usually starting with the rise in activity followed by subsequent decline. The mechanism by which the level of enzyme synthesis/activity is regulated by growth regulators is poorly understood in plants. Although several workers (Varner and Ho, 1977; Higgins and Jacobson, 1978; Bewley and Black, 1978; Letham *et al.*, 1978; Moore, 1980) have discussed different aspects of hormonal regulation of enzyme synthesis in plants, the data on the physiological basis of the diverse requirements of orchid seeds during their germination and development and the effects of various growth regulators on the metabolic pathways underlying the process of seed germination and seedling growth is scanty. On the basis of an observed increase in the activity of the peroxidase, as a result of exogenous application of IAA in *Vanda*, Alvarez and King (1969) have suggested that the auxin is capable of stimulating the activity of an enzyme supposed to be involved in its oxidation. The effect of mycorrhizal infection on respiration and activity of some oxidative enzymes of orchid protocorms have been studied by Blackeman *et al.* (1976). The study on the synthesis of phenolic compounds as affected by growth regulators in orchids is insufficient. The growth regulators are also reported to affect the nucleic acid content of the cells of *Cymbidium* (Nagl and Rucher, 1974,

1976) but the detailed information on their effects is lacking.

The establishment and healthier growth of *in vitro* grown orchid plants in the glasshouse requires right container and compost (Hegde, 1984). According to Bose and Bhattacharjee (1980), the potting media differ with the types of orchid and the climate in which they are grown. The composts of osmunda and shagnum moss for *Bulbophyllum, Masdevallia* and *Paphiopedilum,* chunks of hardwood charcoal for some epihytes e.g., *Dendrobium, Laelia, Oncidium, Phalaenopsis, Rhychostylis* and *Vanda,* coconut husks for *Dendrobium, Phalaenopsis,* etc. and charcoal in mixed compost for terrestrial and semi-terrestrial orchids are mainly used. Regular addition of manure and fertilizers is beneficial and depends on the composition of the potting materials and the orchid grown.

Although the North-East India is reported to have the richest reservoir for rare ornamentals, the orchid resources of this region are fast depleting due to ruthless exploitation of orchid plants for commerce and trade and also due to increasing deforestation. On account of this, a few species are extremely scarce or perhaps already extinct and many more are facing the danger of being wiped out. The natural population of *Dendrobium fimbriatum* is on decline and has become rare in certain parts of India (Misra, 1989).

Keeping in mind the conservation and protection of this orchid from extinction, work was undertaken to study some functional and biochemical aspects of its *in vitro* regeneration and growth.

II. ASYMBIOTIC GERMINATION ON DIFFERENT MEDIA

INTRODUCTION

A large number of orchid seeds are produced which are very minute and possess little or no endosperm. Because of their particular fungal requirement, less than 5% of the orchid seeds germinate in nature (Rao, 1977). On the other hand, a very high percentage of seed germination could be achieved asymbiotically in flasks or test-tubes. Asymbiotic techniques of orchid seed culture are useful in tracing the seedling development from undifferentiated embryos. Seed culture can be applied not only to several artificial and natural hybrids, but also to certain desirable species and

forms which are not available in quantity. Seedling production *in vitro* and further development is also an effective means of saving many orchid species from extinction (Clements and Ellyard, 1979; Clements *et al.*, 1986). Using different nutrients and culture conditions, seed germination in a large number of orchid species has been accomplished (Arekal and Karanth, 1980; Stoutamire, 1981; Arditti, 1982; Arditti and Ernst, 1984; Sharma and Tandon, 1987; Yam and Weatherhead, 1988; Yam *et al.*, 1989). However, *in vitro* germination methods successful for one species are not always applicable to others and also procedures for orchids from one region may not be suitable for those from another (Arditti *et al.*, 1981).

MATERIALS AND METHODS

Dendrobium fimbriatum var. *oculatum* Hk.f., is an orchid of considerable ornamental and horticultural importance. The plant is a hardy cane-type orchid which produces a beautiful bunch of yellow flowers (Plate 1a,b). The stem is usually erect, shorter, slender and bearing somewhat smaller flowers, which are mildly scented, orange-yellow in colour with orbicular fimbriate lip having a large dark reddish-brown blotch at the base. This variety is remarkably floriferous, as many as 123 racemes bearing 1,216 flowers have been counted on a single plant. The plant species is

Plate 1. (a) *Dendrobium fimbriatum* var. *oculatum* blooming in natural habitat

(b) A closer view of the flowers.

Plate 1 b

distributed both in tropical and subtropical zones, in partially exposed areas of trees and rocks. It is also found in tropical valleys of the Himalayas from Nepal to Bhutan upto 1200 m and extends to Khasi Hills in North-East India, Burma, Thailand, Vietnam and Malaya.

About four-months old capsules of *D. fimbriatum* var. *oculatum* were collected from the plants growing in University Botanical Garden. These were surface sterilized with sodium hypochlorite solution (1.25% available chlorine) for about 15 min and slit open under aseptic conditions to expose the seeds (Kumaria and Tandon, in press). The seeds were germinated aseptically on six different nutrient media viz. Knudson C (1946), Vacin and Went (1949), Murashige and Skoog (MS, 1962), White (1963), Nitsch (1969) and Mitra *et al.* (1976). The pH of the media was adjusted to 5.5 prior to autoclaving. Before using, the transfer table of the laminar flow cabinet was thoroughly scrubbed with cotton soaked in 70% (v/v) ethanol. The ultra-violet tube light of 30 Watt was switched on for 10-15 min after which the transfer area was left as such for about 5 min with the flow of ultra-filtered sterile air to remove the ozone. The velocity of the air flow was 27 ± 3 m/min which helped in preventing air-borne contamination. Forceps, needles, surgical blades, etc. were sterilized by dipping in alcohol and flamed before use for proper sterilization. The culture tubes were incubated at

$25\pm2^{\circ}C$ under 8 h of 1500 lux light.

After two months, percentage germination in different media was determined by examining the seeds microscopically. The seeds were considered to have germinated upon emergence of the embryo from the testa. The percentage germination was calculated using both green and white protocorms. The protocorm volume was determined using the formula given by Stoutamire (1981) for an oblete spheroid ($4/3\ \pi\ a^2b$, where a and b are minor and major semi-axes, respectively). Ten replicates were used for each treatment and the experiments were repeated twice.

RESULTS

The best germination was recorded on Nitsch medium (91%), followed by MS (85%) and Mitra *et al.* (67%). The seeds showed poor germination on Vacin and Went, Knudson C and White media (Table 1). The time required for germination on different media also varied (Table 2). On MS and Nitsch media germination was faster than others. The protocorm stage was attained within the first 4-5 weeks on MS, Nitsch and Vacin and Went media; but this was delayed in other media tried. The largest protocorm volume was recorded in MS medium followed by Vacin and Went and Nitsch (Plate 2). The colour of the developing protocorms was different on all the six media tried. Healthy and dark green protocorms were

Plate 1. (a) Dendrobium lituiflorum Lindl. blooming in natural habitat

(b) Closer view of the flowers

Pictures to be taken (1). MS, NN, B_5 Mitra et al Kw C'

(2) IAA – 0.5, 2.5, 5, 10.

NAA → 0.5, 2.5, 5, 10.

2,4D → ~~Kin~~ 0.5, 2.5, 5, 10.

BAP → 0.5, 2.5, 5, 10.

Kw → 0.5, 2.5, 5, 10.

Combinations :-

IAA + BAP → 2, → 0.5 g BAP + 2.5 + BAP + 0

NAA + BAP → 2. → ~~2.5 g~~ A

2,4D + BAP → 2.

IAA + KN – 2

~~NAA~~ + Kw – 2

2,4D + KN – 2.

pictures to be taken!

Table 1 : Effect of different media on seed germination and protocorm differentiation

Media	Germination(%)	Remarks
Murashige and Skoog	85	Protocorms were round and green and exhibited differentiation into healthy root and shoot
Nitsch	91	Protocorms were small and green without any differentiation
Mitra *et al*	67	The germinating seeds formed a yellow callus-like mass
Vacin and Went	48	The developing protocorms showed some signs of differentiation
Knudson C	35	The protocorms turned brown and did not differentiate
White	20	Very small green protocorms were produced

Table 2 : Effect of different media on the development of protocorms

Media	Developmental stage* (weeks) I	II	III	IV	Protocorms colour	Dimensions** Length (mm)	Width (mm)	Volume (mm^3)
Murashige and Skoog	2	2	8	12	Dark green	0.079	0.090	$26.0x10^{-4}$
Nitsch	2	4	10	15	Green	0.056	0.064	$9.4x1-^{-4}$
Mitra *et al.*	12	15	-	-	Light yellow	0.045	0.049	$4.2x10^{-4}$
Vacin and Went	3	5	12	15	Green	0.058	0.071	12.0x10-4
Knudson C	15	-	-	-	Brown	0.024	0.032	$1.2x10^{-4}$
White	2	12	-	-	Green	0.016	0.012	$0.001x10^{-4}$

* I, Non-germinated seeds, embryo slightly swollen and white but still covered with its seed coat or testa;

II, Germinating seeds, embryo greatly swollen forming an ovoid tear-drop shaped protocorm without seed coat or testa;

III, Young protocorms showing pointed vegetative apex;

IV, Protocorms enlarged having root and leaf initials;

** Mean of ten values.

Plate 2. Protocorms produced from seeds cultured on (a) Nitsch, (b) MS, (c) Vacin and Went, (d) Mitra *et al.*, (e) Knudson C, and (f) White media

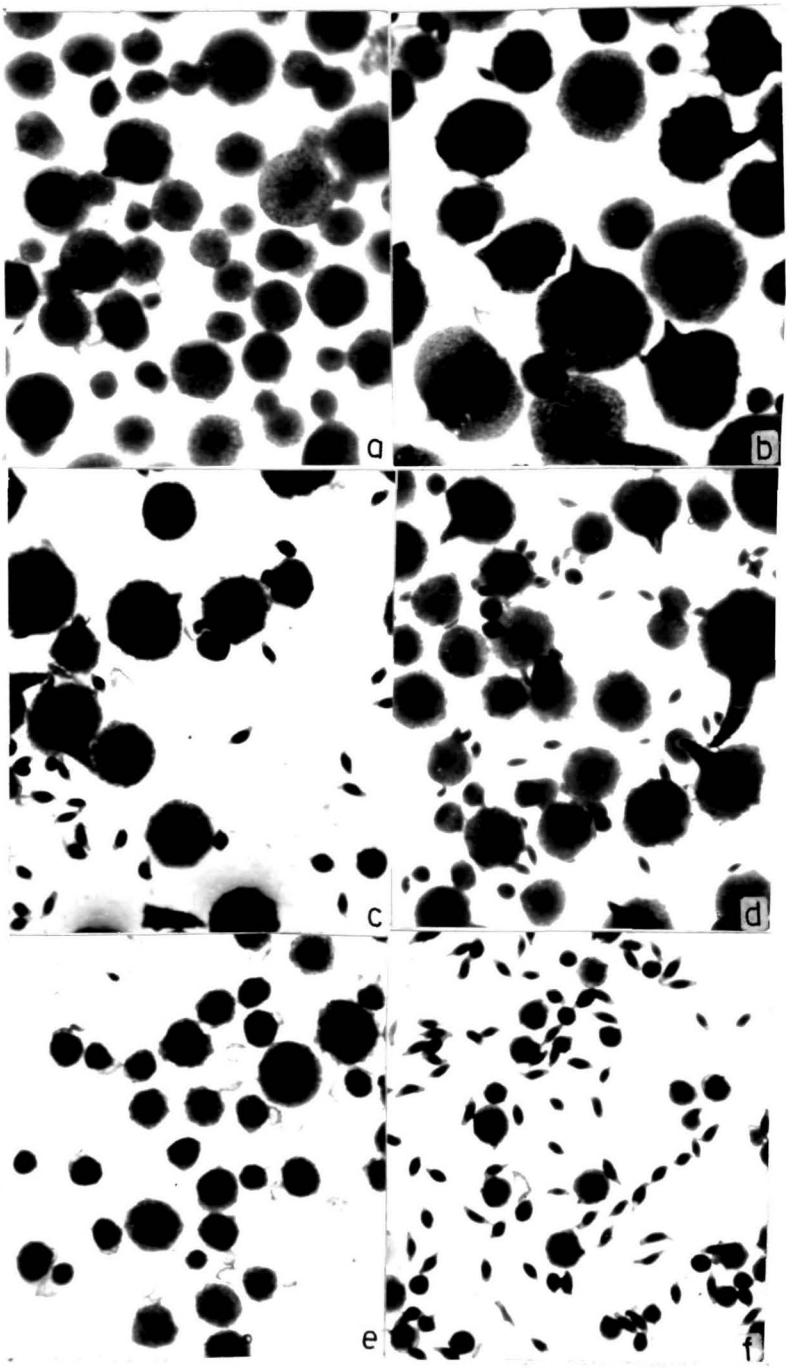

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Plate 2

observed on MS medium. On Mitra *et al.* and Knudson C media, the protocorms failed to turn green (Table 2).

DISCUSSION

The orchid seeds respond differentially to different media used for germination. The development of orchid seeds requires a balanced supply of both organaic and inorganic nutrients, growth factors and suitable light and temperature (Arditti, 1982; Zeigler *et al.*, 1985; Van Waes and Debergh, 1986). In the present study, Nitsch medium was found to be the best for seed germination followed by MS and Mitra *et al.* Ammonium nitrate present in the former two media, is considered to be the most suitabale source of nitrogen for orchid seed germination (Arditti and Ernst, 1984). Mitra *et al.*, Vacin and Went and Knudson C media contain both calcium nitrate and ammonium sulphate which did not support good germination. The difference in responses to nitrogen sources used may be due to varied requirements of this species. Mixture of vitamins in the medium has been reported to improve germination and growth of orchid seeds (Arditti and Harrison, 1977). The seeds of *D. fimbriatum* showed poor germination on Vacin and Went, Knudson C and White media as these do not contain sufficient amounts of nutrients and vitamins. Yam and Weatherhead (1988) reported that the nutritional requirements of germinating orchid seeds vary due

to their physiological state. The protocorms produced on MS medium were quite large compared to those developed on other media. Healthy growth of orchid protocorms in medium containing balanced supply of organaic and inorganic nutrients has been reported (Arditti and Ernst, 1984). The protocorms developed on Vacin and Went medium were quite large as compared to the ones growing in other media except MS. This could be due to the availability of more nutrients to the few developing protocorms. The development of protocorms to the next stage with pointed vegetative apex was observed in case of MS, Nitsch and Vacin and Went media only. It has been reported that mortality rate is quite high at protocorm stage (Arditti *et al.*, 1981). However, it could be reduced under suitable nutritional and evnironmental conditions enabling further development of protocorms to the next stage. The last stage of development with leaves and rhizoids/roots was observed within three to four months. The seeds of this orchid require a rich medium (containing higher concentrations of nutrient salts and vitamins) for both germination and protocorm development.

III. EFFECT OF PHYSICAL FACTORS ON SEED GERMINATION AND SEEDLING GROWTH

INTRODUCTION

With the discovery that germination of orchid seeds was possible *in vitro* without fungal association by providing balanced organic and inorganic nutrition for the developing seedlings (Knudson, 1925), the asymbiotic germination of orchid seeds gained importance. But the success of *in vitro* orchid seed germination and seedling growth is not only restricted to nutrient supply but also to physical factors like temperature, light and pH of the medium which greatly influence the physiology and development of orchid seedlings (Ueda and Torikata, 1972; Arditti and Ernst, 1984). However,

the information on the influence of the physical factors on orchid seed germination is scanty.

The orchid seeds exhibit differential responses to light for germination whereas others can germinate in its complete absence (Van Waes and Debergh, 1986). It has been reported that orchid seeds of most of the epiphytes and some terrestrials can germinate both in light and darkness but for further growth and development, the requirement of light is essential (Arditti, 1967a, 1979; Ueda and Torikata, 1972; Fast, 1976; Voth, 1976). The influence of both qualitative and quantitative light including photoperiod on orchid seed germination and growth has been studied to some extent (Withner, 1959; Zeigler *et al.*, 1967). Stimart and Ascher (1981) reported that *Cattleya* seeds germinated at different rates of illumination or darkness. However, in case of *Cymbidium*, the seeds germinated in dark with the development of leaves, the initiation of roots was totally absent (Yates and Curtis, 1949; Kohl, 1962). Temperature is a major factor in seed germination and development of orchid seedlings. Temperature sensitivity of *in vitro* seedling development has been reported in *Dactylorhiza majalis* (Rasmussen *et al.*, 1990). The range between 20-25^{o}C was found suitable for most orchid species (Harvais, 1973; Stoutamire, 1974; Arditti, 1982; Van Waes and Debergh, 1986). However, the temperature range extends from 6^{o}C to 40^{o}C in

case of orchids (Arditti, 1967a, 1967b; Mukherjee *et al.*, 1974; Thompson, 1977). The tolerance of pH by the orchid species ranges from 3.6 to 7.6 (Arditti, 1967a; 1979). But some workers recommend the specific pH value between 5.0 and 6.0 for orchid seed germination (Knudson, 1946; Israel, 1963; Goh, 1971; Raghuwanshi *et al.*, 1986).

MATERIALS AND METHODS

The immature four-months old capsules of *D. fimbriatum* var. *oculatum* were sterilized following the procedure described in chapter II. The seeds were exposed aseptically and inoculated on MS medium without growth regulators. The cultures were subjected to different physical factors such as temperature (15, 20, 25, 30 and 35^{o}C), photoperiod (0, 8, 12, 16, 20 and 24 h day), light intensity (0, 1500, 3000 and 5000 lux) and pH of the medium (4.5, 5.5, 6.5 and 7.5). The percentage germination was recorded microscopically after two months. The two-months old protocorms developed under ideal conditions of temperature, light and pH of the medium (25^{o}C, 12 h day of 1500 lux, pH 5.5) were transferred to fresh MS medium and grown under different physical conditions (as described above) to study their effects on seedling growth and development. Shoot length, leaf number and area, root number and length, and dry weight were taken at monthly intervals for four months. From the primary data, net

assimilation rate (NAR) and leaf area ratio (LAR) were determined using the following formulae

$$NAR = \frac{(W_2-W_1)\ 2.303\ (\log_{10}A_2-\log_{10}A_1)}{(t_2-t_1)\ (A_2-A_1)}$$

$$LAR = \frac{(A_2-A_1)\ 2.303\ (\log_{10}W_2-\log_{10}W_1)}{2.303\ (\log_{10}A_2-\log_{10}A_1)\ (W_2-W_1)}$$

where W_1 and A_1 are the dry weight and leaf area at time interval t_1, and W_2 and A_2 are the dry weight and leaf area at time interval t_2.

The plant growth is described by the relative growth rate (RGR) which is the function of the NAR and the LAR

$$RGR = NAR \times LAR$$

while NAR is the increase in plant weight/unit leaf area, LAR is the ratio of leaf area to plant weight.

Five replicates of each of the treatments were taken and the experiments were repeated twice.

RESULTS

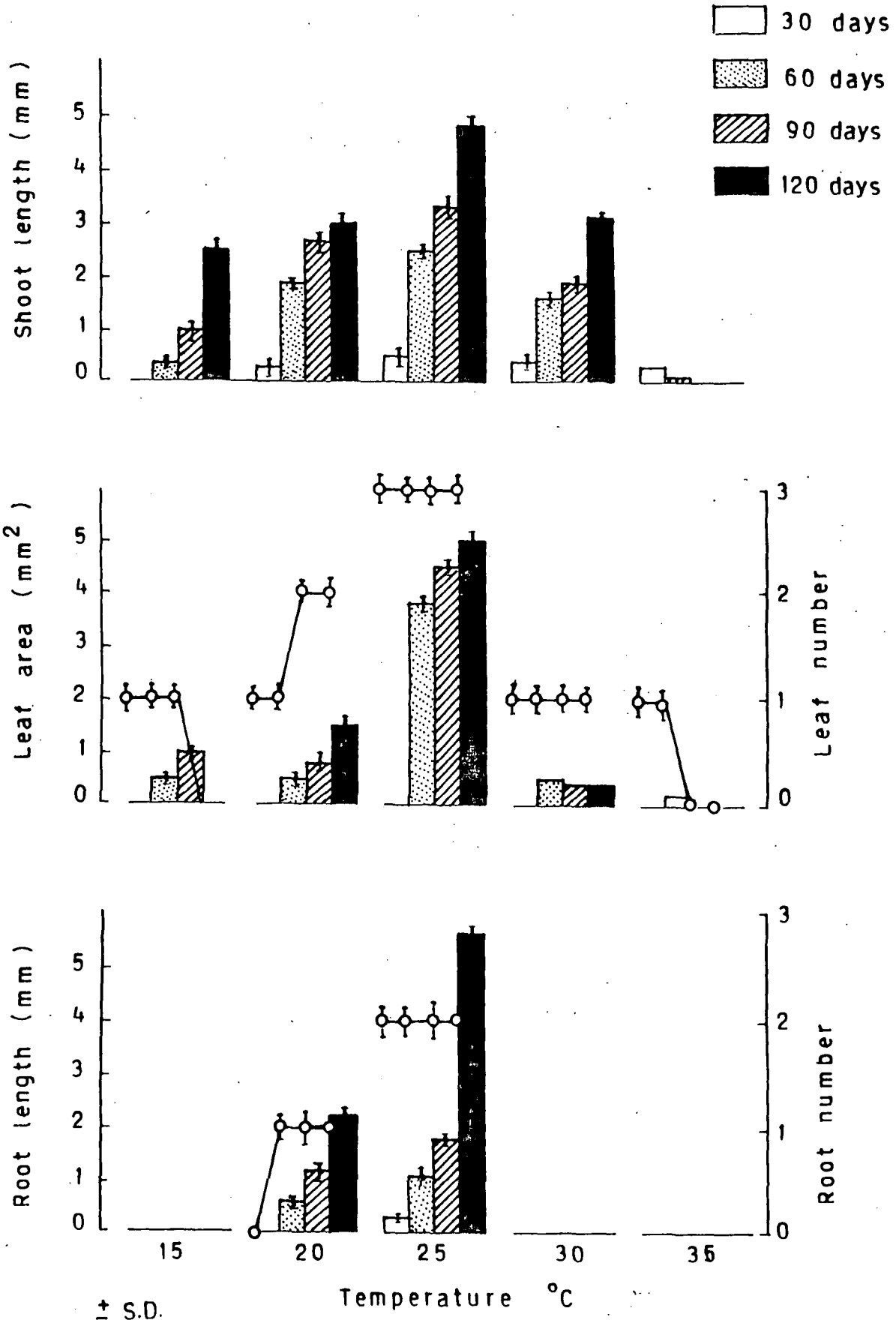
Effect of Temperature

The maximum germination was observed in seeds cultured at 25°C. The percentage germination declined by increasing the temperature (Table 3). On the other hand, there was no seed germination at 15°C. Based on the growth analyses, the optimal temperature for seedling growth was 25°C (Fig. 1;

Table 3. Effect of different physical factors on seed germination

	Treatments	Germination (%)	Remarks
Temperature (°C)	15	-	The seeds failed to germinate
	20	80.0	Good proyocorm development
	25	88.0	Healthy green protocorms
	30	73.0	Developing protocorms differentiate into seedlings
	35	25.0	Green but small protocorms
Photoperiod (h)	0	-	In complete darkness, the seeds failed to germinate
	8	82.0	Green protocorms
	12	89.0	Green protocorms
	16	85.0	Healthier protocorms
	20	70.0	Very small protocorms
	24	15.0	Few green protocorms
Light Intensity (lux)	0	-	No germination recorded
	1500	85.0	Healthy protocorms
	3000	33.0	The seeds show some signs of germination but the protocorms remained brown weak growth
	5000	5.0	
pH of the medium	4.5	62.0	Few small green protocorms emerge
	5.5	88.0	Healthy protocorms
	6.5	10	Seeds turn brown
	7.5	-	No germination

Fig. 1. Effect of temperature on seedling development


30 days
60 days
90 days
120 days
Shoot length (mm)
Leaf area (mm²)
Leaf number
Root length (mm)
Root number
0
1
2
3
4
5
15
20
25
30
35
Temperature °C

± S.D.

Fig. 1

Plate 3. Seedlings developed at different temperatures

Plate 4. Seedlings developed at different photoperiods

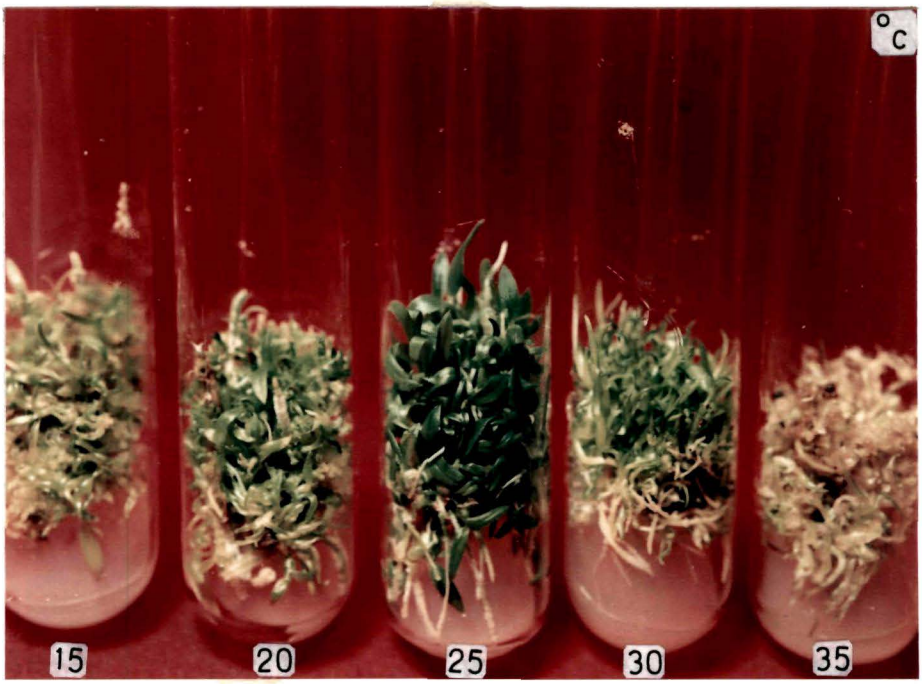


Plate 3

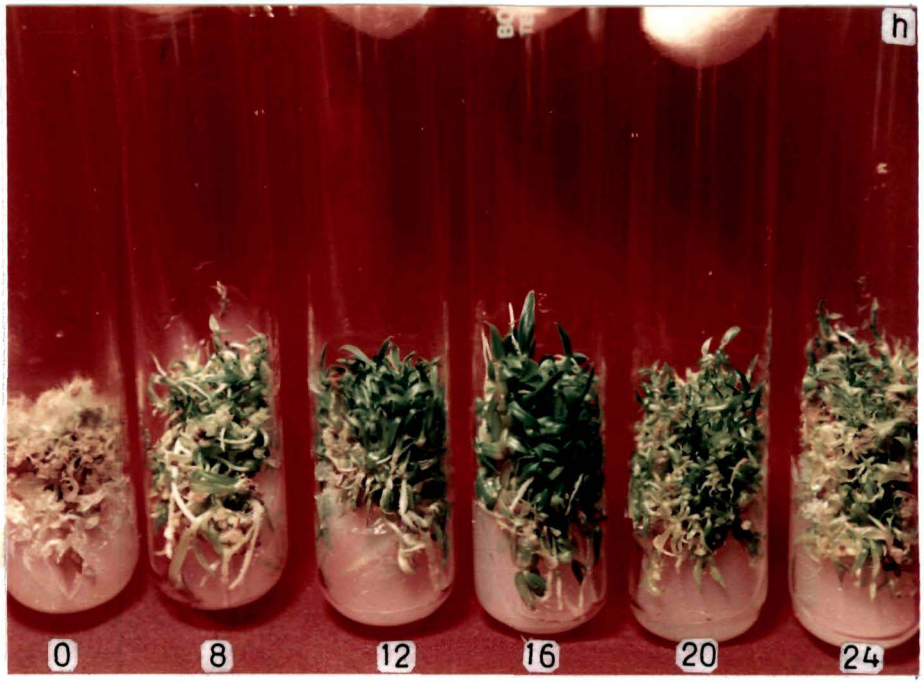


Plate 4

Table 4. Effect of different physical factors on NAR and LAR during seedling development

Physical factors		NAR g/mm^2/Unit time (Days)				LAR (Days)			
		30	60	90	120	30	60	90	120
Temperature ('C)	15	-0.363	-0.0016	-0.0016	-0.0016	-0.067	37.553	35.443	35.441
	20	-0.237	-0.0004	-0.0001	-0.000015	-0.121	15.433	149.29	404.84
	25	-157.32	0.00065	0.00013	-0.0000027	-0.084	26.315	207.48	244.29
	30	-0.311	-0.0007	0.000032	-0.0001	-0.078	18.480	121.47	123.22
	35	-0.347	-0.4633	Dried	Dried	-0.055	-0.067	Dried	Dried
Photoperiod (h)	0	-0.0004	-0.000026	-0.0000016	-0.0000085	86.73	1489.09	1329.75	492.71
	8	-392.59	0.000146	0.0000013	0.000003	-0.000038	28.84	514.17	596.06
	12	-151.15	0.0007	0.000126	0.000033	-0.084	26.32	207.48	244.29
	16	-116.21	-0.0005	0.000001	0.0000011	-0.0002	64.31	1504.56	1499.20
	20	-1243.6	-0.0007	0.0	-0.0000065	-0.000016	28.94	0.0	941.26
	24	-1779.8	-0.0032	-0.000017	-0.0000087	-0.000021	11.60	363.41	482.52
Light intensity (lux)	0	-0.00004	-0.00003	-0.000002	-0.000009	86.48	1489.19	1329.75	482.46
	1500	-157.32	0.00065	0.000126	0.000027	-0.084	26.315	207.48	244.29
	3000	-101.71	0.0052	0.00028	0.00001	0.00003	8.64	100.91	229.59
	5000	0.0	-0.00019	-0.000005	0.0	0.0	1.33	674.43	0.0
pH	4.5	-0.106	-0.000057	-0.0000057	-0.000002	-0.201	33.633	238.94	307.06
	5.5	-157.32	0.00065	0.000126	0.00003	-0.084	26.315	207.48	244.29
	6.5	-56.88	-0.0002	-0.000007	-0.000008	-0.0002	4.559	85.23	206.06
	7.5	-124.60	-0.0012	-0.00001	-0.000018	-0.0001	11.440	209.59	338.42

Plate 3). Even though the seedlings developed in cultures grown at all the temperatures, a considerable reduction in shoot length was observed at 30°C, and beyond this temperature the seedlings dried after 60 days of growth. A temperature of 15°C was inhibitory for the growth of seedlings. Both at 30°C and 35°C, there was no root growth. A drastic increase in leaf area was recorded upto 60 days in all treatments after which the increase was not pronounced. The root/rhizoid number and length were maximum at 25°C followed by 20°C treatment. An increase in NAR was recorded on the 60th day of treatment in seedlings raised at 15°C and 20°C (Table 4). At 25°C, an exponential pattern of growth resulted in the initial period due to higher NAR. However, after 60 days a decline in NAR was recorded. In case of seedlings raised at 30°C, NAR increased upto the 90th day of growth but at 35°C the seedlings dried completely after 60 days. The results show an increase in LAR with time (Table 4).

Effect of Light

a) Light quality : A wide range of photoperiod (8h - 20h) was suitable for germination, but the best germination was recorded at 12 h day (Table 3). No germination was recorded in complete darkness. For the optimum growth of the seedlings, a 16 h photoperiod was suitable (Fig. 2, Plate 4).

Fig. 2. Effect of photoperiod on seedling development

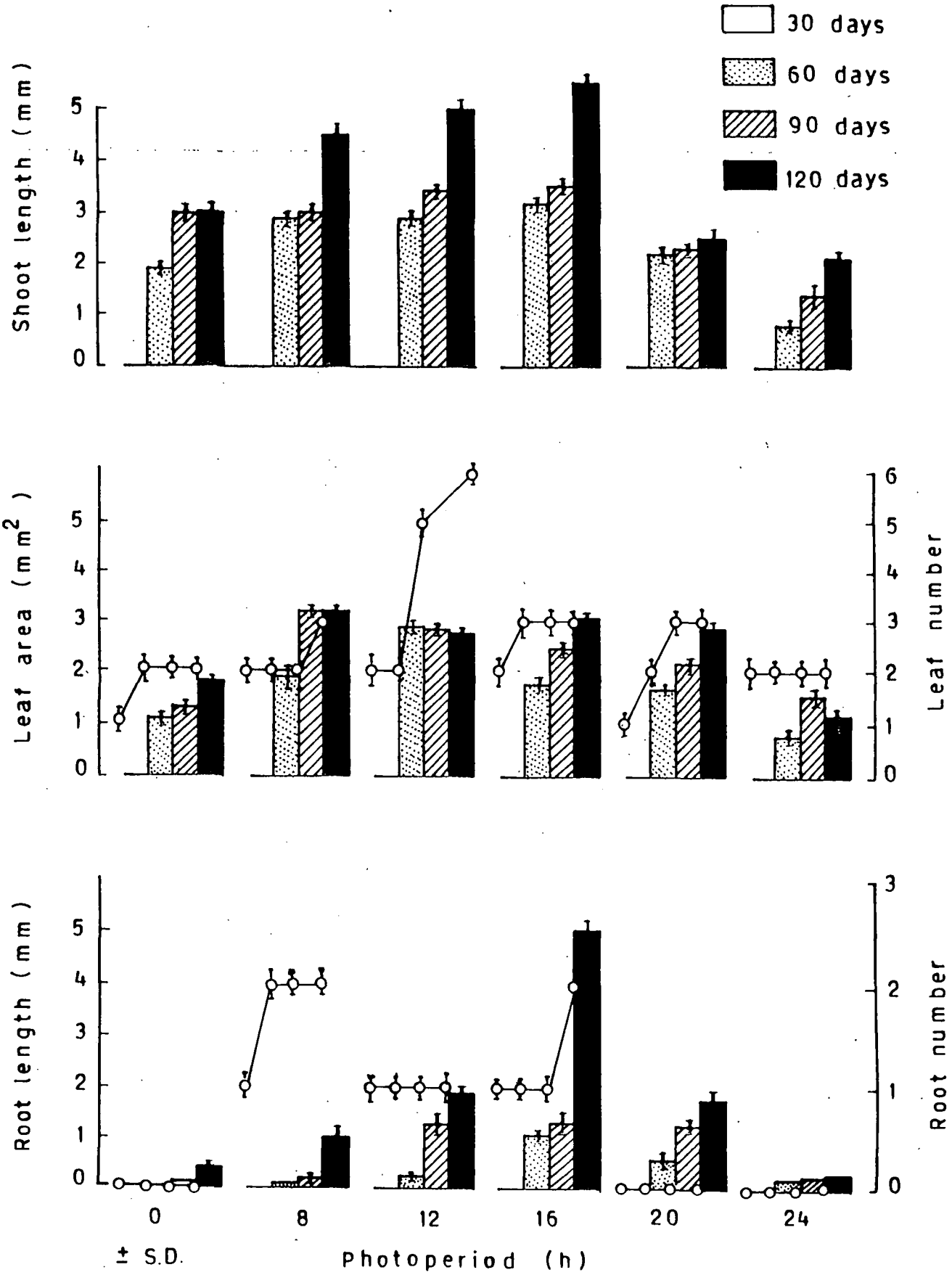

30 days
60 days
90 days
120 days
Shoot length (mm)
Leaf area (mm²)
Leaf number
Root length (mm)
Root number
0
8
12
16
20
24
± S.D.
Photoperiod (h)

Fig. 2

In darkness and continuous light, the seedling growth was poor. The optimum shoot and rhizoid/root length, and number were recorded at 16 h photoperiod. The maximum leaf number was recorded at 12 h photoperiod and the leaf area, at 16 h photoperiod. NAR was negligible in complete darkness from the very beginning till the end of the observation period (Table 4). In case of seedlings raised in the range of 8h-20h photoperiods, NAR increased till the 60th day after which it declined or remained stationary. However, NAR increased at a constant rate with time in seedlings grown at 16h photoperiod. An increase in LAR was recorded with time except in total darkness (Table 4).

b) Light intensity : The maximum percentage of seed germination was recorded at 1500 lux and the lowest at 5000 lux of light (Table 3). The seeds failed to germinate in complete darkness. Shoot length, number and area of leaf primordia/leaves and number and length of rhizoid/root were highest at 3000 lux light (Fig. 3, Plate 5). Both at 1500 and 3000 lux light, a marked increase in NAR was observed on the 60th day after which it declined (Table 4). However, the latter light intensity was found better. At light intensities 0 and 5000 lux, NAR remained negative or almost near zero throughout the growth period. LAR increased throughout the growth of seedlings at 1500 and 3000 lux of light (Table 4).

Fig. 3. Effect of light intensity on seedling development

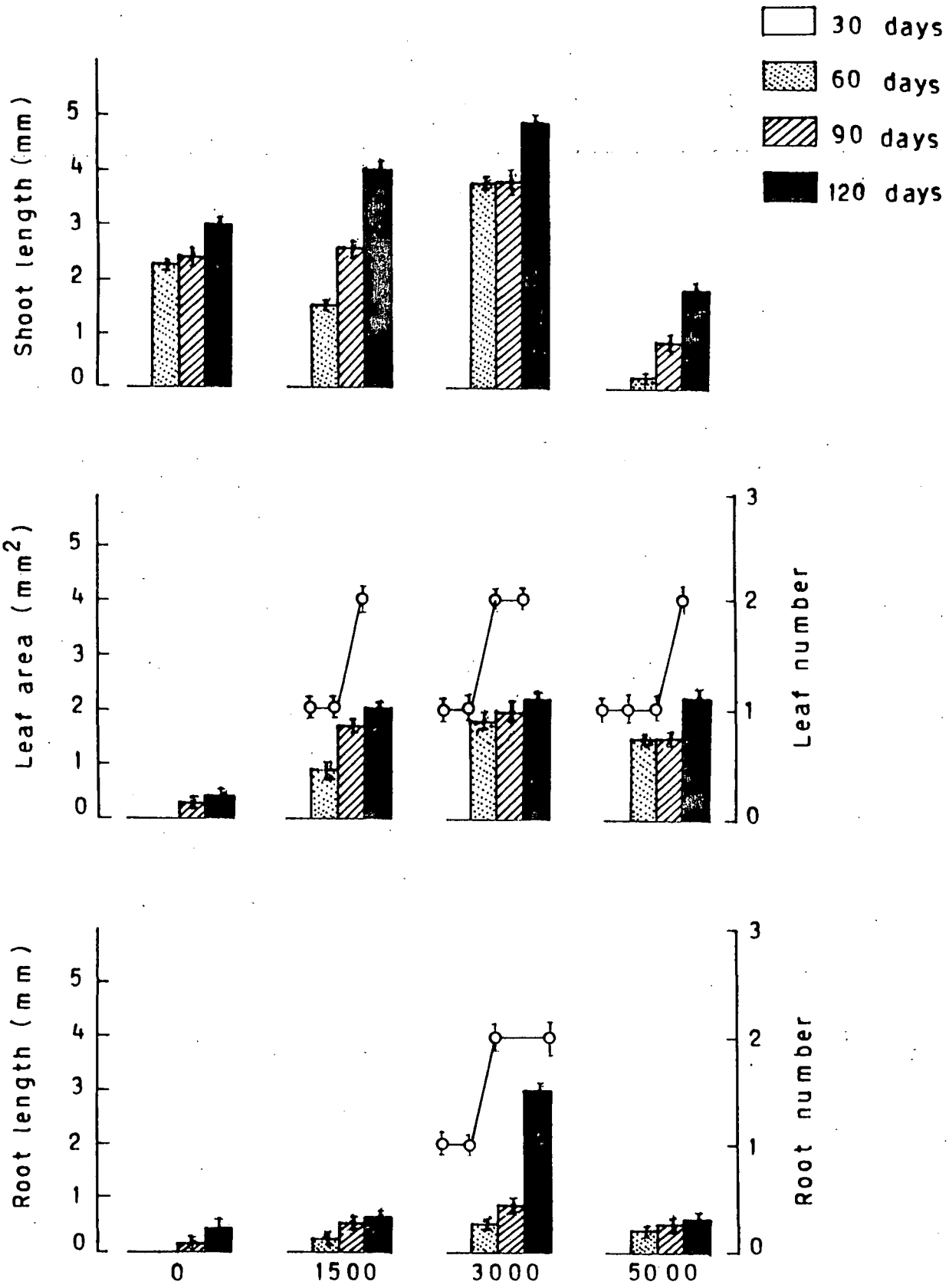

30 days
60 days
90 days
120 days
Shoot length (mm)
Leaf area (mm²)
Leaf number
Root length (mm)
Root number
0
1500
3000
5000
± S.D. Light intensity (lux)

Fig. 3

Plate 5. Seedlings developed at different light intensities

Plate 6. Seedlings developed at different pH of the medium

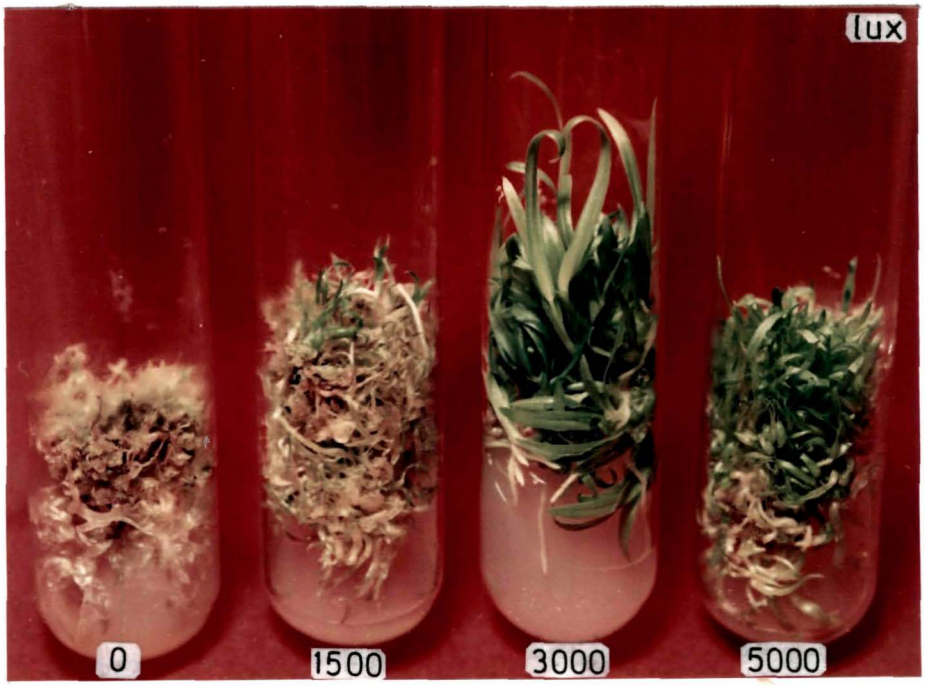


Plate 5

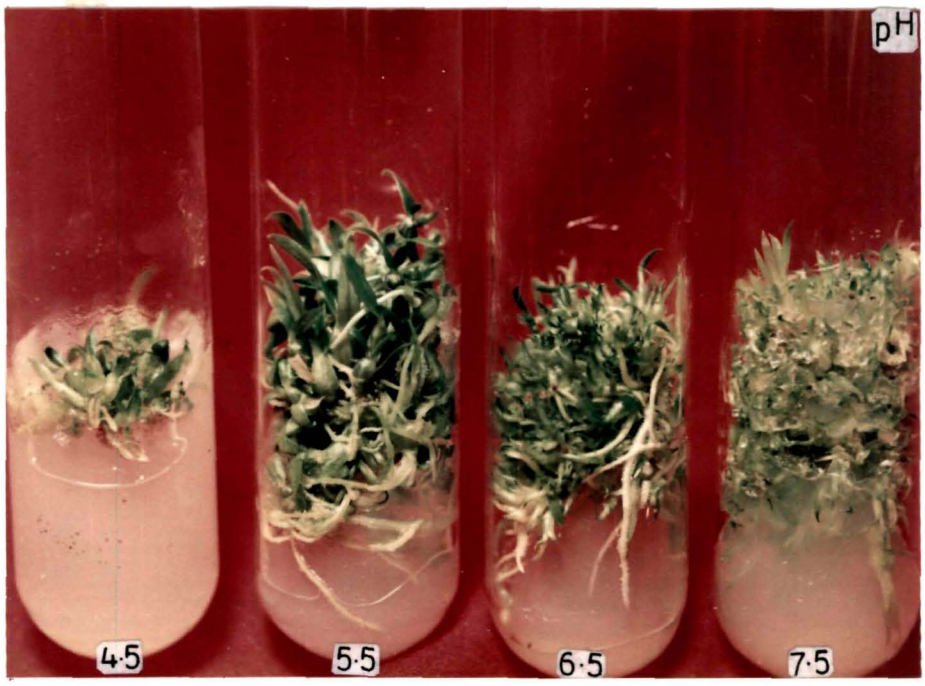


Fig. 4. Effect of pH on seedling development

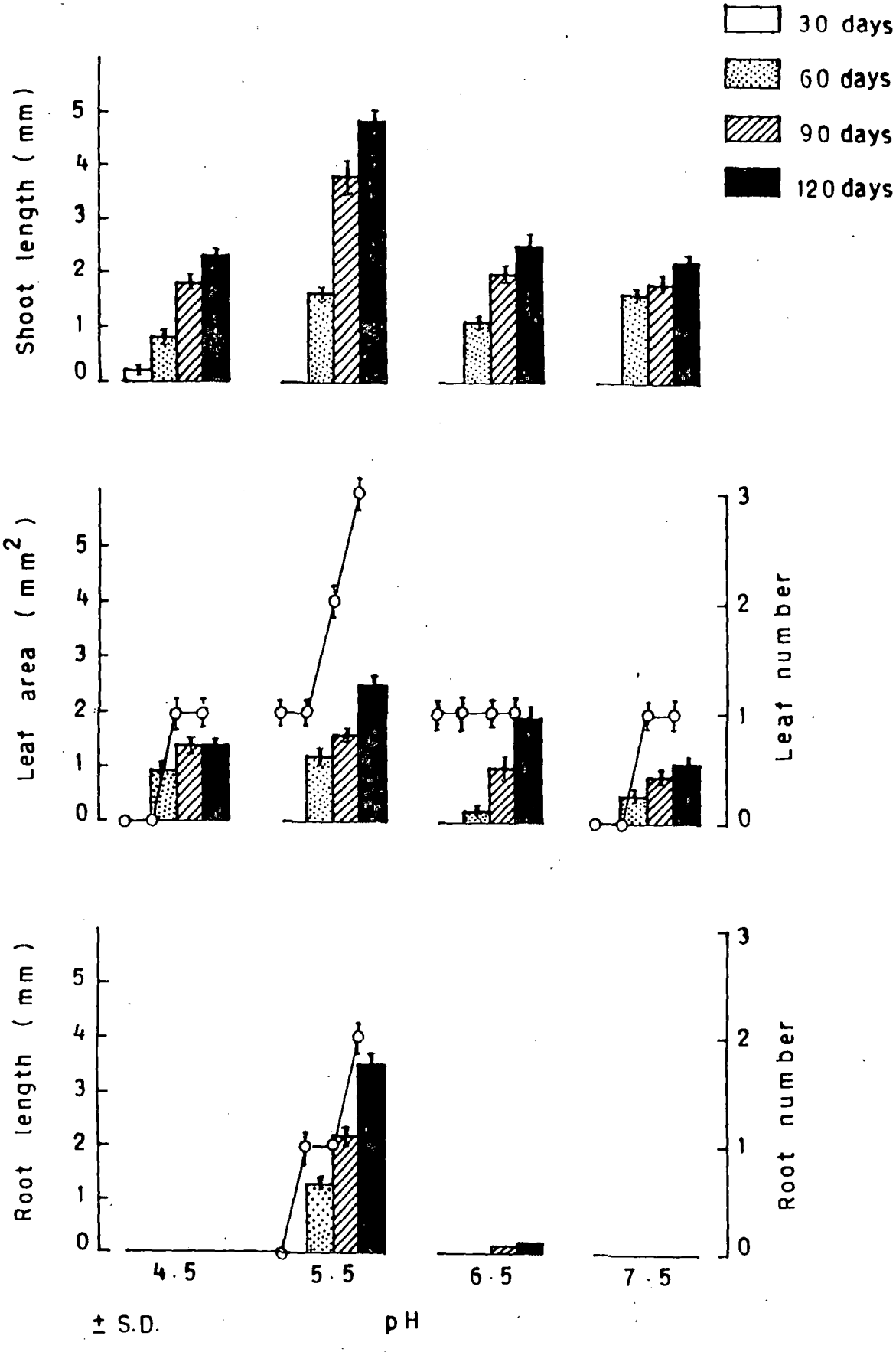

30 days
60 days
90 days
120 days
Shoot length (mm)
Leaf area (mm^2)
Leaf number
Root length (mm)
Root number
4·5
5·5
6·5
7·5
± S.D.
pH

Fig. 4

Effect of pH of the Medium

The germination of seeds and subsequent seedling development varied at different pH levels of the medium. The seeds exhibited the best germination at pH 5.5 followed by pH 4.5. However, germination was inhibited at pH 6.5 and 7.5 (Table 3). The optimum seedling growth was observed at pH 5.5 of the medium (Fig. 4, Plate 6). The highest shoot length was recorded at pH 5.5 and the lowest at pH 7.5. The average number and area of leaf primordia/leaves were also observed to be higher at pH 5.5, followed by those at pH 6.5 and 4.5. Even for the development of root/rhizoid, pH 5.5 was found to be the best throughout the growth. No roots were observed in the medium having pH 4.5, 6.5 and 7.5. At pH 6.5 some minute hairy growths developed. An increase in NAR was recorded on the 60th day of culture at pH 4.5, 6.5 and 7.5 (Table 4). It, however, showed a stationary phase after 60 days. At pH 5.5 of the medium, the NAR was much higher on the 60th day as compared to other treatments. After 60 days, NAR declined at this pH. The increase in LAR with time at different pH was recorded (Table 4).

DISCUSSION

Germination was considered to have occurred on the emergence of the embryo from the testa. Protocorm development and regeneration occurred by the appearance of

rhizome, shoot and root. The environmental factors have been reported to influence the growth of orchids (Hugh and Thomas, 1980).

In the present study, the higher percentage of seed germination and the overall seedling growth of *D. fimbriatum* were recorded in asymbiotic cultures. The optimal temperature recorded for seed germination of this orchid species was 25°C. The temperature range of 20-30°C has been reported to increase the seed germination and seedling growth in several orchids (Knudson, 1950; Miura, 1982; Zeigler *et al.*, 1985) by increasing the metabolic rate of the plants. On the other hand, Zeigler *et al.* (1967) reported the loss of water from the medium as well as from the living system at extreme temperatures (around 30°C) resulting in poor seedling growth. NAR increased on the 60th day of growth in case of seedlings raised at 15°C and 20°C. However, at 25°C NAR increased and exhibited exponential pattern of growth in the initial periods. But after 60 days, a decline in NAR was observed. Higher temperature of 30°C exhibited an increase in NAR till the 90th day after which it became almost constant. However, seedlings raised at 35°C dried at the end of 60 days of growth. Above 30°C, photosynthesis is reported to be negligible (Bazzaz *et al.*, 1970). Although LAR increased with time, it could not compensate for reduced NAR. This is in agreement with the findings of Regnier *et al.*

(1988) who reported that increased LAR did not fully compensate for the reduced NAR in *in vitro* grown plants. According to Leopold and Kriedmann (1975), NAR along with LAR and RGR could be used to analyse the response of plant growth to environmental conditions.

The germinating orchid seeds and developing seedlings vary in their requirements and responses to light and/or photoperiods (Arditti, 1967a, 1979; Arditti *et al.*, 1982; Zeigler *et al.*, 1985). In this study, seed germination was enhanced by light. The range of photoperiod from 8h-20h was found to be quite suitable for seed germination. However, the highest percentage of germination was recorded at 12h photoperiod. Stoutamire (1974) reported that seed germination in many orchids is affected by light which may be stimulatory or inhibitory depending on the wavelength and the plant used. However, the induction or improvement of shoot and/or root formation is invariably promoted by light. No germination was recorded in darkness. For the better growth of seedlings, a 16h photoperiod was found to be the most suitable. In complete darkness and continuous light, the seedling growth was poor. The seedlings turned achlorophyllous in darkness. NAR increased in the range of 8h-20h photoperiod till the 60th day after which it declined or remained stationary. However, at 16h photoperiod, it increased at a constant rate with time. The LAR increased

with time in all treatments except in complete darkness. An increase in growth with increase in photoperiod could be due to improved metabolic processes and higher photosynthetic activity (Zeigler *et al.*, 1967). Although 1500 lux light was found to be the optimum for seed germination, the seedling growth was enhanced at 3000 lux light intensity. Both at 1500 and 3000 lux light, a marked increase in NAR was observed on the 60th day of growth. However, the latter light intensity was found to be better. The negative NAR observed in cases of 0 and 1500 lux light, might indicate the loss of carbon by respiration that exceeded photosynthetic gain (Bourdôt *et al.*, 1985). The decrease in NAR with time could be due to the decrease in the capacity of seedlings to increase dry weight as a result of limited nutrient supply to the developing seedlings.

The seeds subjected to different pH treatments showed the best germination at pH 5.5. However, pH 4.5 was also found to be satisfactory for seed germination. Pierik *et al.* (1988) reported that pH 6.0 promoted maximum germination in *Paphiopedilum ciliolare*. The growth analyses of the seedlings showed that pH 5.5 was most suitable for seedling growth. There was a systematic increase with time in shoot length, leaf number and area, and root number and length at this pH. Conversely, at pH 4.5, 6.5 and 7.5 the roots did not develop except for some small projections at pH 6.5. Goh

(1971) reported better growth of orchid seedlings of *Vanda* and *Dendrobium* at low pH and also suggested that the range of pH 5.0-5.5 might be suitable for maximum absorption of water and nutrients from the medium. This could account for the healthy growth of seedlings at pH 5.5 in the present study. The decrease in the NAR after 60 days of growth was recorded at pH 5.5 whereas at pH 4.5, 6.5 and 7.5 it became almost stationary after this period. The decrease in NAR with increase in age might be due to decrease in photosynthetic activity per unit leaf area as a result of mutual shading of the leaves (Konings *et al.*, 1989). It could also have decreased due to the limitations of space, nutrients or accumulation of end products. The increase in LAR with time could not compensate for the decrease in NAR at pH 4.5, 6.5 and 7.5.

IV. EFFECT OF GROWTH REGULATORS ON SEED GERMINATION AND SEEDLING GROWTH

INTRODUCTION

Asymbiotic seed germination and seedling growth can be manipulated by the addition of various growth regulators in the medium. *In vitro* seed germination and seedling growth are influenced differentially by auxins, cytokinins and gibberellins and the results have been inconsistent and inconclusive (Arditti, 1967a, 1979, 1982; Withner, 1959, 1974). Auxins, cytokinins or ethrel are reported either to inhibit or promote orchid seed germination (Arditti *et al.*, 1981). IAA, indolebutyric acid (IBA), and NAA have been reported to enhance germination and/or seedling growth to

some extent (Kano, 1965; Ernst, 1967; Strauss and Reisinger, 1976; Arditti and Ernst, 1984). However, there are very few reports regarding the inhibition by auxins. In case of excised *Dendrobium* ovaries death occurred in the absence of auxin (Israel, 1963). The inconsistent results indicate that, in general, the *in vitro* germinating orchid seeds and developing seedlings do not require an exogenous source of auxins. The species which require or benefit from an exogenous source probably derive them from their mycorrhizal fungus in nature (Hayes, 1969; Arditti and Ernst, 1984). The growth of orchid seedlings *in vitro* is either enhanced or inhibited/unaffected by cytokinins. Van Waes and Debergh (1986) reported the necessity of cytokinin for *Cypripedium calceolus* and *Epipactis hellebornie* but not for *Dactylorhiza musculata* and *Listera ovata*. The inhibitory effect of BAP on the development and differentiation of *Cymbidium* protocorms was reported (Gailhofer and Thaler, 1975). According to Pierik and Steegmans (1972), numerous plbs, protocorms and plantlets resulted at higher concentrations of BAP in the medium. Kinetin in medium stimulated both germination and seedling growth of *Orchis pupurella* (Hadley and Harvais, 1968), *Dactylorhiza purpurella* (Harvais, 1972), *Cattleya* (Pierik and Steegmans, 1972), *Cymbidium* (Fonnesbech, 1972a), *Cypripedium reginae* (Harvais, 1982) and *Galeola septentrionalis* (Nakamura, 1982). An inhibitory role of KN

was observed in *Dendrobium* and *Laeliocattleya* (Kano, 1965). Although the effects of exogenous gibberellins on the growth of orchid seedlings were reported to be mostly negative (Kano, 1965; Nakamura, 1982; Arditti and Ernst, 1984; Van Waes and Debergh, 1986), germination and seedling growth of *Cattleya* and *Cypripedium* were stimulated in presence of GA_3 in medium (Blowers, 1958; Hirsh, 1959; Harvais, 1982). The information on the effects of hormonal interactions on the growth of orchid seedlings is scanty. However, combinations of auxins and cytokinins may enhance growth but the effects of these combinations vary with the growth regulators used, their concentrations, ratios and the orchid (Kusumoto, 1978, 1979a, 1979b; Uesato, 1978). On the other hand, the inhibitory effects of kinetin and auxins on shoot/root balance in orchids is well documented (Hadley and Harvais, 1968; Rao, 1977; Harvais, 1982).

MATERIALS AND METHODS

The seeds were exposed aseptically and cultured as described earlier chapters. The seeds were inoculated on MS medium containing different growth regulators viz. NAA, IAA, IBA, 2-4-D, BAP, KN and GA_3 separately in the range of 0.0-10.0 μM. The influences of IAA+BAP and NAA+BAP incorporation in the medium were also studied on seed germination. The pH of the medium was adjusted to 5.5 before

autoclaving. The cultures were maintained under a daily regime of 12 h darkness and 12 h light of 1500 lux intensity at a temperature of 25±2°C. The percentage germination was recorded after two months by examining the seeds microscopically.

Two-months old protocorms (developed on MS medium without growth regulators) were transferred to fresh MS medium containing above mentioned growth regulators separately or in combination to study their influence on seedling growth. The cultures were incubated at 25±2°C temperature and 16 h photoperiod of 3000 lux light. These were observed regularly at 30-day interval for 120 days and different parameters like shoot length, number and area of leaves, number and length of roots, and dry weight were recorded. From the primary data, NAR and LAR were calculated. Five replicates of each treatment were taken and experiments were repeated twice.

RESULTS

Effects of IAA

The maximum seed germination (89%) was recorded at 0.5 µM concentration of IAA in medium (Table 5). With increase in its concentration, the percentage germination was inhibited and the protocorms (brown in colour) failed to grow further. When compared to the control, shoot length, leaf

Table 5. Effect of different growth regulators on seed germination

Treatments	Germination (%) Growth regulator concerntration (μM) 0.5	2.5	5.0	10.0	Remarks
Control	-	-	-	-	85% germination
IAA	89	45	33	-	The protocorms developed at higher concentrations were mostly brown which failed to grow further
NAA	48	25	12	-	The brownish green developing protocorms showed poor growth and failed to turn green
IBA	36	29	28	16	Very small and poor protocorms were developed which turned brown eventually
2',4-D	12	9	5	-	Poor germination response of the seeds
BAP	90	68	65	47	Healthy and green protocorms were developed
KN	59	43	17	8	Small and brown protocorms were developed which failed to grow further
GA_3	31	27	10	-	Poor germination response of the seeds

Table 8. Effect of growth regulators on NAR and LAR during seedling development

Treatments (μM)		NAR g/mm^2/unit time (Days) 30	60	90	120	LAR (Days) 30	60	90	120
Control		0.0	-0.55	-0.0012	-4×10^{-6}	0.0	-0.042	14.57	186.54
IAA	0.5	-0.236	-0.0004	-27×10^{-6}	59×10^{-6}	-0.12	15.305	113.08	285.38
	2.5	-0.363	-0.0014	-20×10^{-6}	0.0	-0.07	31.860	691.85	0.0
	5.0	-0.042	-920×10^{-6}	-16×10^{-6}	-70×10^{-6}	-0.46	30.470	172.26	339.67
	10.0	-0.238	-260×10^{-6}	-22×10^{-6}	-76×10^{-6}	-0.08	20.520	105.93	-130.98
NAA	0.5	0.0	0.0	0.00016	0.0	0.0	0.0	56.73	0.0
	2.5	0.0	-195×10^{-6}	-2.4×10^{-6}	-2.4×10^{-6}	0.0	43.290	700.91	-30907.10
	5.0	-785.965	-810×10^{-6}	-3.3×10^{-6}	-11.3×10^{-6}	-31.2×10^{-6}	29.480	460.59	1002.69
	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
IBA	0.5	-1778.0	-0.0025	0.0	0.0	-20×10^{-6}	9.885	0.0	0.0
	2.5	-120.5	-690×10^{-6}	-5.7×10^{-6}	0.9×10^{-6}	190×10^{-6}	38.563	797.52	0.0
	5.0	-1243.6	-594×10^{-6}	-1.5×10^{-6}	0.0	-16.2×10^{-6}	32.278	-498.98	0.0
	10.0	0.0	16×10^{-6}	-7.6×10^{-6}	-7.8×10^{-6}	0.0	1489.330	725.45	319.35
2,4-D	0.5	-0.288	-362×10^{-6}	-59×10^{-6}	0.0	-0.069	22.920	0.0	0.0
	2.5	-0.173	-280×10^{-6}	0.0	0.0	-0.066	43.342	0.0	0.0
	5.0	-0.188	-0.024	-0.024	-0.024	-0.065	0.990	1.5	0.0
	10.0	-0.299	-0.024	Dried	Dried	-0.049	1.070	Dried	Dried
BAP	0.5	0.0	-1.435	0.0016	0.0	0.0	-0.0048	0.489	0.0
	2.5	-0.23	-0.0005	-0.00011	-14×10^{-6}	-0.121	16.385	164.960	430.57
	5.0	-994.59	-0.0007	-2×10^{-6}	-1×10^{-6}	-12.5×10^{-6}	33.520	746.340	880.45
	10.0	-397.90	-0.0016	-68×10^{-6}	0.0	0.0	1.467	-11.205	0.0
KN	0.5	-1197.56	-0.0013	-10.6×10^{-6}	-10.8×10^{-6}	-16.6×10^{-6}	12.350	284.996	567.20
	2.5	-0.105	-0.001	-120×10^{-6}	-700×10^{-6}	-0.332	39.820	222.0	452.31
	5.0	-116.3	-0.0004	0.0	0.0	-0.00019	54.080	0.0	0.0
	10.0	0.0	39×10^{-6}	-0.00008	Dried	0.0	-177.860	119.720	Dried
GA_3	0.5	-0.331	-0.00056	-55×10^{-6}	-45×10^{-6}	-0.076	17.782	69.160	185.34
	2.5	-0.249	-0.00068	-18.7×10^{-6}	-8.6×10^{-6}	-0.143	34.280	331.630	444.67
	5.0	-611.1	-49×10^{-6}	-1.3×10^{-6}	-6.3×10^{-6}	-0.020	31.170	572.370	725.94
	10.0	-388.92	-0.00057	-19×10^{-6}	-19×10^{-6}	-32×10^{-6}	17.450	362.720	-400.52

Fig. 5. Effect of different growth regulators on seedling development

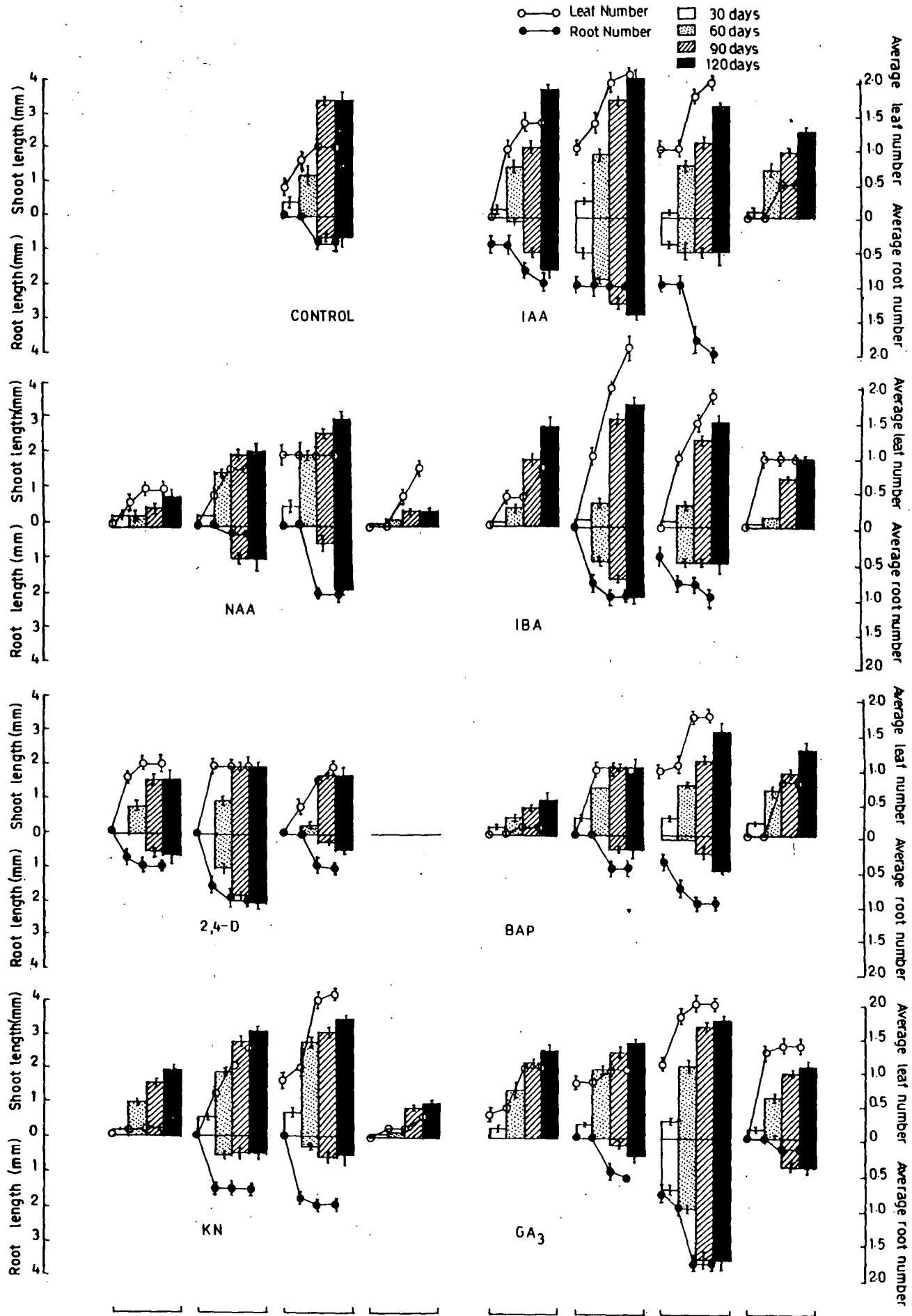

Leaf Number
Root Number
30 days
60 days
90 days
120 days
Root length (mm)
Shoot length (mm)
Average leaf number
Average root number
CONTROL
IAA
NAA
IBA
2,4-D
BAP
KN
GA3
0·5
2·5
5·0
10·0
(µM)
± S.D.
Treatments

Fig. 5

number and root length increased with time at 0.5, 2.5 and 5.0 µM of IAA. However, at 10.0 µM, the seedlings showed poor growth with no root formation. At 2.5 µM of IAA in the medium, the root number remained the same throughout the observation period whereas at 5.0 µM treatment the number increased with growth. The overall seedling growth was optimum at 2.5 µM IAA in medium (Fig. 5, Plate 7). NAR of seedlings increased several folds to a positive value during 120 days in the medium containing 0.5 µM of IAA (Table 8). At the same concentration, NAR increased to zero by the 120th day. Both at 5.0 and 10.0 µM treatments, NAR increased till the 90th day after which it declined. A similar picture was obtained for LAR except for 5.0 µM treatment where it increased further upto 120 days.

Effect of NAA

Seed germination was poor in the medium containing different concentrations of NAA (Table 5). The developing protocorms (brownish green in colour) failed to grow further. Except at 5.0 µM of NAA in medium, the seedling growth was inhibited at other concentrations tried. At 5.0 µM of NAA, the shoot length, root number and root length increased with time as compared to the control. However, both at 0.5 and 10.0 µM of NAA in the medium, the roots failed to develop (Fig. 5, Plate 8). At 0.5 µM concentration of NAA, the NAR

Plate 7. Effect of IAA on seedling development

Plate 8. Effect of NAA on seedling development

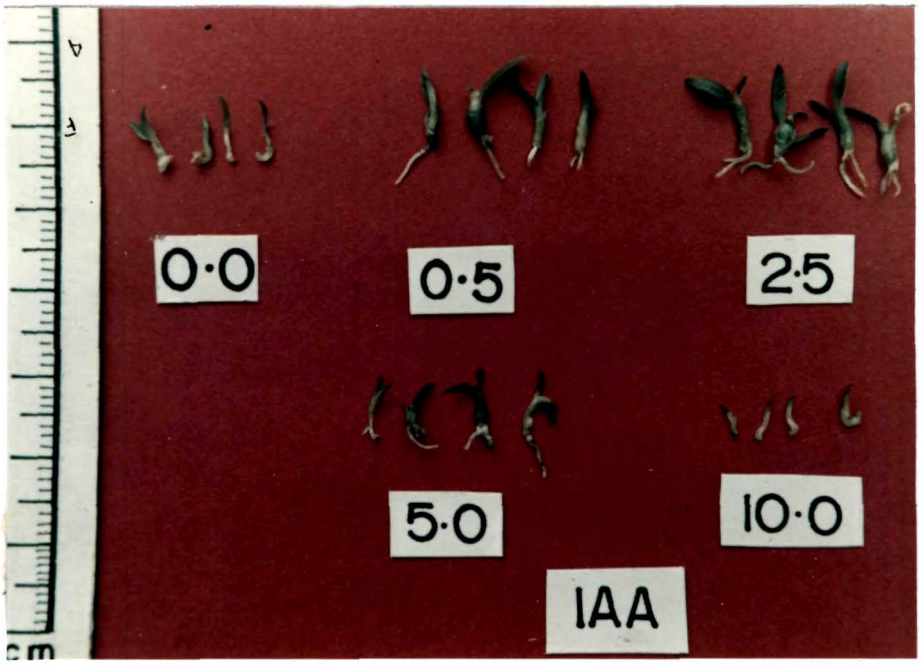


Plate 7

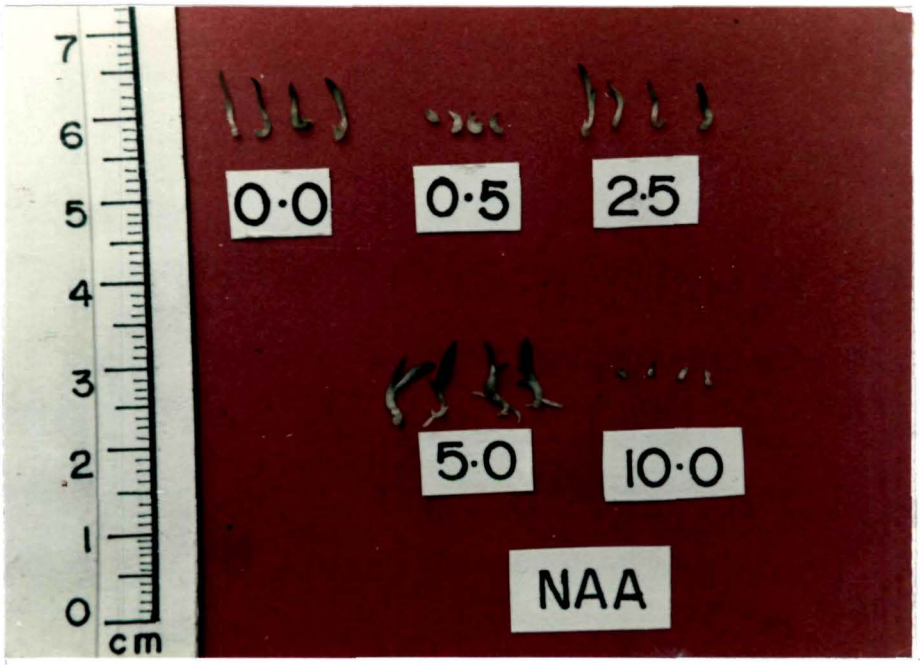


Plate 8

increased slightly on 90th day of growth whereas it remained zero throughout the observation period (Table 8). The LAR decreased on 60th day and increased subsequently in 2.5 µM NAA treatment. A gradual increase in NAR was recorded in case of seedlings raised at 5.0 µM NAA in the medium. However, on 120th day a decline was observed. LAR also exhibited an increase throughout the growth period at 5.0 µM concentration. Both NAR and LAR remained zero throughout the growth period at 10.0 µM concentration.

Effect of IBA

The seeds showed poor germination in the medium containing IBA (Table 5). The seedling growth was enhanced at 2.5 and 5.0 µM concentrations. The shoot length and leaf number increased with time at the above concentrations. The root length increased with time at 2.5 µM, whereas the number was constant after 90 days. On the whole, better growth of the seedlings was observed in 2.5 µM treatment. No roots were formed at 0.5 and 10.0 µM of IBA in the medium (Fig. 5, Plate 9). In the 0.5 µM IBA treatment, the NAR increased till the 60th day and subsequently became zero. However, at 2.5 µM concentration, it increased gradually and exhibited a positive value by the end of the observation period. A similar picture was obtained for LAR except for the 120th day where it declined to zero. At 5.0 µM of IBA in the medium,

Plate 9. Effect of IBA on seedling development

Plate 10. Effect of 2,4-D on seedling development

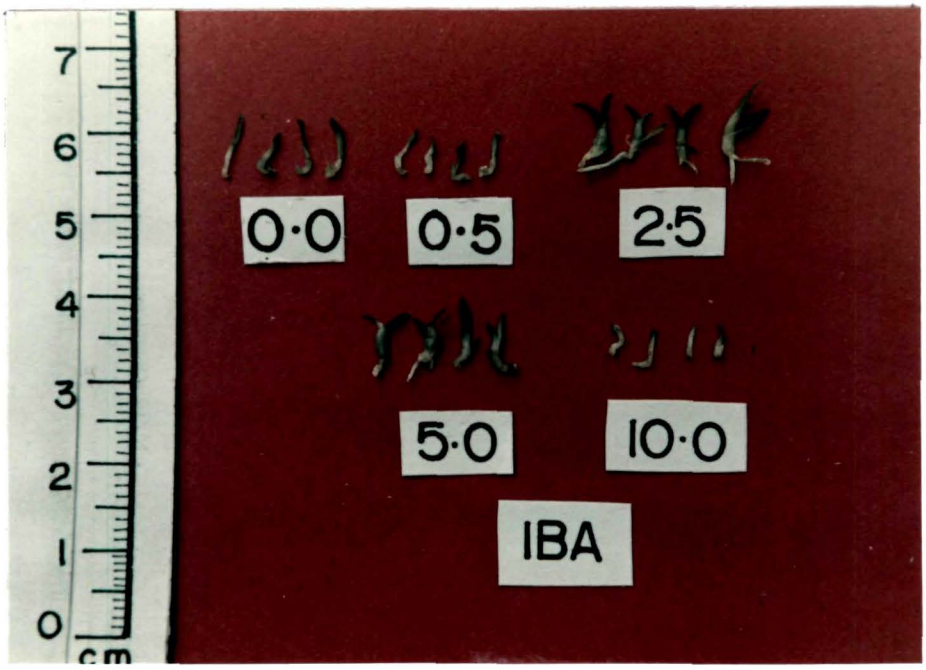


Plate 9

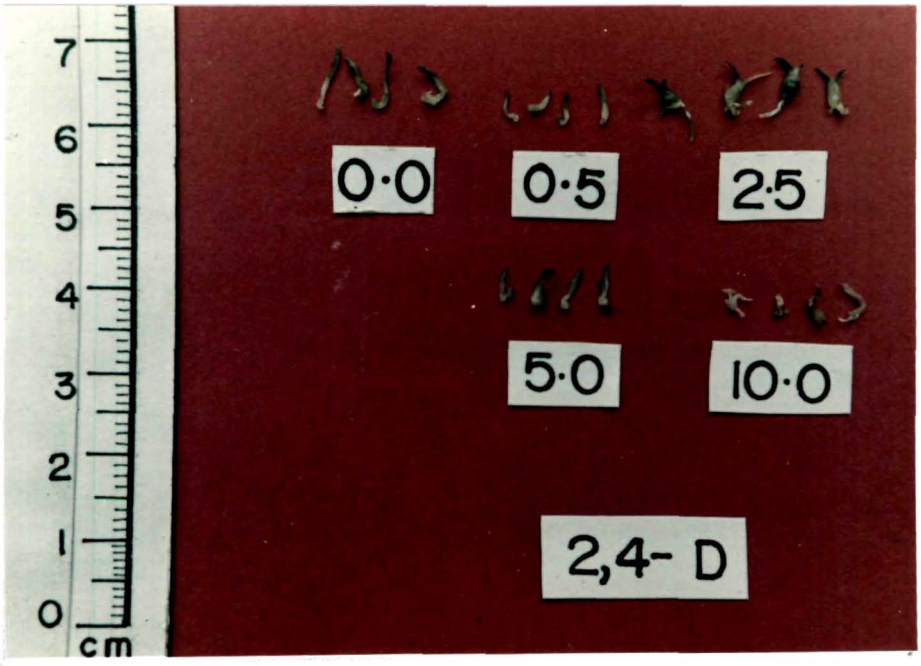


plate 10

NAR increased gradually throughout the observation period, but the LAR declined after 60 days. A slight increase in NAR was recorded on the 60th day which declined subsequently in case of seedlings raised at 10.0 µM. The LAR also followed the same pattern (Table 8).

Effect of 2,4-D

The incorporation of 2,4-D in the medium was inhibitory for seed germination (Table 5). As compared to the control, the shoot length was less in seedlings raised at 0.5 and 2.5 µM of 2,4-D. The leaf number was the same as in control. However, the root number and length were higher at the above mentioned concentrations. With increase in the concentration of 2,4-D in the medium, the seedling growth was inhibited (Fig. 5, Plate 10). At 0.5 and 2.5 µM of 2,4-D in the medium, both NAR and LAR increased from negative to zero at the end of the observation period. However, at 5.0 µM, the NAR was constant and negative throughout (Table 8). While a slight decline in NAR was observed on the 60th day, the LAR was higher in case of the 10.0 µM 2,4-D treatment. In the same treatment, however, on the 90th day the seedlings were dead and dry.

Effect of BAP

The optimum seed germination of 90% was recorded at 0.5 µM BAP (Table 5) in the medium. The shoot length, leaf

Plate 11. Effect of BAP on seedling development

Plate 12. Effect of KN on seedling development

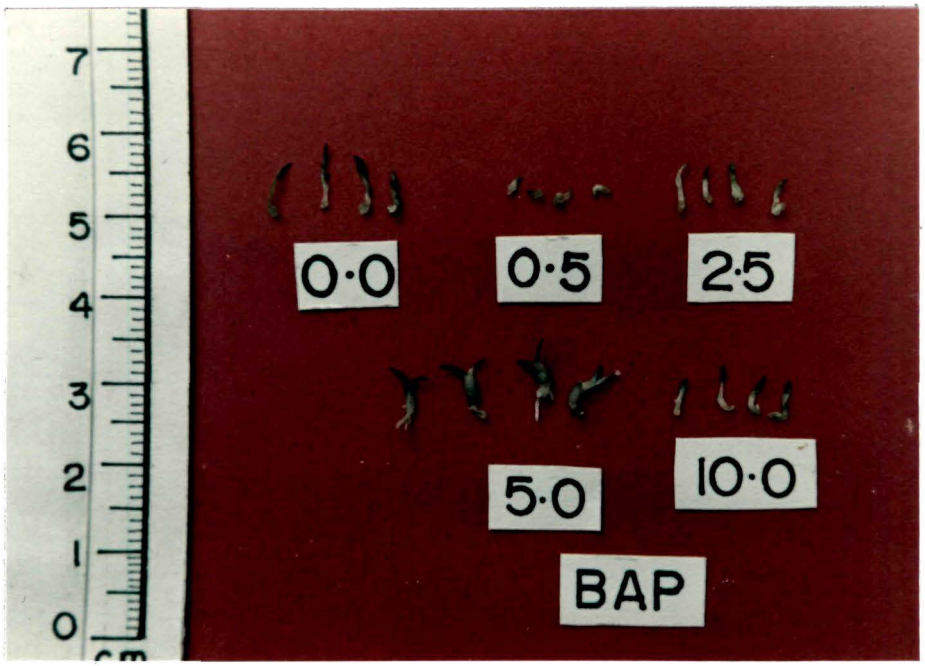


Plate 11

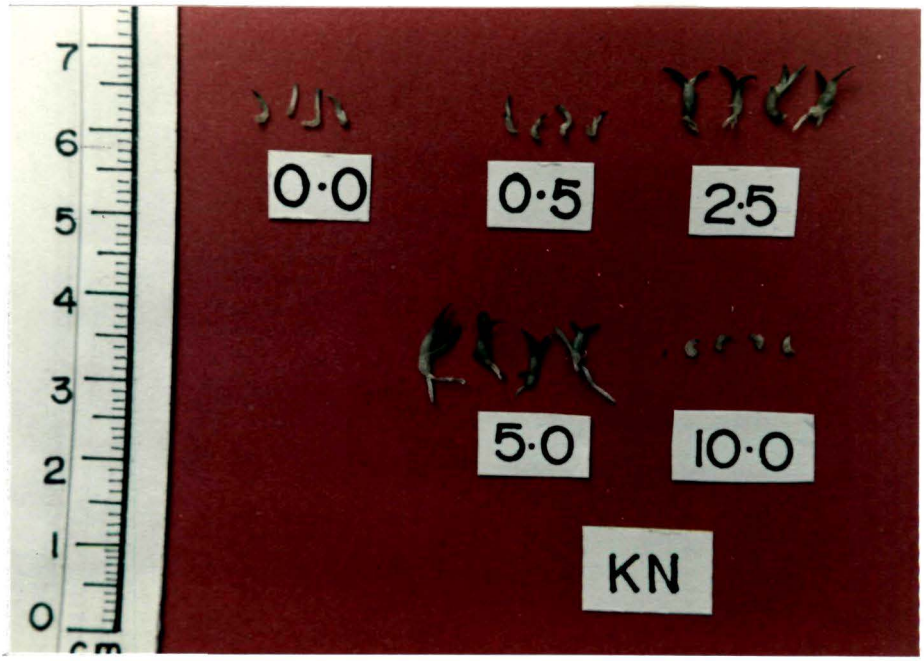


Plate 12

number, root number and root length were optimum and increased with time in 5.0 µM of BAP treatment. There was no root formation at 0.5 and 10.0 µM concentrations (Fig. 5, Plate 11). Both NAR and LAR increased gradually with time at 2.5 and 5.0 µM concentrations of BAP in the medium. On the other hand, slight increase in NAR was recorded at 0.5 µM of BAP till the 90th day after which it declined. By the end of the observation period, both NAR and LAR were zero at 10.0 µM of BAP in medium (Table 8).

Effect of KN

Although germination was 59% at 0.5 µM of KN in the medium, the protocorms failed to grow further and turned brown (Table 5). Both 0.5 and 10.0 µM concentrations of KN were inhibitory for seedling growth. However, at concentrations of 2.5 and 5.0 µM in the medium, the shoot length, leaf number, root number and root length increased with time (Fig. 5, Plate 12). A gradual increase with time was recorded in both NAR and LAR at 0.5 and 2.5 µM KN in the medium (Table 8). Both NAR and LAR were higher after 60th day in case of 5.0 µM treatment. In the medium containing 10.0 µM KN, the seedlings were completely dry on 120th day of observation.

Effect of GA_3

The seed germination was quite poor in the presence of

GA_3 in medium (Table 5). As compared to the control, the seedling growth was markedly higher at 5.0 µM concentration of GA_3 in the medium. However, other concentrations were slightly inhibitory as compared to the control. No roots were observed at 0.5 µM treatment (Fig. 5, Plate 13). The NAR and LAR increased with time in case of 0.5 and 2.5 µM treatment (Table 8). At 5.0 µM of GA_3 in the medium, NAR increased upto the 90th day, the LAR increased throughout. At 10.0 µM concentration, the NAR became constant after 90 days whereas the LAR decreased.

Effect of growth regulators in combination

The best germination (96%) was recorded at 0.5 µM each of IAA and BAP in combination. The germination was also quite pronounced in treatments having 0.5 µM of IAA and different concentrations of BAP (2.5, 5.0 and 10.0 µM) (Table 6). The germination was markedly higher at 0.5 µM each of NAA and BAP in combination (Table 7). However, the germination percentage at other concentrations was lower than the control. The optimal seedling growth was recorded at 0.5 µM IAA + 5.0 µM BAP (Plate 14) followed by 0.5/2.5 µM IAA + 0.5 µM BAP in medium (Fig. 6). At higher concentrations of these in the medium, the growth was inhibited. Although NAR gradually increased with time in most of the combinations, the optimum NAR was recorded at

Table 6. Effect of different concentrations of IAA and BAP in combination

IAA+BAP (µM)	Germination (%)	Remarks
0.5+0.5	96	The protocorms were green in colour
2.5+0.5	84	"
5.0+0.5	65	"
10.0+0.5	19	Poor germination
0.5+2.5	85	Healthy and green protocorms were developed
2.5+2.5	64	Protocorms were green
5.0+2.5	61	"
10.0+2.5	45	-
0.5+5.0	80	Round and Green
2.5+5.0	66	Protocorms were developed
5.0+5.0	65	"
10.0+5.0	31	Both green and brown protocorms were observed
0.5+10.0	82	Green protocorms were developed
2.5+10.0	58	-
5.0+10.0	25	-
10.0+10.0	19	-

Table 7. Effect of different concentrations of NAA and BAP in combination

NAA+BAP (μM)	Germination (%)	Remarks
0.5+0.5	92	Healthy green protocorms were developed
2.5+0.5	86	Both brown and green protocorms were observed
5.0+0.5	57	The developed protocorms were healthy and brown
10.0+0.5	44	-
0.5+2.5	80	The protocorms were mostly green. Some unhealthy brown protocorms were also observed
2.5+2.5	66	"
5.0+2.5	42	"
10.0+2.5	10	Mostly brown protocorms were produced
0.5+5.0	77	Green healthy protocorms were developed
2.5+5.0	55	"
5.0+5.0	42	Half the protocorms were unhealthy
10.0+5.0	18.2	-
0.5+10.0	55	The protocorms were green in colour
2.5+10.0	45	Unhealthy protocorms, were developed which turned brown
5.0+10.0	30	"
10.0+10.0	-	-

Plate 13. Effect of GA_3 on seedling development

Plate 14. Effect of the interactions of NAA+KN and IAA+BAP on seedling development

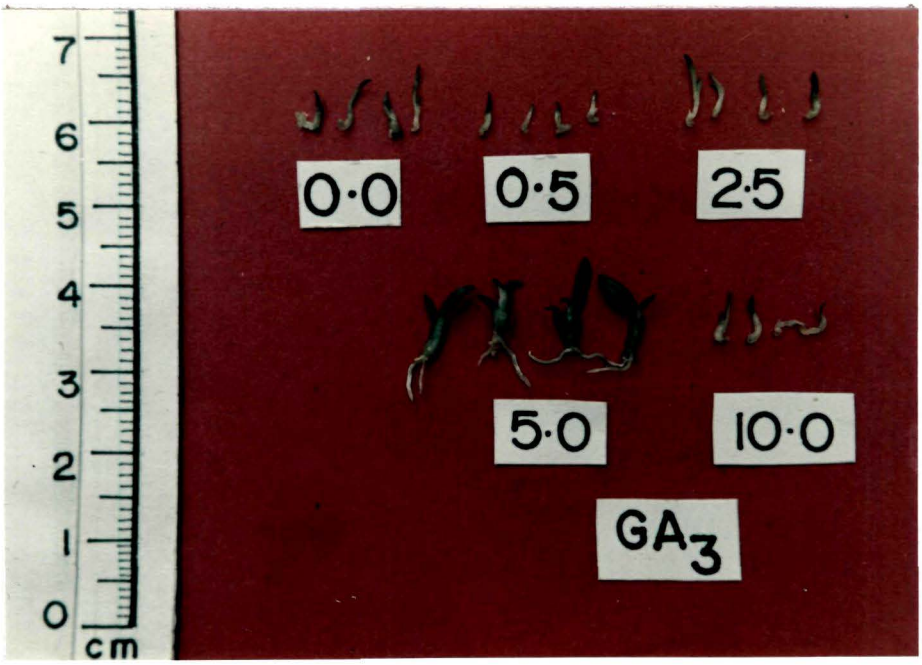


Plate 13

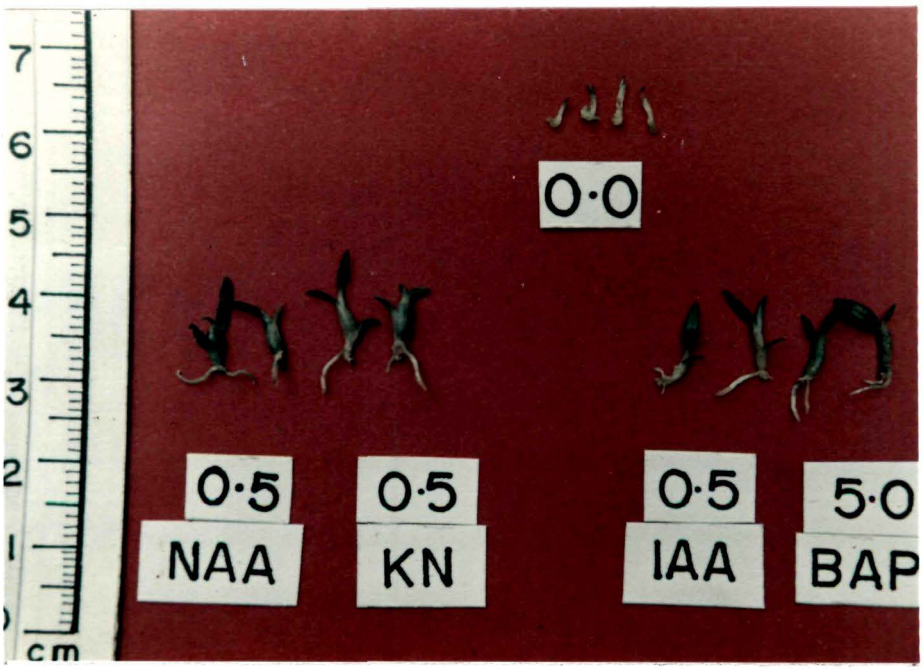


Plate 14

Fig. 6. Effect of IAA+BAP on seedling development

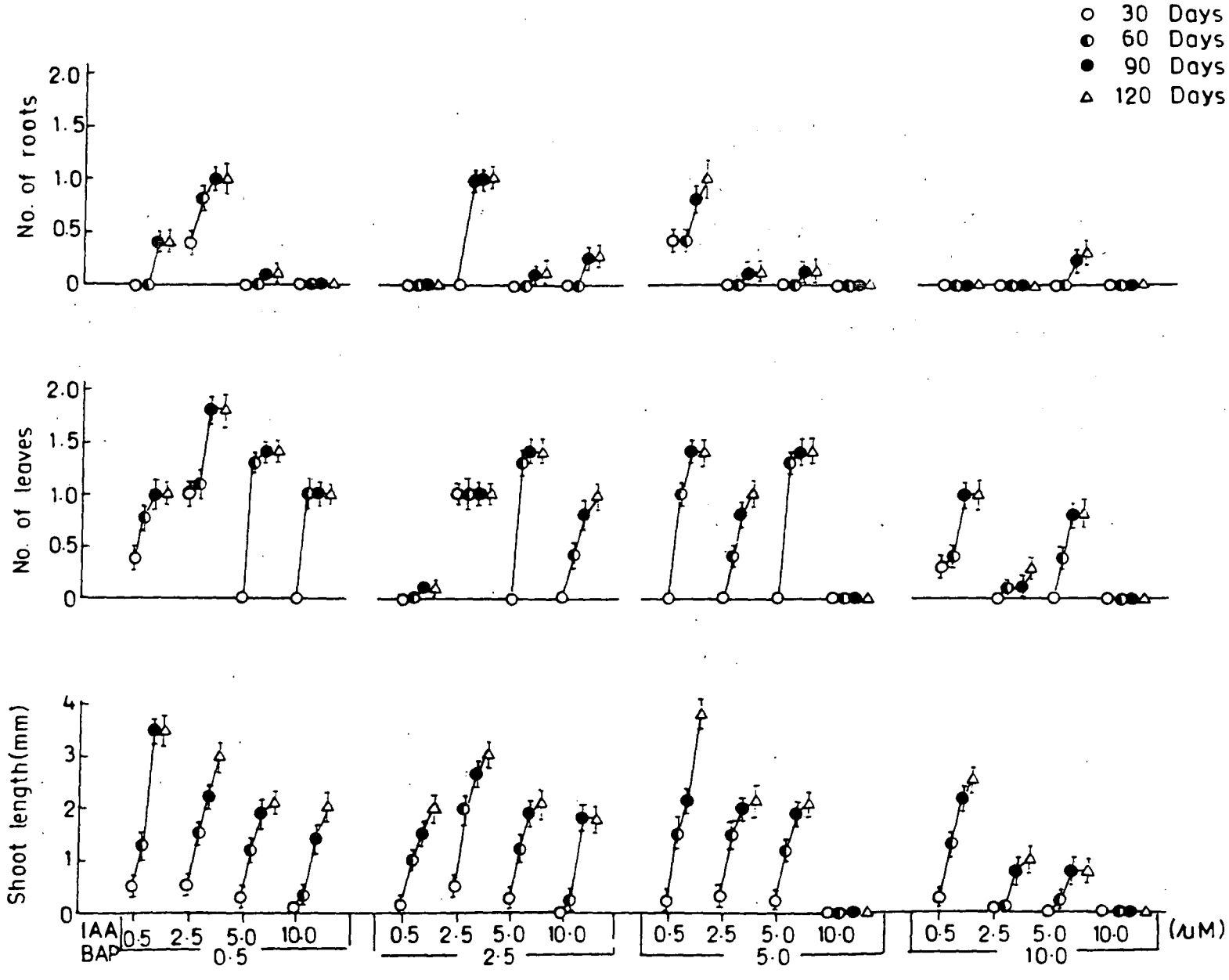

30 Days
60 Days
90 Days
120 Days
No. of roots
No. of leaves
Shoot length(mm)
IAA
BAP
0·5 2·5 5·0 10·0
0·5
2·5
5·0
10·0
(µM)
± S.D.
Treatments

Fig. 6

Table 9. Effect of IAA+BAP on NAR and LAR during seedling development

Treatments (µM)		NAR g/mm^2/unit time Days				LAR Days			
IAA	BAP	30	60	90	120	30	60	90	120
0.5		-282.5	-0.0005	-0.00001	-1.3×10^{-6}	-0.00006	32.72	327.69	550.70
2.5		-391.68	-0.003	-7.4×10^{-6}	-5.5×10^{-6}	-28×10^{-6}	40.51	518.17	826.08
5.0	0.5	-0.11	-277×10^{-6}	-8.3×10^{-6}	-4.4×10^{-6}	-0.31	85.87	963.66	1329.98
10.0		-0.24	-0.0004	-0.0001	-27×10^{-6}	-0.12	18.50	170.81	507.20
0.5		-111.90	-0.0005	-6.9×10^{-6}	0.0	-0.0002	52.24	1094.38	0.0
2.5		-1694.60	-0.0025	-32×10^{-6}	-11.4×10^{-6}	-22.5×10^{-6}	10.59	294.76	540.05
5.0	2.5	-173.65	-0.00026	-0.00002	-0.00001	-115×10^{-6}	15.25	299.92	550.13
10.0		-179.02	-0.0005	Dried	Dried	0.0001	13.61	Dried	Dried
0.5		-62.06	0.0027	-39×10^{-6}	$-65\times^{10}-6$	-615×10^{-6}	4.26	79.37	257.25
2.5		-146.73	0.0	-53×10^{-6}	-2.8×10^{-6}	-156×10^{-6}	0.0	174.09	4266.58
5.0	5.0	-1924.65	-0.0028	-34×10^{-6}	19×10^{-6}	-19.8×10^{-6}	9.55	278.76	553.99
10.0		-1817.80	-0.0036	Dried	Dried	-16.9×10^{-6}	7.10	Dried	Dried
0.5		-188.87	-0.001	-68×10^{-6}	-26×10^{-6}	-119.7×10^{-6}	8.81	179.39	416.46
2.5		-0.35	-0.0014	0.0	Dried	-0.07	41.37	0.0	Dried
5.0	10.0	-0.23	-326×10^{-6}	Dried	Dried	-0.08	23.09	Dried	Dried
10.0		0.0	0.462	Dried	Dried	0.0	-0.07	Dried	Dried

Fig. 7. Effect of NAA+KN on seedling development

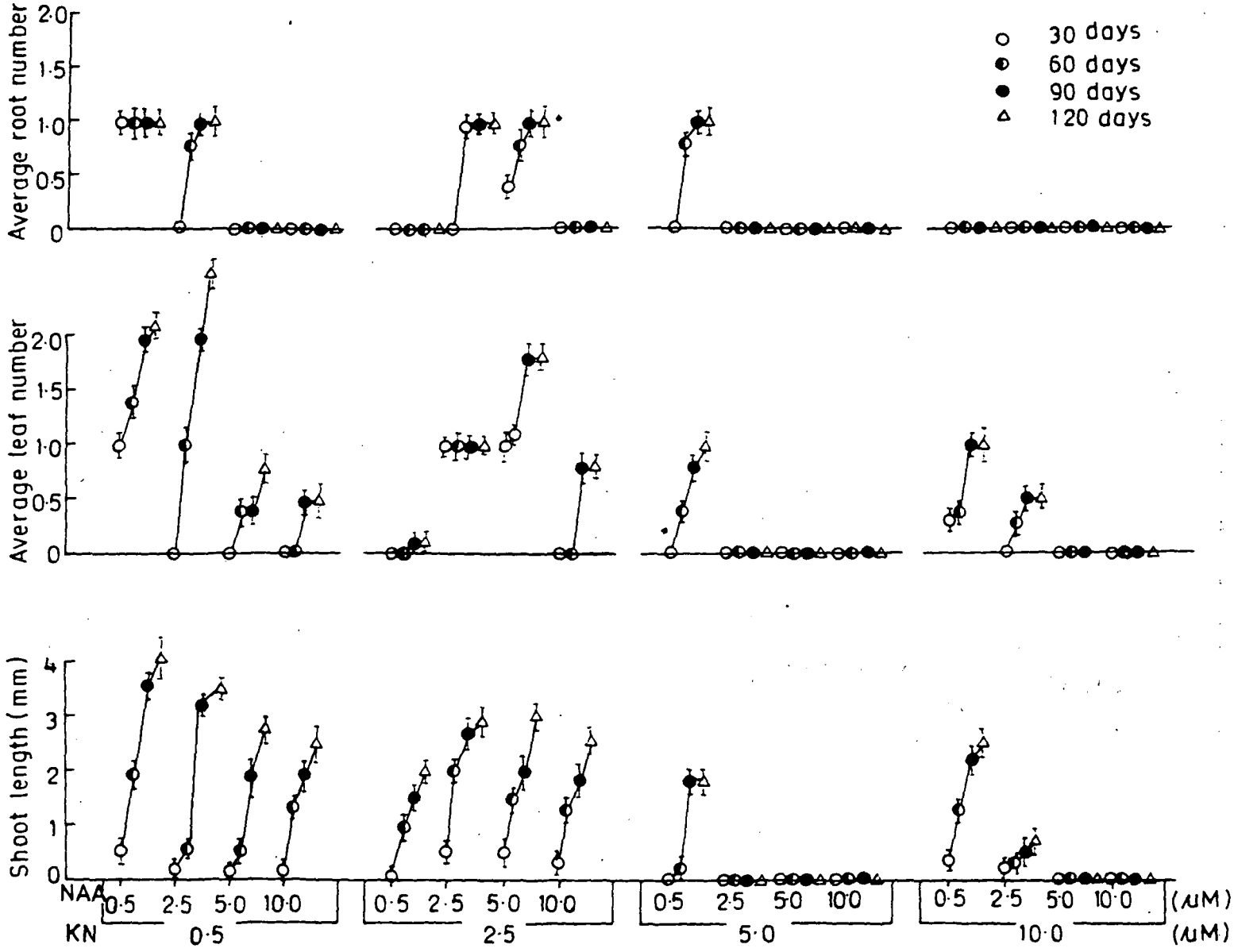

Average root number
2·0
1·5
1·0
0·5
0
30 days
60 days
90 days
120 days
Average leaf number
2·0
1·5
1·0
0·5
0
Shoot length (mm)
4
3
2
1
0
NAA
0·5 2·5 5·0 10·0
0·5 2·5 5·0 10·0
0·5 2·5 5·0 10·0
0·5 2·5 5·0 10·0
(µM)
KN
0·5
2·5
5·0
10·0
(µM)
± S.D.
Treatments

Fig. 7

Table 10. Effect of NAA+KN on NAR and LAR during seedling development

Treatments (μM)		NAR g/mm^2/Unit time Days				LAR Days			
NAA	KN	30	60	90	120	30	60	90	120
0.5		-0.071	-0.00005	-7×10^{-6}	-1.25×10^{-6}	-0.034	76.57	762.87	982.61
2.5		-0.085	-27×10^{-6}	-21.2×10^{-6}	0.0	-0.395	28.34	269.87	0.0
5.0	0.5	-0.022	-78×10^{-6}	-5.5×10^{-6}	0.0	-1.070	107.94	688.98	0.0
10.0		-1704.2	-0.014	-0.0014	-89×10^{-6}	-15.6×10^{-6}	0.0	11.62	103.03
0.5		-90.450	-0.0004	-0.1×10^{-6}	-0.000041	-158×10^{-6}	25.51	0.0	1491.15
2.5		-0.875	-0.0013	13.6×10^{-6}	-11.4×10^{-6}	-0.033	10.44	279.65	641.0
5.0	2.5	-0.407	-0.021	-39×10^{-6}	-0.0001	-0.068	0.357	19.64	130.14
10.0		-0.129	-0.0009	-68×10^{-6}	-0.0003	-0.137	11.218	75.57	139.81
0.5		-921.20	-0.0015	Dried	Dried	-18.10^{-6}	10.41	Dried	Dried
2.5		-1959.66	-0.0022	-198×10^{-6}	-51×10^{-6}	-13.2×10^{-6}	4.25	88.45	115.65
5.0	5.0	Dried	Dried	Dried	Dried	Dried	Dried	Dried	Dried
10.0		Dried	Dried	Dried	Dried	Dried	Dried	Dried	Dried
0.5		0.0	-0.025	-0.0008	-41×10^{-6}	0.0	-0.031	20.11	188.57
2.5		0.0	0.0	0.0	0.114	0.0	0.0	0.0	-0.074
5.0	10.0	Dried	Dried	Dried	Dried	Dried	Dried	Dried	Dried
10.0		Dried	Dried	Dried	Dried	Dried	Dried	Dried	Dried

5.0 µM each of IAA and BAP in the medium (Table 9). At 0.5 µM each of IAA and BAP, NAR increased upto the 60th day and declined subsequently. Except at 0.5 µM IAA + 2.5 µM BAP, LAR increased with time. Seedling growth was greatly enhanced at 0.5 µM NAA + 0.5 µM KN (Plate 14) followed by 2.5 µM NAA + 0.5 µM KN in combination. An exponential increase in leaf number was recorded at 2.5 µM NAA + 0.5 µM KN in the medium (Fig. 7). The higher concentrations of NAA and KN were inhibitory for the growth of seedlings. There was a gradual increase in NAR at 0.5 µM KN in combination with NAA (Table 10). However, LAR became zero at 120 day in treatments having 2.5 µM NAA + 0.5 µM KN and 5.0 µM NAA + 0.5 µM KN in the medium. At 2.5 µM of KN in combination with different concentrations of NAA, the NAR increased upto the 90th day and declined subsequently. However, LAR increased with time in most of the combinations. At 2.5 µM NAA and 2.5 µM KN, NAR was comparatively higher on the 90th day.

DISCUSSION

The germination of seeds and seedling growth of *D. fimbriatum* were greatly affected by the incorporation of growth regulators in the medium. Auxins have been reported to influence germination and/or seedling growth but the reports are inconclusive. In the present study, the seed germination was enhanced at 0.5 µM of IAA in medium as

compared to the control. However, the germination response was poor on media containing other auxins. Harvais (1972) reported inconclusive results with IAA in medium for *Dactylorhiza (Orchis) purpurella* seeds and seedlings. In the present study, compared to the control, the seedling growth was prominently higher at 2.5 µM of IAA in medium. At 5.0 µM of IAA, the root number was quite high. Auxins have been reported to enhance root formation in plants (Bhojwani and Razdan, 1983). Promotion of seedling growth as a result of IAA in medium is reported in many orchid species (Kano, 1965; Ernst, 1967; Strauss and Reisinger, 1976; Arditti and Ernst, 1984; Sharma and Tandon, 1986). The enhancement of seedling growth by NAA has also been reported in *Dendrobium* (Israel, 1963), *Cymbidium* (Torikata *et al.*, 1965), *Cattleya aurantiaca*, *Cymbidium madidum*, *Bletilla* species and *Chondrorhyncha discolor* (Strauss and Reisinger, 1976), *Cattleya* (Kusumoto, 1979a) and *Galeola septentrionalis* (Nakamura, 1982). Consistant with the earlier reports, seedling growth in *D. fimbriatum* was promoted by incorporation of NAA (5.0 µM) in the medium. The growth of seedlings was also promoted at 2.5 and 5.0 µM of IBA in the medium. Similar results have been reported in *Dendrobium* protocorms by Pages (1971). 2,4-D in the medium inhibited the seedling growth. At 2.5 µM of 2,4-D the root growth was, however, promoted as compared to the control. The inhibitory

effects of 2,4-D were also observed in other orchids like *Vanda* Miss Joaquin (Goh, 1970), *Galeola septentrionalis* (Nakamura, 1982), *Cymbidium reginae* (Harvais, 1982), *Cymbidium elegans* (Sharma and Tandon, 1986). In the present study, the influence of cytokinins on seed germination was quite pronounced. Maximum germination was recorded at 0.5 µM of BAP in medium. On the other hand, KN was inhibitory for seed germination. The inhibitory effect of KN has been reported in *Dendrobium*, *Laeliocattleya* (Kano, 1965) and *Coeloglossum viride* (Hadley, 1970). The seedling growth of *D. fimbriatum* was slightly enhanced at both 5.0 µM of BAP and KN incorporated separately in the medium. Pierik and Steegmans (1972) reported the formation of plbs, protocorms and plantlets at higher concentrations of BAP in *Cattleya aurantiaca*. The enhancement of seedling growth by KN in medium was also reported in *Cypripedium reginae* and *Galeola septentrionalis* (Harvais, 1982; Nakamura, 1982). The present study suggests that the seeds show differential germination response to cytokinins. BAP at lower concentrations promoted seed germination whereas KN inhibited it at all concentrations tested. The seeds were more sensitive to higher cytokinin levels than the developing protocorms. It can be assumed that the germinating seeds are able to synthesize enough of the cytokinins to satisfy their own need and destroy the hormone at a slow rate than do the

protocorms. The exogenous supply of GA_3 to the medium was inhibitory for seed germination. But the seedling growth was markedly enhanced at 5.0 uM concentration of GA_3. Both the inhibitory (Kano, 1965) and stimulatory (Blowers, 1958; Hirsh, 1959; Harvais, 1982) action of GA_3 for germination and/or seedling growth are reported.

In the present study, combinations of both auxins and cytokinins enhanced seed germination. As compared to the control, germination was greatly enhanced at 0.5 μM IAA + 0.5 μM BAP and 0.5 μM NAA + 0.5 μM BAP in the medium. The optimal seedling growth was recorded at 0.5 μM IAA + 5.0 μM BAP and 0.5 μM NAA + 0.5 μM KN. The promotion of seedling growth as the result of interactions between different growth regulators has been reported (Kusumoto, 1978, 1979a, 1979b; Uesato, 1978). But the effects of these combinations are inconclusive as they vary with the growth regulators, their concentrations and the orchids used. The influence of auxin and cytokinin on shoot/root balance in orchids has been emphasized (Pierik and Steegmans, 1972; Rao, 1977; Harvais, 1982). The changes in the growth of the seedlings treated with growth regulators can be explained by the changes in the efficiency of the leaves to produce dry matter (NAR) and changes in the leafiness of the seedlings (LAR). The highest NAR indicates the best response of the seedlings to the particular growth regulator treatments and successful growth.

In the present study, an increase in LAR was recorded in many treatments inhibitory to seedling growth. The increased LAR in these cases could not fully compensate for the reduced NAR. This is in agreement with the report of Regnier *et al.* (1988) who suggested that in *in vitro* grown plants the increase in LAR did not compensate for reduced NAR. The negative NAR observed in most cases might be due to the excessive loss of carbon by respiration. The decrease in NAR with time could also be due to the limitations of space, nutrients or the accumulation of end products.

The results on the effect of growth regulators on seed germination and seedling growth are quite widely documented. According to Arditti and Ernst (1984), there could be various reasons for the differential response of the growth regulators on the germination and growth of the orchids. The possible reasons could be, (i) interactions might have occurred between different combinations of growth regulators, culture conditions and seedlings used, (ii) variations occurred in the physiological responses and requirements of species, (iii) differences existed in the forms and analogues of hormones, (iv) culture conditions varied, (v) a wide range of dosage concentrations might have been used, and (vi) the age of the seedlings varied.

V. BIOCHEMICAL STUDIES AT DIFFERENT STAGES OF PROTOCORM DEVELOPMENT AS INFLUENCED BY GROWTH REGULATORS

INTRODUCTION

The role of enzymes in relation to germination has been focussed by many workers (Scandalios, 1974; Blackeman *et al.*, 1976; Gaspar *et al.*, 1977; Dendsay and Sachar, 1982; Barendse, 1983; Pitel *et al.*, 1984; Miller, 1985). There are numerous reports on synthesis of enzymes during germination of agricultural crops and tree species (Pitel *et al.*, 1984), but only a few are concerned with orchids (Blackeman *et al.*, 1976). The available data on the physiological basis of the diverse requirements of orchid seeds during their germination and the effects of various growth regulators on the metabolic

pathways underlying the process of seed germination and seedling growth are scanty. However, the reports on other plants suggest that growth regulators do regulate the enzyme activity in several instances (Sachaar *et al.*, 1975; Varner and Ho, 1977; Bewley and Black, 1978; Letham *et al.*, 1978; Moore, 1980). The biochemical changes occurring in developing seeds in response to growth regulators play an important role in controlling seed germination by affecting the rate of hydration, enzyme release, ion transport and concentration, pH and inhibitor contents (Tao and Khan, 1977; Penel *et al.*, 1984).

The changes in enzyme levels cause developmental changes during germination and growth. The differentiation processes are controlled by the interactions between several hormones (Ashton, 1976). The presence of a single hormone in different tissues or in the same tissue during different stages of its development may influence enzyme activity/synthesis through different mechanisms. The role of oxidative enzymes has been studied during early growth of orchid seedlings having mycorrhizal infection (Blackeman *et al.*, 1976). The IAA level in plant tissues is regulated by IAA-oxidase/peroxidase. The oxidation of IAA results in the inactivation of the hormone and hence the control of IAA-oxidase and/or peroxidase activities, that may have repercussions for IAA synthesis and degradation. Thus, it

may be involved in plant growth response to auxins (Barendse, 1983). A large number of enzymes are affected by growth regulators and they cover a wide range of metabolic activities (Letham *et al.*, 1978, Penel *et al.*, 1984; Miller, 1985).

DNA synthesis has been observed to occur prior to differentiation and is affected by growth regulators (Roberts, 1976). Auxin and KN treatment is reported to bring about DNA synthesis (Torrey and Fosket, 1970). Nagl and Rucher (1972) observed DNA amplification during histogenesis and morphogenesis of *Cymbidium* protocorms. The role of nucleic acid and protein synthesis during lateral root initiation has been studied in *Marsilea quadrifolia* (Bai-Ling and Raghavan, 1991). It is generally believed that RNA and protein synthesis in many plants are regulated by growth regulators (Key and Hanson, 1961; Maas and Klambt, 1977; Simpson and Torrey, 1977; Meyer *et al.*, 1984; Mohen *et al.*, 1985). The role of phenolics in building up host resistance against potential pathogens has been studied extensively (Mayer and Harel, 1979; Bell, 1981; Friend, 1981; Zucker, 1982; Beart *et al.*, 1985; Rosenthal, 1986). The influence of phenols on growth and differentiation of plant tissues has been emphasized recently (Kefeli, 1985; Joshi and Tandon, 1989). Many studies have been conducted which show that the changes in oxidative enzymes and phenolics are associated

with both normal and abnormal growth of the tissues (Tandon and Arya, 1982; Tandon, 1985; Joshi *et al.*, 1985; Joshi and Tandon, 1990). The phenolic content of the tissues is reported to be affected by the growth regulators (Shah *et al.*, 1976). However, very few studies have been conducted on the phenols in relation to growth period of cell cultures (Shailaja and Mehta, 1980).

MATERIALS AND METHODS

One-month old developing green protocorms of *D. fimbriatum* (cultured on MS medium without growth regulators) were transferred to fresh MS medium supplemented with different growth regulators viz. IAA, NAA, IBA, 2,4-D, BAP and KN incorporated separately and IAA and BAP in combination at concentrations ranging from 0.0-10.0 μM.

Analytical Procedures

For the biochemical studies, four different stages of protocorms were recognised :

Stage I : Developing protocorms (2 weeks after treatment)

Stage II : Young protocorms showing pointed vegetative apex (4 weeks after treatment)

Stage III : Enlarged protocorms showing leaf initials (6 weeks after treatment)

Stage IV : Protocorms at 2-leaf stage showing root initials (8 weeks after treatment)

The quantitative assays of peroxidase (PRO), polyphenol-oxidase (PPO), IAA-oxidase and estimations of soluble protein, phenols and nucleic acids (DNA and RNA) were carried out in the developing protocorms at each of the developmental stage described above. The qualitative assays of peroxidases and proteins were done in protocorms at 2-leaf stage only.

Tissue Extraction

A) For enzyme assays

One gram each of the treated tissues was homogenised separately in 15 ml of pre-chilled phosphate buffer (0.1 M, pH 6.0). The homogenates were squeezed through double-layered cheese cloth and centrifuged at 16,000 rpm for 20 min at 0-2^{o}C. The supernatants were used for the assay of enzymes.

i) Peroxidase

For peroxidase (EC 1.11.1.7) activity different hydrogen donors like pyrogallol and O-dianisidine were used. The method of Mahadevan (1974) was followed when pyrogallol was used as a hydrogen donor. In a 3.0 ml reaction mixture, 2.5 ml pyrogallol (0.05 M in 0.1 M PO_4 buffer) and enzyme were taken in appropriate amount. The absorption was calibrated to zero. To this, 0.5 ml of H_2O_2 (1%) was added and the tube inverted immediately to mix the contents. The

change in absorbance was recorded at 15 sec interval for 3 min at 420 nm. The enzyme activity is expressed as change in absorbance per min per 100 mg fresh weight of tissue.

The procedure given in Worthington Enzyme Manual (1972) was followed using O-dianisidine as hydrogen donor. The reaction mixture of 3.0 ml contained 0.4 ml of 1 mM H_2O_2 (in PO_4 buffer), and appropriate amounts of phosphate buffer (0.1 M; pH 6.0) and enzyme. After calibrating the absorbance to zero, 0.2 ml of O-dianisidine (2 mM dissolved in methanol) was added to the reaction mixture and mixed quickly. The absorbance was recorded at 15 sec interval for 3 min at 460 nm. The enzyme activity is expressed as change in absorbance per min per 100 mg fresh weight of tissue.

ii) Polyphenol oxidase

The polyphenol oxidase (EC 1.10.3.1) activity was measured using the method of Ponting and Joslyn (1948). In a 3.0 ml reaction mixture, phosphate buffer (0.1 M; pH 6.0) and 1.0 ml catechol (0.5 M in water) were taken. After calibration of absorbance to zero, the appropriate amount of enzyme extract was added. The change in absorbance was recorded at 15 sec interval for 3 min at 420 nm. The activity is expressed as change in absorbance per min per 100 mg fresh weight of tissue.

iii) IAA-oxidase

The activity of this enzyme was measured by the method of Tandon and Arya (1982). In the reaction mixture, first 2,4-dichlorophenol (DCP) was added to 0.1 M phosphate buffer (pH 6.0), then manganese chloride ($MnCl_2$) and enzyme, and, at last IAA. The total volume of the reaction mixture was kept at 5 ml and the final concentrations of DCP, $MnCl_2$ and IAA were 0.2 mM. The reaction mixture was incubated at 37°C in a shaking water bath. After 1 h 2 ml of Salkowski reagent [1 ml of 0.5 M ferric chloride ($FeCl_3.6H_2O_2$) in 50 ml of 35% perchloric acid ($HClO_4$)] was added to each tube to terminate the reaction and following 1 h wait, the absorbance of the mixture was measured at 530 nm. The amount of IAA destroyed was calculated from a standard curve for IAA. The enzyme activity is expressed as ug IAA oxidised per 100 mg fresh weight of tissue per h at 37°C.

B) For soluble protein estimation

For protein (soluble) estimation the method of Lowry *et al.* (1951) was followed. 500 mg each of the treated tissue was extracted in alcohol and macerated in chilled mortar with pestle. The volume was made upto 5 ml with 80% alcohol and centrifuged at 5000 rpm for 20 min. The supernatant was discarded and the residue suspended in 5% trichloroacetic acid (TCA) for 15 min after which 2 ml of its aliquot was

taken and resuspended in 10% TCA in the ratio of 1:1 (v/v) for 15 min to precipitate proteins at 0-2°C, and centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the residue 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 taken and 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) was added and allowed to stand for 10 min at room temperature. After incubation, 0.5 ml Folin-Phenol reagent (equally diluted with water) was added with immediate shaking and held at room temperature for 30 min and the colour was read at 750 nm. Protein is expressed as µg per 100 mg fresh weight of tissue using standard curve prepared by bovine serum albumin.

Polyacrylamide gel electrophoresis (PAGE)

The enzyme extract was subjected to PAGE for localizing isozymes of PRO and soluble proteins according to the method of Davis (1964) and Ornstein (1964). The different solutions for electrophoresis were prepared in the following manner :

Solution A: NHCl - 41.0 ml

Tris (hydroxymethyl) amino methane - 36.6 g

N N N N-tetramethylethylene diamine- 0.32 ml

Total volume was made upto 100 ml with double-distilled water.

Solution B: Acrylamide - 30.0 g

Methylene-bis-acrylamide - 0.8 g

Final volume was made upto 100 ml with double-distilled water.

Solution C: Ammonium per sulphate - 0.14 g
in double-distilled water to make up the volume to 100 ml.

All the stock solutions except solution C (prepared freshly each time) were kept in thoroughly washed amber-coloured bottles in the refrigerator at $4^{o}C$.

Tank buffer: Tris-glycine (0.025 M; pH 8.3) was made by dissolving 0.6 g tris (hydroxymethyl) amino methane and 2.88 g glycine in one litre of double-distilled water.

Preparation of gel

Before mixing, the stock solutions were brought to the room temperature to avoid bubble formation in the gels. The isozymes and protein were separated by using 7.5% polyacrylamide gels prepared by mixing gently 1 part of solution A, 1 part of solution B and 2 parts of solution C. The mixture was loaded in the gel tubes (7 cm length and i.d.5 mm) leaving 15 mm space on the upper side. A layer of water was loaded on top of the gel to prevent contact with the atmospheric oxygen. Polymerization of gels was completed within 30-40 min.

Electrophoresis

Electrophoresis was done at $4^{o}C$. Sample density was increased by 20% sucrose solution. Bromophenol blue

(500 mg/10 ml distilled water) was used as the marker dye by simply adding 1 or 2 drops of it to the extract. In each gel tube, suitable extract (containing 200 to 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 and subsequently increased to 4 mM 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 glass wall.

Staining of gels

Peroxidase : PRO isozymes were localized by using equal amounts of benzidine (1.5% in 25% acetic acid) and H_2O_2 (1%). The gels were stained for 15 min when isozymes of PRO showed up as blue bands. The gels were stored in 7% glacial acetic acid.

Proteins : The proteins were localized by staining the gels with amido black (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.

In each case, the gels were zymogrammed immediately. The positions of the bands on the gels were expressed by their respective R_m values taking the distance travelled by bromophenol blue as 0.0.

(C) For estimation of phenols

The method of Mahadevan and Sridhar (1982) was used for extraction and measurement of both O-dihydroxy and total phenols. 500 mg each of the treated tissues was immediately plunged into 10 ml of 96% boiling ethanol and boiled for 5-8 min. After cooling, the tissue was crushed thoroughly in a mortar and pestle for 5-10 min and squeezed through two-layered cheese cloth. The residue was re-extracted in 80% ethanol. Both extracts were pooled and filtered through Whatman No.41 filter paper. The final volume of the extract was made to 2 ml with 80% ethanol for every 500 mg of tissue.

i) Total phenols

An appropriate amount of alcohol extract was diluted to 1 ml with distilled water and to this 1 ml of Folin-Phenol reagent (1 part diluted with 9 parts of distilled water) and 2 ml of 20% (w/v) Na_2CO_3 solution were added. The reaction mixture was boiled exactly for a min and cooled. The final volume was made to 10 ml with distilled water and the intensity of blue colour was read at 650 nm. A blank containing all the reagents minus Folin-Phenol reagent was used to adjust the absorbance to zero. The total phenols are expressed as ug per 100 mg fresh weight of tissue using a standard curve prepared with catechol.

ii) O-dihydroxyphenols

The appropriate amount of alcohol extract was diluted

to 1 ml using distilled water. To this were added 2 ml of 0.05 N HCl, 1 ml of Arnow's reagent (10 g sodium nitrite and 10 g sodium molybdate dissolved in 100 ml distilled water; kept in amber-coloured bottle in a refrigerator), and 2 ml of 1N NaOH. The final volume was made up to 10 ml with distilled water. The absorbance of the solution was recorded at 515 nm using a blank containing all the reagents minus Arnow's reagent. O-dihydroxyphenols are expressed as ug per 100 mg fresh weight of tissue, using a standard curve prepared with caffeic acid.

D) For estimation of nucleic acids

DNA and RNA contents of the tissues were estimated by means of diphenylamine and orcinol reactions respectively, as described by Plummer (1988). TCA precipitates of the plant tissues (obtained during the preparation of samples for protein estimation) were used for the estimation of DNA and RNA. The residue was suspended in suitable volume of KOH (0.3 N) and incubated for 16-18 h at 37^{o}C. It was then centrifuged at 5000 rpm for 15-20 min and the supernatant was collected. The residue was washed twice with distilled water, centrifuged and the supernatant collected each time. The supernatants were pooled together and the final volume made up to 15 ml with distilled water. This extract contained the nucleic acids. For the separation of DNA and

RNA, the nucleic acid extract was acidified to pH 1.5 with $HClO_4$. This precipitated DNA and excess of potassium chlorate. The extract was centrifuged at 5000 rpm for 15-20 min and the supernatant containing RNA and residue containing DNA were collected.

The RNA present in the supernatant was estimated by orcinol reaction whereas DNA was estimated by diphenylamine reaction after solubilizing the residue in $HClO_4$ and removing the excess of perchlorate.

i) Estimation of RNA

An appropriate amount of the RNA extract (supernatant solution) was mixed with 3 ml of orcinol reagent [1 g of $FeCl_3.6H_2O$ dissolved in 1 l of conc. HCl and to it was added 35 ml of 6% (w/v) orcinol in alcohol]. It was then heated on a boiling water bath for 20 min, cooled and the absorbance read at 665 nm against an orcinol blank. The total RNA content is expressed in ug per 100 mg fresh weight.

Clarification of DNA

The residue containing DNA was suspended in suitable aliquots of 5% of TCA at $0^{o}C$ and centrifuged at 5000 rpm for 15 min. The supernatant was discarded and the process was repeated twice. The residue was washed with absolute ethanol and then with ethanol : ether (3:1) mixture. The residue was then suspended in 0.5 N $HClO_4$ and incubated at $90^{o}C$ for 7 min in a constant temperature water bath, centrifuged and the

supernatant was collected. The residue was washed twice with water and the supernatants were collected. All these supernatants were pooled and the volume made to 15 ml. Equal volume of potassium hydroxide (1 N) was added to precipitate excess perchlorate as $KClO_4$. The extract was centrifuged at 5000 rpm for 15 min and the supernatant which contained DNA was collected.

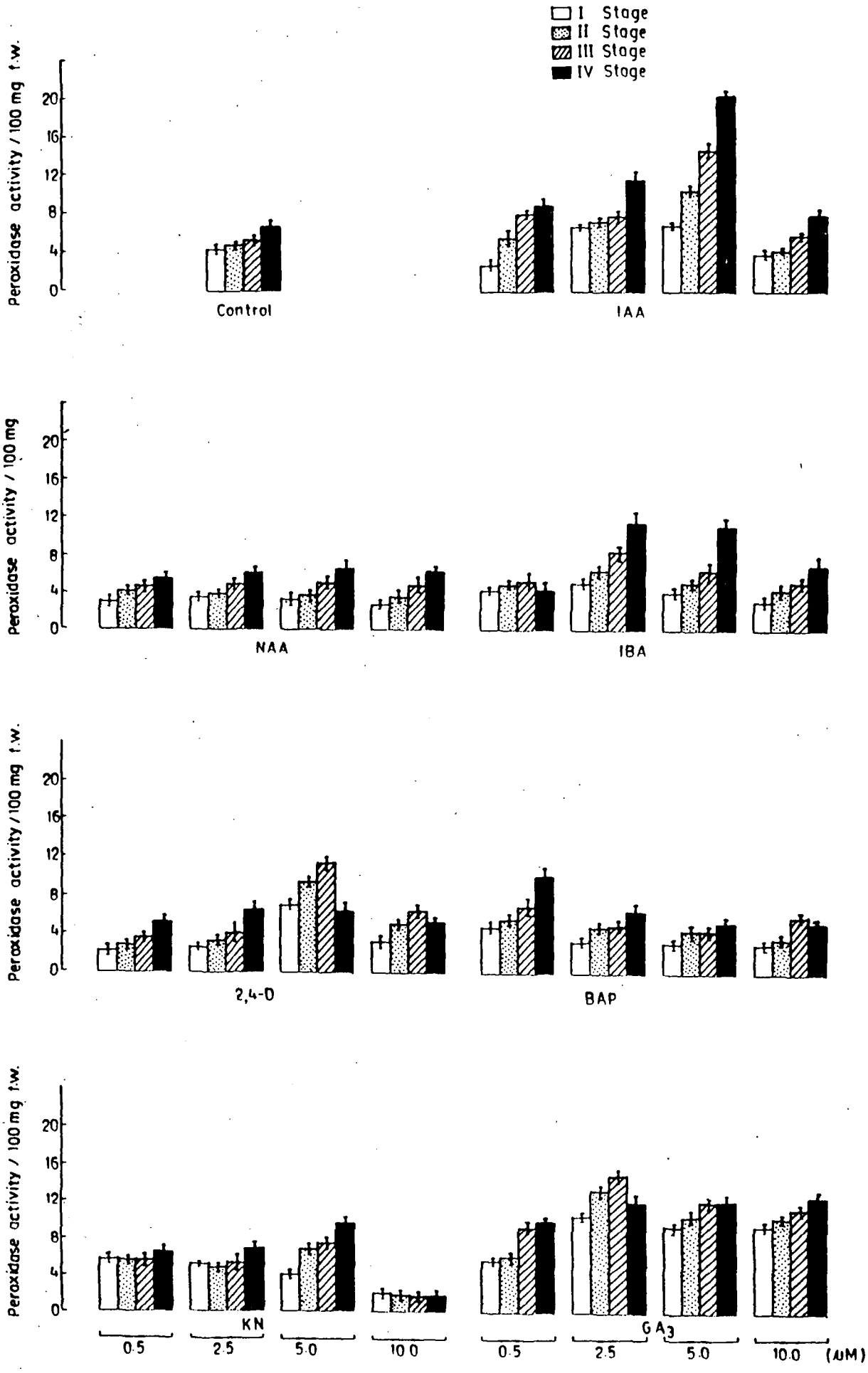
ii) Estimation of DNA

To an appropriate amount of DNA extract, 4 ml of freshly prepared diphenylamine reagent (10 g of pure diphenylamine dissolved in 1 l of glacial acetic acid and to it added 25 ml of conc. H_2SO_4). It was then heated on a boiling water bath for 10 min and cooled. The absorbance was read at 595 nm. The DNA content present was calculated from a standard curve and is expressed in µg per 100 mg fresh weight.

RESULTS

The PRO activity in case of IAA, IBA, 2,4-D and GA_3 treated protocorms was influenced differentially as development proceeded. In case of IAA treatment, it was highest at 5.0 µM concentration and decreased at higher concentrations. At 5.0 µM of 2,4-D in the medium, PRO activity increased upto the stage III after which it declined. The activity was not much different from the

Fig. 8. Effect of growth regulators on peroxidase activity (ΔA/min/100 mg. fresh wt.) at different stages of protocorm development


I Stage
II Stage
III Stage
IV Stage
Peroxidase activity / 100 mg f.w.
Control
IAA
Peroxidase activity / 100 mg
NAA
IBA
Peroxidase activity / 100 mg f.w.
2,4-D
BAP
Peroxidase activity / 100 mg f.w.
KN
GA3
0.5
2.5
5.0
10.0
0.5
2.5
5.0
10.0
(µM)
Treatments

± S.D.

Fig. 8

control in protocorms treated with NAA, BAP and KN. However, higher concentration of KN (10.0 μM) was inhibitory for PRO activity. In all GA_3 treatments, the activity of PRO was slightly higher than the control (Fig. 8).

An increase in the PPO activity with growth occurred in all the treated protocorms except for KN and GA_3 treatments where a reverse picture was obtained. The higher concentrations (5.0 μM and 10.0 μM) of IAA, NAA and IBA were slightly inhibitory whereas in cases of 2,4-D and BAP only 10.0 μM concentration was inhibitory for the activity of PPO. In KN and GA_3 treated protocorms, the activity declined drastically during development after stage I (Fig. 9).

The activity of IAA-oxidase increased slightly with the increase in the concentrations of IAA, NAA and GA_3 as compared to the control. However, it decreased in protocorms treated with higher concentrations (5.0 μM and 10.0 μM) of IBA and BAP. In all the concentrations of 2,4-D used, the IAA-oxidase activity declined drastically after stage III. A similar picture was observed at 10.0 uM of KN in the medium (Fig. 10).

As compared to the control, the soluble protein content markedly increased with the incorporation of growth regulators in the medium. On the other hand, it decreased with growth in the untreated protocorms. The higher concentrations (5.0 μM and 10.0 μM) of 2,4-D, IBA and BAP

Fig. 9. Effect of growth regulators on polyphenol oxidase activity (ΔA/min/100 mg. fresh wt.) at different stages of protocorm development.

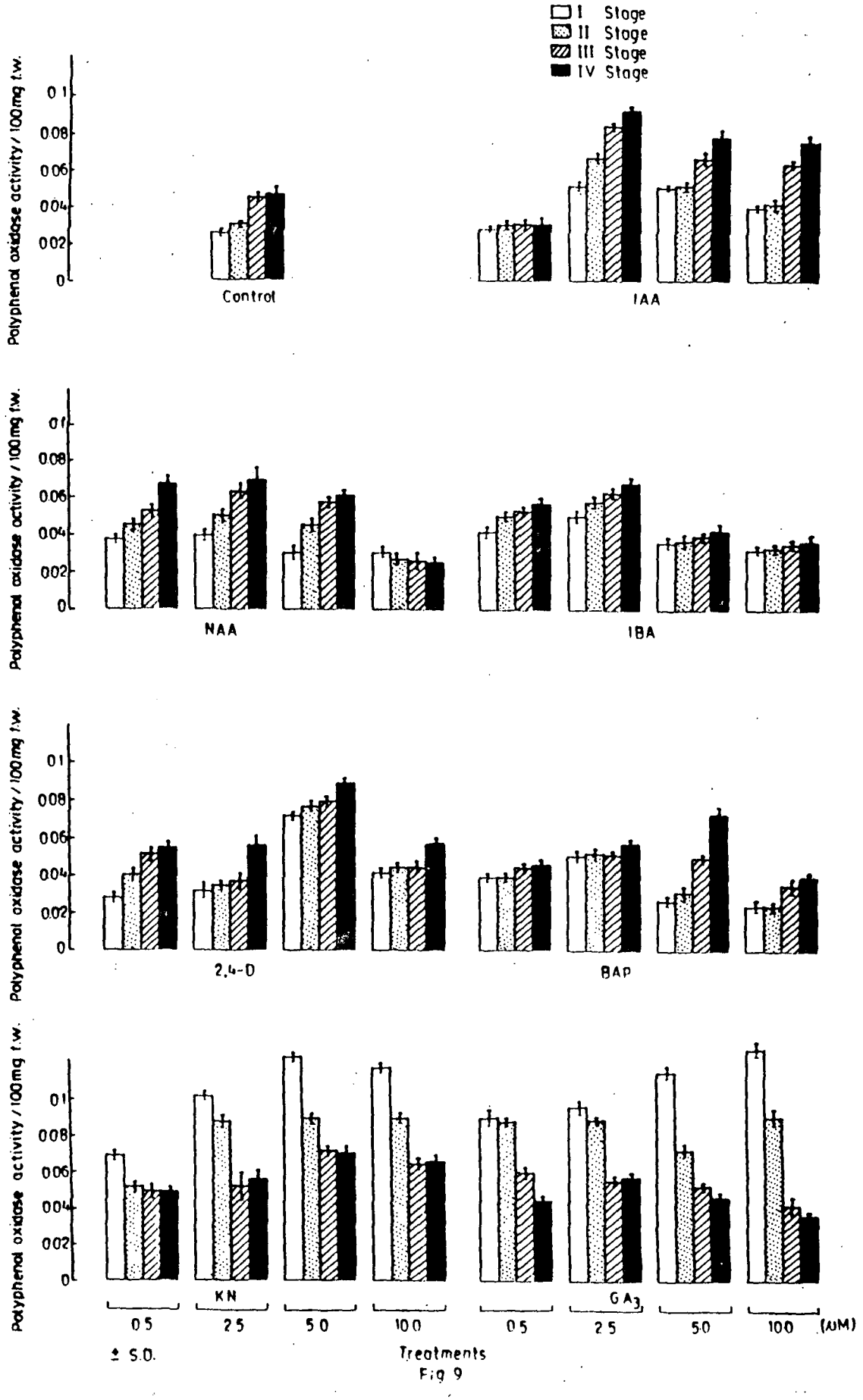

I Stage
II Stage
III Stage
IV Stage
Polyphenol oxidase activity / 100mg f.w.
0.1
0.08
0.06
0.04
0.02
0
Control
IAA
NAA
IBA
2,4-D
BAP
KN
GA3
0.5
2.5
5.0
10.0
(µM)
± S.D.
Treatments

Fig 9

Fig. 10. Effect of growth regulators on IAA-oxidase activity (µg IAA oxidised/100 mg fresh wt./h) at different stages of protocorm development

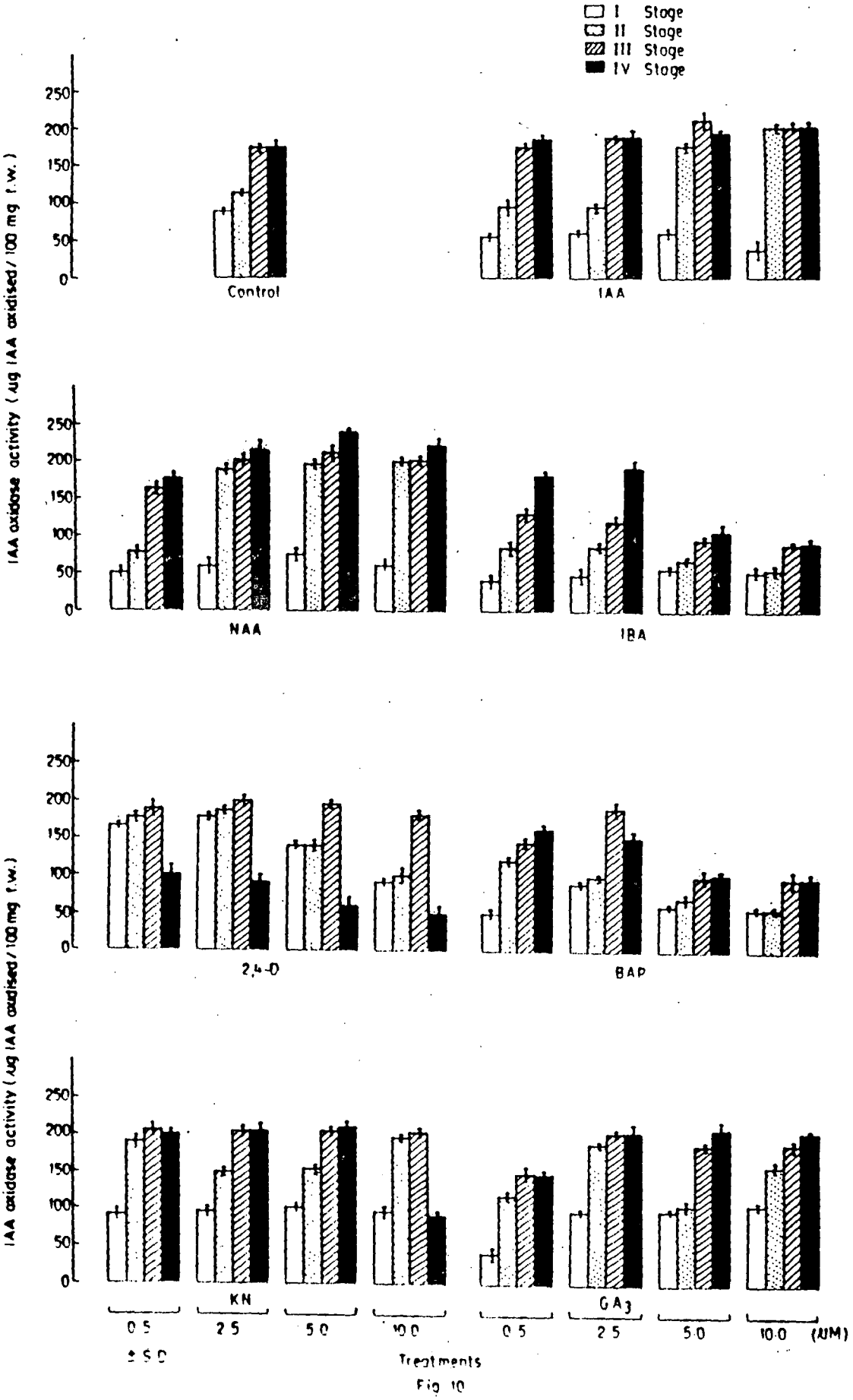

I Stage
II Stage
III Stage
IV Stage
IAA oxidase activity (Ag IAA oxidised / 100 mg f.w.)
250
200
150
100
50
0
Control
IAA
NAA
IBA
2,4-D
BAP
KN
GA3
0.5
2.5
5.0
10.0
(MM)
± S.D.
Treatments

Fig. 10

Fig. 11. Effect of growth regulators on soluble protein content (µg/100 mg. fresh wt.) at different stages of protocorm development

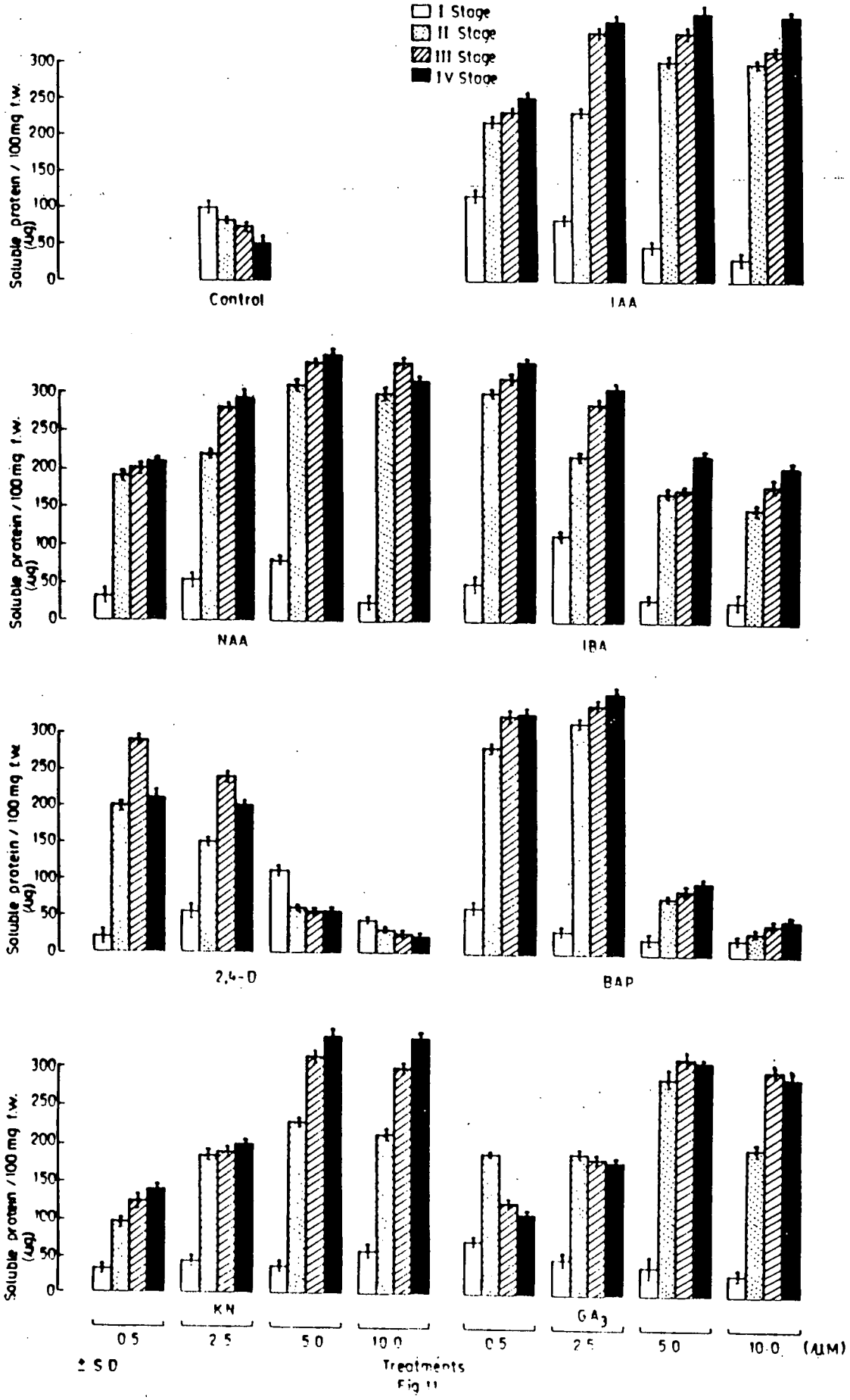

I Stage
II Stage
III Stage
IV Stage
Soluble protein / 100 mg f.w. (µg)
Control
IAA
NAA
IBA
2,4-D
BAP
KN
GA3
0.5
2.5
5.0
10.0
(µM)
± S.D.
Treatments

Fig 11

were inhibitory for the accumulation of soluble protein. The protocorms treated with lower concentrations of 2,4-D showed a decline in protein content after stage III. However, at its higher concentrations, a decline was observed right after stage I. In case of GA_3 (0.5 µM) treated protocorms, the soluble protein content declined after stage II (Fig. 11).

Determination of the electrophoretic profile for proteins revealed the presence of seven major bands in all the treated and untreated protocorms. The bands differed in their R_m, colour intensity and width (Fig. 12). Irrespective of the growth regulator treatments, four isozymes of peroxidase were detected in the protocorms. The isozyme bands as detected on the gels differed from each other in their R_m, intensity and width (Fig. 13).

The contents of total phenols increased with growth in most of the treated protocorms. However, in the control, it decreased as the growth of protocorms proceeded. At 0.5 µM of IAA in the medium, the contents of total phenols were lower at stage II and then remained stationary. NAA, IBA, 2,4-D and BAP at 10.0 µM concentration were inhibitory for the accumulation of total phenols. The KN and GA_3 treated protocorms showed a decline in the total phenolic content with growth. The O-dihydroxy phenols decreased with growth in the treated tissues except for IAA (10.0 µM), NAA (5.0 µM) and BAP (5.0 µM) treatments. In case of all KN treatments

Fig. 12. Electrophoretic profile for proteins

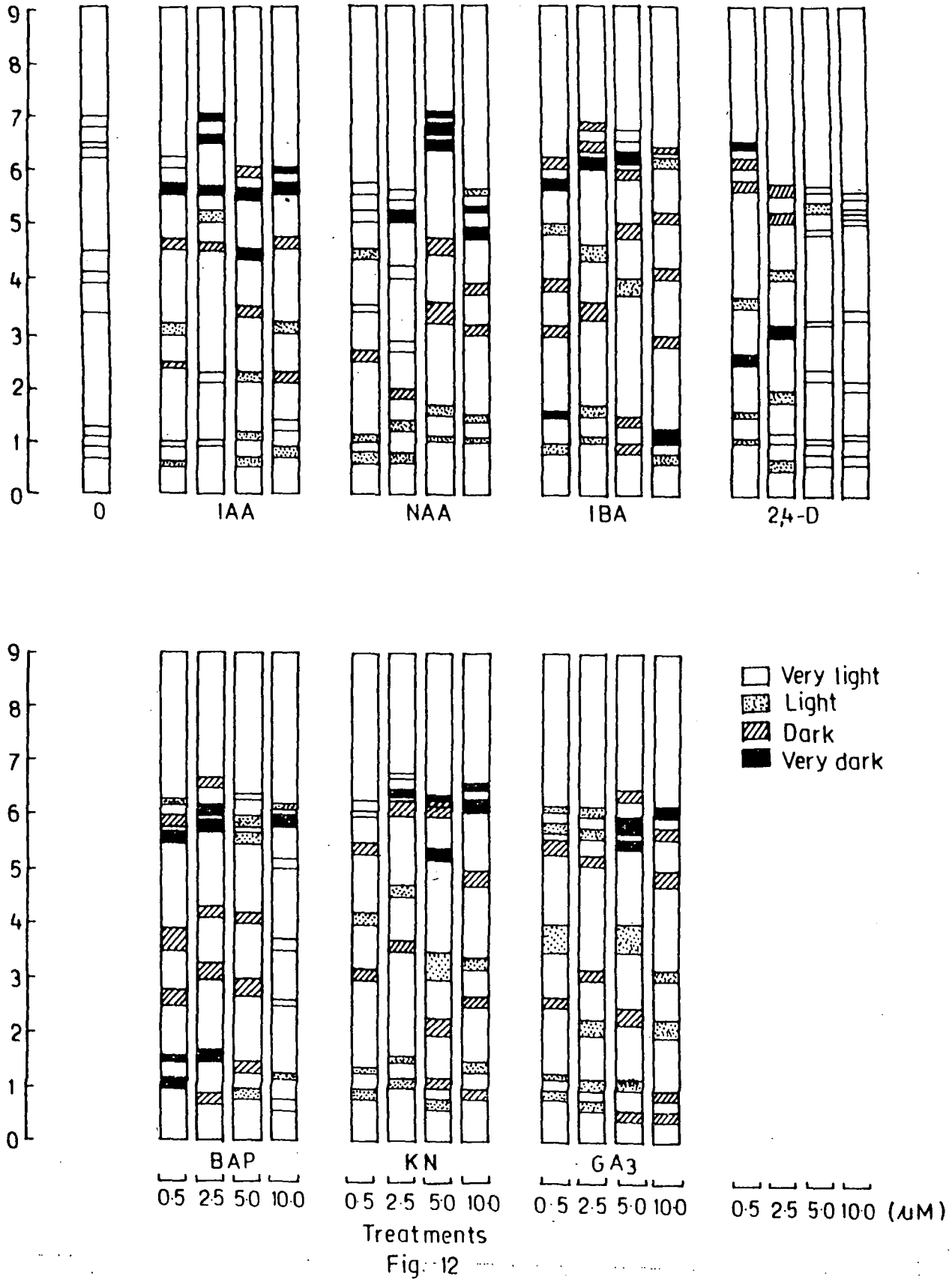

9
8
7
6
5
4
3
2
1
0
0
IAA
NAA
IBA
2,4-D
BAP
KN
GA3
Very light
Light
Dark
Very dark
0·5 2·5 5·0 10·0
0·5 2·5 5·0 10·0
0·5 2·5 5·0 10·0
0·5 2·5 5·0 10·0 (μM)
Treatments

Fig. 12

Fig. 13. Electrophoretic profile for isozymes of peroxidases

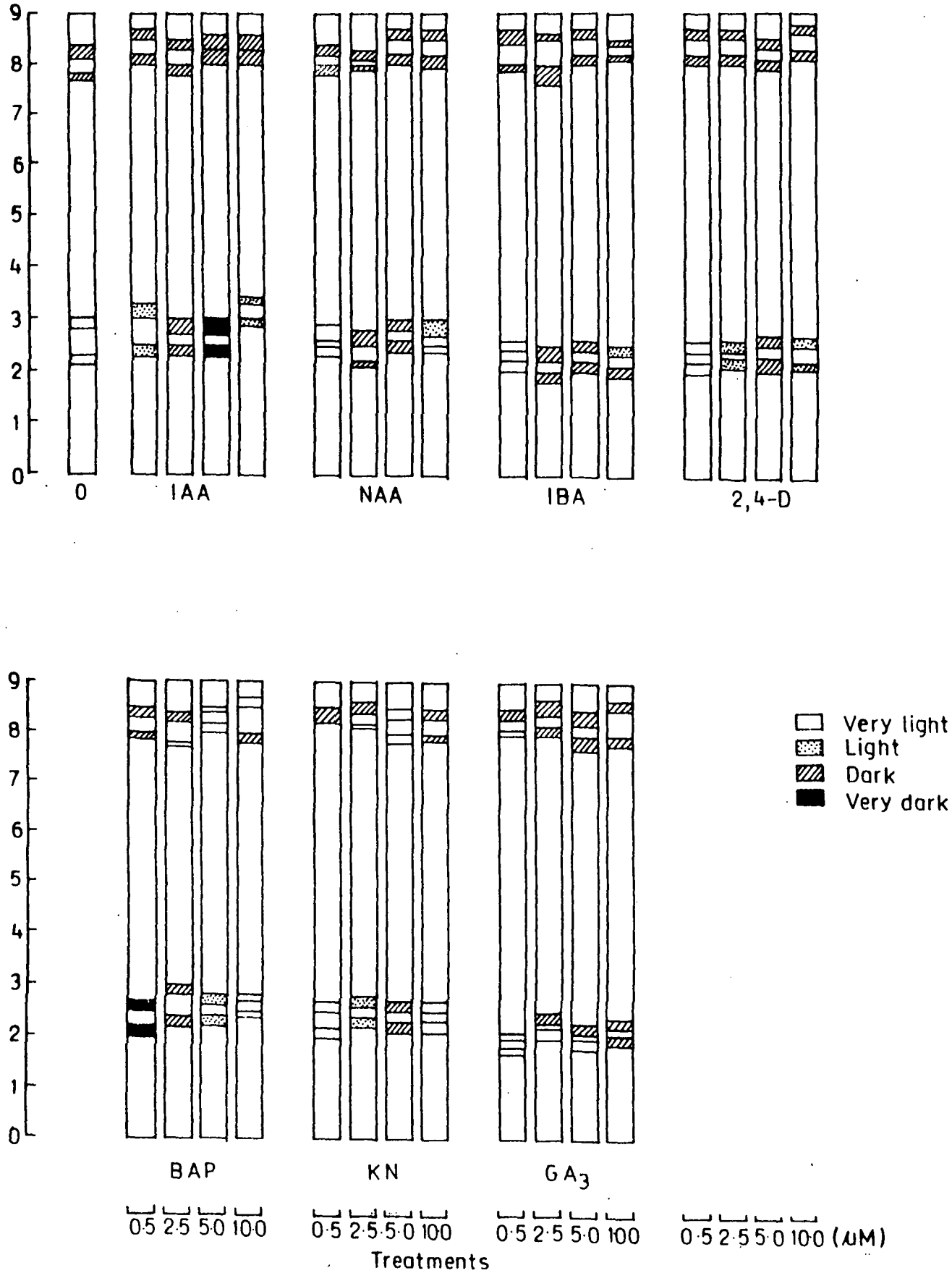

9
8
7
6
5
4
3
2
1
0
0
IAA
NAA
IBA
2,4-D
BAP
KN
GA3
Very light
Light
Dark
Very dark
0·5 2·5 5·0 100
0·5 2·5 5·0 100
0·5 2·5 5·0 100
0·5 2·5 5·0 100 (μM)
Treatments

Fig. 13

Fig. 14. Effect of growth regulators on total phenols (µg/100 mg. fresh wt.) and O-dihydroxy phenols (µg/100 mg. fresh wt.) accumulation at different stages of protocorm development

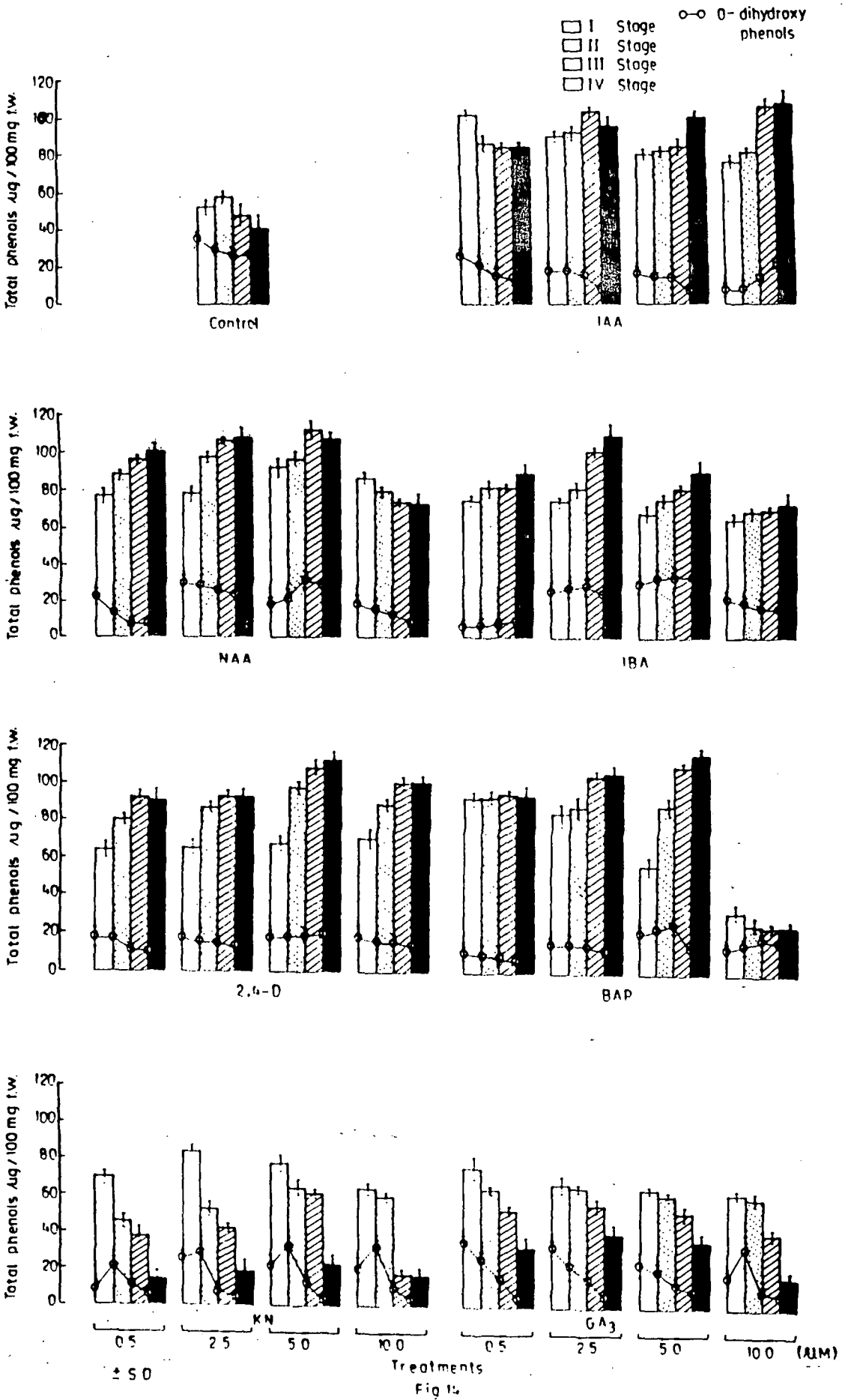

I Stage
II Stage
III Stage
IV Stage
o–o 0- dihydroxy phenols
Total phenols Aug / 100 mg f.w.
Control
IAA
NAA
IBA
2,4-D
BAP
KN
GA3
0 5
2 5
5 0
10 0
± S D
Treatments
(ALM)

Fig 1.

and 10.0 μM of GA_3, the O-dihydroxy phenol contents increased at stage II and then declined subsequently (Fig. 14).

The DNA and RNA contents were markedly affected by the incorporation of growth regulators in the medium. In untreated controls, the contents of both the nucleic acids increased markedly upto the stage II and subsequently exhibited a decline. On the other hand, the DNA contents increased during all the developmental stages of IAA, NAA, IBA, BAP and KN treated protocorms. However, at 10.0 μM concentration of NAA and KN, the DNA content showed a decline at stage IV. The 2,4-D and GA_3 treated protocorms showed a marked initial increase in the DNA content which declined towards the development of protocorms (Fig. 15). The RNA content increased with growth in IAA, NAA, IBA, BAP and KN treated protocorms. At 10.0 μM of NAA, however, it declined after stage III. In 2,4-D and GA_3 treated protocorms, a decline in RNA content was observed in course of the development of protocorms (Fig. 15).

The PRO activity increased markedly at 2.5 μM each of IAA and BAP in combination. As compared to the control, the PRO activity increased upto stage III and declined subsequently in protocorms treated with 5.0 uM each of IAA and BAP. In all other combinations of IAA+BAP, the PRO activity was close to the control. However, at 10.0 μM BAP in combination with 2.5, 5.0 and 10.0 μM IAA, a decrease in

Fig. 15. Effect of growth regulators on DNA and RNA accumulation (µg/100 mg. fresh wt.) at different stages of protocorm development

Fig. 16. Effect of IAA+BAP in combination on peroxidase and polyphenol oxidase activities at different stages of protocorm development

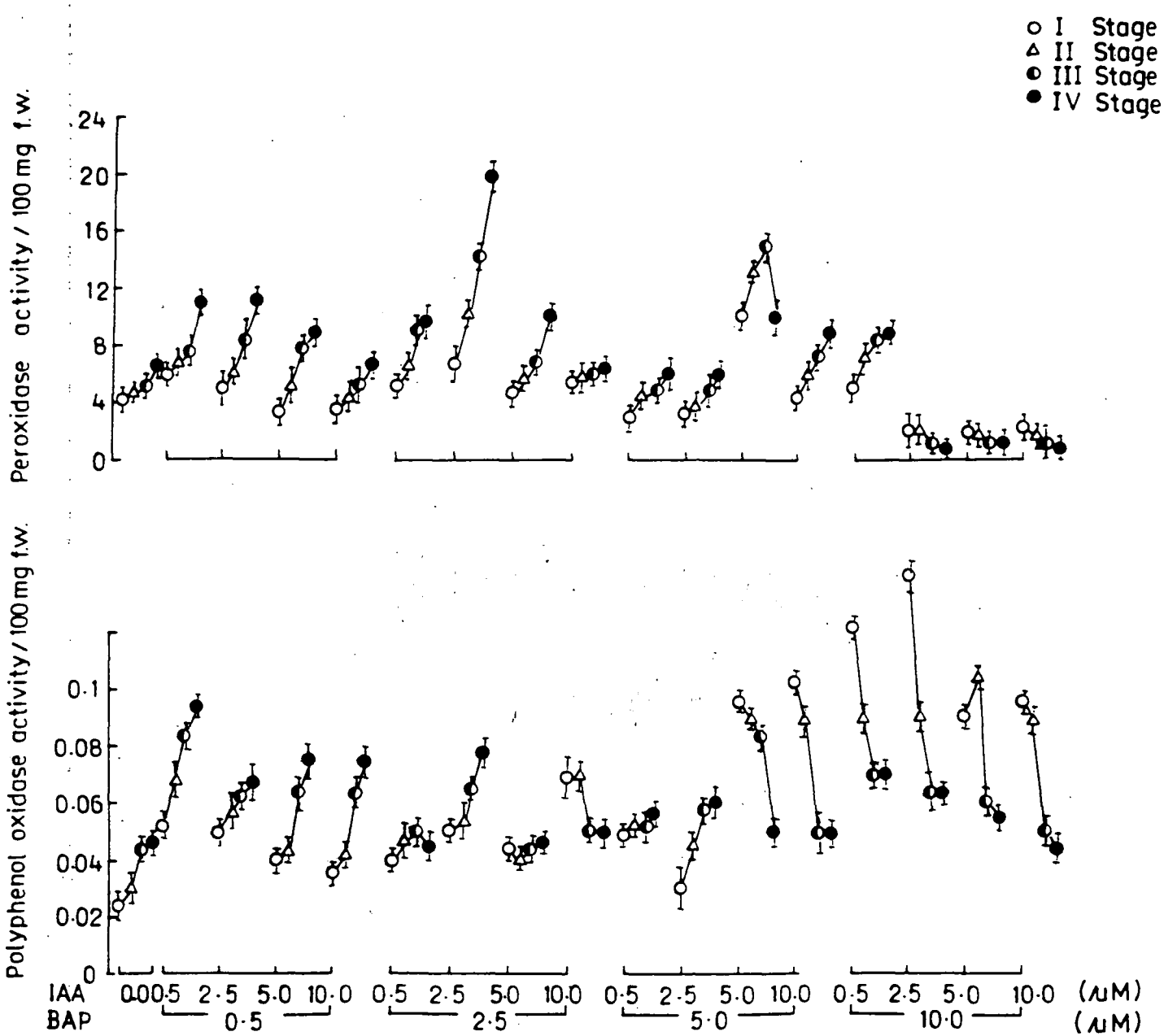

I Stage
II Stage
III Stage
IV Stage
Peroxidase activity / 100 mg f.w.
24
20
16
12
8
4
0
Polyphenol oxidase activity / 100 mg f.w.
0·1
0·08
0·06
0·04
0·02
0
IAA 0·0 0·5 2·5 5·0 10·0 0·5 2·5 5·0 10·0 0·5 2·5 5·0 10·0 0·5 2·5 5·0 10·0 (µM)
BAP 0·5 2·5 5·0 10·0 (µM)
± S.D.
Treatments

Fig. 16

Fig. 17. Effect of IAA+BAP in combination on IAA-oxidase and soluble protein content at different stages of protocorm development

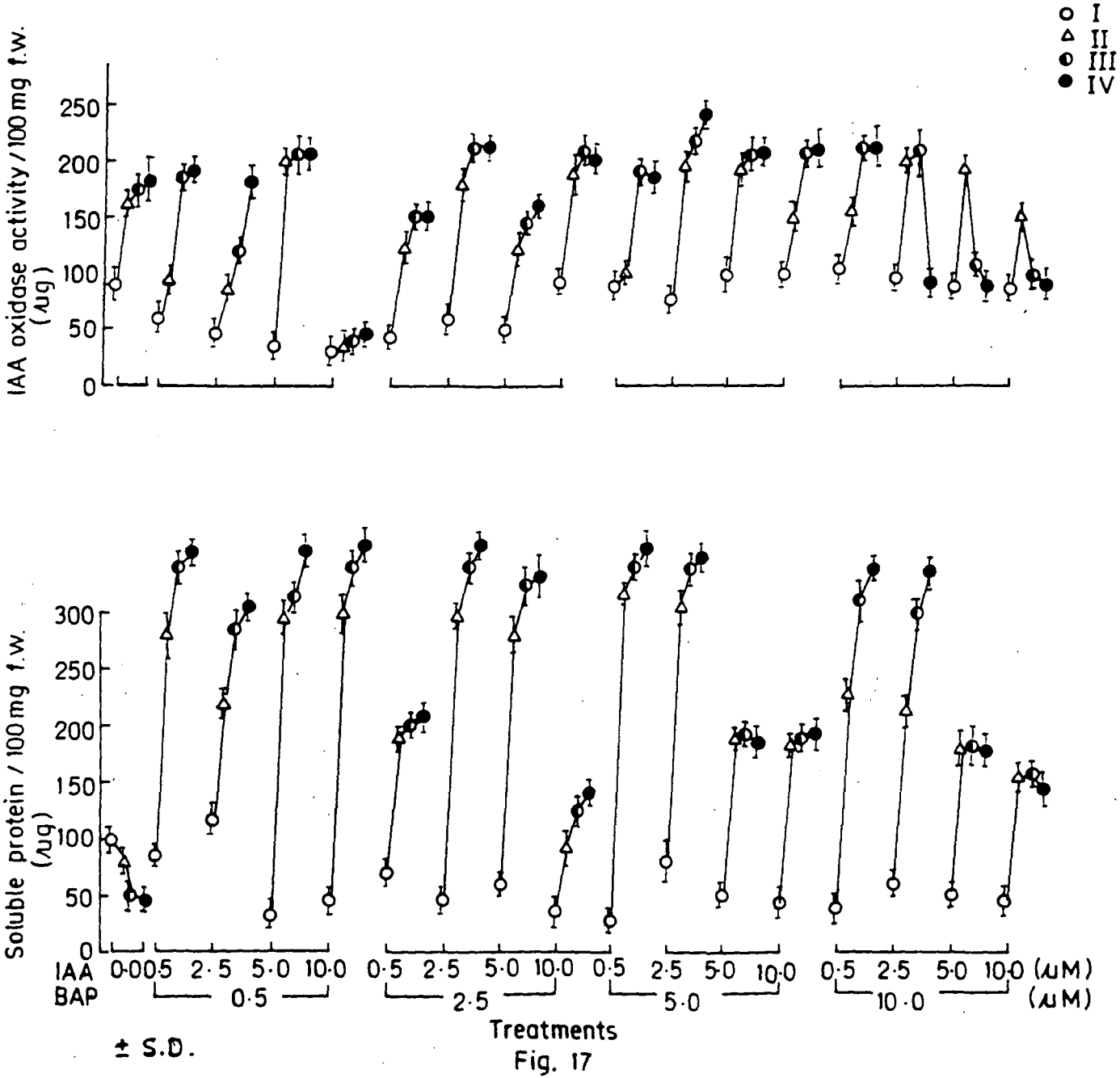

I Stage
II Stage
III Stage
IV Stage
IAA oxidase activity / 100 mg f.w. (λug)
0
50
100
150
200
250
Soluble protein / 100 mg f.w. (λug)
0
50
100
150
200
250
300
IAA 0·0 0·5 2·5 5·0 10·0 0·5 2·5 5·0 10·0 0·5 2·5 5·0 10·0 0·5 2·5 5·0 10·0 (λM)
BAP 0·5 2·5 5·0 10·0 (λM)
± S.D.
Treatments

Fig. 17

the activity of PRO was observed with growth (Fig. 16). The PPO activity was relatively higher at initial stages of growth in treated protocorms. The activity of PPO increased with growth in treatments having 0.5 µM BAP with 0.5, 2.5, 5.0 and 10.0 µM IAA, 2.5 µM BAP with 2.5 µM IAA, and 5.0 µM BAP with 2.5 µM IAA in combination. However, a completely reverse picture was obtained in case of treatments of 5.0 µM BAP with 5.0, 10.0 µM IAA, and 10.0 µM BAP with 0.5, 2.5, 5.0 and 10.0 µM IAA (Fig. 16). The IAA-oxidase activity was not much affected by the incorporation of IAA and BAP in combination. However, at 10.0 µM IAA and 0.5 µM BAP in combination, the IAA-oxidase activity was much lower as compared to the control. A decrease in the activity of IAA-oxidase with growth was observed at 10.0 µM BAP with 2.5, 5.0 and 10.0 µM IAA (Fig. 17). The soluble protein content increased markedly as the result of interactions between growth regulators. However, the soluble protein contents declined after stage II in protocorms treated with 5.0 µM BAP with 5.0 µM IAA and 10.0 µM BAP and 5.0 and 10.0 µM IAA in combinations (Fig. 17).

DISCUSSION

The treatment of whole plants, isolated plant organs or tissues with growth regulators results in changes in the activity of several enzymes (Galston and Davies, 1969; Lee,

1971, 1972; Vernon and Strauss, 1972; Schneider and Wightman, 1974; Shah *et al.*, 1976; Saleh, 1981; Singh *et al.*, 1982; Tandon and Arya, 1982; Barendse, 1983). It is accepted that changes in enzyme levels cause developmental changes, although the precise role of the particular enzyme is not fully understood. The role of plant growth regulators in influencing the level of phenolics and activities of PRO, PPO and IAA-oxidase which are involved in growth and development of plants is studied to a great extent (Galston and Davies, 1969; Tandon and Arya, 1982). In the present study, the activity of both PRO and PPO increased on fresh weight basis in most of the treated tissues. No appreciable difference in PRO activity was observed between different treatments except IAA treated tissues where an increase was recorded at 2.5 µM and 5.0 µM concentrations. The increase in PRO activity as a result of exogenous supply of IAA is in agreement with the earlier reports (Alvarez and King, 1969; Kumaria *et al.*, 1990). Moreover, Dendsay (1989) has also found the increase in PRO activity by 2 to 3 folds in mung cotyledons due to exogenous supply of IAA. On the other hand, the PRO activity has been found to be the exact reciprocal of IAA production by *Vanda* seedlings (Alvarez, 1968; Alvarez and King, 1969). The PPO activity increased with growth in protocorms treated with auxins. However, the higher concentrations (5.0 µM and 10.0 µM) were found to be inhibitory. Auxins are reported to

increase the PPO activity in tobacco tissue cultures (Vernon and Strauss, 1972). A slight increase in IAA-oxidase was recorded in the IAA, NAA and GA_3 treated protocorms. Brunner (1978) reported an increase in IAA-oxidase and PRO activities with the progressive regeneration of *Phaseolus vulgaris* tissue by IAA and IBA. IAA-oxidase system is involved in the control of endogenous auxin levels and thus regulates various physiological processes such as cell growth and differentiation (Sembdner *et al.*, 1980). The changes in PRO, PPO and IAA-oxidase during growth can be attributed largely to the ability of these enzymes in the regulation of IAA. A negative correlation has been found between growth rate and IAA-oxidase activity of various organs and tissues (Wareing and Phillip, 1982; Zolfia, 1984).

The accumulation of soluble proteins was greatly influenced by growth regulators. The soluble protein content was higher at the initial stages of differentiation during protocorm development. A perusal of literature suggests that the effects of growth regulators on growth and differentiation are conditioned by an undisturbed protein synthesis. It may be assumed that the protocorm showing no signs of development/differentiation has less protein (soluble) synthesis. Protein synthesis is a pre-requisite for the growth to continue. In the present study, the auxins stimulated the accumulation of soluble protein contents.

However, higher concentration of IBA and 2,4-D were inhibitory. The incorporation of BAP and KN enhanced protein synthesis but higher concentrations of the former were inhibitory. The cytokinin treatment may not be a pre-requisite for the induction or enhancement of protein synthesis in several plants as most plant cells contain sufficient cytokinin to keep protein synthesis going (Jouanneau, 1968, 1970; Fosket and Short, 1973). However, it seems that *D. fimbriatum* requires an exogenous supply of both auxins and cytokinins for protein synthesis and growth. Here, differentiation of the protocorm, that is, emergence of the vegetative apex coincides with a drastic accumulation of soluble proteins in the tissues. Reynolds (1990), has reported that *in vitro* morphogenesis of stem segments of *Solanum carolinense* is associated with nature quantitative changes in protein expression.

The electrophoretic profile for proteins showed a similar pattern for all the treated protocorms at the developmental stage IV. The incorporation of the growth regulators in the medium had pronounced effect on the oxidative enzymes studied. It may be assumed that some of the proteins produced *de novo* as a result of the treatments belong to the oxidative enzyme pathway. The difference in the intensity and width of the isozyme bands of the PRO at stage IV accounts for the differential effects of the growth

regulators on the isozyme pattern.

The phenolic compounds occurring in the tissues are not necessarily inhibitory to growth but also possess stimulatory properties and some of them are inert or participate in processes such as respiration and photosynthesis (Kefeli and Kutacek, 1977). The total phenolic contents increased in developing protocorms at different concentrations of auxins and BAP in medium. Inhibition of growth as a result of higher phenolic contents was observed in case of 2,4-D and BAP (0.5 μM) treated protocorms. The influence of auxins on phenols synthesis has been studied to some extent (Shah *et al.*, 1976). However, the role of phenols in relation to growth period of cell cultures is poorly understood (Shailaja and Mehta, 1980). Monophenols are regarded as co-factors of IAA-oxidase, while O- and p-dihydroxyphenols and polyphenols as IAA-oxidase inhibitors (Schneider and Wightman, 1974). The enzyme PPO converts monophenols (electron acceptors) to O-diphenols (electron donors) to quinones (strong electron acceptors) and thus regulates the level of different phenolics. The phenolic compounds (auxin protectors) in turn affect the activities of PRO and IAA-oxidase responsible for IAA destruction (Tandon and Arya, 1982). The phenolic compounds and changes in the enzyme activities are involved in building up host resistance against potential pathogens (Manibhushanrao *et al.*, 1988).

The synthesis of RNA and proteins is regulated by growth regulators in many plants (Key and Hanson, 1961). For any growth process to be sustained, RNA and protein synthesis should be continued. The response of the plant tissues to auxins for RNA accumulation has been reported for many plant species (Meyer *et al.*, 1984; Mohen *et al.*, 1985; Walker *et al.*, 1985). In the present study, the auxins except for 2,4-D and higher concentration (10.0 µM) of NAA, stimulated RNA synthesis. The RNA content increased with growth in BAP and KN treated protocorms. GA_3 treatment was found to be slightly inhibitory for RNA and DNA synthesis. The synthesis of DNA was markedly increased by growth regulators except for all concentrations of 2,4-D and 10.0 µM of NAA where it declined with growth. The effect of growth regulators on DNA synthesis has been studied in several plants (Roberts, 1976). It has been reported that the synthesis of DNA takes place prior to differentiation. In the present study also it was found that the DNA content was reduced or remained almost constant by the time the 2-leaf stage was reached. DNA content in plants is related to sizes of cells, tissues and organs, cell cycle time and duration of S phase, duration of meiosis, pollen maturation time and minimum generation time (Thompson and Murray, 1981). The extremely slow growth rate of the orchid, *Taeniophyllum aphyllum* (Mutsuura *et al.*, 1962) and the much faster rates in *Zeuxine strateumatica* might

reflect the differences in the rates of DNA synthesis (Arditti and Ernst, 1984). According to the different reports, the action of several plant growth regulators on *Cymbidium* protocorms could be explained in terms of their effects on the DNA contents of the cells (Nagl and Rucher, 1974, 1976).

It is obvious from the results that the growth regulators have a pronounced effect on the growth and physiology of the development of protocorms of *D. fimbriatum*. It also appears that besides their effects on the activities of PRO, PPO and IAA-oxidase, and the contents of proteins, phenols and nucleic acids, the treatments influence some other enzymes which are involved in other metabolic pathways.

VI. CLONAL MICROPROPAGATION

INTRODUCTION

Clonal propagation of orchids by means of explants is gaining wide importance in the tissue culture industry. It is particularly valuable in perpetuating clones of special merit. Morel (1960) for the first time noted the formation of plbs around the shoot tips of *Cymbidium* cultured *in vitro*. These plbs when cut into small sections and sub-cultured on fresh nutrient medium, multiplied and, on being left undisturbed, developed into complete plantlets. This work led to the mass propagation of desirable virus-free varieties at a very high rate. Many pioneer workers have applied the

technique of meristem culture to either eliminate virus infection from clonal plants or the production of asexual seedlings on a large scale (Ilsley, 1965; Blowers, 1966, 1967; Lindeman, 1967a,b; Taylor, 1971; Thompson, 1971). Tissue culture techniques have also been used to save many orchid species from extinction.

Knudson's (1951) discovery that the developing orchid seedlings could be grown on a medium supplemented with organic and inorganic nutrients led to the formation and utilization of a number of media for tissue culture of orchids (Withner, 1959; Arditti, 1967a, 1977; Butenko, 1968). The medium used differs from genus to genus and species to species. The incorporation of certain additives like coconut milk, tomato juice, banana extract, different fruit juices, fish emulsion, beef extract and even beer (Alberts, 1953; Withner, 1959; Arditti, 1967a) and growth factors (Arditti, 1977) proves to be beneficial for orchid tissue culture. However, the physiology of nutrition of orchids is difficult to explain. Besides defining the medium, other conditions like suitable pH, minimizing the fungal infection, sterilization of the inoculum have also been considered in tissue culture studies of orchids (Rao, 1977). But the available information is insufficient because the medium requirements of orchids vary, several media can be suitable for one genus, and more than one genus can be cultured on a

specific medium.

Tissue culture methods for the propagation of orchids were introduced by several workers using various plant parts and a number of media (Bergman, 1972a, 1972b; Wang, 1989). *Cymbidium, Dendrobium, Vanda, Spathoglottis* and allied genera are widely propagated by tissue culture throughout the world (Vajrabhaya, 1977; Oliva *et al.*, 1985). According to Murashige (1978), important orchids except *Paphiopedilum* are clonable *in vitro*. Kusumoto (1979) used the shoot apexes of *Cattleya* buds for the production of plbs. Clonal propagation of *Phalaenopsis* by means of flower stalk, bud culture and shoot-tip culture has also been reported (Intuwong and Sagawa, 1974; Yoneda *et al.*, 1983). Explants from the floral stalk have been used in studies on *in vitro* propagation of orchids (Koch, 1974; Lay, 1978; Tanaka and Sakanishi, 1978; Homma and Asahira, 1985; Yoneda, 1986; Momose and Yoneda, 1988). Reports concerning the propagation of orchids through leaf segments have been published (Tanaka *et al.*, 1975; Vij *et al.*, 1984; Tanaka, 1987; Vij and Pathak, 1990). Phillip and Nainar (1988) reported the *in vitro* transformation of root meristem to shoot and plantlets in *Vanilla planifolia*. Sanchez (1988) carried out micropropagation of *Cyrtopodium* cf. *punctatum* seedlings, grown asymbiotically *in vitro*, through root tip culture. Yoneda and Momose (1988) reported plbs' and plantlets' formation by root tip cultures in

Phalaenopsis. *In vitro*, shoot regeneration from root tips of an orchid, *Mormodes histrio*, has also been reported by Holters and Zimmer (1990).

Different tissue culture techniques have been developed to promote the selected clones' multiplication in Dendrobiums (Kim *et al.*, 1970; Mosich *et al.*, 1974; Kukulczanka and Wojciechowska, 1983). But these techniques differ from species to species. It has been reported that the explants employed for the tissue culture respond selectively depending on their source, physiological state and nutrient environment (Vij *et al.*, 1983, 1984). The following chapter deals with tissue culture of *D. fimbriatum* var. *oculatum* for mass propagation of identical genotypes of this orchid.

MATERIALS AND METHODS

Different explant sources such as leaf parts, root tips, axillary buds and apical meristems were obtained from the plants maintained in the Botanical Garden of North-Eastern Hill University, Shillong. About 1-2 cm segments of the explants were cut and cleaned gently scrubbing with a soft brush and mild detergent. These were then washed in running tap water for about 15-20 min, and were rinsed with distilled water. The surface-sterilization of the explants was done by 20% (v/v) of sodium hypochlorite solution (4-6% available chlorine in the stock solution). The explants were

thoroughly rinsed with sterile distilled water to remove the sterilant. Leaf bases, leaf tips and root tips measuring about 2 mm were cut from the larger sections of the explant sources. The nodal sections containing the axillary buds were also cut into 3-4 mm size after the removal of the leaves, dry sheaths and other external tissues. The apical meristems measuring about 1 mm were excised from the shoot tips aseptically and cultured on different media.

A number of media were tried for the clonal propagation of *D. fimbriatum*. A new medium comprising inorganic and organic nutrients along with the vitamins was developed. The composition of the medium is given in Table 11. The sterilized explant pieces were inoculated on this medium aseptically. The cultures were maintained at 24±2°with 16h illumination of 3000 lux light intensity. The pH of the medium was adjusted to 5.5 prior to autoclaving. The medium was supplemented with different growth regulators both separately and in combination so as to initiate the formation of plbs and shoot buds on the explants. The different combinations of growth regulators tried were 2,4-D + KN, IAA + KN, NAA + KN, NAA + BAP and IAA + BAP in a range of 0.0-10.0 µM.

RESULTS

Out of the different explant sources tried, the apical meristems and axillary buds were found to be suitable for

Table 11. Basal medium devised for clonal propagation

Macro and micro nutrients	mg/l
NH_4NO_3	825
KNO_3	950
KH_2PO_4	200
H_3BO_3	0.5
KI	0.02
$Na_2MoO_4.2H_2O$	0.025
$CoCl_2.6H_2O$	0.02
$MgSO_4.7H_2O$	275
$MnSO_4.4H_2O$	6.8
$ZnSO_4.7H_2O$	1.0
$CuSO_4.5H_2O$	0.05
$CaCl_2.2H_2O$	88.0
$CaHPO_4$	400.0
N/1 HCl	6 ml
Na_2EDTA	3.72
$FeSO_4$	2.785
$AlCl_3$	0.03
$NiCl_3.6H_2O$	0.03
Vitamins	
Inositol	100
Thiamine HCl	0.4
Pyridoxine HCl	0.3
Nicotinic acid	0.3
Glycine	2.0
Sucrose	20 g
Agar	9 g

Table 12. Effect of IAA and BAP individually and in combination on the formation of plbs and caulogenesis in the apical meristem

Medium* + Growth regulator	Conc. µM	% response	Nature of response		Remarks
			plbs	Caulogenesis	
Control	-	-	-	-	No morphogenetic response observed
IAA	0.5	15	-	+	The apical meristem differentiated into a solitary shoot bud
	2.5	10	-	+	1-2 shoot buds emerged from the meristem
	5.0	20	-	++	Few shoot buds appeared on the surface of the tissue
	10.0	50	+	+	Small green plbs and shoot buds appeared
BAP	0.5	30	-	+	Green protuberances, i.e. shoot buds appeared
	2.5	20	-	-	The tissue enlarged and remained green. No further development occurred.
	5.0	15	-	-	No growth noticed
	10.0	15	-	-	The tissue remained green
IAA + BAP	0.5+0.5	20	-	-	Tissue remained green
	0.5+2.5	60	+	+	Both plbs and shoot buds emerged from the meristematic tissue
	0.5+5.0	-	-	-	Tissue dried up
	0.5+10.0	-	-	-	Tissue dried up
	2.5+0.5	40	-	-	The distal end of the explant showed swellings
	2.5+2.5	30	-	++	The emerging shoot buds differentiated into complete plantlets
	2.5+5.0	20	-	+	Few shoot buds appeared from the tissues
	2.5+10.0	-	-	-	Tissue dried up
	5.0+0.5	50	+	-	Very few plbs appeared which turned brown
	5.0+2.5	60	-	+	Single shoot emerged, turned yellow subsequently
	5.0+5.0	40	-	+	Shoot buds differentiated but turned brown
	5.0+10.0	-	-	-	The explant dried up
	10.0+0.5	80	++	+	Numerous healthy plbs developed
	10.0+2.5	50	-	+	Poorly developed shoot buds emerged
	10.0+5.0	20	-	+	Solitary shoot developed
	10.0+10.0	-	-	-	The explants dried up

* Basal medium used; - no response; + moderate; ++ appreciable

Table 13. Effect of NAA and BAP individually and in combination on the formation of plbs and caulogenesis in the axillary buds

Medium* + Growth Regulator	Conc. µM	% response	Nature of response		Remarks
			plbs	Caulogenesis	
Control	-	50	-	+	Single solitary shoot developed from the axillary bud
NAA	0.5	30	-	+	The bud proliferated into a single shoot
	2.5	60	-	+	2-3 shoots developed
	5.0	70	-	++	The axillary bud enlarged into a swollen mass which developed into small shoots
	10.0	50	++	+	Small shoot buds and plbs formed
BAP	0.5	35	-	-	Bud development into a single shoot took place
	2.5	20	-	-	Signs of shoot initiation developed no further development
	5.0	-	-	-	The explant remained green. No growth noticed
	10.0	-	-	-	" "
NAA+BAP	0.5+0.5	30	-	+	2-3 shoots developed
	2.5+0.5	80	-	+++	A large number of small shoots emerged
	5.0+0.5	60	-	++	Multiple shoots developed
	10.0+0.5	40	+	+	Small shoots and plbs emerged out
	0.5+2.5	20	-	+	2-3 shoots developed
	2.5+2.5	30	-	+	"
	5.0+2.5	20	-	+	The developing shoots turned yellow subsequently
	10.0+2.5	20	-	+	Poor development of shoots
	0.5+5.0	40	-	+	Development of single shoot
	2.5+5.0	50	-	+	Development of single shoot
	5.0+5.0	40	-	+	Developing shoots turned brown
	10.0+5.0	-	-	-	The explant dried up
	0.5+10.0	10	-	+	Formation of a small and poor plant
	2.5+10.0	-	-	-	The explant dried up
	5.0+10.0	-	-	-	"
	10.0+10.0	-	-	-	"

* Basal medium used; - no response; + moderate; ++ appreciable; +++ good

clonal propagation of this dendrobe. The apical meristem enlarged and produced the protocorm mass in about 3-4 weeks time in the medium supplemented with IAA and BAP both separately and in combination (Table 12). IAA at 10.0 µM concentration resulted in the formation of plbs on the the apical meristem (Plate 15a). The maximum number of plbs were formed on the medium supplemented with 10.0 µM of IAA and 0.5 µM of BAP (Plate 15c). For caulogenesis from apical meristem a balanced supply of 2.5 µM each of auxin and cytokinin in combination was found to be effective (Plate 15b). The axillary buds responded better to the medium containing NAA and BAP. It took about 4 weeks for the formation of plbs or shoot buds from axillary buds. A large number of plbs formed in presence of NAA at 5.0 µM in the medium (Plate 16a). NAA (10.0 µM) and BAP (0.5 µM) stimulated the formation of plbs and small shoots (Table 13, Plate 16b). The plbs were multiplied by further cutting and subculturing on fresh medium. The formation of complete plantlets from apical meristems (Plate 17) and axillary buds (Plate 18) took place in about 8 weeks time on leaving the plbs undisturbed. The other combinations of NAA and BAP stimulated the differentiation of the plantlets from the axillary buds (Table 13). Other growth regulators and their combinations tried were found not suitable for plb formation and caulogenesis. The leaf tips dried up in about 2 weeks' time.

Plate 15. Development of plbs and shoot buds from the apical meristem :

(a) 10.0 µM IAA, (b) 2.5 µM IAA+2.5 µM BAP, (c) 10.0 µM IAA+0.5 µM BAP

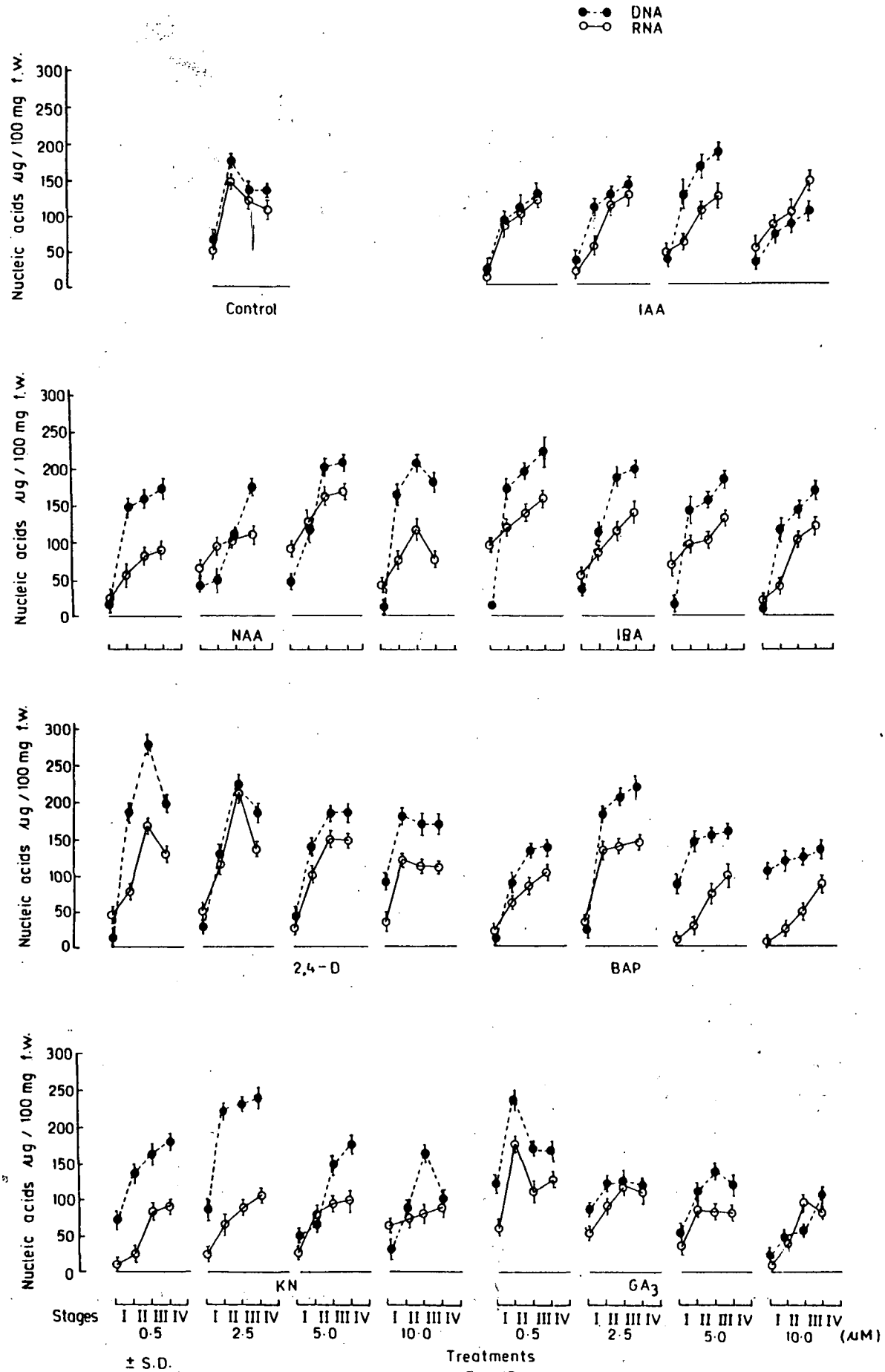

DNA
RNA
Nucleic acids µg / 100 mg f.w.
300
250
200
150
100
50
0
Control
IAA
NAA
IBA
2,4-D
BAP
KN
GA3
Stages
I II III IV
0·5
2·5
5·0
10·0
(µM)
± S.D.
Treatments

Fig. 15

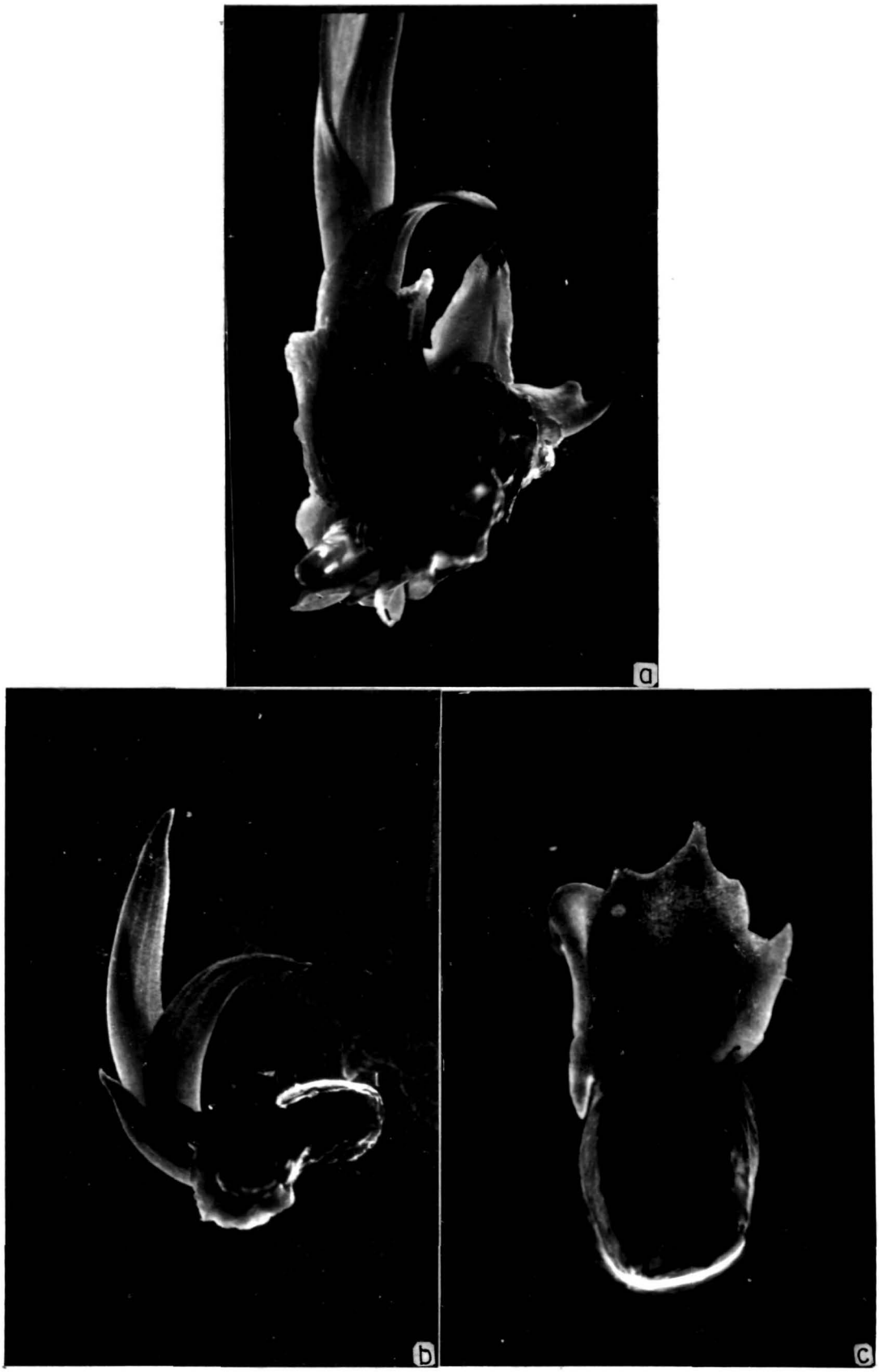


plate 15

1 mm

Plate 16. Development of plbs and shoot buds from the axillary bud :

(a) 5.0 µM NAA (plbs only), (b) 10.0 µM NAA+0.5 µM BAP

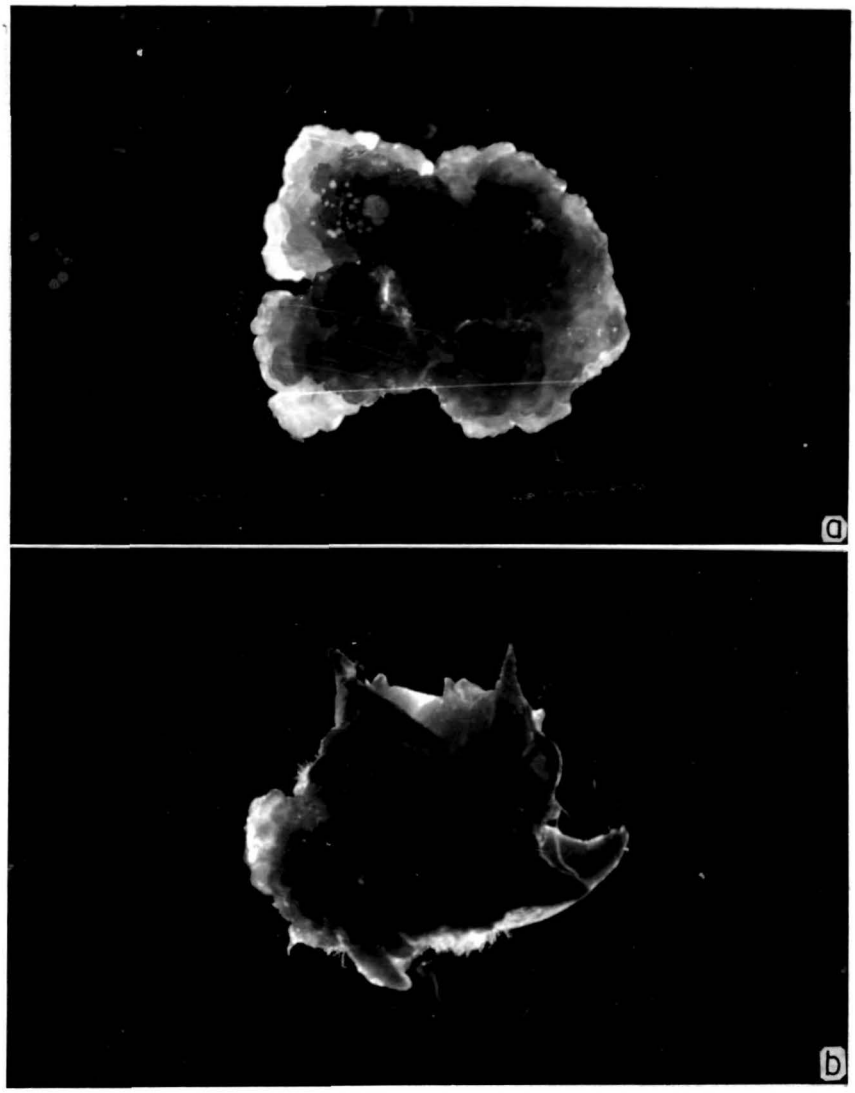


Plate 16

1mm

Plate 17. Development of complete plantlets from the apical meristem :

(a) Proliferating apical meristem
(b) Formation of plbs
(c) Formation of plbs and shoots
(d) Development of shoots
(e) Rooted plantlets
(f) Complete plantlets transferred to flasks

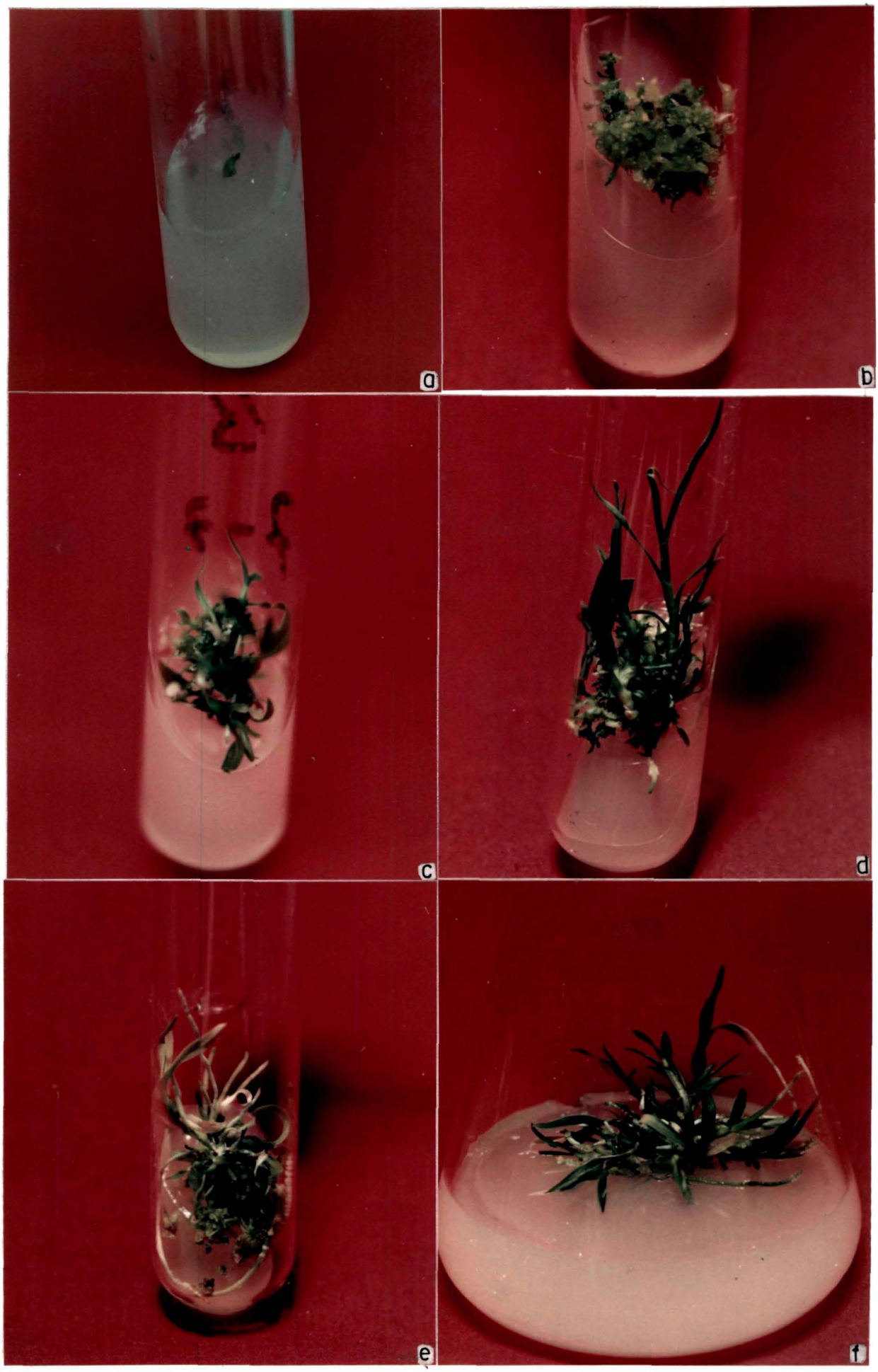


Plate 17

Plate 18. Development of complete plantlets from axillary bud :

(a) Proliferating axillary bud
(b) Formation of plbs
(c) Multiplication of plbs by cutting
(d) Formation of plbs by cutting
(e) Formation of roots
(f) Rooted plantlets in culture

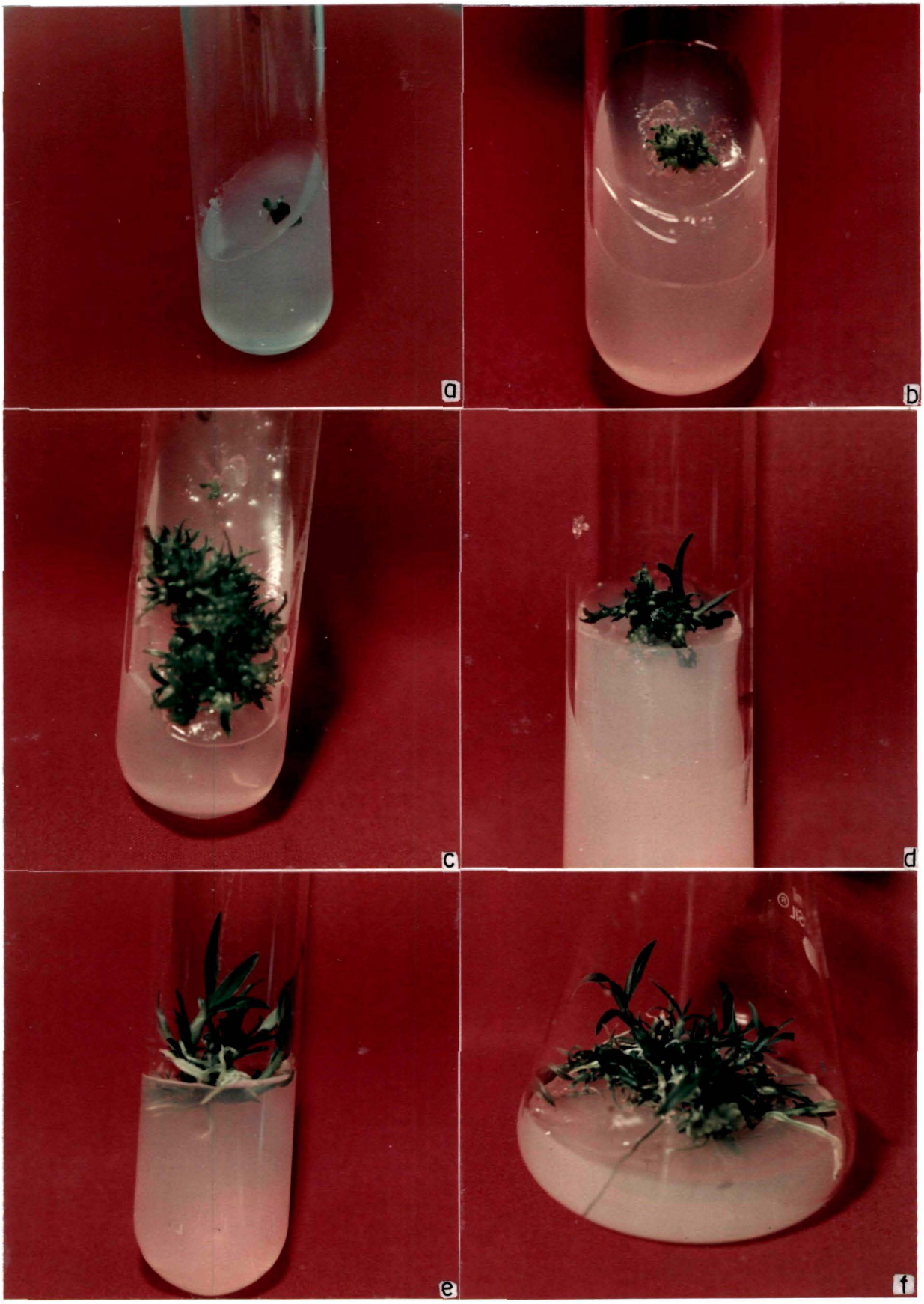


Plate 18

However, the leaf bases remained green for longer time period. The root tips also remained green for some time and showed slight swelling. These dried in course of time.

DISCUSSION

Tissue culture of orchids is a fast means of clonal propagation and can be employed as a research tool for the development of procedures in basic research. The causative factors controlling morphogenesis and regeneration from various plant parts are diverse. Nutritional requirements for the optimal growth of the plants *in vitro* vary from species to species. Arditti (1977) reported the variation in the media used for tissue culture of orchids and suggested that the composition of the medium changes with the same or different genera. Some media contain many components while others are simpler. The concentrations and chemical forms of the components vary considerably. Some media contain components of defined chemical nature (salts, vitamins, hormones, amino acids, organic acids, nucleotides, nucleic acids and chelating agents) while the others have complex, not entirely defined ones (peptone, tryptone, casein hydrolysate, yeast and/or potato extract, and green or ripe banana). Considerable variations are reported to exist between media used for the same or different genera of orchids (Morel, 1970, 1971; Mitra, 1971; Fonnesbech, 1972a,

1972b; Intuwong and Sagawa, 1973; Kako, 1973; Mosich *et al.*, 1973, 1974; Arditti, 1977). In the present study, a new medium was formulated which proved to be highly suitable for micropropagation of *D. fimbriatum*. This medium was composed of nutrient salts and vitamins and was purely synthetic. Although Heller (1953) reported that aluminium and nickel were not essential for tissue culture of plants, in the present case the chlorides of these elements were used and found useful. Increased concentration of chloride ions in the medium might have promoted the induction of plbs. The use of hydrochloric acid in the medium was additional and it maintained the required acidity of the medium.

The axillary buds and apical meristems were found to be the suitable explant sources for the formation of plbs. Clonal propagation of *Dendrobium* through shoot tips and nodes have been successfully accomplished in some other species (Sagawa and Shoji, 1967; Kim *et al.*, 1970; Mosich *et al.*, 1974). The other inoculated explants of *D. fimbriatum* failed to respond. Although Rao (1977) had reported tissue culture of *Dendrobium* through leaf and root explants, the media used were not purely synthetic. The successful propagation through these explants has also been reported in other orchid species (Tanaka *et al.*, 1975; Kerbauy, 1984a, 1984b; Kraus and Kerbauy, 1987; Sanchez, 1988; Yoneda and Momose, 1988; Kraus and Monteiro, 1989; Holters and Zimmer, 1990). The use of

growth regulators in the medium is beneficial and results in different morphogenetic responses. It has been reported that the tissues from different organs or/and parts differ in their growth-substance requirements. These differences are mostly marked in respect of the auxin requirements (Audus, 1972). In the present study, the combinations of IAA and BAP, and NAA and BAP resulted in the formation of plbs from apical meristems and axillary buds, respectively, which is consistent with the earlier reports (Vij *et al.*, 1984; Ponchet *et al.*, 1985; Sanchez, 1988). Fonnesbech (1972a) reported that in low concentration and in combination with auxin, BAP induced the formation of plbs and small shoots from the explants. Similar findings are reported in the present study where the addition of IAA and NAA at higher concentrations brought about the formation of plbs, both when added separately and in combination with BAP. The morphogenetic responses of the explants varied with the concentrations of the growth regulators used. The incorporation of NAA and BAP in addition to 10% coconut milk in the medium was found to increase the rate of plbs formation in *Phalaenopsis*, where internodal sections of the flower stalk were used as the explant source (Yoneda *et al.*, 1983).

The results of the present study have shown that the new nutrient medium with the addition of growth regulators is

suitable for the clonal micropropagation of *D. fimbriatum* through apical meristems and the axillary buds. The plbs and shoot buds developed into complete plantlets in eight weeks time when left undisturbed on the medium devoid of growth regulators.

VII. HARDENING OF THE CLONALLY PROPAGATED PLANTLETS, THEIR TRANSFER AND ESTABLISHMENT

INTRODUCTION

The transfer of the *in vitro* raised orchid seedlings from the culture vessels to the community pots requires a careful, step-wise procedure which can cause the hardening of the seedlings and hence lead to better survival when transplanted to the pots. The first and foremost requirement for successful transplantation is the maintenance of seedlings under very high humidity conditions (90-100%) for the first 10-15 days (Bhojwani and Razdan, 1983). Different types of pots and composts have been used. Glazed pots are not suitable as they do not allow sufficient aeration of the

roots and the compost. Mukherjee (1983) suggested the use of clay pots for many epiphytic orchids like *Cattleya, Epidendrum, Dendrobium,* etc. To facilitate drainage and aeration, the plastic pots are poked for small holes. According to Hegde (1984), four types of containers can be used for orchids, viz., (i) pots, (ii) baskets or cradles, (iii) wooden logs, and (iv) tree fern blocks. The use of wooden or bamboo basket or cradle for epiphytic orchids has been recommended. The use of plastic baskets or copper wire baskets as containers for orchids is also common. Different combinations of containers and compost have been tried for orchids culture (Rao *et al.*, 1979; Rao and Mohanan, 1983). Depending on whether the orchids are terrestrial or epiphytic, a number of composts are used. An ideal compost is the one which is inert, resistant to organic decomposition, porous to ensure adequate aeration for root respiration, less costly and easily available.

Meyer (1951) used fern fibres for the compost. Bark-based compost have been used for the cultivation of *Clowesia rosea, Dendrobium alexandrae* and *Lemboglossum cervantesii* (Stewart, 1988; Robbins and Bell, 1990; Cribb, 1990). Terrestrial orchids like *Cymbidium, Paphiopedilum, Phaius,* etc. are generally potted in a porous media containing loamy soil and adequate organic matter but the epiphytic orchids are held in position by using stake (Bose and Bhattacharjee,

1980). A majority of workers have used the mixture of equal parts of chopped tree-fern fibre, chopped sphagnum moss and crushed bark preparation.

Use of fertilizers proves to be beneficial for healthy growth of orchid seedlings. Addition of the nutrients to the compost varies with the composition of the potting materials and the type of orchids grown. In some cases small amounts of turkey grit or very fine vermiculite is also used in the compost.

Besides the container and the compost, suitable temperature plays an important role in successful transplantation of orchid plantlets. The best temperature range is 18.3°C to 29.4°C. Cribb (1990) reported 18°C and 23°C-25°C as the minimum and maximum temperatures respectively for better establishement of *Dendrobium alexandrae*. In case of *Phragmipedium hesseae* 17°C and 30°C were the required minimum and maximum suitable temperatures (Robbins, 1989). In this chapter, successful hardening of the plantlets of *D. fimbriatum*, their transfer and establishment are described.

MATERIALS AND METHODS

Complete plantlets were regenerated *in vitro* from the plbs derived either from the apical meristems or axillary buds of *D. fimbriatum* var. *oculatum* using the experimental protocol as described in chapter VI. Tiny plantlets

measuring 2.5-3.0 cm in height were used for the transplantation study. The plantlets were removed from the culture tubes/flasks by means of a long handled spoon along with a small amount of the adhering agar. The agar medium sticking to the roots was removed with a brush and care was taken to avoid damage. The plantlets were then transferred to clean clay pots of 10 cm diameter containing different mixtures of compost viz., (i) charcoal chunks and brick pieces (1:1); (ii) charcoal chunks, brick pieces and coconut husks (1:1:1/2); (iii) charcoal chunks, brick pieces (1:1), and a layer of moss; (iv) charcoal chunks, brick pieces, vermiculite (1:1:1/2) and a layer of moss, and (v) charcoal chunks and a layer of moss.

To minimise the spread of disease, pots and crocks were thoroughly washed, soaked in tap water for several hours and then filled with tightly packed composts. The pots were filled 3/4th with compost and watered as planting in the moistened compost is easier. The washed plantlets were picked up with the help of forceps and the roots were carefully placed into the crevices of the compost. About 3-4 plants were potted in each of the pots. In certain cases as described above, a layer of moss was laid on the compost after the planting. The pots were carefully sprayed with water and shifted to the glass-house. The minimum and maximum temperatures of the glass-house at the time of

transplantation were 16-18°C and 20-22°C, respectively. The relative humidity of the glass-house was around 70-80%. The plantlets were watered daily in the morning and fed with MS nutrient salt solution (diluted 10 times) fortnightly. The potted plantlets were kept both in shade and direct sunlight in the glass-house.

RESULTS

Of the various composts used, the combination of charcoal chunks, brick pieces, vermiculite and a layer of moss was found to be the best substratum for the survival and healthy growth of the plantlets (Table 14). The compost having brick pieces, charcoal chunks and vermiculite was also a suitable substratum. The compost comprising charcoal and moss did not support good growth and survival of the plantlets. Without vermiculite, the compost of charcoal, brick and moss layer supported good survival percentage and growth of the transferred plantlets. The substrates comprising charcoal and brick pieces, and charcoal, brick pieces and coconut husks were not suitable for transplantion. Feeding the plantlets with diluted MS nutrient salt solution fortnightly proved to be beneficial for the healthy growth. In 6-7 weeks time the plantlets were hardened and established in the pots (Plate 9a,b). Keeping the plantlets in shade improved their survivability.

Table 14. The response of *in vitro* grown plantlets to different potting media

	Substrate used	% survival	Growth
i)	Charcoal + brick pieces	10	-
ii)	Charcoal + brick + coconut husks	15	-
iii)	Charcoal + brick + moss layer	60	+++
iv)	Charcoal + brick + vermiculite + moss	70	+++
v)	Charcoal + brick + vermiculite	60	++
vi)	Charcoal + moss	45	+

- poor growth; + fair growth; ++ good growth; +++ best growth

Plate 19. Transferred plantlets growing in the glass-house

(a) with a layer of moss, (b) without moss

a

Plate 19

b

DISCUSSION

The transfer of plantlets from the culture vessels to the glass-house conditions requires a careful, step-wise procedure. The successful transplantation depends on the suitable size and growth of seedlings and the compost used. Healthy seedlings showing vigorous growth in the culture vessels were transferred to the pots. It is a well established fact that the hardiest and vigorous seedlings are easier to transplant and these are less susceptible to diseases and mechanical injuries. The transferred plantlets had a healthy and vigorously growing root system which ensured higher establishment and growth. Charcoal chunks, brick pieces, vermiculite and a layer of moss formed the best substratum for the growth of the seedlings as it may have facilitated proper drainage and aeration for root respiration. The layer of moss on top proved to be beneficial due to higher retention of moisture content. Partial defoliation of the plantlets at the time of transplantation is reported to be beneficial in certain cases (Bhojwani, 1980; Tisserat, 1981). However, in the present study there was no need to remove the leaves as they were few in number. Direct sunlight was harmful to the transferred plantlets which may be due to increase in temperature at the leaf surface. On the other hand, pots kept under shade showed high survivability of the plantlets. The plantlets were

hardened in 6-7 weeks of transferring them in the pots. Feeding the plantlets with diluted MS nutrient salt solution was found to be beneficial as it supplied the essential nutrients to the developing plantlets. The promotion of orchid seedling growth by the nutrient solutions has been reported in many instances (Sheehan, 1960; Sander, 1969, 1979; Mukherjee, 1983). The procedure described for the hardening and transplanting of the plantlets of *D. fimbriatum* var. *oculatum* results in 70% survivability and healthy growth of the plantlets. The compost used is easily available, porous and convenient for the transfer and establishment of the *in vitro* grown plantlets.

VIII. SUMMARY

Different media viz. MS, Nitsch, Mitra *et al.*, Vacin and Went, and White were tried for asymbiotic seed germination and protocorm development of *Dendrobium fimbriatum* var. *oculatum*. Nitsch medium was found to be the best for seed germination followed by MS and Mitra *et al.* The seeds were considered to have germinated on the emergence of embryo from the testa. The protocorms produced on MS medium were, however, quite large compared to those developed on other media. To quantify the protocorm growth, the volume of the developing protocorms was determined using the formula given by Stoutamire (1981) for an oblete spheroid. The

protocorms developed on Vacin and Went medium were quite large although the germination percentage of the seeds was quite low on this medium. Development of the protocorms was observed within the first 4-5 weeks on MS, Nitsch and Vacin and Went media whereas it was delayed on other media tried.

The effects of physical factors like temperature, light and pH of the medium were studied on seed germination and seedling growth. For assessing seedling growth, shoot length, leaf area and number, root length and number, and dry weights were measured at 30-day intervals for 4 months. From the primary data, NAR and LAR were calculated to find out the optimal conditions of growth. The best seed germination and seedling growth were recorded at 25°C. The optimal photoperiods for seed germination and seedling development were 12 h and 16 h, respectively. Although 1500 lux light intensity was found optimum for seed germination, the seedling growth was best at 3000 lux light intensity. The pH of the medium 5.5 was most suitable for seed germination and seedling growth.

Asymbiotic seed germination and seedling growth were also greatly affected by the incorporation of growth regulators viz. IAA, NAA, IBA, 2,4-D, BAP, KN and GA_3 singly and in combination at 0.5 uM of IAA in the medium as compared to the control. The germination response of the seeds on media containing other auxins was, however, poor. The

seedling growth was markedly higher at 2.5 μM of IAA in the medium. The root number was quite high at 5.0 μM of IAA as compared to other treatments. The growth of seedlings was also promoted at 5.0 μM of NAA in conjunction with both 2.5 and 5.0 μM of IBA in the medium. 2,4-D incorporation in the medium inhibited the seedling growth. Of the various cytokinins tried, BAP at 0.5 μM was found to be the best for seed germination. On the other hand, KN was inhibitory for seed germination. The seedling growth was slightly enhanced at both 5.0 uM of BAP and KN incorporated separately in the medium. The exogenous supply of GA_3 was inhibitory for seed germination. But the seedling growth was markedly enhanced at 5.0 μM of GA_3 in the medium. The combinations of both auxins and cytokinins enhanced seed germination. Seed germination was greatly enhanced at 0.5 μM IAA + 0.5 μM BAP and 0.5 μM NAA and 0.5 μM BAP in the medium. The combinations of auxins and cytokinins which promoted seedling growth were 0.5 μM IAA+5.0 μM BAP and 0.5 μM NAA+0.5 μM KN.

Biochemical studies at four different developmental stages of protocorm, as influenced by growth regulators, were carried out. The effects of growth regulators on the activities of some oxidative enzymes viz. peroxidase, polyphenol oxidase, IAA-oxidase; tissue concentration of soluble proteins; total and O-dihydroxy phenolic contents; and the contents of nucleic acids, viz. DNA and RNA were

recorded in developing protocorms. As compared to the control, the activities of both peroxidase and polyphenol oxidase increased on fresh weight basis in most of the treated tissues. The peroxidase activity did not differ much between different treatments except for IAA-treated protocorms where an increase was recorded at 2.5 µM and 5.0 µM concentrations. The polyphenol oxidase activity increased with growth in protocorms treated with auxins. However, higher concentrations (5.0 µM and 10.0 µM) were found to be inhibitory. A slight increase in IAA-oxidase activity was recorded in case of protocorms treated by IAA, NAA and GA_3. The soluble protein content increased drastically in treated protocorms. Conversely, it declined with growth in the control. The auxins stimulated the accumulation of soluble protein. However, higher concentrations of IBA and 2,4-D were inhibitory. The cytokinins, BAP and KN, enhanced protein synthesis but the higher concentrations of the former were inhibitory. The electrophoretic profiles of proteins and peroxidases did not show any change in the number of the bands as the result of growth-regulator treatments. Irrespective of the treatments, four bands of peroxidase isozymes were observed. These bands differed in their Rm, width and intensity of colour. Similarly, seven bands of proteins were observed which differed in the intensity of colour and Rm values. The total

phenolic contents increased with protocorm development at different concentrations of auxins and BAP in the medium. Inhibition of growth as a result of higher phenolic contents was observed in case of 2,4-D and BAP (0.5 µM) treated protocorms. RNA and DNA synthesis were stimulated by incorporation of auxins in the medium. However, in protocorms treated with 2,4-D (all concentrations used) and 10.0 µM of NAA, both the RNA and DNA contents decreased as growth of the protocorms proceeded. The RNA content increased in protocorms treated with BAP and KN. GA_3 treatment was found to be slightly inhibitory for both RNA and DNA contents during protocorm development. DNA content was either reduced or remained constant as a result of treatments by the time the 2-leaf stage of the protocorms was attained. These studies show that the growth regulators had a pronounced effect on the growth and physiology of the developing protocorms.

Clonal micropropagation of *D. fimbriatum* was carried out keeping in mind the mass propagation of identical genotypes. A new medium was formulated for the micropropagation of this dendrobe. The medium used was composed of nutrient salts and vitamins and was purely synthetic. Out of the different explant sources tried, axillary buds and apical meristems were found suitable for the formation of plbs and shoot buds. The maximum number of

plbs were formed on the medium supplemented with 10.0 µM IAA and 0.5 µM BAP in case of apical meristem. On the other hand, a large number of plbs were formed from axillary buds in presence of NAA at 5.0 µM concentration. NAA (10.0 µM) in combination with BAP (0.5 µM) stimulated the formation of plbs and small shoots. The plbs were multiplied by further cutting and subculturing on fresh medium. The formation of complete plantlets from apical meristems and axillary buds took place in about 8 weeks time.

Tiny plantlets measuring about 2.5-3.0 cm in height were transferred from the *in vitro* grown cultures to the glass-house conditions. The plantlets were hardened in 6-7 weeks. The mixture of charcoal chunks, brick pieces, vermiculite and a layer of moss on top formed the best substratum for the growth of the plantlets. This easily available and porous compost resulted in 70% survivability of the plantlets in the glasshouse. Direct sunlight was harmful to the transferred plantlets. Feeding the plantlets with MS nutrient salt solution (1/10 strength) was found beneficial for healthy growth of the plants.

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*Original not seen.