SEED GERMINATION AND TISSUE CULTURE STUDIES IN ORCHIDS

S. RAMACHANDRAN NAIR

DIVISION OF HORTICULTURE
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SEED GERMINATION AND TISSUE CULTURE STUDIES IN ORCHIDS

S. RAMACHANDRAN NAIR

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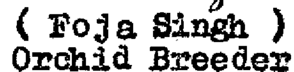
CERTIFICATE

This is to certify that the thesis entitled " SEED GERMINATION AND TISSUE CULTURE STUDIES IN ORCHIDS" submitted by S. RAMACHANDRAN NAIR for the degree of DOCTOR OF PHILOSOPHY IN HORTICULTURE of the University of Agricultural Sciences, Bangalore, is a bonafide research work carried out by him during the period of his study in the University under my guidance and supervision, and the thesis has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles.



BANGALORE

September 30th, 1982.

(Foja Singh)
Orchid Breeder
Indian Institute of Horticultural Research, Bangalore- 560 080

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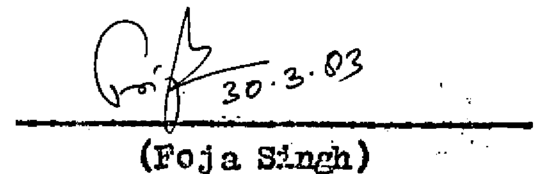

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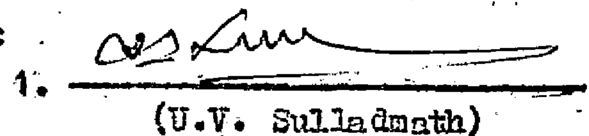
1. 

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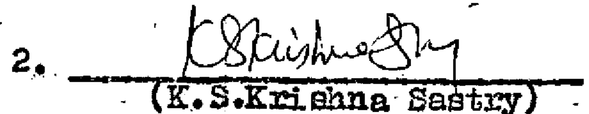
2. 

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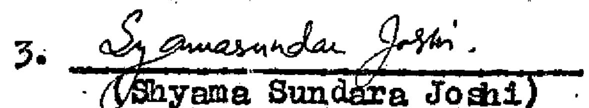
3. 

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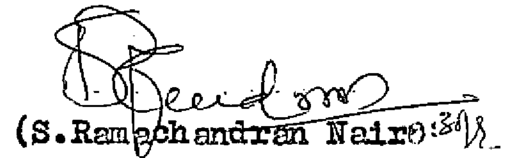

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INTRODUCTION

CHAPTER I

INTRODUCTION

The Orchids belong to one of the largest families of flowering plants (Orchidaceae) comprising of more than 30,000 species and exhibiting an incredible range of diversity in size, shape and colour of flowers.

The oldest record on orchids comes from China. However, anicient Greeks were the first to take note of these strange plants. Theophrastus gave the name orchids to these plants in 370-285 B.C. Indian Vedic scriptures also list orchids as "Vanda" which also include all the other known epiphytes.

Though orchids are found everywhere, majority of the cultivated orchids are native to tropical countries and occur in their greatest diversity in humid tropical forests of South and Central America, India, Ceylon, Burma, China, Thailand, Malysia and Brazil. The Indian _Dendrobium_ and _Cymbidiums_, Mexican _Laelias_ and Brazalian _Cattleyas_ have contributed a lot in the development of present day hybrids (more than 30,000). India has about 1600 species scattered in Himalayas and Western ghats.

The smallest orchid _Eria pusilla_ is hardly one centimeter while the largest _Galeola falconeri_ is 2.5 to 3 meters. The plants are having wide variations in growth and other characteristics. This necessitates numerous adaptive characteristics. Growth habits, nature of growth, form, colour

and shape which are all the results of adaptations. Some orchids contain no chlorophyll, and are saprophytic in nature (*Epipigon aphylla* and *Coralorizha innata*).

In the evolutionary history, orchids represent the most highly evolved group among monocotyledons as proposed by Brown (1931) and also by Darwin (1877). These plants were probably derived from primitive plants whose flowers resembled the lily.

The common method of propagation of orchids is by division, the monopodial orchids however are difficult to propagate by vegetative means. They have single growing tip and do not produce pseudobulb or keikis. Large sections with roots are also used for propagation.

The orchid seed germination is the most interesting adaptive feature. The process of seed germination was noted quite accidentally by Bernard in 1899. He found that orchid seeds germinate only when they are infected by some fungus. Subsequent observations showed that tropical epiphytic orchids also require infection by a fungus for germination (Mc Dougal, 1899). Immediately further investigations were made to study the effect of fungus on orchid seeds, seedlings and mature plants (Burgeff, 1909). A group of fungi were initially found responsible for seed germination and they were named as Orcheomyces (Burgeff, 1909). Later it was found that many more fungi were responsible for seed germination, which include

Rhizoctonia, *Corticium*, *Armillaria*, *Fomes*, *Phytophthora*, *Pencillium*, *Aspergillus*, *Trichoderma* and others. These fungi were chiefly responsible for breaking down complex starch into simple sugars for the germinating seeds.

The work of Knudson, (1922) showed, for the first time that germination of orchid seeds would be possible in vitro without the association of fungi. Subsequently a number of media were developed by many investigators. To date atleast 25 different media are known and are used for the orchid seed germination. Modification on a particular recipe is also proposed for a given genus or species and improverised by the addition of various growth adjuncts to the medium such as different micro elements, growth hormones, coconut milk, tomato juice, banana extract, fruit juices and such others.

Morel in 1960 first reported that it is possible to culture shoot apices of orchids to get virus - free plants. The technique has been further improved to produce hundreds of plants from a single bud. Presently this technique is being widely applied to produce selected clones of *Cymbidium*, *Cattleya*, *Dendrobium* and *Vanda* for the flower trade. The polyploid forms of *Cymbidium*, *Cattleya* and *Phalaenopsis* are also multiplied by the meristem culture technique.

The tissue culture techniques enables the growers to produce large number of plantlets which are genotypically and phenotypically similar to the mother plant.

The development of embryo in many Angiosperms has been described morphologically but very little work has been done on Orchids especially regarding the histochemical aspects of seed development. There is very little information about the chemical changes which take place in the embryo and tissue development. An investigation to these changes will serve to enhance our present understanding of the biochemical relationship existing in seeds and tissues during morphogenesis.

Karyotypic instability is a most common phenomenon in plant tissue culture, the general observation is that the chromosome number in cell cultures is not stable but is subject to variation usually through the process of endoreduplication and nuclear fusion (D'Amato, 1975). Variation in chromosome number can also be due to spindle abnormalities and chromosome fragmentation.

It is a well known fact that plant cell cultures generates wide genetic variability which can be put to different uses in plant improvement.

Considering all these aspects the present study was undertaken with the following objectives.

1. To standardise various nutrient media for the germination of selected species of orchids including Indian species.
2. To determine the effect of different growth regulators and adjuncts along with the media in the germination of orchid seeds.

3. To standardise rapid multiplication of selected orchid species through tissue culture using vegetative parts such as leaf,shoot,root,flower stalk,offshoots or keikis.
4. To standardise the media for callus formation and differentiation of tissues.
5. To study the effects of different adjuncts individually and in combination on tissue culture of orchids.
6. To study the cytological variations and histochemical changes in the cells of the tissues raised through tissue cultures.

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

2.1 Origin and history

Theophrastus (370- 285 B.C) who is often called father of botany was first to give the name "Orchids" from the Greek word "Orchis", on the basis of the resemblence of paired underground tubers of these plants to the testicles. This was finally retained and adopted by Linnaeus in his species Plantarum (1836). Indian Vedic scriptures also mention about these plants under the name "Vanda", the Sanskrit name for epiphytic plants. The name has now been adopted as a generic name of one of the most beautiful monopodial group of orchids.

The orchid plant with its wide variations in growth, flowering, seed production and germination has got many adaptive characteristics. The most suitable example for the adaptive feature is the physiology of orchid seed germination. The first published description of an orchid seed is by Theophrastus (Salisbury, 1804). It was later found that the seeds germinated in the natural condition only when infected by a fungus (Mycorrhizal association) (Bernard, 1899). However, Knudson (1922) could germinate orchid seeds in an artificial medium. A detailed review of work on orchid seed germination was compiled by Arditti (1967) and later in 1977.

Some of the recent Indian publications on Orchids also deal with their culture. (Bose and Bhattacharjee, 1980 and Abraham and Vatsala, 1981).

2.2 Seed germination

The seed germination in orchid is a complex process. A single capsule produces several millions of seeds, however the percentage of germination and number of plants developed are very low due to lack of any functional endosperm.

In orchids, the germination and development of a seedling is not like in any other angiosperm. The rudimentary embryo enclosed in the seed coat develops like a dormant bud. In the process of development, the seed may or may not develop chlorophyll, but it swells in size and burst out of the seed coat. A cone shaped spherical seedling is formed and this is called protocorm stage (Bernard, 1909). The first leaf primordia is formed as a bulge and the protocorm increase in size and subsequently rhizoids, leaf primordia are formed (Arditti and Bills, 1965).

The process of germination proceeds symbiotically in nature, with the association of some root fungus and asymbiotically in aseptic conditions. The significance of fungus and its importance was well established by Bernard (1899). He found that the fungal infection was necessary for germination. Some of the fungi isolated from orchid roots includes Rhizoctonia (Duperrex, 1961), Corticium;

(Downie, 1957), Armillaria; (Burgeff, 1959; Campbell, 1962). Other fungus isolated were Phytophthora, Pencillium, Aspergillus and Trichoderma (Curtis, 1939)

2.2.1 Nature of seed

Orchid seeds are very minute weighing 0.3 to 14 μ_g (Harley, 1951) and measure from 0.25 to 1.2 mm in length (Hoene, 1949) and 0.090 to 0.270 mm in width (Arditti, 1967). Each capsule produces 1300 to 4,000,000 seeds (Arditti, 1961).

Two major groups of orchid seeds are usually distinguished. One group has relatively differentiated embryos, including rudimentary cotyledons as in Bletilla hyacinthina. (Harley, 1951). However majority of the species have relatively undifferentiated embryos and no endosperm (Maheshwari and Narayana Swami, 1952).

2.2.2 Asymbiotic germination

Knudson (1921) demonstrated that orchid seeds germinate freely on a medium containing sugar, mineral nutrionts and agar and reported that no fungus is needed for germination, this revolutionised the basic approach of orchid cultivation and started a new era of asymbiotie seed germination. Now asymbiotio method of orchid seed germination is widely in commercial orchid growing.

2.2.3 Media for seed germination

Consequently to the development of an artificial media by Knudson (1922), attempts were made to develop different media suitable for different species.

A comparative study of 2 media (Burgeff, N_3F and Thomale GD) for the germination of <u>Cypripedium</u> seed revealed that better percentage of germination and subsequent shoot and root growth and colour were superior on Burgeff N_3F medium. But <u>Cattleya</u> seeds germinated and developed more rapidly on Knudson's C. medium (Boesman, 1962 a).

The type of media selected for seed germination are mostly solid media with agar at varying concentrations. But liquid cultures are also used. Seeds of 5 <u>Paphiopedilum</u> hybrids were sown on liquid nutrient medium based on two substrates (Burgeff N_3F and Thomale GD) with supplements. All the 5 hybrids behaved differently, both in their germination rate and growth. In general Thomale GD gave the best results (Flamee, 1978).

2.2.4 <u>The components of various media</u>

Sugar is one of the very important components in the medium. A variety of sugars have been tested to study the preference in seed germination and growth. However, when chemically pure sugar was used, there was no germination (Noggle and Wynd, 1943). In an experiment various carbon sources such as mono and disaccharides, sugars, alcohols and deoxy sugars were compared for orchid seed germination. All the laevo-series of sugars tested, failed to support

seedling growth. Dextroxylose gave satisfactory results, d-ribose supported only marginal growth. All d-hexose sugars except d-galactose proved suitable for germination, d-fructose being the outstanding. The di and trisaccharides tested were not satisfactory (Ernst, 1967 b). Nitrogen is taken up in the inorganic form. Different forms of nitrogen source will have different effect on growth. Organic nitrogen sources such as proteins, peptones, aminoacids, urea and others are superior to inorganic ones. Cypripedium seedlings growing on ammonium nitrate as nitrogen source had better root development and dark green foliage. (Boesman, 1962 b). For Arundina bambusaefolia seedlings ammonium nitrate was the most suitable nitrogen source (Mitra, 1971). While Cattleya seedlings grown on media with NH4, No3, NH_4^{+-}, $No3^{+}$ nitrogen or urea at concentrations between 50 and 800 ppm showed best results with NH_4^{+} and $No3^{+}$ in proportions 2:8 or 3:7 (Uesato, 1973).

The anion cation concentrations in the nutrient media was studied at various concentrations, at 20 mg equivalent per litre. The optimum ranges were 16 to 20 per cent NH4+, 35 to 41 per cent K^{+}, 34 to 37 per cent Ca^{++}, 10 per cent Mg^{++}, 66 to 88 per cent $No3^{-}$, 7 to 23 per cent H2PO4 and 4 to 14 per cent $So4^{--}$. Ammonium ions were not required for germination of Bletilla striata seeds, but they improved seedling growth (Ichihashi and Yamashita, 1977).

Experiments were conducted to find out a suitable iron source. The addition of EDTA to the basal Knudson C medium resulted in healthier and greener seedlings (Miyasaki and Nagamatsu, 1965).

2.2.5 <u>Containers</u>

Selection of suitable containers makes orchid tissue culture successful. Increasing the size (volume) of the container, as the seedlings grow in size is very essential. Containers with poor quality glass gives off alkali to the medium. Plugging the culture flaks also influences the growth sometimes. However, the air tightness of the container had little effect on seed germination and plantlet formation. The size of the container had no effect on percentage of germination but plantlet growth was better in large containers (Hasegawa *et al*. 1928). Seedling growth was much better in flask covered with cotton plugs (Miyazaki and Nagamatsu, 1965).

2.2.6 <u>Culture conditions</u>

<u>pH of the medium</u>: The level of pH in the medium greatly influence the growth of the seedlings. The pH may be critical only during the early stages of germination and the seedlings are less sensitive to difference of pH (Knudson, 1951). Comparative studies on the effect of pH were made; (Kotomori and Murashige, 1965). The pH requirement for orchid seed

germination and growth varies depending upon the species. Cymbidiums can grow at pH ranging from 3 to 6, Dendrobium 4.5 to 5.4 and Epidendrum 4.8 to 5.2

Temperature and light requirements

Orchid seeds germinate best at 20 to 25°C (Arditti, 1967) the requirement of light for orchid seed germination varies depending upon the species. Seedlings of Cattleya, Epidendrum and Oncidium vary greately in their ability to germinate in the dark (Yates and Curtis, 1949). Cymbidium seeds can germinate in dark (Kohl, 1962).

Oncidium seedlings require no light for shoot and root development. Seedlings grown in dark appear normal but growth is more in the light (Yates and Curtis, 1949). Similar results were also reported earlier by Burgeff (1936) for other species.

Unripe seeds of Cattleya aurantiaca germinated and produced protocorms and normal plants on Knudson C medium at 25°C under continuous illumination. Germination was inhibited in darkness (Pierik and Steegmans, 1972).

In general, most orchid seedlings can grow under natural light and photoperiods with occasional supplementary illumination.

2.2.7 Seed storage

The longeivity of the orchid seed is variable, some may loose their viability in few months (Brummit, 1962)

or less (Lindquist, 1965) and others may remain for longer periods (upto 19 years) if allowed to dry and stored in a dessicator and refrigerated (Kano, 1965). However at room temperature most of the orchid seeds loose their viability in a short time (Humphrey, 1960; Davidson, 1964). Quick freezing and storage of seeds in the frozen condition for a long period is also possible (Fehlandt, 1960).

2.2.8 Seed sterilization

The orchid seeds are usually cultured in completely aseptic condition. Hence the seeds are to be sterilized before inoculation into the medium. Orchid seeds can resist chemical treatments for sterilization (Redlinger, 1961, Jordan, 1965), they withstand upto a concentration of 6 per cent hydrogen peroxide for 10 minutes (Breedy, 1953), 1:32 clorox solution upto 15 minutes (Liddel, 1948); Calcium hypochlorite 5 per cent upto 48 hours (Wilson, 1915); 10 per cent Potash for 36 hours (Boriquet and Boiteu, 1937); mercuric bichloride solution (1:2500) for short periods (Willoughby, 1950); and 10 minutes in toluene followed by 30 minutes in 90 per cent alcohol and 30 minutes in 50 per cent calcium hypochlorite (Withner, 1955). Sterilization by radiation is also reported. The seeds can withstand x-ray radiation upto 2400 r without loosing their viability percentage (Kano, 1965).

Seeds of *Vanda* "Miss Jaoquim" treated with 5 per cent clorox for 10 minutes and washed with sterile water and

inoculated on a culture medium produced seedlings in 10 to 12 weeks. Whereas seeds without such treatment did not germinate. Seeds directly transferred to the medium without exposure to outside, germinated well and produced strong seedlings within 8 to 10 weeks. (Rao and Avadhani, 1963). Bletilla capsules sterilized with alcohol and inoculated directly into the medium produced 100% germination and vigorous seedlings (Luks and Shevchenko, 1977).

2.2.9 Effect of growth regulators on seed germination

Orchid seed germination and seedling growth is to a great extent influenced by the growth regulators. Auxins, Gibberellins and Cytokinins plays an important role in seed germination and growth. The result of studies with auxin showed that only traces of auxin have been found in Cypripedium seeds and none at all in Calanthe and Dendrobium seeds (Poddubnaya - Arnoldi, 1960, Poddubnaya, et al, 1961). The noticeable differences in growth and development of both control and treated plants were observed as the plant developed and grew older. This may be possible due to no hormone produced during the early stages of germination and growth but, production is initiated and increased as the seedlings grew older and leaves and roots are produced (Arditti, 1965).

Root development of Cypripedium seedlings on Burgeff N_3F medium were stimulated by the addition of NAA at 1.5 mg/l (Boesman, 1962 b).

In media containing NAA, the roots were healthy and thick, within 15 to 18 days after transfer, shoot growth was less marked. (Bose and Mukherjee, 1974).

Better protocorm proliferation was obtained in 3 interspecific *Vanda* hybrids, when NAA (1ppm) was used along with other additives (Mathews and Rao, 1980).

Experiments with IAA did not give any satisfactory results for orchid seed germination. IAA impeded germination and caused elongation of the protocorms (Hadley and Harvis, 1960). Addition of IAA completely inhibited the seed germination in *Miltonia* and *Odontoglossum* (Hayes, 1969). The seedlings of *Zeuxine strateumatica* remained green coloured when cultured on Knudson's C. medium with IAA and kinetin (1 ppm each) (Arekal and Karanth, 1978). The germination and growth of *Dendrobium nobile* seeds was inhibited at 0.1, 1 and 10 ppm IAA (Miyazaki and Nagamatsu, 1965).

Use of GA in orchid seed germination has given promising results. GA at 1, 10 and 100 ppm induced rapid germination and growth of *Dendrobium* seeds. Highest concentration however, produced toxic symptoms. (Miyazaki and Nagamatsu, 1965). GA enhanced protocorm survival and caused elongation of the emerging shoot, but did not affect the growth and overall size of the protocorms. (Hadley and Harvais, 1968). *[illegible] [illegible]* seedlings cultured on Knudson's C. medium were transferred to same medium but with GA_3, IAA

NAA (1 or 2mg/l) or 2,4-D (0.1 or 0.5 mg/l). GA at both these concentrations caused marked leaf elongation. Plants treated with 2 mg/l produced few roots compared to lower concentrations. All four compounds stimulated protocorm formation and 2,4-D being most effective (Bose and Mukherjee, 1976).

2.2.10 Effect of Vitamins

The effect of vitamins on seed germination has been studied extensively by many workers. It appears that specific vitamins are required for some species. But a general statement of vitamin requirement cannot be made except for niacin, where a growth enhancing effect has been reported (Arditti, 1965). In an experiment with different vitamins like ninacin, adenine, ribose and coenzymes NAD and NADP alone and in combinations have shown that niacin alone or in combination with other additives were capable of enhancing growth in seedlings. NAD and NADP (concentrations 8 and 12 micromoles/1) inhibited germination,growth and development (Arditti,1966 a). Germination of Cattleya seeds were enhanced upto 80 per cent in Knudson's C. medium with niacin, adenine and ribose along with other additives. Seeds cultured on media with biotin produced better chlorophyll than other treatments. The root development was also better with biotin treatment (Lucke, 1971).

In general vitamins have been shown to promote growth of plant tissues and organs in vitro (Bonner, 1937, Schopfer, 1943).

2.2.11 <u>The effect of other complex additives</u>

<u>Coconut water and fruit juices</u>

Coconut water in the media influences the germination and growth of orchid seedlings to a very great extent. <u>Cattleya</u> seed germination was enhanced (80 per cent) on a medium of Knudson C with additives such as coconut milk, banana pulp, tomato juice and vitamins (Lawrence and Arditti, 1964).

The addition of 15 per cent coconut water to the Knudson's C medium increased growth of both epiphytic and terrestrial species, particularly tuber growth in terrestrials and root growth in epiphytes. (Mc Intyre <u>et al</u>. 1974). The seeds of <u>Spathoglottis plicata</u> grown on modified whites agar medium with 2 per cent sucrose had germination rates of 60 per cent and 80 per cent when the medium was supplemented with 10 per cent coconut milk and 1000 ppm casein hydrolysate respectively (Chennaveeraiah and Patil, 1975).

The seed germination and seedling growth of five different genera of orchids (<u>Cattleya</u>, <u>Cymbidium</u>, <u>Paphiopedilum</u>, <u>Phalaenopsis</u> and <u>Eulophidium</u>) on Knudson C medium supplemented with chelated iron, micro elements, coconut milk, banana and charcoal was very satisfactory (Rose <u>et al</u>. 1977).

The effect of banana pulp on seed germination has been reviewed by Anderson (1967). He found that addition of banana pulp to Knudson C medium stimulated the growth of Cattleya seedlings. Further studies indicated that addition of banana pulp increased the number and growth of roots (Arditti, 1968). A comparative effect of additives such as banana pulp, coconut water, tomato juice was studied on the germination of Dendrobium seed on nine different media. After 9 1/2 months of growth, the dry weight of the seedlings were more with a treatment of banana homogenate (150 g in 50 ml distilled water/ liter of medium). The treatment also induced better vegetative growth (Mowe, 1973).

Tomato juice was originally described as an excellent culture medium for orchids (Vacin and Went 1949). But recent investigations have shown that it is inhibitory unless used in very low concentrations (Kano, 1965, Arditti, 1966 b). Seeds of Vanda "Miss Joaquim" when transferred directly from the pod on to a medium containing tomato juice or yeast extract, germinated forming strong seedlings within 8 to 10 weeks (Rao and Avadhani, 1963). Whole tomato juice, reconstituted juice of a mixture of all juice fractions were slightly detrimental to Cattleya seedlings (Arditti, 1966). In Phalaenopsis seed culture Knudson's C medium modified with addition of ripe banana, pineapple, papaya, fig, tomato, raspberry, grape, chinese gooseberry, mushroom,

coconut milk, autolysed fish, tryptone and peptone, accelerated seedling growth and development to a marked degree (Ernst, 1967 a).

2.3 Tissue culture

Plant tissue culture is used for the mass propagation of germplasm.

The use of tissue culture techniques in orchid culture has enabled to obtain quality plants in large numbers by clonal multiplication, establishment of hybrid plants and improvement of orchid trade and industry.

2.3.1 Culture media

For tissue culture both solid and liquid media are used. Majority of the tissues are grown on solid medium. The liquid medium is best suited for root cultures (Street, 1969). The use of liquid medium is very common for all tissues for the production of Callus. Shaking the media helps in the incorporation of oxygen to the media for the growing tissues (Heller, 1953; White, 1963). Recent studies have indicated that the liquid medium is the most effective for the induction of proliferation, particularly in protocorm or callus. The growth rate of orchid protocorms was much greater when the aeration of the liquid medium was increased by forcing sterile air into it (Cheng *et al.* 1978). Apical meristems of *Cymbidiums*, were excised from young shoots and inoculated on

a liquid medium. Cultures were agitated for four weeks. Protocorm like bodies were produced within 2/2 months. Many shoots were developed when they were transferred to the solid medium (Wimber, 1963; 1965). Apical and axillary bud sections of Dendrobium " James Storie " were cultured on both solid and liquid vacin and went medium and on modified Knudson's C medium, with coconut water. The best growth and longest survival rates were recorded on liquid modified Knudson's C medium (Iravati *et al.* 1977). The tissue masses can later be sub divided and transferred to flasks containing fresh nutrient solution for further proliferation. The process can be repeated several times and finally transferred to solid medium for differentiation (Wimber, 1963). In case of Vanda hybrids (V. insigne x V. tessellata) apical or axillary buds were first cultured on liquid Vacin and Went medium with the addition of 15 per cent coconut water. Later when the shoot primordia appeared it was transferred to solid media for proliferation. Greener tissues were obtained when sub-cultures were made on liquid Vacin and Went media (Teo *et al.* 1973). The process of culturing and sub-culturing described above was reverse when Vanda " Miss Joaquim " was cultured on Vacin and Went medium, where the axillary buds were made to grow first in a solid medium and later transferred to a liquid medium for proliferation. Again the proliferated tissues were transferred to solid medium for differentiation(Kunisaki *et al.* 1972). The shoot proliferation and differentiation did

not behave as above in case of Haemaria discolor. When the sterilized shoots of H.discolor were cultured on liquid Knudson C medium, no protocorm tissues were produced, but numerous white out growths were produced and when these were transferred to solid Knudson C medium they turned yellowish green and formed nodes and leaves (Teo, 1978).

A semi solid media was found suitable for Rhynchostylis gigantea and Dendrobium. (Vajrabhaya and Vajrabhaya, 1970).

The characteristics of the media greatly depend upon the kind and quantity of the soluble salts present in it. The suitability of the genus or the species to a particular medium is based on the above properties. High salt concentration of the media is beneficial to certain species. Similarly changing the form of a particular salt may also influence the growth and behaviour. The carbon source for almost all media is sucrose (Freson, 1969). Further studies on the suitability of carbon source indicated that sucrose is better than maltose, glucose and fructose. D-Manose was less effective than all the four other sugars (Fonnesbech, 1972). Elimination of sugars from the medium had produced healthy tissues in certain plants. Various concentrations of sucrose, glucose, fructose and manitol were used along with Knudson C medium and the protocorms of Holttumara. "Loke Tuck Yip" were inoculated to the medium the control received no sugar. After 2 weeks all tissues had multiplied two fold.

But at the end all protocorms which received sugars were yellow and necrotic (Teo, 1978). In a nitrate free medium of Murashige-Skoog for Cymbidium protocorms, many large protocorms without leaf or root primordia were produced during the first 5 weeks. Omission of magnesium favoured the development of leaf and root primordia (Bruijne and Debergh, 1974).

Several culture media were tried for the tissue culture studies on the species selected for this study. Different species of Cymbidiums were successfully cultured on Knudson C and other media (Champagnat et al. 1966; Steward and Mapes, 1971; Thompson, 1971; Morel, 1965; Wilfret, 1966; Wimber, 1963; 1965 Wimber and Vancolt, 1966 and Kano, 1968).

Different species of Dendrobium were successfully cultured on various media. Vacin and Went was found the best by many investigators (Gilliland, 1958; Nimoto and Sagawa, 1961; Israel, 1963; Sagawa Valmayor, 1966; Sagawa et al, 1967; Kim et al. 1970; Singh and Sagawa, 1972; Sanguthai et al. 1973 and Valmayor, 1974).

Epidendrum species were successfully cultured on different media like Murashige-Skoog Vacin and Went, Heller, Knudson C and Ojima and Fujiwara (Churchill et al.1970; 1972a; 1972 b; 1973; Sagawa and Valmayor, 1966; Valmayor and Sagawa, 1967; Rudolph et al. 1972).

Vanda species were successfully cultured on different media like Vacin and Went. (Rao and Avadhani, 1963, 1964;

Sagawa and Sehgal, 1967; Sanguthai and Sagawa, 1973; Valmayor, 1974), Vacin and Went medium (Kunisaki et al. 1972, Sanguthai, Sagawa, 1973, Teo et al. 1973), White media (Goh, 1970). Knudson C (A) (Valmayor, 1974).

The pH of the medium also influences the growth and development of the tissue. In Cymbidium culture the optimum pH is 5 to 5.5 (Sagawa, et al. 1966). The pH requirement of media recommended by Wimber (1963) is 5.2 to 5.5. Fonnesbech (1972) recommended a still higher pH for Cymbidium culture (5.5 to 5.8). Mosich et al. (1974) recommended a pH of 5.5 for Dendrobium. The same authors reported a higher pH (5.8) when modified Murashige-Skoog media was used for Dendrobium culture.

Epidendrum leaf tips are best grown at pH 5.5 on modified Murashige-Skoog's medium (Churchill, et al. 1970). Whereas Epidendrum roots were reported to grow on modified Ojima and Fujiwara medium with a pH of 5 (Churchill et al. 1972 a).

Still lower pH (4.8 to 5) was recommended for Phalaenopsis culture on modified Vacin and Went medium by Intuwong et al. (1972).

Cattleya shoot tips when cultured on a solid medium, turned brown and died eventually. Tests were conducted for Polyphenoloxidase activity and the leaves tested resulted in browning. The activity was greatest

at pH 6.5 and inhibited at lower pH (Ichihashi and Kako, 1977).

Vanda explants were best grown on White's medium with a pH of 5.5 (Sagawa and Sehgal, 1967).

Selection of an appropriate vegetative part is an important aspect in tissue culture.

Wilfret (1966) reported that in Cymbidium hybrids, protocorm like out growths developed on the meristems 6 to 8 mm away from apex, and also from the apical dome. Johansen (1967) used both micro and macro meristems (Apical and lateral buds) of Cymbidium for culture. The macro meristems grew well on Knudson C medium and formed protocorms. But the apical meristems did not grow at all. Apical and axillary meristems of Cymbidium, Dendrobium and Cattleya are the best explants for tissue culture (Sagawa and Kunisaki, 1969). The apical meristem of old and young pseudobulbs of Cymbidium were used for culturing. Early development was good in both. But failures from old pseudo bulbs reached 35 per cent, compared with only 15 per cent from young pseudobulbs (Alpi and Garibaldi, 1969). The growth rate of Cymbidium protocorm depends mainly on the size of the explant materials and also on the number of cut surfaces. Those with more cut surfaces increased relatively faster than that of large intact ones. (Zimmer et al. 1971).

Clonal propagation of Dendrobium was made easy with explant materials such as terminal and axillary buds or stem internodal section (Sagawa et al. 1967; Mosich et al. 1974; Intuwong and Sagawa, 1975) and new growths from previously cut rhizome or keikis (Kim et al. 1970).

With stem and flower stalk explants of Epidendrum hybrids, dormant bud at the nodes developed successfully into plantlets. Upto 20 plantlets obtained from a single cane (Stewart and Button 1976). Clonal propagation in Vanda can be easily achieved by shoot tip culture.

Use of leaf tip as a source of explants has the advantage of not endangering or even seriously damaging a plant (Arditti et al. 1972, Churchill, et al 1973). Epidendrum leaf tips formed callus on Murashige-Skoog's medium and differentiated on Knudson C medium. Laelio-Cattleya leaves cultured on Hellers medium produced callus and plantlets (Churchill et al. 1973). Leaf segments of Vanda and Phalaenopsis cultured on only agar medium produced protocorm like bodies. The proximal tissues formed protocorm like bodies more easily than distal tissues. Bettor development of protocorm like bodies were with young seedling tissue than on mature tissue particularly in Vanda (Tanaka et al. 1975). Leaf segments of Phalaenopsis amabilis hybrids produced protocorm like bodies on Murashige-Skoog's medium with supplements (Tanaka and Sakanishi, 1977).

The ideal method of clonal propagation would utilise an organ or small mass of tissue which can be removed without damaging the plant. Leaves and aerial roots can be used for this purpose. Orchid root tip contain definite meristematic zones, they can be made to grow in vitro. *Epidendrum* root tips can be cultured, but all they produce is roots, albeit longer roots (Churchill *et al.* 1972 a).

Root tips of *Phalaenopsis amabilis* hybrid seedlings cultured on 2 solid media proved satisfactory for callus growth and development of protocorm like bodies. Transferring these bodies to Kyoto nutrient solution resulted in plantlet formation (Tanaka *et al.* 1976).

2.3.2 Growth regulators in tissue culture

Subsequent to the development of different media for orchid tissue culture, several modifications were made to the media by changing the ingredients and their quality and quantity. The most important development in the culture media was the incorporation of growth substances which includes auxins, gibberellins, cytokinins and vitamins.

The beneficial effect of Indole acetic acid (IAA) was reported by Boesmann (1962 b) on *Cattleya*. The *Cattleya* meristems were cultured in a solid media with napthaline acetic acid (NAA), gibbrellic acid (GA_3) and kinetin at 1 μM each with vitamins and coconut water. NAA was found to stimulate growth of the tissues. A concentration of $5 \times 10^{-7} M$

was optimum and concentrations about 10^{-5} appeared to be toxic to plant (Lindemann, 1967). In Dendrobium meristems, a concentration of 1 ppm NAA with 25 per cent coconut water was most successful in producing protocorm like bodies. Young stems however do not respond to this treatment (Sagawa et al. 1967). Cymbidium protocorms cultured on Knudson C medium with Nitsch micro element supplement, produced plantlets within 2 months. The growth and development was promoted by NAA, bacto kryptone, L-arginine and L-aspartic acid. When the medium was supplemented with 0.1 mg/l NAA, about half of the protocorms produced shoots (Ueda and Torikata, 1968). The same authors reported the addition of low concentration of NAA (below 0.1 mg/l), promoted shoot formation in Cymbidiums but with increasing concentration upto 0.6 mg/l roots were formed and the number of shoots decreased. Shoot formation was also encouraged by addition of 0.01 mg/l 2,4-D, 0.01 mg/l GA, 10^{-3} M. L- arginine, 10^{-3}M. L-aspartic acid or 1 mg/l ascorbic acid. Knietin had no effect (Ueda and Torikata, 1969). Matsui et al. (1970) reported that in Cymbidiums the protocorm formation was not affected by the addition of NAA at rates upto 1 ppm. But a higher concentration of BA (10 ppm) with NAA increased the number of shoots. Root formation was slightly affected by 0.1 ppm NAA, but BA inhibited it at higher concentrations.

In a study to compare the effect of auxins, cytokinins and GA alone or in combination on growth of <u>Cymbidium</u> protocorms indicated that IAA alone had no effect, NAA resulted optimal fresh weight at 10 M and the protocorms were vigorous. 2,4-D caused a high weight increase at 1 μM but protocorms were abnormal. Kinetin induced growth of many small shoots and also promoted callus formation and increased fresh weight in liquid medium and GA alone promoted shoot and leaf growth. NAA and kinetin resulted in maximal fresh weight increase (Fonnesbech, 1972). A report by Kusumoto (1979) had shown that, in <u>Cymbidium</u> protocorms consistent shoot growth was obtained in the media containing GA_3 at 1.0 mg/l and NAA at 0.01 to 0.1 mg/l. For root formation a combination of 1 mg/l GA_3 and 0.1 mg/l NAA was most satisfactory.

A higher concentration of NAA(1.0 mg/l) and BA (5 mg/l) induced maximum proliferation of protocorms in <u>Cattleya.</u> Shoot formation and propagules were stimulated in a medium containing BA at 0.1 mg/l and 2,4-D at 0.1 mg/l. 2,4-D alone at a concentration of 0.5 mg/l killed the protocorms (Kusumoto, 1979).

The concentration of growth substance needs changes at each stage of development. The medium most effective for plantlet growth contained 0.1 to 1.0 mg/l kinetin and 1.0 to 5.0 mg/l NAA or 0.1 to 0.5 mg/l kinetin

and 0.1 mg/l 2,4-D. Shoot formation of propagules were best promoted in a medium containing 1.0 mg/l BA and 0.5 mg/l NAA or 0.1 mg/l kinetin and 0.1 mg/l 2,4-D. High concentration of growth regulators induced the formation of protocorms or callus on root tips and leaf surfaces (Kusumoto, 1979).

Higher concentration of NAA (6 mg/l) and kinetin (2 mg/l) and 2,4-D (2 mg/l) with coconut water(5 percent) in Murashige-Skoog medium produced good callus tissue in the rhizomatous portion of Spathoglottis (Bapat and Narayana Swami, 1977). Growth initiationof Cattleya explants were optimum with NAA or 2,4-D at a concentration of 0.1 ppm in the media (Ichihashi and Kako, 1975).

Before the discovery of cytokinins by Miller et al.(1955), the cultivation of the tissue of many species, like tobacco and soyabean, were possibly only by adding to the media complex factors of unknown composition, such as coconut milk, yeast extract and corn endosperm. The main compound in these substances responsible for cell division is cytokinin.

Cytokinins are responsible for differentiation (bud formation). The cell division or cell differentiation is also associated with auxins (Skoog and Miller, 1967).

Different forms of cytokinins tested so far (Benzylamino Purine, N-Benzyl adenine, N-benzyl adenosine and Kinetin) had the similar effects at 0.1 ppm. However higher concentration inhibited organ differentiation (Rucker, 1974).

2.3.3 Gibbrellins

Gibberellins(GA_3) are mainly responsible for stem elongation. In certain plants, meristems will not develop without GA. (eg. Potato, *Chrysanthemum*). But in orchid meristems, the exogenous application of GA_3 produces deleterious effects, resulting in very thin, thread like and chlorotic stems. However in *Cattleya* GA_3 concentration of 0.5 μM associated with 1M. NAA and Kinetin had produced better results (Lindemann, 1967).

2.3.4 Miscellaneous additives for tissue culture

A variety of ingredients are incorporated into the media for orchid culture. Some of the additives have very striking effect in tissue culture. Coconut water was first introduced in tissue culture by Von Overbeek *et al.* (1941). Since then several investigators observed the stimulating effect of coconut milk in tissue culture. Its effect on the apical meristem of *Cattleya*, *Dendrobium* and *Vanda* are very striking. It helps in the division of epidermal cells and formation of protocorms. The optimum concentration of coconut water in the media is 10 to 15 per cent

and it is added before autoclaving (Morel, 1965; Intuwong and Wagawa, 1973).

In *Vanda* shoot tip culture, use of 15 per cent coconut water without sugar induced the production of dark green protocorm like bodies. Those without the supplement were brown and those with sucrose alone were light green. Continuous prliferation occured when agar and coconut water were used in the medium (Kunisaki *et al*. 1972)

The role of coconut water has been further emphasised by Mc Intyre *et al*. (1974). The addition of 15 per cent coconut water to basic Knudson C medium produced increased growth of both epiphytic and terrestraial species.

The stimulatory effect of coconut milk on the protocorms may be due to its complex composition, which includes amino acids, Sorbitol, Inositol and cytokinins (Pollard *et al*. 1961 Van Staden and Drewes, 1975). The greater production of chlorophyll in the protocorms cultivated with coconut milk may be due to cytokinins as these growth regulators are involved in the differentiation of lamellar system in chloroplasts.

2.4 Histochemical studies

2.4.1 Orchid seeds

Swamy (1949) described two fundamental types of seed development in the members of orchidaceae. In the first , the zygote divides by a transverse wall to form two

cells, of which the basal one again divides transversely and results into a proembryo of three cells. The upper cell adjacent to the wall of the embryo sac frequently gives rise to one or more suspensor haustoria, which become very prominent and aggressive structures in some species. The terminal cell divides vertically to form two daughter cells, which by further divisions gives rise to the greater part of the embryo, the rest being contributed by the middle cell. In the second type of development the zygote may divide by either a transverse or an oblique wall. Further divisions, do not follow any definite pattern and give rise to a mass of 5 to 10 cells, some of which begin to enlarge and assume a haustorial function. One or two cells divide transversely to form a filament of variable length whose lower portion gives rise to the embryo proper. Thus the young proembryos of orchids have been grouped under the category of unorganised and reduced embryos (Maheshwari, 1950).

The orchid seeds have differentiated and undifferentiated embryos (Withner, 1959). At maturity the seed does not possess either endosperm or cotyledons. Food reserves are stored in the embryo itself. Under favourable conditions the embryo swells and bursts the membraneous seed coat. Further enlargement follows, forming a top or cone shaped body called protocorm. The interval between germination and production of elaborated leaves may extend for several months.

The embryo utilizes the stored carbohydrates and lipids as well as exogenous nutrients during its differentiation (Alvarez and Sagawa, 1965). The protocorm consists of parenchymatous tissue which disappears, consequent to the production of photosynthetically active primordial leaves. The parenchymatous region of the protocorm accumulates good amount of metabolic reserves in the form of protein, lipids and high molecular weight carbohydrates and serves as a reservoir in the absence of endosperm.

When the embryo sections were stained with Feulgen stain for locating the DNA, it was observed that the large nuclei in the parenchymatous region stained darker than those of the meristem indicating an increase in DNA content during differentiation stage (Alvarez & Sagawa, 1965).

Alvarez (1968) measured the nuclear DNA cyto-photometrically in sections and isolated nuclei of the developing embryo of Vanda . The amount of DNA in the nuclei of the parenchymatous region was shown to increase in direct proportion to the distance of the nucleus from the meristem.

The ultrastructural examination of developing Vanda protocorms indicated that a substantial amount of lipid, protein, and carbohydrate reserves, which disappear gradually with the senescence of the parenchymatous regions. The proteinaceous reserves appear initially as discrete bodies which become initially associated with clusters of

small, tubules. These tubules later disperse throughout the cytoplasm and disappear. The lipid reserves then appear as discrete bodies and later disappear. The plastids in the meristematic cells differentiate a well-developed thylakoid system and contain little amount of starch. The parenchymal cells contain large starch grains and numerous osmiophilic droplets and develop meager thylakoid systems (Ricardo and Alvarez, 1971).

Harrison (1977) reported the presence of large amount of reserve materials in the orchid seed in comparison of the over all size of the seed. The ultra structural examination and histochemical study on the protocorm of *Cattleya aurantiaca* showed that the mature unimbibed seeds contain numerous protein and lipid bodies, but no carbohydrate reserves. Protein bodies were observed only in the upper two-thirds of the embryo. Lipid reserves were utilized slowly in seeds sown aseptically on the medium lacking a carbohydrate source but more rapidly on a medium containing sucrose.

2.5 Cytological evaluation of [illegible]

Plant cell culture has been hailed as one of the most significant and potential adjunct to plant propagation and improvements.

Clonal propagation is very essential and only mean of mass propagation of orchids as the genotypes of most of the commercial hybrids are highly heterozygous and segregate if raised through seeds.

In propagating orchids by tissue culture, explants from different plant parts are taken and grown in different medium. Tissues from inner layer taken for further development may become heterogeneous after differentiation. (Murashige and Nakano, 1967). This may be due to the artificial environment in vitro which has a considerable affect on the cell development and differentiation and leads to somaclonal variation.

The variability in chromosome number in regenerated plant is a widespread phenemenon (Larkin and Scowcroft, 1981) and gross karyotype changes such as aneuploidy-polyploidy or myxoploidy have been reported in many crops (Murashige, 1974, Thomas, 1979) indeed gross karyotype alterations have been observed in tissue culture plant cells (Murashige and Nakano, 1967; Kao and Michayluck,1980; Bennici, 1974; Sreeramulu et al.1976; Skirvin, 1978 and Roy, 1980). Aneuploids in rye grass (Ahloowalia, 1976) trisomics in tobacco (Nishiyama and Taira, 1976).

A perusal of earlier literature reveals that chromosome variability among regenerated plants has been more in original callus cells than in regenerated plants, (Orton, 1980) which clearly indicates that there is a selection force in favour of specific chromosome constitution during the process of regeneration. In Hawarthia (Ogihara, 1981) selection for eudiploid and tetraploid plants were

rather stronger than for plants with abnormal karyotypes (with deletion and translocation).

Chromosome breakage and reunion, multicentrics and translocations have also been observed in plants derived from callus cultures in barley (Orton, 1980). In plants from rye grass cultures the meiotic chromosome behaviour suggested that the presence of reciprocal translocation, deletion and inversions, and other chromosome rearrangements do results in loss of genetic material which may result in phenotypic variants as well as affecting the gene in which the chromosome break occurs. Cryptic changes can not only result in the loss of genes and the functions but also the expression of genes which hitherto have been silent.

MATERIAL AND METHODS

CHAPTER III

MATERIAL AND METHODS

The experiments were conducted at the Orchid Laboratory of the Indian Institute of Horticultural Research, Hessaraghatta, Bangalore. The plants were maintained in the Orchidarium attached to the laboratory. The Orchidarium provides optimum condition for the growth of orchid plants.

The Orchid laboratory has a culture room and an inoculation room. The cultures were maintained at a temperature of 25°C and under artificial light of 600 lux for 12 to 16 hours a day depending upon the stage of germination and differentiation.

3.1 Orchid species used for the experiments

The following orchid species were selected for the propagation studies.

Cymbidium pendulum (Roxb.) SW

An epiphytic species with short stout stems. Leaves thick leathery, ensiform, obtuse retuse apically furrowed, 50 to 100 centi meter long and 5 to 10 centi meter broad. Inflorescence racemose, very long and pendulous (Plate 1.1). Flowers small dull yellowish green.

Bletilla hyacinthina Rechb.f.

A terrestrial species with round compressed pseudobulbs typically subterranean. Leaves stalked at base, folded above, light or dark green 30 to 60 centi meter tall. Inflorescence solitary appearing from the middle of the

expanding shoots. Flowers 4 to 6 in number, rose-purple.

Phaius wallichi Hk.f. (Syn. Phaius tankervillae)

A cultivated terrestrial orchid with tightly clustered rather irregular dull green pseudobulbs. Leaves 3 to 4, 90-100 centi meter long, folded, narrowly elliptic, acuminate. Inflorescence stout, borne from the base of the pseudobulb, more than 125 centi meter tall. Flowers 10 to 25 opening in succession. Flowers white outside and red brown inside, often with yellowish margins, bract large fragrant. Lip large mostly whitish outside (Plate 1.2).

Spathoglottis plicata: Bl

It is an ever blooming terrestrial cultivated orchid. The pseudobulbs are tightly clustered, avoid to rather conical, often irregular, 45 centi meter tall, bright or dull green, strongly ringed. Leaves sheathing, linear-lanceolate, acute or acuminate, more than 60 centi meter long, 5 to 7 centi meter wide, folded. Inflorescence errect, 65 to 75 centi meter tall and with 15 to 25 flowers. Flowers large, pink bracts persistant, flowers opening successively over a very long time. Four different varieties with different colours are known (Plate 1.3).

Epidendrum radicans: Pav. ex Ldl

Also known as Epidendrum ibaguense, the plant reed-like, having tall, slender stem with rather widely spaced short leaves and long aerial roots. It is usually trained on supports, The tall erreot stem bear dense cluster of a spear shaped head of 2 to 5 centi meter brilliantly coloured red

PLATE NO. 1.

DIFFERENT ORCHID SPECIES.

1. *Cymbidium pendullum*

2. *Phaius wallichii*

3. *Spathoglottis plicata*

4. *Epidendrum radicans*

5. *Vanda coerulea*

6. *Dendrobium moschatum*

7. *Dendrobium pierardii*

PLATE - I

7

5

2

6

flowers (Plate 1.4)

Vanda coerulea Griff. ex Ldl.

An epiphytic orchid native of Himalayas, stem robust, 150 centi meter tall, very densely leafy, leaves-leathery, usually yellowish-green, 25 centi meter long and 2 to 3 centi meter wide, linear-ligulate, deeply channelled above and keeled beneath, irregularly cut and toothed at apex. Inflorescence-errect or arching, to 60 centi meter tall. Flowers 5 to 15 in number highly variable in shape, colour and size. Flowers pale blue with dark reticulate marking. It flowers mostly in autumn-winter (Plate 1.5)

Dendrobium moschatum: (Willd,)SW.

This is an epiphytic orchid native to Himalayas. Pseudobulbs arching or pendulous, strongly striped with dull brownish purple stripes. Leaves oblong to xoblong-ovate, strongly veined, leathery, acuminate to 15 centi meter long, deciduous. Inflorescence- a spike with 5 to 10 flowers pendulous, produced from near tip of old pseudobulbs. Flowers 8 centi meter across, heavy textured, musk scented creamy-buff flushed with rose purple (Plate 1.6).

Dendrobium pierardii:Roxb.

This species is native of India and China and distributed in Himalayas to Burma. Pseudobulbs very slender stem like, pendulous or drooping to 150 centi meter long. Leaves-deciduous, rather soft textured to 120 centi meter long,

sessile, lanceolate acuminate. Inflorescence-1 to 3 flowers, short stalked, produced from old leafless pseudobulbs. Flowers 5 centi meter across, fragrant, very fragile in texture, often semi-transparent. Flower-Pale blush white or rosy-bluish. It flowers during spring to early summer (Plate 1.7).

Vanda teres (Roxb) Ldl.

The species is highly ornamental and is used as cutflower, native of Himalayan foot hills and Western ghats. It is almost ever-blooming in tropics. Stem terete, often profusely branched near base and above and forming dense tangled masses frequently few meters in length. Leaves-terete, similar to the stem, 10 to 20 centi meter long, distichously and alternatively arranged at an acute angle to the stem, errect or rather sharply ascending. Inflorescence- 15 to 30 centi meter long borne opposite to the leaf bases from upper part of the stem bearing 3 to 6 flowers, which are loosely arranged. Flowers 5 to 10 centi meter in diameter, long lasting, variable in colour, lip funnel shaped yellow lined with purplish brown and speckled with red lobe is deeply cleft and rose purple, fragrant.

3.2 Seed germination studies

The flowers of the selected species were self-pollinated and mature undehised capsules were collected. The maturity is attained in 28 days in Spathoglottis plicata and in ten months in Vanda teres. Viability of the seed was

tested by differential staining method (Singh, 1981) and only those capsules in which the viability was more than 75 per cent were used. In most of the cases green pod culture technique was used for seed culture. However, when the capsules had dehised the seeds were sterilised with 2 per cent chlorine water before sowing.

3.3 Tissue culture studies

The different vegetative parts were collected from the selected species.

3.3.1 Meristem culture

The apical and axillary meristems of *Cymbidium pendulum* were obtained from young sprouts and pseudobulbs. The sprouts and pseudobulbs were separated from the mother plant, washed throughly with water and teepol and surface sterilized with 5 per cent chlorine water for 15 minutes. The outer enclosing leaf was removed and again dipped in 2.5 per cent chlorine water for 10 minutes. The lateral and apical buds were excised and finally washed thoroughly in sterile distilled water 2 to 3 times and used for culturing. Sterilization technique of *Vanda teres* and *Epidendrum radicans* were almost same as *Cymbidium*, but the *Vanda* and *Epidendrum* stems are very hard at base and soft at the top, the axillary meristems were enclosed with only one leaf sheath and only the top 6 to 7 nodes were used for extracting explants. The stem sections with the bud were washed thoroughly with water and teepol and surface sterilized with 2.5 per cent chlorine water for 10 minutes. The leaf sheaths were seperated

carefully and the buds were exposed. Single node cuttings 1 to 2 centimeter long were made . The cuttings were passed successfully through 5 per cent and 2.5 per cent chlorine water for 5 minutes in each. They were finally washed with sterile distilled water and used for culturing.

The same procedure was adopted for Dendrobium pierardii except that the chlorine water used for initial sterilization was only 5 per cent and time of treatment was 15 minutes. The thin scale leaves were removed and made into nodal cuttings as in Vanda teres.

3.3.2 Preparation for root cultures

Root explants of Dendrobium moschatum were collected from the young aerial roots. Root tips (2 centimeter) long were removed and washed in distilled water with teepol. Surface sterilization was done with 3 per cent and 1 per cent sodium hypochlorite solution for 10 to 15 minutes. Finally washed in sterile distilled water and cultured.

3.3.3 Media used for culture studies

The following five different media were used for the experiments.

1. Knudson C medium
2. Murashige-Skoog medium
3. Vacin and Went medium
4. Burgeff N_3F medium
5. Modified Vacin and Went medium.

Further modifications to these media were made by adding growth substances and other organic complex additives. The composition of the media and the method of preparation are given in the table (Table 1 to 5).

Table 1. Composition of Knudson C medium.

Components	Quantity per litre of culture medium
Calcium nitrate, $Ca(NO_3)_2\ 4\ H_2O$	1000 mg
Monopotassium phosphate, KH_2PO_4	250 "
Magnesium sulphate, $Mg\ SO_4\ 7H_2O$	250 "
Ammonium sulphate $(NH_4)_2\ SO_4$	500 "
Ferrous sulphate, $FeSO_4\ 7H_2O$	25 "
Manganese sulphate, $MnSO_4\ 4H_2O$	7.5 "
Sucrose	20 g.
Distilled water to make upto	1000 ml
Agar	9 g

Table II. Composition of Murashige-Skoog medium

Components	Quantity per litre of the culture medium
<u>Macroelements</u>	
Ammonium nitrate, $NH_4 No_3$	1650 mg
Potassium nitrate, KNO_3	1900 mg
Calcium chloride, $Cacl_2 . 2H_2O$	440 mg
Magnesium sulphate, $MgSo_4 . 7 H_2O$	370 mg
Potassium phosphate, $KH_2 PO_4$	170 mg
Iron chelate (Na_2 EDTA)	37.39 mg
Ferrous sulphate, $FeSo_4 . 7 H_2O$	27.80 mg
<u>Microelements</u>	
Boric acid, H3 BO_3	6.20 mg
Manganese sulphate, $MnSO_4 . 7 H_2O$	22.30 mg
Zinc chloride, $Zncl_2$	3.93 mg
Potassium iodide, KI	0.83 mg
Sodium molybdate, $Na_2 MO_4 . 2 H_2O$	0.25 mg
Copper sulphate, $CuSo_4 . 5 H_2O$	0.025 mg
Cubalt chloride, $Cocl_2 . 5 H_2O$	0.025 mg
Sucrose	30 g
Distilled water to make upto	1000 ml
Agar	9 g

Table III. Composition of Vacin and Went medium.

Components	Quantity per litre of culture medium
Tricalcium phosphate, $Ca_3(PO_4)_2$	200 mg
Potassium nitrate, KNO_2	525 mg
Potassium phosphate, KH_2PO_4	250 mg
Ammonium sulphate, $(NH_4)_2SO_4$	500 mg
Ferric tartarate, $Fe_2(C_4H_4O_6)_3$	28 mg
Manganese sulphate, $MnSO_4 \cdot 4H_2O$	7.5 mg
Magnesium sulphate, $MgSO_4 \cdot 7H_2O$	250 mg
Sucrose	20 g
Distilled water to make upto	1000 ml
Agar	9 g

Table IV. Composition of Burgeff N_3F medium.

Components	Quantity per litre of culture medium
Solution A.	
Calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$	1000 mg
Ammonium sulphate, $(NH_4)_2SO_4$	250 mg
Magnesium sulphate, $MgSO_4 \cdot 7H_2O$	250 mg
Ferrous sulphate, $FeSO_4 \cdot 7H_2O$	20 mg
Distilled water	500 ml
Solution B.	
Monopotassium phosphate KH_2PO_4	250 mg
Dipotassium acid phosphate K_2HPO_4	250 mg
Distilled water	500 ml
Solution A and B were mixed together after preparing separately and to the combined mixture added	
Sucrose	20 g
Agar	9 g

Table V. Composition of modified Vacin and Went medium

Components	Quantity per litre of culture medium
Tricalcium phosphate, $Ca_3(PO_4)_2$	200 mg
Potassium nitrate, KNO_3	525 mg
Potassium phosphate, KH_2PO_4	250 mg
Ammonium sulphate $(NH_4)_2SO_4$	500 mg
Ferric tartarate $Fe_2(C_4H_4O_6)_3$	28 mg
Manganese sulphate, $MnSO_4 \cdot 4H_2O$	7.5 mg
Magnesium sulphate, $MgSO_4 \cdot 7H_2O$	250 mg
Sucrose	20 g
Coconut water	250 ml
Distilled water to make upto	1000 ml
Agar	9 g

3.3.4 Preparation of media

A stock solution of nutrients were prepared first by dissolving the known quantity of a chemical separately in one litre of distilled water and stored in coloured bottle and kept in a refrigerator. Solutions containing ammonium and nitrate may become contaminated while in storage. Hence no stock solution was prepared. A uniform concentration of 1000 ppm was prepared both for macro and micro nutrients. The quantity of nutrients as detailed in Table 1 to 5 were measured and the media were prepared. Sucrose and agar were added directly into the prepared medium after adjusting the pH between 5.2 to 5.5. The mixture was then heated for dissolving the agar into the media. After the agar was dissolved the medium was poured into the culture tubes and flasks for autoclaving.

For preparation of Murashige-Skoog media, Na_2 EDTA and $FeSO_4 \cdot 7H_2O$ (Table 2) were added to one litre of distilled water and kept at 60°C for few hours, ten ml of this solution was used per litre of the medium.

While preparing Vacin and Went and modified Vacin and Went media, Tricalcium phosphate (Table 3 and 5) were first dissolved in minimum quantity of 1 N HCl and the volume was made upto 1000 ml

Liquid medium was prepared without adding agar.

3.3. 5 Adjustment of pH

The pH is very critical only during the early stages of germination when the seedlings are less sensitive to differences in pH (Knudson, 1951). Hence the pH of all media were maintained between 5.2 and 5.5. The level of pH was measured with 'ELICO' electronic pH meter. The adjustment of pH was made by using IN HCl or NaOH.

3.3.6 Autoclaving

The autoclave in the laboratory is a horizontal type which can hold about 30 to 40 flasks or 200 tubes at a time. The flasks with the media were autoclaved at 15 pounds per square inch pressure for a period of 20 minutes. The flasks were then taken out and allowed to cool and solidify. The inoculation was done after 3 to 4 days to ensure that the flasks were free from contamination.

3.3.7 Sowing of seeds

The sowing of seeds were done inside the inoculation chamber fitted with an ultra violet tube. The mature undehised capsules were surface sterilized with alchol. The capsules were flamed and then cut open with a blade and the seeds were sown on the medium and the flasks closed with cotton plug and kept in the culture chamber.

3.4 Growth regulators

The standardisation of media was done with five basic media except the modified Vacin and Went medium which contain coconut water also.

The additive includes growth substances, including, hormones and other complex additives. Three important group of growth substances (auxins, gibbrellins and cytokinins) were used in these experiments.

3.4.1 Auxins

In the present study four auxins (both natural and synthetic) were used.

Indole-3-acetic acid (IAA)

A stock solution of IAA 1000 ppm was prepared by dissolving 500 mg of IAA (Sigma) in a smallquantity of ethanol. The volume adjusted to 500 ml with distilled water. This hormone was incorporated into the media at three concentrations of 1, 3 and 5 ppm for seed culture studies.

Naphthaline acetic acid (NAA)

A stock solution was prepared by dissolving 500 mg of NAA powder (SIGMA) in a small quantity of ethanol and the volume was adjusted to 500 ml with distilled water. Required amount of stock solution was mixed with culture medium before autoclaving. Three concentrations 1 , 3 and 5 ppm were used for seed and meristem culture studies.

2,4-Dichlorophenoxyacetic acid (2,4-D)

A stock solution of 1000 ppm was prepared by dissolving 500 mg of powder (SIGMA) in minimum quantity of ethanol and the volume adjusted to 500 ml with distilled water and stored in a coloured bottle in a refrigerator. Three concentrations 1, 3 and 5 ppm were used for seed germination studies.

3.4.2 Gibberellin Acid (GA_3)

A stock solution of 1000 ppm was prepared by dissolving 500 mg GA_3 powder (SIGMA) in minimum quantity of NaOH and the volume made up to 500 ml with distilled water and stored in coloured bottle in a refrigerator. Different concentrations 0.1, 1 and 3 ppm were used for the experiments. Adequate amount of the stock solution was incorporated with the media before autoclaving.

3.4.3. Cytokinins

Two cytokinins used for the experiments were kinetin and benzyl adonine (BA). A stock solution of both substances were prepared at 1000 ppm and stored in a coloured bottle in refrigerator. 500 mg of the power (SIGMA) was dissolved in a small quantity of 1N HCl and the volume made to 500 ml with distilled water and adjusted the pH. Concentrations 1, 3 and 5 ppm were used for various experiments. Adequate quantity of the stock solution was incorporated with the prepared medium before autoclaving.

3.4.4 Other complex additives

Other complex additives such as coconut water (the liquid inside the nut) mature unripe banana and tomato juice were also used along with the media to study their effect on germination, growth and development. Coconut water was

collected from tender coconuts and filtered through cheese cloth and stored in a freezer. Mature but unripe banana fruits were peeled and blended in a small quantity of distilled water before use. Ripe tomato fruits were blended in a mincer and the seeds and peels were removed by straining through muslin cloth.

3.4.5 Vitamins

Vitamins such as nicotinic acid, pyridoxin and thiamin were used to find out their effect on growth and development of orchid seedlings. Five hundred milligrams each of the vitamins were dissolved in 500 ml of water and stored in a bottle under refrigeration. Three concentration (0.5, 1 and 3 ppm) were used for seed culture.

3.5 Culture conditions

The flasks after culturing were arranged on a rack and illuminated at 600 lux for 12 to 14 hours a day. The temperature of the culture room was maintained at 25°C $\pm$ 2°C, and the humidity range was 60 to 70 per cent. The culture room and the surroundings were kept clean and aseptic.

3.6 Observations made

The following observations were recorded periodically.

1. Number of days taken for greening
2. Germination percentage
3. Number of days taken for first, second, third and fourth leaf stage.

4. Plant height in 120 to 135 days
5. Number of leaves in 120 to 135 days
6. Number of roots in 120 to 135 days
7. Root length in 120 to 135 days
8. Fresh and dry weight in 120 to 135 days.

3. 7 Tissue culture

Tissue culture studies were conducted with different vegetative parts of four species. The species are *Cymbidium pendulum*, *Vanda teres*, *Dendrobium pierardii* and *Epidendrum radicans*. The vegetative parts used were apical and axillary buds, roots and off shoots.

3.7.1 Preparation of explant material for culturing *Cymbidium pendulum*. BL

Young pseudobulbs emerging from the base of the old plants were cut and removed, they were first washed with water and a few drops of detergent (Teepol). The surface sterilization was done with 5 per cent sodium hypochlorite solution. The leaf sheaths enclosing the meristems were removed one by one. Removal of each sheath was followed by a dip in lower concentration of sodium hypochlorite. The concentration was gradually reduced and finally the meristems were washed thoroughly in sterile distilled water. The plant material was then inoculated into the liquid medium selected for the study. The media was continuously agitated on a rotary shaker at 160 rpm. The liquid medium was changed after every two months.

Vanda teres. Griff. ex Idl.

The top most 6 or 7 nodes were used for culturing. The cylindrical leaves were cut and removed and the stem sections were surface sterilized with 5 per cent sodium hypochlorite solution for 5 to 15 minutes. The sections were then taken out and the leaf sheath covering the axillary buds were removed and again dipped in lower concentration of sodium hypochlorite (3 and 1 per cent). Single node sections were cut to a length of 1 to 2 centi meter and thoroughly washed in sterile distilled water and inoculated onto the selected medium. The apical meristems were also used for culturing. The aerial roots on the opposite side of the axillary buds were cut at the base and removed before inoculation.

Dendrobium pierardii

The mature stem before flowering was used for culture studies. Only the top 5 to 6 axillary buds were used. The leaves were first removed and the stem sections dipped in Sodium hypochlorite solution (5 per cent) for 5 minutes. The sections were then taken out and the leaf sheath surrounding the axillary buds were peeled off and cut into single node cuttings of 1 to 2 centi meter long. Single node sections were further sterilized with lower concentrations of sodium hypochlorite (3 and 1 per cent) and finally washed thoroughly in sterile distilled water and then inoculated onto the medium.

Epidendrum radicans. L.

The top 5 to 6 buds were used for culturing. Leaves were removed and surface sterilized with sodium

hypochlorite (5 per cent) for 10 minutes. The axillary buds were removed, single node sections were further sterilized in lower concentration of sodium hypochlorite (3 and 1 per cent) and finally washed thoroughly in sterile distilled water. The axillary buds on the flower stalks were also used. These buds were more developed than the axillary buds on the stem and later develop to form keikis or offshoots.

3.7.2 Root culture

Root culture studies were conducted with Dendrobium moschatum roots. The aerial roots half to one centimeter long were surface sterilized with 1 per cent sodium hypochlorite solution for a period of 10 minutes, roots sheaths were removed, and washed thoroughly in sterile distilled water and inoculated on different media.

3.7.3 Culture media for the study

Three types of media were prepared. A basal liquid medium for callus initiation, a solid medium with additives for callus proliferation and a solid medium with additives for differentiation of shoot and root.

A liquid medium was prepared and poured into 150 ml erlenmayer conical flasks (30 ml in each). The flasks were closed with cotton plug and autoclaved at 15 PS1 for 20 minutes and then cooled.

The explants were inoculated on the liquid medium and the flasks were kept on a rotary shaker (EMENVEE) and agitated at 160 rpm.

A solid medium was used for the proliferationof the tissue. Sufficiently grown tissue in the liquid medium was sub-cultured in a solid medium containing other additives for proliferation. A solid media was prepared and filled in 200 x 25 mm culture tubes at the rate of 25 ml in each tube and closed with a screw cap and sterilized by autoclaving at 15 psi pressure for 20 minutes.

A third type of solid medium with the additives were prepared for differentiation of the callus developed in the liquid and solid medium.

3.7.4 Sub-culturing for callus proliferation and differentiation

The callus proliferated on liquid medium/is ready for sub-culturing in about 2 to 3 months time. Solid medium with growth substances and other complex additives were used for differentiation. NAA at the rate of 1 ppm and coconut water at 15 per cent were used along with the solid medium.

By using sterile scalpel the callus tissue was cut into small pieces. These small pieces were taken out of the flask with a sterile forceps and put aseptically into the tubes with solid media. The tubes were kept in the culture room under a light illumination of 600 lux for 12 hours a day for differentiation into plantlets.

3.7.5 Culture condition

The inoculated tubes were labelled and arranged in culture tube racks and placed in the culture room where the light intensity was maintained to 600 lux. and a temperature

of 25°C ± 2°C. The humidity was maintained at 60 to 70 per cent and the culture room was kept clean and aseptic.

3.8 Observations recorded

The data were collected on the following aspects.

Meristem culture

1. Number of days taken for swelling of the meristems
2. Number of cultures which produced callus
3. Number of leaf and root primordia initiated at monthly intervals.
4. Height of plant at 4 leaf stage
5. Green and dry weight

Nodal culture

1. Number of days taken for swelling of the nodes
2. Number of explants which produced callus or plantlets
3. Number of leaf and root primordia produced at monthly intervals
4. Height of plants at 4 leaf stage

Root culture

1. Roots extended at monthly intervals
2. Roots initiated callus
3. General growth and development

3.9 Histochemical studies

It has been reported by many workers that the parenchymal cells of the protocorms accumulated substantial quantities of carbohydrates (insoluble polysaccharides),

insoluble proteins,RNA, DNA and lipids, which disappeared gradually with the senescence of the parenchymatous region (Ricardo and Alvarez, 1971). Various tissues from Bletilla hyacinthina and Dendrobium pierardii were subjected to histochemical studies to study the changes taking place at different stages of plant development. Callus obtained from seeds (PLB's), stems and the Pseudobulbs at different stages of growth were used for the study.

3.9.1 Preparation of plant materials for histochemical studies

The tissue used for histochemical studies were taken from the developing seeds of Bletilla hyacinthina and the callus from the lateral buds of Dendrobium pierardii . The various cell components and their variations at different stages of development give a comprehensive account of differentiation of tissues and organs during plant development.

3.9.2 Plant material

The seeds, seed callus (Protocorm like bodies(PLB's), pseudobulbs and stem callus at various stages of development were used for histochemical studies.

3.9.3 Killing and fixing

The materials were fixed in a fixative after killing. Two different types of fixatives were used during the present studies . Formaldehyde, acetic acid, and ethyl alcohol(FAA) was used as fixative for the materials used for localising insoluble Polysaccharides, while Carnoy's B fixative was used

for the tissues used for localising insoluble proteins, DNA and RNA. The composition of FAA and Carnoy's B are as below:

FAA		Carnoy's. B.	
Ethyl alcohol (50 per cent)	-50 ml	Ethyl alcohol	- 60 ml
Glacial acetic acid	- 5 ml	Chloroform	- 30 ml
Formal dehyde (40 per cent)	- 5 ml	Acetic acid	- 10 ml

The plant material was immersed for 24 hours in FAA and washed in 50 per cent alcohol and 1 hour in Carnoy's Solution B and washed in 75 per cent ethyl alcohol before subjecting to dehydration.

3.9.4 Dehydration

Ethyl alcohol and N.butanol were used for dehydration. The plant materials were passed through 70, 80, 90, 95 per cent alcohol and finally two changes in absolute ethyl alcohol. The materials were then changed to 3:1, 1:1 and 1:3 alcohol-butanol mixtures and finally two changes of pure butanol were given. In each grade the materials were kept for a period of 4 to 6 hours.

3.9.5 Infiltration

A mixture of paraffin wax and bee's wax (9:1) was used both for infiltration and embedding. Thin peelings of wax were put into the tubes containing tissue and butanol. The wax gradually dissolved in the butanol till the solution became saturated. These tubes containing the materials and

wax-butanol mixture was poured off and replaced with fresh molten wax. This procdss was repeated several time till butanol was completely replaced with wax.

3.9.6 Embedding

Paper boat method was employed for embedding. Small square paper boats were placed on a glass plate kept over an ice block. The molten wax and the plant materials were poured into the boat and a label was fixed. The wax was allowed to solidify by immensing the boat slowly in cold water.

3.9.7 Sectioning

The plant materials arrangedin the groups were cut into small wax cubes of about one centi meter size. Section of 5 μM thickness were taken, throughErma microtome and kept ready for staining.

3.10 Staining

The sections were stained with the following staining techniques.

i. Periodic acid schiff's reagent method of staining insoluble polysaccharide bodies.
ii. Mercuric bromophenol blue method for insoluble proteins.
iii. Feulgen staining for DNA containing bodies.
iv. Toludine blue method for nuclein acids
v. Sudan IV test for lipids.

3.10. 1 Periodic acid schiff's reagent method

The total insoluble polysaccharide distribution was studied by this method using 0.5 per cent HIO_4 in distilled water as an oxidant. Periodic acid exposes aldehyde groups from the polysaccharides. These aldehyde groups with fuchsin and sulphurous acid yields a typical magenta colour.

Preparation of stains and staining

Schiff's reagent was prepared by mixing 1 g basic fuchsin and 2 g potassium metabisulphite with 100 ml of 0.15 NHCl in an air tight contained and the contents were agitated repeatedly for 24 hours. A straw yellow coloured solution was obtained. The solution was further cleared by adding 100 mg activated charcoal and filtered after shaking.

The slides containing the sections were deparaffinised by passing through 2 grades of xylol for a period of 10 minutes each. The sections were hydrated stepwise by passing through butanol, alcohol, grades of alcohol (80, 70 and 50 per cent) and water by keeping in each grade for 5 minutes.

The slides were immersed in 1 per cent solution of periodic acid at room temperature for 15 minutes, washed in running water for 10 minutes and stained with schiff's reagent for 15 minutes at room temperature. Again the slides were rinsed in water for 10 minutes. The sections were bleached with 2 per cent potassium metabisulphite for one minute to

remove superfluous acid and rinsed in running water for 10 minutes.

Sections were dehydrated by passing through different grades of alcohol (50, 70, 80, 90, 95 per cent and absolute alcohol, alcohol butanol mixtures (3:1, 1:1 and 1:3) and finally through butanol. The sections were further cleared by passing through xylol and finally mounted in DPX.

3.10.3 Iodine-Potassium iodide (IKI) test for starch:

This method was used specifically to locate starch bodies in the cells. The deparaffinised and hydrated sections were immersed in IKI solution for 10 minutes and washed mildly and mounted in water.

3.10.4 Mercuric bromophenol blue method

The mercuric bromophenol blue method was used to localise total proteins in the cells ( ). The stain was prepared by dissolving 10 g mercuric chloride and 500 mg bromophenol blue powder in 100 ml of ethanol. The deparaffinised slides were kept in the stain for 10 minutes. The slides were then passed through acidified water, dehydrated in alcohol-butanol series, cleared in xylol and mounted in DPX.

3.10.5 Feulgen staining for DNA

This method was used to localise DNA using the classical Feulgen reaction. On hydrolysis with normal HCl

the purine containing fraction of DNA is separated from the sugar, unmasking the aldehyde groups of the latter. Further the exposed aldehyde groups enter into combination with fuchsin sulphurous acid to yield a typical magenta colour.

The slides were deparaffinised brought upto alcohol level, coated with celloidin, and hydrated. The sections were hydrolysed in 5 N HCl at room temperature for a period of 15 minutes. The slides were washed thoroughly in running water and stained with leucobasic fuchsin for 10 minutes. After bleaching with 2 per cent potassium metabisulphite they were washed in running water. Sections were then dehydrated, cleared and mounted in DPX.

3.10.6 <u>Toludine blue method for DNA and RNA</u>

The DNA and RNA are located by the use of toludine blue with mercurin chloride or potassium iodide. The mordanted, dried dye yields a very intense colour staining both the types of nucleic acids. The sections were deparaffinised, hydrated and immersed in one per cent aqueous solution of toludine blue (pH 6.7 to 7.0) for 15 minutes. The slides were washed in running water, dehydrated, cleared in xyloland mounted in DPX and observed.

3.10.7 <u>Suddan III test for lipids</u>

Whole mounts of seeds were used for staining the lipids. Seeds were spread on a slide with a drop of gelatin and covered with cover slip, which was pressed gently

for proper spreading. The cover slip then removed carefully and the seeds were stained with a saturated alcoholic solution of Sudan III. Slides were washed inalcohhol and mounted in gycerine.

3.11 Photomicrography

Various stages of development were photomicrographed with the help of Olympus binocular microscope and Olympus camera PM6. by using slow speed film DK 5.

3.12 Cytological analysis of callus cultures

Well grown fresh callus bodies were taken out of the flaks and thoroughly washed in distilled water, the callus was pretreated with 0.002 M 8 hydroxyquinoline for 2 to 3 hours at 14°C. After the pretreatment the callus was fixed in a mixture of 1:1:1 chloroform, 95 per cent ethyl alcohol and glacial acetic acid for 1 hour and squashed and stained in 1 per cent acetoorcein.

The actively deviding cells were scored for the number of chromosomes at M1 and behaviour at A1. A minimum of 25 cells were scored in a slide to know the percentage of different chromosome numbers in different cells.

3.13 Statistical analysis

The results of the experiments were analysed statistically adopting completely randomised design as described by Sunderaraj et al.(1972). The experiments for standardisation of media were replicated four times and all the treatments were replicated three times.

RESULTS

CHAPTER IV

EXPERIMENTAL RESULTS

The results of the experiments conducted are reported in this chapter under three major heads.

4.1 Seed culture studies

The effect of different nutrient media on germination and growth of seeds, and the effect of growth regulators and other additives on growth of seedlings of different orchid species are reported in the follbwing paragraphs.

4.1.1 Seed structure

The orchid seeds are minute weighing 0.3 to 14 µg. The seeds of the selected species for the experiment varied in size and structure. Bletilla hyacinthina and Epidendrum radicans have comparatively large seeds. While Dendrobium moschatum seeds are very small and the seed coat is almost filled with embryo (Plate 2.1 to 5).

4.1.2 Bletilla hyacinthina

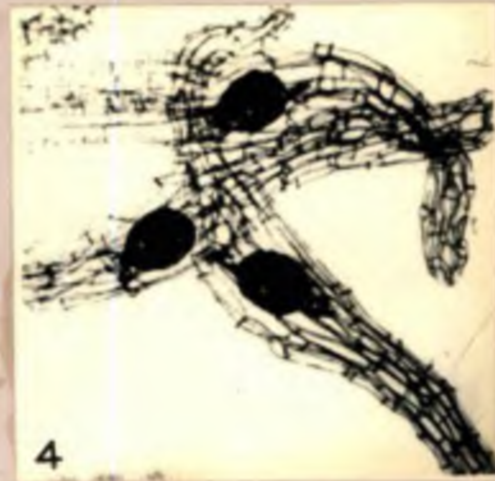
Nature seeds of Bletilla hyacinthina from undehisced capsules were tested for viability. About 83 per cent of the seeds contained viable embryos and the remaining 17 per cent were either sterile or with undeveloped embryos. Seeds were sown on different media (KC, MS, VW, BN and NVW) for standardising a suitable medium for germination and growth. The number of days for greening and further growth were recorded.(Table 6). The seeds took shortest period of seven

PLATE NO. 2.

<u>Seed Structure in different orchid species</u>

1. *Dendrobium moschatum*
2. *Vanda Coerulea*
3. *Epidendrum radicans*
4. *Spathoglottis plicata*
5. *Bletilla hyacinthina*

PLATE-2



days for greening in Vacin and Went medium. This was followed by KC medium (9 days) and the longest time was taken on BN medium (14 days). The seeds prior to germination imbibed water, swelled and formed into a protocorm like body (PLB) which later differentiated into shoot, root and pseudobulb.

The rate of leaf production was faster on Vacin and Went media.

The growth measurements of seedlings were recorded (Table 6). The seedling height was maximum on VW media (4.57 centi meter) so also the mean number of roots (2.41), fresh and dry weight and mean number of pseudobulbs (0.58) were maximum on VW medium. The mean number of leaves were maximum on BN medium(3.85) followed by MVW medium (3.82). The root length was maximum on MVW medium (1.256 centi meter) followed by VW medium (1.24 centi meter). Based on the above results Vacin and Went media was selected as a basic medium for further studies.

Effect of growth regulators and other additives

The treatments were applied inidividually and also in combinations. The individual treatments were IAA, NAA, IBA and 2,4-D at 1, 3 and 5 ppm, GA 0.1, 0.3 and 0.5 ppm BA and Kinetin 1, 3 and 5 ppm, Pyridoxin and Thiamin 0.5, 1 and 3 ppm Coconut water and Banana pulp 10 and 15 per cent and control.

The results of the experiments are given in Table 8. IAA at all 3 levels were superior to control. Maximum plant growth was obtained at 3 ppm.

Table 6. Effect of different nutrient media on seed germination and subsequent growth of *Bletilla hyacinthina* seedlings

Nutrient media	No. of days taken for				
	Greening	First leaf	Second leaf	Third leaf	Fourth leaf
Knudson C. (KC)	9	32	57	80	104
Murashige-Skoog (MS)	11	35	60	81	116
Vacin and Went (VW)	7	28	50	70	89
Burgeff N_3F (BN)	14	38	63	86	114
Modified Vacin and Went (MVW)	12	33	56	79	101

Table 7. Effect of nutrient media on growth and development of *Bletilla hyacinthina* seedlings

Nutrient media	Mean						Percentage
	Plant height cm	Leaf number	Root number	Root length cm	Fresh wt. mg	Dry wt. mg	
Knudson C. (KC)	2.754	2.99	1.86	0.649	41.78	2.98	7.13
Murashige-Skoog (MS)	2.534	3.16	1.75	0.841	63.63	3.88	6.09
Vacin and Went (VW)	4.570	3.55	2.41	1.240	88.79	5.50	6.19
Burgeff N_3F (BN)	1.961	3.85	1.64	0.985	46.81	2.95	6.30
Modified Vacin and Went (MVW)	1.924	3.82	1.70	1.256	46.50	3.00	6.45
F-Test	**	**	*	**	*	*	
S-Em±	0.195	0.195	0.133	0.042	0.243	0.62	
C.D.	0.60	0.301	0.409	0.130	0.743	1.91	

* Significant at 0.05 ** Significant at 0.01 NS Not significant

NAA at 3 levels produced better results. Maximum plant height (36.59 mm), leaf and root number (3.86 and 1.41), root length (.8.27 mm) and fresh and/dry weight were obtained with 1 ppm NAA. NAA at 3 and 5 ppm produced more callus along with few plantlets IBA did not give any good results at all the 3 levels.

No plantlets were formed on treatments with 2, 4-D, while heavy callussing was observed at all 3 levels and with optimum at 1 ppm. Higher levels of 2, 4-D caused blackening of the callus bodies followed by degeneration.

GA treatments at 0.1 and 0.5 ppm levels were superior to control in characters like plant height (28.65 mm), leaf number (3.0), root number (1.1), root length (6.6 mm) and green and dry weight. BA at any level is not comparable with control. But kinetin at higher levels (3 and 5 ppm) were slightly superior in plant height, and green and dry weight. The best results were obtained with 3 ppm kinetin on plant height (15.67 mm), root number (0.52 mm), root length (1.42 mm) and green and dry weight. The leaf number was maximum at 5 ppm level (2.66).

Effect of vitamins

Vitamins such as nicotinic acid, pyrodoxin and thiamin at all the three levels did not give any significant results on growth and development of seedlings.

Effect of additives

Coconut water at 15 per cent was significantly superior to control. Treatment with 10 per cent ~~akk~~ was slightly

superior to control. Maximum plant height (23.71 mm), leaf (3.27) and root (2.32) number, root length (5.87 mm) and green and dry weight was obtained on treatments with 15 per cent coconut water.

Tomato juice was treated at 10 and 15 per cent levels. The germination and initial development of seeds were very poor at all levels. All seedlings were stout at the base and distorted in appearance. However treatment with 10 per cent tomato pulp was slightly superior to control in plant height (14.75 mm), leaf number (3.36) and green and dry weight. All other treatments with tomato juice were inferior to control.

Treatment with banana pulp at both levels (10 and 15 per cent) produced significantly superior results in growth and development of seedlings. Banana pulp in the medium had delayed germination for 7 to 10 days in both treatments, however, the subsequent growth and development was faster. Treatment with 15 per cent banana pulp had produced maximum plant height (41.74 mm), root length (5.06 mm) and green and dry weight. Addition of banana pulp to an extent of 10 per cent produced more number of leaves (3.14) and roots (1.2) compared to control.

A treatment combination was made with the following additives on basic medium. NAA 1 ppm + IAA 3 ppm + GA 3 ppm + Coconut water 15 per cent and banana pulp 15 per cent. The combined effect of growth substances with and without coconut water and banana pulp were studied. The results are given in (Table 9)

Table 8. Effect of individual treatments with different levels of growth substances and other additives on growth of *Bletilla hyacinthina* seedlings

Treatments with Vacin and Went media		Mean: Height of plants mm	Mean: Number of leaves -	Mean: Number of roots -	Mean: Root length mm	Mean: Green weight mg	Mean: Dry weight mg	Percentage of dry weight
1		2	3	4	5	6	7	8
IAA	1 ppm	17.42	1.82	0.6	2.20	6.80	0.41	6.03
	3 "	29.75	3.0	1.4	5.0	12.50	0.74	5.92
	5 "	14.84	2.38	0.66	4.13	9.10	0.56	6.15
NAA	1 "	36.59	3.86	1.41	8.27	22.72	1.38	6.07
	3 "	12.32	1.30	0.25	1.70	10.14	0.70	6.90
	5 "	10.25	1.40	0.75	1.50	13.12	0.75	5.72
IBA	1 "	11.74	2.10	0.96	1.84	7.20	0.60	6.94
	3 "	9.37	1.80	1.40	2.35	13.70	0.86	6.25
	5 "	9.00	1.90	1.33	2.50	12.96	0.80	6.05
2,4-D	1 "	Produced only Callus, no plantlets						
	3 "	Good callussing at 1 ppm						
	5 "	"						
GA	0.1 ppm	12.70	1.70	1.04	3.20	7.65	0.47	6.14
	0.3 "	28.65	3.00	0.80	5.50	8.00	0.50	5.80
	0.5 "	14.50	2.90	1.10	6.60	12.80	0.87	6.79

contd.

1		2	3	4	5	6	7	8
BA	1 ppm	8.20	1.47	1.20	1.70	11.04	0.75	6.80
	3 ppm	8.36	1.23	1.14	1.45	8.33	0.54	6.48
	5 ppm	6.75	1.04	0.75	1.21	7.50	0.45	6.00
Kinetin	1 "	6.52	1.30	0.37	1.40	2.10	0.14	6.66
	3 "	15.67	2.55	0.52	1.42	6.75	0.45	6.67
	5 "	14.33	2.66	0.18	0.51	6.50	0.45	6.92
Nicotinic Acid								
	0.5ppm	8.27	1.04	0.60	1.10	5.45	0.38	6.97
	1.0 "	9.11	1.15	0.32	1.34	6.95	0.52	6.47
	3.0 "	10.71	1.70	0.85	2.20	7.40	0.51	6.89
Pyridoxin								
	0.5 "	11.62	1.52	0.68	0.74	7.80	0.49	6.28
	1.0 "	12.71	2.10	1.04	0.54	8.45	0.55	6.51
	3.0 "	9.54	1.92	0.86	0.92	7.20	0.45	6.25
Thiamin	0.5 "	10.27	2.20	3.34	1.20	7.15	0.45	6.29
	1.0 "	8.01	1.85	0.52	0.77	6.65	0.40	6.01
	3.0 "	6.90	1.76	0.95	0.25	5.25	0.35	6.66
Coconut water								
	10%	15.31	2.34	0.28	1.68	6.00	0.40	6.66
	15%	23.77	3.27	2.32	5.84	16.87	1.15	6.81

contd.

1	2	3	4	5	6	7	8
Tomato juice 10%	14.75	3.36	0.03	0.13	21.87	1.32	6.03
15%	7.24	2.38	0.84	1.44	7.85	0.51	6.49
Banana Pulp 10%	35.74	3.14	1.20	4.54	10.00	0.62	6.20
15%	41.74	2.51	0.98	5.06	18.18	1.12	6.16
Control(VW)	9.53	1.50	0.28	1.06	12.87	0.77	5.98
F-test	**	**	*	*	**	**	
S.Em±	1.99	0.243	0.273	0.927	1.248	0.030	
C.D.	6.149	0.672	0.843	2.854	3.443	0.222	

*Significant at 0.05

**Significant at 0.01

NS Not significant

The seedling growth was faster and quick banana pulp 15 per cent and coconut water 15 per cent was used along with NAA 1 ppm, IAA 3 ppm and GA 3 ppm. The plant height (47.25 mm), root number (2.60) and green and dry weight were maximum with this treatment. The leaf number (3.45) and root length (9.15 mm) were also maximum with tr eatment of NAA 1 ppm, however, all the treatments proved superior to control (individual treatment of additives).

4.1.3 Phaius wallichii

The self pollinated flowers of Phaius wallichii took 8 months for maturity. The mature capsules were 2.5 to 4 centimeter long and 2 to 3 centimeter thick and contained 61 per cent viable seeds. Mature seeds from undehisced capsuled were sown on different media (KC, MS, VW, BN and MVW). The time taken for germination and growth are given in Table.10. In general the seed germination was very slow in Phaius, compared to other species. Greening of the seeds were faster on Vacin and Went medium (40 days) followed by Knudson C medium (51 days). Murashige-Skoog medium took maximum time of 59 days and in Burgeff N_3F seeds did not germinate(Table 10).

The maximum rate of leaf production was observed on Vacin and Went medium followed by modified Vacin and Went medium. While it was very slow on MS medium. The growth measurements of the seedlings have varying results on different media(Table 11). The mean height of seedlings(12.70 mm), leaf number (1.85), root

Table 9. Effect of growth regulators and other additives in combination on the growth of <u>Bletilla hyacinthina</u> seedlings

Treatment combinations on Vacin and Went medium	Mean						Percentage of dry weight
	Plant height mm	Leaf No. -	Root No. -	Root length mm	Fresh weight mg	Dry weight mg	
IAA 3 ppm	25.75	2.40	1.37	5.11	14.50	0.91	6.27
NAA 1 ppm	39.70	3.45	1.17	9.15	20.40	1.22	5.98
GA 0.3 ppm	26.90	2.75	1.01	6.10	15.40	0.93	6.04
Banana pulp 15%	40.30	3.11	1.22	7.14	20.12	1.21	6.01
Coconut water 15%	26.42	3.15	2.21	6.18	18.42	1.29	7.00
Vacin and Went media	18.20	2.10	1.20	3.20	10.70	0.66	6.16
IAA 3 ppm+ NAA 1 ppm+ GA 0.3 ppm	22.70	2.14	1.10	7.20	12.15	0.72	5.92
Coconut water 15% + IAA 3 ppm + NAA 1 ppm+ GA.3 ppm	37.20	2.65	1.25	7.40	18.10	1.16	6.41
Banana pulp 15% + IAA 3 ppm+ NAA 1ppm + GA 0.3 ppm	39.10	3.15	2.10	6.50	19.10	1.25	6.54
Coconut water 15%+ banana pulp 15% +NAA 1 ppm + IAA 3 ppm+ GA 0.3 ppm	47.25	3.42	2.60	7.55	24.20	1.45	5.99
F-Test	**	NS	**	**	**	*	
S.Em±	3.546	0.351	0.125	0.705	1.384	0.917	
C.D.	10.534	1.044	0.372	2.09	4.111	0.272	

* Significant at 0.05 ** Significant at 0.01 NS Not significant

numbers (0.96), root length (0.75 mm) green and dry weight were maximum on Vacin and Went medium. This was closely followed by modified Vacin and Went medium. Seeds cultured on Burgeff N_3F medium did not germinate even after 120 days of sowing.

Based on the results, Vacin and Went medium was selected as a basic medium for further treatments. Treatments with growth regulators and other additives were made individually and in combinations.

Effect of growth regulators

Individual treatments were made with 3 levels each (1, 3 and 5 ppm) of NAA, IAA, IBA and 2, 4-D. 3 levels of GA and BA (0.5, 1, 3 ppm) and 15 per cent coconut water and banana pulp. The results are given in Table 12. Growth on all the 3 levels of IAA were quite satisfactory. Plant height (23.57 mm), leaf number (3.1), root length (0.97 mm) and green and dry weight were recorded maximum with a treatment of 5 ppm IAA. IBA at higher levels (3 and 5 ppm) had produced promising results. IBA 1 ppm produced mostly callus and few plantlets. The plant height (19.76 mm), leaf number (2.74), root number (1.74), root length (0.95 mm) and green and dry weight were recorded maximum on 5 ppm IBA. Growth performance at different levels of NAA were not significant. Treatment with different levels of 2, 4-D, GA and BA did not produce any seedling even after 120 days. Some of the treatments produced very little callus, but no plantlets.

Table 10. Effect of nutrient media on seed germination and leaf production in *Phaius wallichii* seedlings

Nutrient media	Mean number of days for greening	Ist leaf	IInd leaf	IIIrd leaf	IVth leaf
Knudson C (KC)	51	74	106	124	152
Murashige-Skoog(MS)	59	83	112	137	164
Vacin and Went(VW)	40	66	92	113	140
Burgeff N_3F (BN)	No germination				
Modified Vacin and Went (MVW)	48	72	102	118	145

Table 11. Effect of different nutrient media on growth and development of *Phaius wallichii* seedlings

Nutrient media	Mean						Percentage of dry weight
	Height of plants (mm)	Number of leaves (-)	Number of roots (-)	Root length (mm)	Fresh wt. (mg)	Dry wt. (mg)	
Kundson C	6.13	1.05	0.72	0.55	5.21	0.31	5.95
Murashige-Skoog	5.85	0.73	0.42	0.21	4.13	0.24	5.81
Vacin and Went	12.70	1.85	0.96	0.75	10.27	0.66	6.42
Burgeff N_3F	-	-	-	-	-	-	-
Modified Vacin and Went	10.27	1.24	0.82	0.63	7.26	0.47	6.47
F-Test	**	**	**	**	**	**	
S.Em±	0.743	0.108	0.059	0.039	0.417	0.026	
C.D.	2.290	0.332	0.184	0.119	1.283	0.082	

*Significant at 0.05 ** Significant at 0.01 NS Not significant

Treatment with 15 per cent banana pulp was superior to control. Satisfactory growth measurements were obtained on plant height (12.70 mm), leaf number (2.46), root number (1.02), root length (.096), and green dry weight. Treatment with 15 per cent coconut water did not produce significant results. However, the plant height and fresh and dry weight were superior over control.

Combination of treatments were fixed based on the promising results obtained with individual treatment of growth substances and other additives. A combination of growth substances, IAA 5 ppm and IBA 5 ppm were used with and without coconut water (15 per cent CW) and banana pulp (15 per cent). The treatment and its effect are given in Table 13. The treatment combinations were compared with individual treatments.

Growth regulators with banana pulp (15 per cent) produced maximum plant height (22.20 mm) and fresh and dry weight. Combination of all the 4 additives recorded plant height (20 mm) and fresh and dry weight (17.11/1.13 mg), which is closely followed by the above treatment.

Root number was ~~maximum~~ highest with banana pulp alone (1.26) followed by IAA 5 ppm (1.25).

Root length was maximum with banana pulp (1.21 mm) and which is followed by growth substances with banana pulp (1.05 mm).

Table 12: Effect of individual treatment of growth regulators and other additives on growth and development of <u>Phaius wallichii</u>

Treatments with Vacin and Went basic media	Mean						Percentage of dry weight
	Plant height mm	Leaf number	Root number	Root length	Fresh weight mg	Dry weight mg	
IAA 1 ppm	16.75	2.40	1.20	0.87	12.30	0.74	6.01
IAA 3 ppm	12.75	2.69	1.07	0.83	11.70	0.72	6.15
IAA 5 ppm	23.57	3.10	1.30	0.97	17.40	1.14	6.55
NAA 1 ppm	7.59	2.87	1.17	0.52	4.18	0.24	5.74
NAA 3 ppm	5.47	1.95	0.75	0.47	3.12	0.19	6.08
NAA 5 ppm	9.76	2.20	1.32	0.84	5.40	0.31	5.74
IBA 1 ppm	7.59	1.24	0.60	0.23	3.20	0.19	5.93
IBA 3 ppm	16.40	2.40	1.25	0.75	9.11	0.59	6.47
IBA 5 ppm	19.76	2.74	1.74	0.95	14.21	0.95	6.68
2,4-D 1,3 and 5 ppm		No germination					
GA 0.5,1 and 3 ppm		"					
BA 0.5,1 and 3 ppm		"					
Coconut water 15%	9.96	1.04	1.12	0.83	11.70	0.70	5.98
Banana pulp 15%	12.70	2.46	1.02	0.96	13.20	0.86	6.51
Control(VW)	6.32	1.45	1.26	0.93	9.25	0.54	5.83
F-test	**	**	**	**			NS
S-Em±	1.35	0.162	0.144	0.118		NS	
C.D.	4.18	0.501	0.476	0.363			

* Significant at 0.05   ** Significant at 0.01   NS Not significant

3. Spathoglottis plicata (BL)

Mature seeds of Spathoglottis plicata were sown on different media (KC, MS, VW, BN and MVW) for standardisation of the media. The number of days taken for greening, germination and further development were recorded (Table 14) (Plate 3.1). The data indicated that, rapid seed germination is with MS medium (19 days) followed by KC medium (19.3 days). All the other 3 media took more than 3 weeks for greening. The embryo at this stage imbibe water and nutrients from the medium, enlarge and break open the seed coat.Initially they turn dark brown and later turns to green and forms protocorm like bodies (PLBs). This in turn produces shoot apex and grow into a plantlet with leaves and pseudobulbs.

The rate of leaf production was maximum on MS medium, followed by modified Vacin and Went medium and Knudson C medium. Other two media had taken more number of days for leaf production. The data also indicate that, with modified Vacin and Went medium there is growth suppression at germination level, but later when the seeds germinated the rate of leaf production was more fast.

The growth measurements of the seedlings recorded indicate varying response in different media. The mean seedling height was maximum with basic MS medium (5.57 cm). whereas the plant height on all the other media were below 3.1 centi meter. The leaf number was also more with MS medium (5.5) followed by Vacin and Went medium (5.07). The root number

PLATE No.3

Seed Culture Studies

1. Effect of different media on germination and growth of *Spathoglottis plicata* seeds

2. Effect of coconut water and banana pulp on root formation in *Spathoglottis plicata*

3. Plant height and growth of *Spathoglottis plicata* as influenced by auxins and banana pulp in the medium.

PLATE - 3

1

2

3

Table 13. Effect of treatment combinations with growth regulators and other additives on growth and development of *Phaius wallichii* seedlings

Treatment combinations on Vacin and Went medium	Mean						Percentage of dry weight
	Plant height mm	Leaf No. -	Root No. -	Root length mm	Fresh weight mg	Dry weight mg	
IAA 5 ppm	19.70	3.30	1.25	0.85	16.46	1.07	6.50
IBA 5 ppm	16.75	2.33	1.16	0.68	9.45	0.58	6.13
Banana pulp 15%	14.78	3.01	1.26	1.21	15.84	1.06	6.69
Coconut water 15%	11.33	1.15	0.86	0.76	9.35	0.57	6.09
IAA 5 ppm +IBA 5 ppm	15.21	2.11	1.67	0.73	8.24	0.52	6.31
IAA 5 ppm +IBA 5 ppm + Banana pulp 15%	22.20	3.25	1.30	1.05	19.45	1.30	6.68
IAA 5 ppm +IBA 5 ppm + Coconut water 15%	12.23	1.70	0.76	0.60	8.30	0.51	6.14
IAA 5 ppm +IBA 5ppm + Coconut water 15% + Banana pulp 15%	20.00	2.26	1.11	0.84	17.11	1.13	6.35
F-Test	**	**	*	NS	**	**	
S-Em±	1.275	0.214	0.148	0.138	1.975	0.744	
C.D.	3.87	0.650	0.449	0.420	5.990	0.402	

* Significant at 0.05   ** Significant at 0.01   NS Not significant

increased in modified Vacin and Went medium (4.41#), but the root length was more with MS medium (14.0 centi meter). The green and dry weight was also maximum with MS medium (Table 15).

Based on these results MS mediumwas used as a basic medium for further treatment. The treatments were applied individually and in combinations (IAA, NAA, IBA and 2,4-D at 1,3 and 5 ppm, GA and BA at 0.5, 1 and 3 ppm, Coconut water and Banana pulp 15 per cent).

Among the auxins NAA at 1 ppm level had produced maximum plant height (4.89 centi meter), followed by IBA at 5 ppm (3.88 centi meter) and IAA at 1 ppm (3.47 centi meter). All the other treatments with different level of auxins were inferior to control (Table 16).

Treatments with 2, 4-D at all the three levels induced excellent callus growth. The rate of callussing was more at 1 and 3 ppm levels. At 5 ppm level severe blackening of the tissues were noticed. NAA at 5 ppm also produced good amount of callus along with few number of seedlings.

More number of leaves were produced with 5 ppm IBA, followed by 1 ppm NAA. IAA at two levels (1 and 5 ppm) produced more number of leaves than control. All other treatments were inferior to control. Maximum number of roots were produced with 1 ppm NAA (1.85), followed by NAA 3 and 5 ppm (1.52 and 1.21 numbers respectively) and IBA 3 ppm (1.14).

Table 14. Effect of different nutrient media on germination and leaf production in <u>Spathoglottis plicata</u>

Nutrient media	Number of days for greening	Production of leaves			
		Ist leaf	IInd leaf	IIIrd leaf	IVth leaf
Knudson C (KC)	19.3	42	78	105	133
Murashige-Skoog(MS)	19.0	40	65	87	116
Vacin and Went(VW)	23.5	68	98	126	154
Burgeff N_3F(BN)	22.5	57	89	116	168
Modified Vacin and Went (MVW)	25.0	58	84	110	131

Table 15. Effect of different nutrient media on growth and development of <u>Spathoglottis plicata</u> seedlings

Nutrient media	Mean						Percentage of dry wt.
	Plant height mm	Leaf No. -	Root No. -	Root length mm	Fresh wt. mg	Dry wt. mg	
Knudson C(KC)	3.02	4.5	2.30	45.50	59.0	3.60	6.10
Murashige-Skoog (MS)	5.57	5.5	3.50	60.40	140.0	9.30	6.60
Vacin and Went (VW)	1.78	3.3	1.52	13.50	52.4	3.28	6.25
Burgeff N_3F(BN)	2.71	4.4	1.50	16.66	40.7	2.50	6.14
Modified Vacin and Went (MVW)	2.70	5.07	4.41	41.20	55.1	3.66	6.64
F-Test	**	**	**	**	**	**	
S-Em±	2.22	0.349	0.281	2.746	3.924	0.32	
C.D.	0.685	0.766	0.867	7.342	12.091	0.986	

** Significant at 0.01

Table 16. Effect of different growth regulators and additives on growth of *Spathoglottis plicata* seedlings

Treatments with MS medium		Means: Height of plants Cm	No.of leaves –	No.of roots –	Root length Cm	Fresh wt. mg	Dry wt. mg	Percentage of dry weight
1		2	3	4	5	6	7	8
IAA	1 ppm	3.47	3.94	0.84	0.53	25.00	1.710	6.84
IAA	3 ppm	2.73	3.45	0.47	0.32	25.45	1.860	7.30
IAA	5 ppm	2.61	3.90	0.16	0.22	21.69	1.475	6.80
NAA	1 ppm	4.89	4.00	1.85	1.42	24.00	1.632	6.80
NAA	3 ppm	3.03	3.53	1.53	1.40	17.00	1.190	7.00
NAA	5 ppm	2.67	3.60	1.21	1.35	17.60	1.214	6.89
IBA	1 ppm	2.39	3.64	0.54	0.29	15.00	1.056	5.83
IBA	3 ppm	3.42	3.88	1.14	0.81	47.00	3.055	6.50
IBA	5 ppm	3.88	4.18	0.98	0.99	61.81	3.860	6.24
2,4-D	1 ppm	No growth-callussing moderate						
2,4-D	3 ppm	-do-	Good callussing					
2,4-D	5 ppm	-do-	Callus blackened					
GA_3	0.5"	3.22	3.21	1.74	1.730	28.10	1.854	6.59
GA_3	1 ppm	3.46	3.01	3.00	2.47	31.60	2.085	6.60
GA_3	3 ppm	2.21	2.90	0.70	0.47	14.70	0.985	6.70

contd.

1	2	3	4	5	6	7	8
BA 0.5 ppm	2.27	3.10	1.20	1.63	13.20	0.871	6.60
BA 1.0 ppm	Moderately callussed no growth						
BA 3.0 ppm		-do-					
Banana pulp 15%	4.05	3.54	1.56	1.22	30.26	2.320	7.06
Coconut water 15%	3.87	3.62	1.20	1.07	25.10	1.681	6.68
Control(MS)	3.38	3.66	0.85	0.57	27.27	1.840	6.74
F-Test	**	**	**	**	**	**	
S-Em±	0.239	0.485	0.129	0.112	2.48	0.740	
CD.	0.736	1.492	0.398	0.346	3.411	0.484	

** Significant at 0.01

Maximum root length was noticed with 1 ppm. NAA (1.42 centimeter) followed by 3 ppm (1.4 centi meter) and 5 ppm (1.35 centi meter) NAA and 5 and 3 ppm (0.99 centi meter and 0.81 centi meter) IBA. Other treatments were inferior to control.

The fresh and dry weight were maximum with 5 ppm IBA followed by 3 ppm IBA. All the other treatments had lower fresh and dry weight compared to control.

Treatments with three levels of GA did not produce any significant difference in plant height or leaf number. But a significant difference was noticed in root number (3) and root length (2.04 centi meter) (Table 15). Treatment with BA at all the three levels were inferior to control, however they formed moderate callus instead of producing plantlets.

Treatments with 15 per cent banana pulp produced more plant height (4.053 centi meter), root numbers (1.56) and root length (1.225 centi meter), compared to control. The fresh and dry weight was also more compared to control (Table 15).

Treatment with coconut water (15 per cent) also produced increased plant height (38.74 centi meter), root number (1.2) and root length (1.07 centi meter). However the fresh and dry weight were inferior to control.

The combined effect of auxins with and without banana pulp and coconut water were also studied (Table 17). MS media with coconut water at all the levels with 3 auxins produced maximum plant height (11.06 centi meter) and leaf number (2.96).

Table 17. Effect of different growth regulators and other additives in combination on growth of *Spathoglottis plicata* seedlings

Treatment combinations on MS medium	Mean						Percentage of dry weight
	Plant height cm	Leaf No. -	Root No. -	Root length cm	Fresh weight mg	Dry weight mg	
NAA 1 ppm	6.475	2.32	2.11	2.27	32.1	2.17	6.76
IAA 1 ppm	5.865	2.10	1.16	1.91	29.7	1.85	6.22
IBA 5 ppm	6.251	2.25	2.40	3.10	39.3	2.78	7.00
NAA 1ppm +IAA 1ppm + IBA 5ppm	7.728	2.92	2.84	3.27	72.3	4.75	6.56
Coconut water 15%+ NAA 1ppm+IAA 1ppm + IBA 5ppm	6.108	2.96	2.23	3.32	30.5	1.95	6.39
Banana pulp 15%+ NAA and IAA 1 ppm + IBA 5ppm	8.660	2.63	2.73	2.24	36.7	2.31	6.29
Banana pulp 15%+ Coconut water 15%+ NAA and IAA 1 ppm + IBA 5ppm	11.430	2.84	3.44	2.17	81.40	5.02	6.18
Coconut water 15%	5.470	1.86	1.14	1.98	19.41	1.17	6.02
Banana pulp 15%	10.70	2.63	2.52	2.44	26.31	1.71	6.49
F-Test	**	**	**	**	*	*	
S-Em±	0.859	0.158	0.340	0.175			
C.D.	2.649	0.489	1.049	0.540			

*Significant at 0.05 ** Significant at 0.01

Whereas the same medium with banana pulp produced more number of roots (3.56). (Plate 3.2), root length (4.25 centi meter), fresh weight (89 mg) and dry weight (6.13 mg).

A combination of all the growth substances had results on par with individual treatments. A treatment with coconut water and growth substances produced results identical to individual treatments. The combined effect of banana pulp with growth substances had also given promising results (Plate 3.3).

4.1.5 Dendrobium moschatum

The seeds were sown in different media for standardisation. The percentage of germination was high on Murashige-Skoog medium (83 per cent) followed by Burgeff N_3F (77 per cent) Knudson C (74 per cent), Vacin and Went (72.5 per cent) and modified Vacin and Went (68 per cent).

The germination was faster on Burgeff N_3F medium, followed by MS medium. The rate of leaf production was also faster with Burgeff N_3F medium, which was closely followed by MS medium. Very slow rate of leaf production was observed with Knudson C and Vacin and Went medium (Table 18.)

The Burgeff N_3F medium was used as the basic medium for the experiments along with additives. Individual treatments were applied at 3 levels of IAA, NAA, IBA and 2,4-D (1, 3 and 5 ppm each), GA and BA (0.1, 1 and 3 ppm), Coconut water 15 per cent and Banana pulp 15 per cent. The

Table 18. Effect of different nutrient media on germination and leaf production in Dendrobium moschatum

Nutrient media	Mean number of days for				
	greening	Ist leaf	IInd leaf	IIIrd leaf	IVth leaf
Knudson C(KC)	16.5	42.6	60.50	79.50	-
Murashige-Skoog(MS)	20.0	47.25	70.20	90.25	-
Vacin and Went(VW)	25.5	55.30	75.20	96.20	-
Burgeff N_3F (BN)	12.5	38.50	51.25	70.50	-
Modified Vacin and Went(MVW)	27.0	57.50	75.80	93.25	-

Table 19. Effect of different media on growth and development of Dendrobium moschatum seedlings

Nutrient media	Mean						Percentage of dry weight
	Plant height mm	Leaf No. -	Root No. -	Root length mm	Fresh weight mg	Dry weight mg	
Knudson C (KC)	6.13	2.05	-	-	0.65	0.039	6.00
Murashige-Skoog (MS)	12.75	2.37	-	-	1.22	0.070	5.73
Vacin and Went (VW)	3.47	1.07	-	-	0.25	0.015	6.00
Burgeff N_3F(BN)	14.75	2.42	0.40	0.05	1.75	0.113	6.45
Modified Vacin and Went(MVW)	7.75	1.15	0.15	-	0.68	0.042	6.17
F-Test	**	**			**	*	
S.Em±	0.613	0.153	-	-	0.227	0.004	
C.D.	1.88	0.472			0.686	0.013	

*Significant at 0.05 ** Significant at 0.01

Effect of treatments are given in (Table 20). The plant height, leaf number, root number and root length were significantly high with a treatment of IAA, GA, banana pulp and coconut water. Maximum plant height (32.7 mm) was obtained on Burgeff N_3F medium with 1 ppm GA followed by banana pulp (32. 21 mm), Coconut water (29.30 mm) and IAA. 1 ppm (28.43 mm). Other treatments were not significantly superior.

The leaf number was maximum on medium with banana pulp (3.3) followed by IAA 1 ppm (3.21), GA 1 ppm (3.1) and coconut water 15 per cent (2.96). Root production was high with IAA, GA_3, banana and coconut water. Maximum root number and root growth was noticed on medium with banana pulp (1.77 nos/0.14 mm), followed by GA_3 1 ppm and coconut water.

The fresh and dry weight were maximum on media with banana pulp (2.45/0.159 mg), followed by coconut water (1[illegible]73/ 0.197 mg) and GA_3 1.0 ppm (1.45/0.089 mg) (Table 20).

Banana pulp induced stout, healthy seedlings with dark green foliage, whereas only coconut water induced pale and thin seedlings. NAA and IBA at 3 levels did not give any significant results. 2, 4-D at all the 3 levels did not produce seedlings, but produced few number of callii which later became black. BA at all the 3 levels resulted in very poor growth.

Based on the above results a combination of treatments were fixed and applied with and without coconut water and banana pulp. The treatment combination of IAA 1 ppm, GA 1 ppm, banana

pulp 15 per cent and coconut water 15 per cent were made. A significantly high rate of growth and development was obtained with a combination of IAA 1 ppm GA 1 ppm and banana pulp 15 per cent. None of the other treatments were significantly superior to individual treatments. However, a treatment combination of all the four additives had given healthy seedlings with dark green foliage (Table 21).

4.1.6 Epidendrum radicans

The flowers of Epidendrum radicans were selfed. The capsules took 55 to 60 days for maturity. The mature seeds from undehisced capsules were sown on different media for standardisation. The highest percentage of germination was obtained with Vacin and Went medium (79 per cent) followed by modified Vacin and Went medium (72 per cent), MS medium (68 per cent), Knudson C medium (61 per cent) and Burgeff N_3F medium (58 per cent).

The number of days taken for greening, and leaf production were recorded (Table 22). The shortest period taken for greening was with Vacin and Went medium (17 days), followed by KC medium (19 days) and MS medium (21 days). The other two media took the maximum time (24 days) for germination. The rate of leaf production recorded was maximum on modified Vacin and Went medium, followed by Vacin and Went medium. However, the germination and initial development of leaf on modified Vacin and Went medium was slow compared to plants on Vacin and Went medium. The rate of leaf development on other 3 media were very slow.

PLATE NO. 4

Seed Culture Studies

1. Effect of GA, NAA and coconut water on plant height in *Vanda coerulea*

2. Effect of banana pulp on growth and development of Vanda coerulea seedlings.

PLATE - 4

1

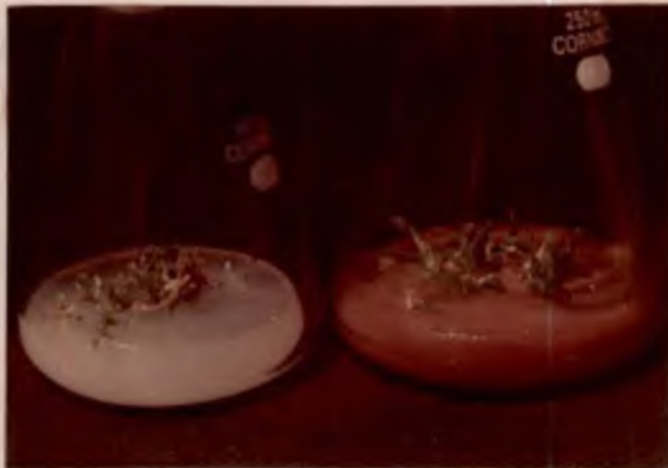


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Table 20. Effect of different growth regulators and other additives on the growth of *Dendrobium moschatum* seedlings

Treatments with Burgeff N_3 F medium.	Mean						Percentage of dry wt.
	Plant height	Leaf No.	Root No.	Root length	Fresh weight	Dry weight	
	mm	-	-	mm	mg	mg	
1	2	3	4	5	6	7	8
IAA 1 ppm	28.43	3.21	0.40	0.09	1.27	0.082	6.45
IAA 3 ppm	22.25	2.85	0.21	0.04	0.93	0.057	6.13
IAA 5 ppm	23.80	2.26	0.16	0.02	0.87	0.055	6.32
NAA 1 ppm	11.70	2.21	-	-	0.65	0.039	6.00
NAA 3 ppm	15.40	2.17	-	-	0.67	0.042	6.26
NAA 5 ppm	12.35	1.40	-	-	0.42	0.024	5.71
IBA 1 ppm	10.32	1.13	-	-	0.62	0.018	6.12
IBA 3 ppm	18.24	1.10	-	-	0.55	0.041	7.45
IBA 5 ppm	16.11	0.96	-	-	0.63	0.040	6.35
2,4-D 1,3 and 5ppm	No germination and growth						
GA_3 0.1 ppm	26.35	2.64	0.86	0.02	1.17	0.071	6.07
GA_3 1.00ppm	32.70	3.10	1.42	0.05	1.45	0.089	6.14
GA_3 3.0 ppm	20.20	2.25	0.33	0.02	0.76	0.045	5.92

Contd.

1	2	3	4	5	6	7	8
BA 0.1 ppm	9.40	1.02	-	-	0.57	0.033	5.79
BA 1 ppm	8.75	0.79	-	-	0.32	0.019	5.94
BA 3 ppm	8.00	0.85	-	-	0.35	0.019	5.43
Coconut water 15%	29.30	2.96	1.05	0.09	1.73	0.107	6.18
Banana pulp 15%	32.21	3.30	1.77	0.14	2.45	0.159	6.49
Control (BN)	20.70	2.15	0.82	0.06	0.88	0.052	5.90
F-Test	**	**	**	**	**	**	
S-Em±	2.255	0.210	-	-	-	-	-
C.D.	6.95	0.649	-	-	-	-	-

** Significant at 0.01

Table 21. Effect of different growth regulators and other additives in combination on growth and development of *Dendrobium moschatum* seedlings

Treatment combination with Burgeff N_3 F medium	Means						Percentage of dry weight
	Plant height mm	Leaf No. -	Root No. -	Root length mm	Fresh weight mg	Dry weight mg	
IAA 1 ppm	24.17	2.74	0.94	0.04	0.85	0.05	5.88
GA_3 1 ppm	29.42	2.55	0.85	0.03	0.92	0.06	6.52
Coconut water 15%	22.25	1.44	0.60	0.01	0.63	0.03	5.87
Banana pulp 15%	30.30	2.92	1.23	0.09	1.34	0.08	5.97
IAA 1ppm+$GA_3$1ppm	26.26	1.33	0.77	0.06	0.62	0.03	5.97
IAA 1ppm+$GA_3$1ppm+ Banana pulp 15%	35.46	3.33	1.55	0.19	2.26	0.14	5.38
IAA 1ppm+$GA_3$1ppm + Coconut water 15%	24.30	1.47	0.65	0.08	0.78	0.05	6.41
IAA 1ppm+ $GA_3$1ppm+ Coconut water 15%+ Banana pulp 15%	30.75	2.67	1.14	0.14	1.45	0.09	6.20
F-test	**	**	**	**	**	**	
S-Em±	2.105	0.141	-	-	-	-	
C.D.	6.387	0.448	0.321	-	-	-	

**Significant at 0.01

The growth measurement of the seedlings were recorded. The height of the seedlings were maximum on Vacin and Went medium (44.3 mm) which was immediately followed by modified Vacin and Went medium (38.21 mm). The maximum leaf number was recorded on modified Vacin and Went medium (4.36) followed by Vacin and Went medium (4.20). Root number was more on Vacin and Went medium (2.48) followed by modified Vacin and Went medium (2.12). But root length was more on Vacin and Went medium (21.20 mm). The green and dry weight was also more with Vacin and Went medium.

The Vacin and Went medium was selected as the basic medium for further treatments. Individual treatments with NAA, IAA, IBA, 2,4-D, GA and BA at 5 levels and banana pulp and coconut water at 15 per cent were applied. The results are given in Table 24. The growth was not satisfactory at all the three levels of IAA. Treatment with 3 ppm IAA was slightly better than others and was on par with control. NAA at lower levels (1 and 3 ppm) produced excellent growth (Plate 5.1,2). Maximum growth was observed with 1 ppm NAA. At 3 ppm the growth of plantlets were reduced and the amount of callus increased. Callussing was further increased at 5 ppm level (Plate 5.3). It was found that higher concentration of NAA induces only callussing. The growth of seedlings with 1 ppm 2,4-D was on par with control, but the root formation was poor. Higher levels of 2,4-D induced only callus and no plantlets. IBA at all the 3 levels produced satisfactory growth. Maximum growth was

PLATE No.5

Effect of auxins on growth and development of *Epidendrum radicans* seedlings

1. Treatment with NAA, 1 ppm.

2. Treatment with NAA, 3 ppm showing callus initiation

3. Treatment with NAA 5 ppm showing callus formation.

PLATE-5

1

2

3

Table 22. Effect of different media on seed germination and leaf production in Epidendrum radicans

Nutrient media	Mean number of days for greening	Ist leaf	IInd leaf	IIIrd leaf	IVth leaf
Knudson C(KC)	19	48	83	115	144
Murashige-Skoog(MS)	21	54	82	110	142
Vacin and Went(VW)	17	39	65	93	125
Burgeff N_3F (BN)	24	44	79	110	148
Modified Vacin and Went (MVW)	24	48	70	82	119

Table 23. Effect of different nutrient media on growth and development of Epidendrum radicans seedlings

Nutrient media	Mean Plant height mm	Leaf No. -	Root No. -	Root length mm	Fresh wt. mg	Dry wt. mg	Percentage of dry wt.
Knudson C(KC)	32.10	3.12	1.60	10.20	14.21	0.87	6.12
Murashige-Skoog(MS)	34.27	3.60	1.01	13.27	17.46	1.11	6.35
Vacin and Went(VW)	44.30	4.20	2.48	21.20	22.46	2.15	6.30
Burgeff N_3F(BN)	26.17	2.18	1.87	8.26	8.29	0.51	6.15
Modified Vacin and Went (MVW)	38.21	4.36	2.12	12.85	19.06	1.22	6.40
F-Test	**	**	**	**	**	**	
S-Em±	1.916	0.153	0.131	2.460	0.925	0.078	
C.D.	5.905	0.472	0.405	1.744	2.830	0.241	

** Significant at 0.01

Table 24. Effect of individual treatment of growth regulators and other additivies on growth and development of <u>Epidendrum radicans</u> seedlings

Treatments with Vacin and Went medium		Mean						Percentage of dry weight
		Height of plants	Number of leaves	Number of roots	Root length	Fresh wt.	Dry wt.	
		mm	-	-	mm	mg	mg	
1		2	3	4	5	6	7	8
IAA	1 ppm	7.40	2.15	0.60	2.40	1.40	0.085	6.07
IAA	3 ppm	13.40	2.80	0.76	2.65	7.19	0.44	6.11
IAA	5 ppm	9.11	2.32	0.22	1.22	2.70	0.18	6.66
NAA	1 ppm	54.00	4.92	3.46	40.60	22.11	1.43	6.46
NAA	3 ppm	43.16	4.07	2.20	28.30	19.22	1.22	6.34
NAA	5 ppm	Heavily callused. Only 2-3 seedlings produced.						
IBA	1 ppm	16.30	3.10	2.70	22.16	6.31	0.39	6.18
IBA	3 ppm	47.60	4.10	3.00	34.50	20.25	1.31	6.47
IBA	5 ppm	40.25	3.55	2.40	36.25	18.70	1.19	6.36
2,4-D	1 ppm	11.20	2.40	0.15	0.21	4.20	0.25	5.95
2,4-D	3 ppm	No plantlets produced, only darkened callus.						
2,4-D	5 ppm	No plantlets produced, very heavy callussing						

contd.

1	2	3	4	5	6	7	8
GA_3 0.1 ppm	13.20	2.30	1.05	2.75	5.27	0.32	6.07
GA_3 1.0 ppm	8.40	2.40	1.60	2.63	2.11	0.13	6.16
GA_3 3.0 ppm	6.74	1.10	0.12	0.17	2.07	0.11	5.34
BA 0.1 ppm	No plantlets produced, only greening PLB's.						
BA 1.0 ppm	"						
BA 3.0 ppm	"						
Banana pulp 15%	42.40	3.76	3.30	36.47	18.95	1.26	6.64
Coconut water 15%	36.20	3.28	2.86	31.40	16.70	1.06	6.34
Control(VW)	12.70	2.64	1.60	3.50	4.32	0.26	6.01
F-test	**	**	**	**	**	**	
S-Em±	3.96	0.256	0.196	2.43	-	-	
C.D.	11.80	0.765	0.586	7.45			

** Significant at 0.01

obtained at 3 and 5 ppm. Treatments with 1 ppm IBA was on par with the control. GA at three levels produced very poor growth. BA at all levels induced no growth at all.

The plant height was maximum with 1 ppm NAA (54 mm) followed by 3 ppm IBA (47.6 mm), 3 ppm NAA (43.16 mm), banana pulp (42.4 mm), 5 ppm IBA (40.25) and coconut water (36.20). No significant results were obtained with other treatments. Number of leaves were more with 1 ppm NAA (4.92) followed by IBA 3 ppm (4.1), NAA 3 ppm (4.07), banana pulp (3.76), IBA 5 ppm (3.55) and coconut water (3.28). Maximum number of roots were obtained with 1 ppm NAA (3.46) followed by banana pulp (3.30) and IBA 3 ppm (3.0). Other treatments were not significantly superior. Maximum root length was obtained with 1 ppm NAA (40.60 mm) followed by banana pulp (36.47 mm), IBA 5 ppm (36.25 mm), IBA 3 ppm (34.50 mm) and coconut water (31.4 mm). NAA at 3 ppm and IBA at 1 ppm also produced satisfactory growth of roots. Green and dry weight were recorded maximum with 1 ppm IAA followed by 3 ppm IBA, [illegible], banana pulp, IBA 5 ppm and coconut water.

A combination of treatments were applied to the basic media and the germinated seeds were subcultured. The individual treatment with NAA 1 ppm, IBA 3 ppm, banana pulp and coconut water produced maximum growth effects. The auxins were combined and used with and without banana pulp and coconut water. The results are given in Table 25.

Table 25. Effect of growth regulators and other additives in combination on growth and development of *Epidendrum radicans* seedlings

Treatment combination with Basic medium (VW)	Mean						Percentage of dry weight
	Plant height mm	Leaf No. -	Root No. -	Root length mm	Fresh wt. mg	Dry wt. mg	
NAA 1ppm	46.11	2.72	1.97	30.19	22.10	1.28	5.81
IBA 3 ppm	43.42	2.63	2.95	32.71	19.46	1.26	6.47
Banana pulp 15%	36.37	2.86	2.80	28.81	18.27	1.11	6.07
Coconut water 15%	28.30	2.76	2.12	14.40	17.11	1.02,	5.96
NAA 1ppm +IBA 3 ppm	41.40	2.95	2.70	20.40	20.43	1.30	6.36
Coconut water 15% + NAA 1ppm +IBA 3ppm	74.20	4.30	3.52	48.70	27.70	1.75	6.31
Banana pulp 15% + NAA 1ppm+ IBA 3 ppm	62.10	3.56	2.70	35.40	26.39	1.66	6.29
Coconut water 15%+ Banana pulp 15% + NAA 1 ppm + IBA 3 ppm	66.25	3.48	3.10	39.37	27.15	1.79	6.59
F-Test	**	**	NS	**	NS	NS	-
S-Em±	3.09	0.142	0.427	2.056	-	-	-
C.D.	9.38	0.431	1.297	6.237	-	-	-

** Significant at 0.01 NS Not significant

The combined effect of NAA and IBA were not superior to the individual treatments of NAA and IBA. Maximum growth was obtained with a combination of NAA 1 ppm with IBA 3 ppm and banana pulp. Plant height (74.2 mm), leaf number (4.3), root number (3.52), root length (48.7 mm) and green and dry weight were also more compared to others. A combination of NAA 1 ppm with IBA 3 ppm and coconut water produced more number of leaves (3.56) compared to the above treatment. A combination of all the four additives (NAA 1 ppm, IBA 3 ppm, Coconut water and banana pulp) produced more plant height (66.25 mm), root numbers (3.10), root length (39.37 mm) and green and dry weight. Plants on treatment with banana and growth regulators were dark green and very healthy. Banana pulp in combination with coconut water induced very healthy dark green seedlings.

4.1.7 Vanda Coerulea

Capsules of Vanda coerulea take 8 to 9 months for full maturity. The ripe capsules were cut open and the seeds were cultured on different media (KC, MS, VU, BN and MVW). for standardisation. Initially the seeds do not show any symptom of germination. After a period of 3 weeks, the seed mass turned yellow and formed into callus. The callus growth was continued upto a period of 2½ to 3 months and then differentiated into plantlets. The germination and growth was faster on MS medium (Table 26). The data indicate that the shortest time required for callus formation was with MS medium (22 days), followed by Vacin and Went medium (22.5 days) and

Table 26. Effect of different nutrient media on germination and leaf production in *Vanda coerulea*

Nutrient media	Mean number of days for				
	Callus formation and greening	Callus differentiation	Appearance of 1st leaf	Appearance of 2nd leaf	Appearance of 3rd leaf
Knudson C (KC)	27.20	94.00	153.00	186.00	201
Murashige-Skoog(MS)	22.00	79.50	92.50	122.50	138
Vacin and Went(VW)	22.50	99.00	144.00	176.00	201
Burgeff N_3F(BN)	24.00	97.50	120.00	158.00	177
Modified Vacin and Went(MVW)	23.50	84.00	116.00	152.00	172

Table 27. Effect of different nutrient media on growth and development of *Varda coerulea*

Nutrient media	Mean						Percentage of dry wt.
	Plant height mm	Leaf No. -	Root No. -	Root length mm	fresh wt. mg	Dry wt. mg	
Knudson C (KC)	12.20	2.79	1.75	4.48	47.00	2.82	6.00
Murashige-Skoog (MS)	18.72	3.35	2.42	4.33	56.12	3.76	6.69
Vacin and Went (VW)	13.11	2.54	1.87	3.95	29.20	1.57	5.37
Burgeff N_3F(BN)	11.76	3.04	1.04	3.24	26.40	1.58	5.98
Modified Vacin and Went (MVW)	14.70	2.93	1.22	3.85	33.70	2.09	6.20
F-test	NS	*	**	**	**	**	
S-Em±	2.53	0.143	0.032	0.129	2.10	0.134	
C.D.	7.82	0.442	0.252	0.398	6.49	0.411	

* Significant at 0.05 ** Significant at 0.01 NS Not significant

modified Vacin and Went medium (23.5 days). The shortest time callus differentiation was 79.5 days on MS medium, followed by modified Vacin and Went medium (84 days). The rate of leaf production was also faster with MS medium followed by modified Vacin and Went medium. The media VW and MVW behaved almost similarly in callus formation. But the subsequent growth and development was faster with MVW medium. The seedlings in MS medium was more vigorous with dark green foliage. The leaves were broad and thick.

The growth measurement of the seedlings are recorded (Table 27). The MS medium was found to influence plant height (18.72 mm), leaf number (3.35), root number (2.42), green and dry weight. The maximum root length was obtained with Knudson C medium (4.48 mm). Next to MS medium modified Vacin and Went medium induced more plant height (14.70 mm). Burgeff N_3F medium induced more number of leaves (3.04) and Knudson C induced more root number (1.75), fresh and dry weight.

Based on the above results, MS medium was selected as a basic medium for further studies. The callus bodies (PLBs) produced on a basic medium were sub-cultured on medium with different additives. The treatments were applied individually and in combinations. The individual treatments were as follows. IAA, NAA, IBA and 2, 4-D at 1, 3 and 5 ppm GA and BA at 0.1, 1 and 3 ppm, Banana and coconut water 15 percent.

The results are presented in Table 28. Growth measurements of the plantlets on medium with two levels of NAA, two levels of GA and banana pulp were significantly superior to control. Maximum plant height was obtained with NAA 1 ppm (16.5 mm) followed by GA_3 1 ppm (14.85 mm) NAA 3 ppm (13.25 mm) and Banana pulp 15 per cent (12.00 mm). Maximum number of leaves were produced on medium with NAA 1 ppm (3.75) followed by GA 1 ppm (3.67), GA 3 ppm (3.52) and banana pulp 15 per cent (3.0). Other treatments were not significantly superior over control. The maximum number of roots were produced with NAA 1 ppm (2.85), which is closerly followed by GA 1 ppm (2.70), NAA 3 ppm (2.54), IBA 3 ppm (2.25) and GA 0.1 ppm (2.20) treatments. The maximum root length was recorded in treatment with NAA 1 ppm (5.8 mm) followed by GA 1 ppm (4.75 mm), NAA 3and 5 ppm (4.2 and 4.1 mm) treatments. The green and dry weight were also maximum on treatments with 1 ppm NAA, 3 ppm NAA, 1 ppm GA and 0.1 ppm GA. Other treatments were not significantly superior over control.

Based on the results obtained with the individual treatments a combinationsof treatments were made and the seedlings were sub-cultured. The treatment combinations were using basic medium (MB) with NAA 1 ppm and GA 1 ppm. The combinations were used with and without 15 per cent coconut water and banana pulp. The results obtained are given in Table 29 .

The plant height was maximum with a combination of basic medium with GA 1 ppm, NAA 1 ppm and coconut water 15 per cent (16.66 mm) (Plate 4.1) and this was followed by a

Table 28. Effect of different growth regulators and other additives on growth and development of *Vanda coerulea* seedlings

Treatments with Murashige-Skoog medium		Mean: Height of plants	Mean: Number of leaves	Mean: Number of roots	Mean: Root length	Mean: Fresh weight	Mean: Dry weight	Percentage of dry weight
		mm	-	-	mm	mg	mg	
1		2	3	4	5	6	7	8
IAA	1 ppm	10.40	2.43	1.44	3.25	18.75	1.21	6.45
IAA	3 ppm	9.70	2.89	1.87	3.10	16.65	1.00	6.00
IAA	5 ppm	8.16	2.35	2.05	2.85	17.40	1.01	5.80
NAA	1 ppm	16.50	3.75	2.85	5.80	48.44	3.19	6.58
NAA	3 ppm	13.25	2.84	2.54	4.20	43.22	2.80	6.47
NAA	5 ppm	6.35	2.40	2.10	4.10	29.12	1.77	6.08
IBA	1 ppm	8.25	2.75	1.93	3.25	18.77	1.14	6.27
IBA	3 ppm	9.35	2.25	2.25	3.54	9.12	0.49	6.03
IBA	5 ppm	7.25	2.95	2.05	3.10	11.29	0.65	6.02
2,4-D 1, 3 and 5 ppm		No growth						

contd.

1	2	3	4	5	6	7	8
GA_3 0.1 ppm	9.10	2.20	2.20	3.70	37.14	2.33	6.27
GA_3 1.0 ppm	14.85	3.67	2.70	4.75	40.12	2.44	6.08
GA_3 3.0 ppm	11.45	3.52	1.45	3.65	35.12	2.28	6.49
BA 0.1 ppm	7.15	2.15	1.64	2.58	20.40	1.18	5.78
BA 1.0 ppm	8.50	2.20	1.35	3.10	19.12	0.97	6.12
BA 3.00 ppm	6.15	1.62	1.44	3.15	22.10	1.21	5.92
Coconut water 15%	10.00	2.66	1.33	3.40	26.66	1.67	6.26
Banana pulp 15%	12.00	3.00	1.40	3.45	26.40	1.58	5.98
Control(MS)	9.55	2.25	2.10	3.25	22.18	1.35	6.08
F-test	**	**	**	**	**	**	
S-Em±	2.25	0.245	0.228	0.499	-	-	-
C.D.	6.735	0.730	0.680	1.488	-	-	-

** Significant at 0.01

treatment of GA 1 ppm with coconut water (15.37 mm) and NAA 1 ppm with coconut water (15.25 mm). Maximum leaf number was obtained on a treatment with NAA 1 ppm alone (3.81) and followed by a treatment with NAA 1 ppm and banana pulp (3.62). Maximum root number was obtained with a combination of basic medium with NAA 1 ppm, GA 1 ppm and coconut water 15 per cent (2.4) and it was followed by a treatment with NAA 1 ppm, and coconut water 15 per cent (1.85) and a treatment of NAA 1 ppm with banana pulp 15 per cent (1.75).(Plate 4.2). The root length (8 mm) was maximum on medium with GA 1 ppm, NAA1ppm and coconut water. This was followed by NAA 1 ppm with banana pulp (6.25 mm) and NAA 1 ppm with coconut water (6.20 mm), other treatments were not significantly superior.

The green and dry weight were maximum on a treatment with GA 1 ppm, NAA 1 ppm and coconut water followed by NAA 1 ppm and banana pulp and NAA 1 ppm and coconut water 15 per cent.

4.2 <u>Tissue culture</u>

4.2.1 <u>Cymbidium meristem culture</u>

Meristems of Cymbidiums were tested on different media and the best results were obtained on Vacin and Went medium. Protocorm like bodies were formed on VW medium within 15 days while it took 27 days on Murashige and Skoog medium. There was no development on Burgeff N_3F and on Knudson C medium. Protocorm like bodies with rhizoids and tiny leaves were further slices and put on fresh medium for sub-culturing

Table 29. Effect of different growth regulators and other additives in combination on the growth of *Vanda coerulea* seedlings

Treatments with Murashige-Skoog (MS) medium	Mean Plant height mm	Leaf Number -	Root number -	Root length mm	Fresh weight mg	Dry weight mg	Percentage of dry weight
NAA 1 ppm	9.62	3.81	1.37	5.00	18.75	1.22	6.50
GA_3 1 ppm	11.20	3.12	1.12	4.31	17.50	1.12	6.40
Coconut water 15%	10.00	2.66	1.30	3.40	16.66	1.01	6.06
Banana pulp 15%	12.00	3.00	1.40	3.45	26.40	1.76	6.66
NAA 1 ppm+ Banana pulp 15%	11.62	3.62	1.75	6.25	50.00	3.05	6.10
NAA 1ppm+ Coconut Water 15%	15.25	3.30	1.85	6.20	45.60	2.87	6.29
$GA_3$1 ppm+Banana pulp	9.05	2.10	0.89	2.58	8.95	0.54	6.37
$GA_3$1 ppm+Coconut water	15.37	2.94	1.44	3.06	18.75	1.16	6.18
$GA_3$1 ppm+ NAA 1ppm	12.61	3.07	1.30	3.54	26.92	1.72	6.39
$GA_3$1 ppm+NAA 1ppm+ Banana pulp 15%	12.29	2.63	0.90	3.11	29.40	1.85	6.29
$GA_3$1ppm+NAA 1ppm+ Coconut water 15%	16.66	3.50	2.40	8.00	51.20	3.43	6.70
GA_3 1ppm+ NAA 1ppm+ Coconut water 15%+ Banana pulp 15%	15.48	3.00	1.35	4.43	21.70	1.43	6.59
F-test	**	**	**	**	**	**	
S-Em±	1.00	0.208	0.118	0.349	-	-	-
C.D.	2.[illegible]43	0.82	0.348	1.026	-	-	-

** Significant at 0.01

if left undisturbed the differentiation started within 35 to days and small plantlets were obtained. When the experiments were terminated after 40 days each tissue segment was rinsed in water and dried for 15 minutes over filter paper before each weight determination.

Media	Fresh weight
Vacin and Went	85 mg
Murashige-Skoog	67 mg
Burgeff N_3F	11 mg *
Knudson C	11 mg *

* Original weight of the tissue = 1 mg

Effect of growth regulators

Media Vacin and Went + IAA (1, 3 and 5 ppm)
" + NAA (1, 3 and 5 ppm)
" + IAA + NAA (1,3 and 5 ppm each)

Experiments were also conducted to see the effect of different growth regulators alone and in combination

VW + IAA (1, 3, 5 ppm each)

VW + NAA (1, 3, 5 ppm each)

It was observed that IAA alone did not induce any increase in fresh weight, while in combination with NAA (1 ppm) the growth was much faster and the PLBs were much more green.

2, 4-D (1, 3, 5 ppm)

At 3 and 5 ppm, 2,4-D inhibited the growth and developed. However at 1 ppm, the fresh weight was very high as compared to

control. The fresh weight records were taken after 45 days.

Media	Fresh weight
Vacin and Went (VW)	85 mg
VW + 2, 4-D (1 ppm)	117 mg
VW + 2, 4-D (3 ppm)	85 mg
-- 2, 4-D (5 ppm)	Browning of tissue

inal weight of tissue = 9.5 mg

4.2 Nodal culture

4.2.1 Dendrobium pierardii

The nodal sections were cultured on different media (KC, MS, VW, BN and MVW). The Vacin and Went medium took shortest time (18 days) for swelling of the lateral buds. This was followed by modified Vacin and Went medium (27.5 days). No growth was observed on Burgeff N_3F medium. Explants produced both callus and plantlets on Knudson C medium. Vacin and Went medium produced more number of plantlets as compared to other media (Plate 6.4). This medium also showed over all superiority in getting more number of multiple shoots, plant height, leaf number and green dry weight.

Knudson C (liquid) medium was used for further treatments with growth substances (2, 4-D 0.1 ppm) and other additives (coconut water 15 per cent) for proliferation of callus. A treatment combination of 2, 4-D (0.1 ppm) and coconut water (15 per cent) induced better proliferation of callus bodies with in a period of 60 days (Plate 6.2).

The callus bodies were sub-cultured on solid Knudson C medium with IAA (1 ppm), BA (1 ppm), coconut water (15 per cent)

PLATE No.6

Tissue culture studies

1. Preparation of plant material for tissue culture studies. Dendrobium piererdii.

2. Callus formation in Dendrobium piererdii and differentiation of plantlets.

3. Effect of coconut water on differentiation of callus tissue.

4. Plantlet formation on Vacin and Went medium

5. Development of lateral buds in Vanda teres on different media.

6. Growth of Vanda teres plantlets on medium with NAA 1 ppm and coconut water 15 per cent.

PLATE-6

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and its combinations. Treatment with 15 per cent coconut water alone produced plantlets in minimum number of days (Plate 6.3). Combination of all the three components also produced satisfactory results.

Epidendrum radicans

The nodal sections cultured on different media produced plantlets in all the media except Knudson C medium (Plate 7.1). Out of 10 cultures, Knudson C medium produced 4 callus and Vacin and Went medium produced 1 callus. All the remaining treatments produced plantlets alone. The initiation was fast in Knudson C and Vacin and Went medium compared to other treatments. However, more explants initiated growth in Knudson C medium. Multiple shoots were produced on treatments with Knudson C and Vacin and Went medium (Plate 7.4). Maximum number of sprouts were obtained with Knudson C medium. Knudson C medium proved superior over other media in all growth characteristics (Table 32).

The callus bodies obtained were sub-cultured on solid Knudson C medium with growth substances and other additives for proliferation of callus tissue. Treatment with coconut water 15 per cent alone and in combination with 2,4-D 0.1 ppm induced callus proliferation. However, treatment with coconut water alone produced more callus compared to other treatments. The callus bodies grew approximately to .5 to 1.0 cu. centi meter size within 60 days after culturing.

PLATE No.7

Tissue culture studies

1. Effect of different media on growth and development of *Epidendrum radicans* nodal cuttings

2. Effect of different media on plantlet formation in *Epidendrum radicans*

3. Multiple shoot production in *Epidendrum radicans* on Knudson C medium

4. Plantlet formation in *Epidendrum radicans* on Vacin and Went medium.

5. Shoot differentiation in *Epidendrum radicans* callus bodies influenced by NAA and coconut water.

PLATE - 7

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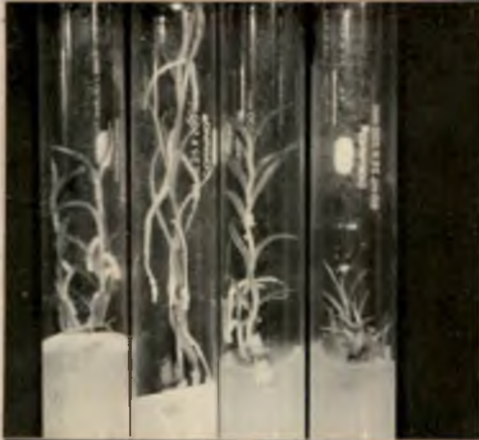

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Table 30. Effect of different media on growth and development of nodal sections of *Dendrobium pierardii*

Nutrient media	Mean number of days for swelling of nodes.	Number of node callus formed.	Number of plant-lets formed	Mean number of multiple shoots	Mean height of plants	Mean leaf number	Mean root number
Knudson C	36.50	2	2	1.50	14.75	1.5	-
Murashige-Skoog	36.50		3	2.00	10.45	2.0	0.33
Vacin and Went	18.00		6	2.50	33.50	3.5	3.00
Burgeff N_3F	-	-	-	-	-	-	-
Modified Vacin and Went	27.50		3	1.33	28.50	2.5	3.00

Number of nodal sections taken for each experiment is 10

The callus bodies obtained were sub-cultured on solid Knudson C medium with IAA (1 ppm), BA (1 ppm) and coconut water (15 per cent) and its combination for shoot and root initiation. The combined effect of coconut water and NAA on shoot initiation was more, compared to treatment with NAA (1 ppm) and coconut water. The plantlets produced were dark green and very healthy. None of the other treatments, initiated shoot and root (Table 33). Treatments with NAA and coconut water produced aerial roots in almost all nodes in addition to the basal roots. NAA treatment produced less number of aerial roots.

Vanda teres:

The nodal sections of Vanda teres were cultured on different media. The lateral buds developed in Vacin and Went medium (Plate 6.5). Whereas no bud development was observed in Knudson C and Murashige-Skoog medium. Among the 3 media responded growth, maximum growth was obtained with Vacin and Went medium followed by modified Vacin and Went medium and Burgeff N_3F medium (Table 34). The buds expanded and grew into pale yellow semispherical structure which continued to grow and formed into plantlets in each tube.

Some of these semispherical bodies were excised from the explant material and the leaves and apical buds were removed and again cultured on a liquid medium (VW) for proliferation Treatment with NAA (1 ppm) alone

Table 31. Effect of growth regulators and other additives on growth and differentiationof *Dendrobium pierardii* callus

Treatments with Knudson C medium	Mean number of days for initiation	Number of plantlets formed	Mean number of leaves produced			Mean number of roots produced			Height of plants cm
			60 days	90 days	120 days	60 days	90 days	120 days	
Basic medium (control)	(Callus growth continued)								
NAA - 1ppm	42	3	1	3	3.3	0.33	[illegible]	3	3.10
BA - 1ppm	55	2	0	1.3	3	0.	0.50	2	2.30
Coconut water 15%(CW)	28	6	2.5	3	5	2.5	4	4	6.50
NAA+BA-1ppm	53	2	0	2	3	0	2.5	3	2.10
NAA+BA-1ppm +CW 15%	35	3	1	2.66	4	1.33	2.66	3	3.50

Table 32. Effect of different media on the growth and development of *Epidendrum radicans* nodal cultures

Nutrient media	Number of days for initiation	Number of plantlets formed	Number of callus formed	Number of multiple shoots formed	Height of plants cm	Number of leaves	Number of roots
Knudson C	50.50	4	4	3.50	1.50	2.25	1.12
Murashige-Skoog	56.30	3	-	-	0.25	1.33	-
Vacin and Went	51.00	5	1	2.20	1.15	1.00	0.40
Burgeff N_3F	61.00	2	-	-	0.55	0.50	-
Modified Vacin and Went	60.50	2	-	-	0.50	0.50	-

Table 33. Effect of growth regulators and other additives on growth and differentiation of *Epidendrum radicans* callus

(Number of cultures in each treatment = 10).

Treatments with Knudson C medium	Mean Number of days for initiation of shoots	plantlets formed	leaves produced 30 days	60 days	90 days	roots produced 30 days	60 days	90 days	Height of plants cm
Knudson medium (control)	Callus turned brown and dead								
NAA 1ppm	37.50	2.75	-	1	3	-	1.25	2.50	1.0
BA 1ppm	No callus growth								
Coconut water (15%) (CW)	28.00	5.00	1	3	4	-	2.30	3.50	3.5
NAA+BA 1 ppm	No callus growth								
NAA+BA 1ppm + CW	Callus slightly developed								
NAA 1ppm+CW	23.00	4.50	1	2	5	-	3.50	4.25	3.75
BA 1ppm+CW	Callus turned down								

Table 34. Effect of different nutrient media on growth and development of *Vanda teres* nodal sections *

Nutrient media	Mean		Number of plantlets formed	Mean				Green dry weight ratio
	No. of days for swelling	Days for semi-spherical sprouts		Height of plants mm	Leaf number	Root number	Root length mm	
Knudson C	-	-	-	-	-	-	-	-
Murashige-Skoog	45.25	-	-	-	-	-	-	-
Vacin and Went	35.85	47.20	7	1.530	2.85	1.00	12.75	
Burgeff N_3F	43.10	55.00	9	0.970	2.44	0.55	5.27	
Modified Vacin and Went	47.25	54.25	5	1.150	2.20	-	-	

* Number of nodal cultures in each treatment = 10

Table 35. Effect of growth regulators and other additives on growth and differentiation of <u>Vanda teres</u> callus

Treatments with solid Vacin and Went medium	Number of								Height of plants
	days for initiation	plantlets formed	Leaves produced			Roots produced			
			60	90 days	120	60	90 days	120	
NAA 1 ppm	Callus growth continued								
BA 1 ppm	No growth and no callus differentiation								
Coconut water 15% (CW)	Callus growth continued								
NAA +BA (1ppm)	No growth or differentiation								
NAA+BA(1ppm)+ CW 15%	Callus slightly developed								
NAA 1ppm+CW 15%	29	3	1	3	4	1	1	1	3.00
BA 1ppm+CW 15%	No growth								
VW medium (Control)	No callus growth obtained								

Roots cultured on MS medium and treated with NAA 1.0 ppm took the shortest time for initiation of growth and produced maximum root length. This was followed by a treatment with coconut water 15 per cent alone. Treatments with 2,4-D 0.1 ppm and its combinations killed all explants. Root extension with other treatments were not significant.

A treatment combination of NAA (1ppm) and kinetin (1 ppm) with basic medium produced short, stout and thick roots, which resembled the callus but not the real callus. They also eventually died after two months.

Histochemical studies

Developmental changes during germination of *Bletilla hyacinthiana* seeds

The orchid seed has an undifferentiated embryo and it lacks the endosperm. Upon examination of the seed sections, two distinct regions could be marked. One region which is in continuity with suspensor consists of large vacuolated cells and the other consists of small and dense cells. The former one develops into the base and the latter develops to form the apical meristem. The rhizoids originate from the basal portion.

The seed has got a transparent seed coat enclosing only the embryo inside it. On contact with suitable media the mature embryo imbibes water along with the nutrients, enlarges and breaks open the seed coat (Plate 8.1). The enlarged embryo does not assume any specific shape. However

Table 36. Effect of nutrient media on root culture of *Dendrobium moschatum* *

Nutrient media	Number of days for initiation	Roots produced callus	Number of roots extended	Mean length of root growth		
				30 days (mm)	60 days (mm)	90 days (mm)
Knudson C	47	-	3	0	0.15	2.47
Murashige-Skoog	25	-	6	0.25	4.13	14.57
Vacin and Went	53	-	2	0	0.20	7.24
Burgeff N_3F	42	-	[illegible]	0	0.31	5.23
Modified Vacin and Went	50	-	3	0	0.22	8.55

* Number of roots cultured for each treatment. =10

Table 37. Effect of growth regulators and other additives on root cultures of Dendrobium moschatum

Treatments with MS media	Mean No.of days for initiation	No.of roots produced callus	No.off roots extended	Mean length of root growth		
				30 days (mm)	60 days (mm)	90 days (mm)
NAA 1 ppm(NAA)	22	-	6	4.0	9.31	16.25
2,4-D 0.1 ppm	-	-	-	-	-	-
Kinetin 1 ppm	44	-	4	-	0.50	2.50
Coconut water 15 per cent	39	-	5	-	1.00	6.75
Kinetin 1 ppm + 2,4-D 0.1 ppm	27	-	4	-	1.50	2.50
NAA 1ppm + Kinetin 1 ppm	27	-	2	-	2.00	4.25
Kinetin+ 2, 4-D 0.1 ppm + coconut water	-	-	-	-	-	-
NAA 1ppm+ Kinetin 1ppm + Coconut Water 15 per cent	37	-	2	-	1.25	3.50
NAA 1ppm + 2,4-D 0.1ppm + Kinetin 1 ppm + Coconut Water 15 per cent	-	-	-	-	-	-

Number of roots cultured for each experiment = 10.

PLATE No.8

Orchid seed development

1. Swelling of embryo
2. Development of embryo showing polarity
3. A Protocorm like body with rhizoids
4. Shoot apex differentiation in Protocorm like body
5. A pseudobulb attached with Protocorm like body
6. Distribution of Protocorm like body positive bodies in pseudobulb tissues.

PLATE - 8

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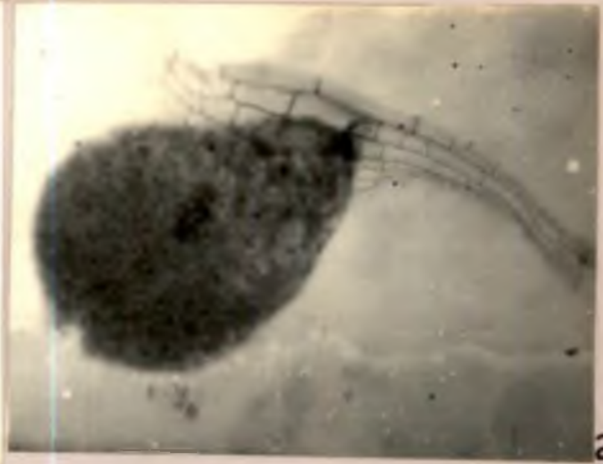
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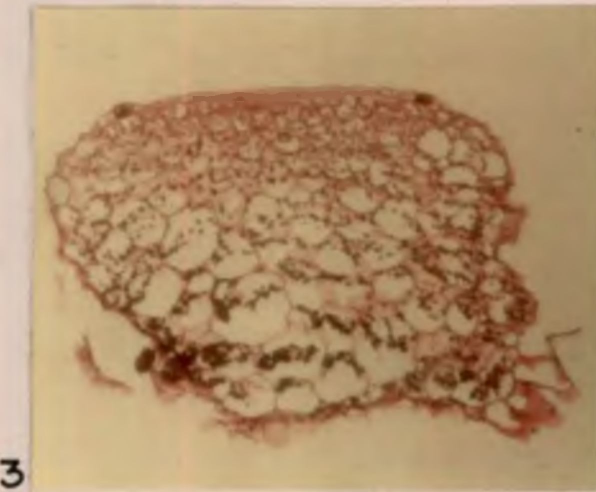
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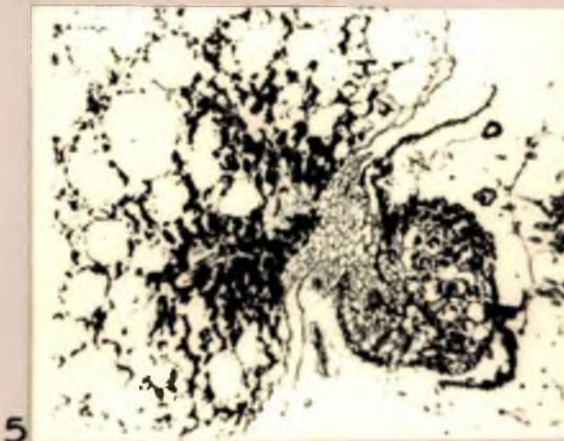
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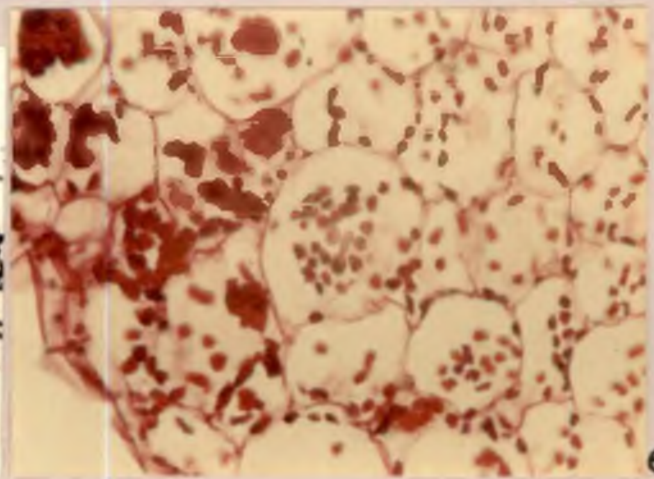
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a polarity seems to have maintained right from the greening stage (Plate 8.2). At the stage of differentiation, the basal and apical ends become more clearly visible.

Initially absorbing hairs are formed at the posterior end. These are the rhizoids which absorb water and nutrients. The enlarged embryo with the rhizoids is called protocorm like body (PLB) (Plate 8.3). The PLB further enlarges and later shoot apex differentiates from its apical end (Plate 8.4). The PLB has got a broad and flat surface at its tip earlier to the differentiation of the shoot apex. A group of narrow cells rich in cytoplasmic contents seems to act as conducting cells in the epidemis. In the epidemis near the top portion of the PLB, stomata get differentiated which help in the exchange of gases.

The leaf primordia differentiate laterally near the shoot apex. With the differentiation of leaves an autotrophic plantlet comes into existence. The plantlets at its early stage of growth are nourished by the PLB, upto the development of chlorophyll in the leaf primordia. The rhizoids are responsible for the absorption of water and nutrients required by the PLB and the young plantlets. With the further development of plantlet a bulbous structure, the pseudobulb develops near the base of the tuft of leaves. The pseudobulbs serves as a storage organ for the plantlet. It consists of large cells and small cells distributed in a

characteristic way. The PLB nourishes the plantlet in the beginning, but when the downward flow of metabolite starts from the photosynthesising leaves the former eases the nutritional function and remains as a vestigeal structure for some more time (Plate 8.5).

The pseudobulbs develops vascular tissues for the upward and downward translocation of nutrients and metabolites. In the meanwhile true roots with well developed vascular strands are also produced from the region of junction of PLB and pseudobulb.

4.3.2 Changes in the macromolecular substances

Following is an account of the changes in the macromolecular substances such as Insoluble polysaccharides, proteins, RNA, DNA and lipids during the differentiation of plantlets from orchid seeds and callus.

In the PLB the lower parenchymatous cells contain rich accumulation of large sized PAS positive granules, which were confirmed as starch grains by Iodine Potassium iochide test (Plate 8.6). The basal cells of the rhizoids contains the starch grains while their terminal cells were devoid of them. Towards the apex of the PLB, in the parenchymatous cells the starch grains gradually diminish in size and they are completely absent in the cells near the apical end of the PLB (Plate 7.4). Thus a gradient is created from base to the apex in the distribution of insoluble of polysacharides in PLB. However, the cells of the

shoot apex contain PAS positive cytoplasm free from starch grains. In the storage cells the starch grains are generally located around the nuclei (Plate 9.1).

Both the leaf primordia and the differentiated leaves contain cells with PAS Positive cytoplasm but no starch grains. The guard cells of the stomata near the top of the PLB contain starch grains (Plate 9.2).

4.3.3 Pseudobulb

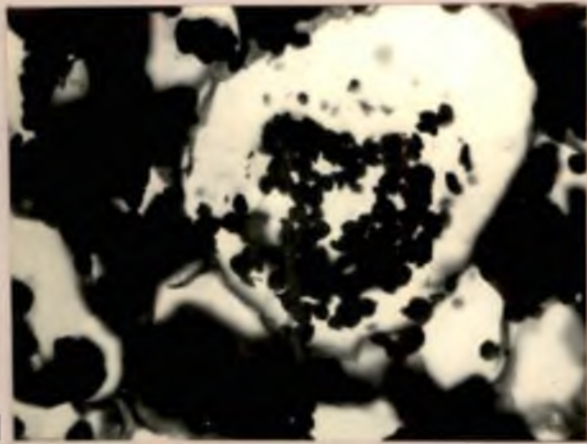
The pattern of starch accumulation in the pseudobulb is different from that observed in case of PLB. Pseudobulb is made up of two kinds of cells. Some of them are large and they are surrounded by small cells (Plate 8.6). The small cells contain a very rich accumulation of densly stained starch grains. While the large cells contain relatively less densly stained starch grains. The relatively less number of large starch grains present in the large cells are distributed around the nuclei and they appear to be at various stages of disintegration. The cells of pseudobulb surrounding the vascular strands have a rich accumulation of starch. The cells in the crown of leaves do not contain any starch; however, their cytoplasm is PAS Positive. Further at the base of the pseudobulb adjacent to the tip of PLB the cells are small and they do not contain any PAS Positive bodies or starch. The roots, lateral shoots and their primordia are free from starch accumulation but their cells contain PAS Positive cytoplasm.

PLATE No.9

Cell structure and contents in protocorm like body and pseudobulbs.

1. Distribution of starch grains in a cell around nucleus
2. Stomata and guard cells in Protocorm like body
3. Presence of cytoplasmic RNA in growing point
4. A pseudobulb with a lateral shoot differentiated at the base and formation conducting cells.

PLATE-9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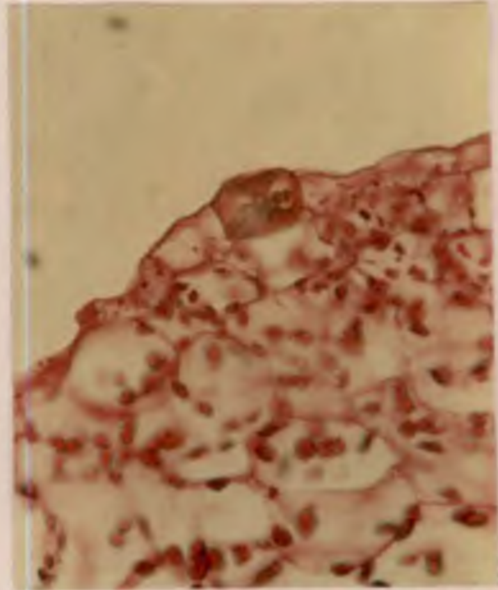
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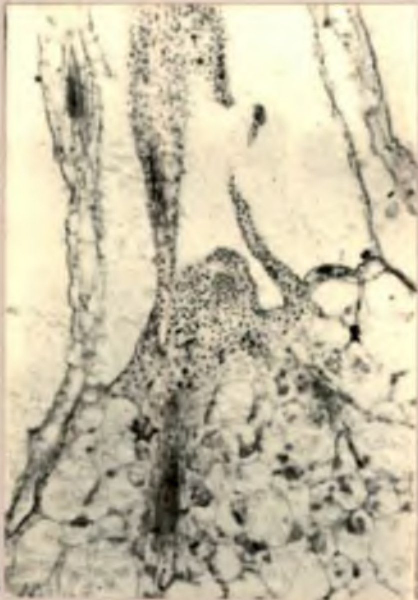
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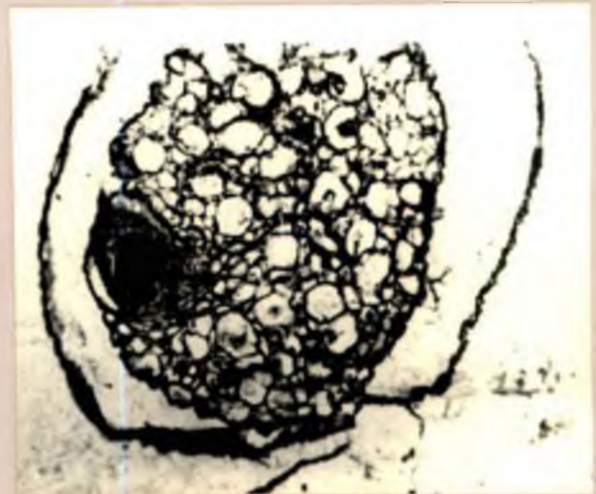
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4.3.4 Insoluble proteins

The cytoplasm of almost all the cells in the seed show a rich accumulation of insoluble proteins. After protrusion, the large basal cells of protocorm like bodies show very low concentration of cytoplasmic protein. Whereas the small apical cells possess a high protein content. Thus an increasing gradient is observed from the base to the apex in the distribution of proteins in protocorm like bodies (Plate 9.3). The rhizoidal cells contain protein rich cytoplasm. The primordia of shoot apex and leaf have small cells rich in cytoplasmic proteins. The narrow conducting cells possess a rich concentration of protein (Plate 9.3).

In pseudobulb cells, only the shoot, root and leaf primordia have protein rich cytoplasm. In the storage region the large cells possess relatively more cytoplasmic protein than the small cells.

4.3.5 Nucleic acids

The distribution pattern of RNA in PLB and pseudobulb is comparable to that of proteins. The conducting cells in the PLB and the primordial cells of shoot and leaf contain rich cytoplasmic RNA.

In pseudobulb the cells of shoot and root primordia possess rich cytoplasmic RNA (Plate 9.4,5). Among the storage cells of the pseudobulb the large ones possess a

higher concentration of RNA than the small cells surrounding them.

DNA is found localised only in the nuclei. In PLB, the cells at shoot and leaf primordia have small spherical nuclei richly stained for DNA. The cells near the base of the PLB possess considerably large nuclei rich in DNA. A decreasing gradient from base to the apex is noticed in the size of nuclei in cells of PLBs. The nuclei in the conducting cells are narrow and elongated.

4.3.6 Lipids

All the cells of the embryo before germination contained rich accumulation of oil globules (Plate 10.1). With the onset of germination the oil globules gradually disappear and by the time the shoot apex is differentiated and all the cells are completely depleted of their oil content. Sudanophilic substances are completely absent in the tissues of Pseudobulb.

4.3.7 Developmental changes in the callus of *Dendrobium pierardii* during plantlet differentiation

The proliferating callus of *Dendrobium* does not possess any regular shape. The tissues grow indefinitely and proliferate in all directions. The callus tissue consists of parenchymatous cells of different size. The peripheral cells are almost uniform in size, while the inner

PLATE No.10

Orchid seeds and cell contents

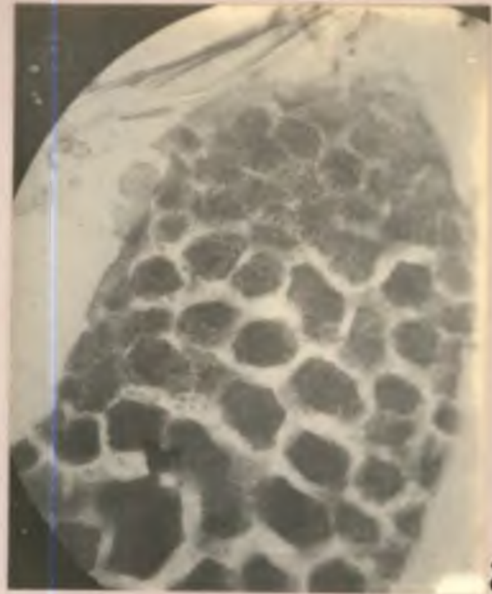
1. Seed sections showing oil globules
2. Seed sections stained for proteins
3. Seed showing suspensor end

Callus differentiation and plantlet formation

4. Peripheral cells of callus
5. Vascular strand formation
6. Interior cells with starch grains.

PLATE - 10

1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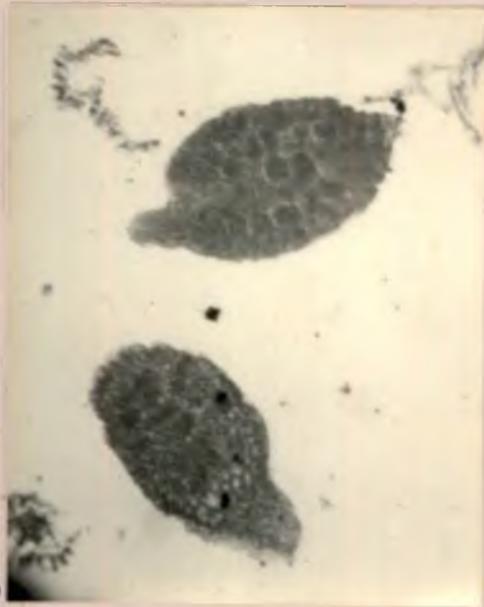
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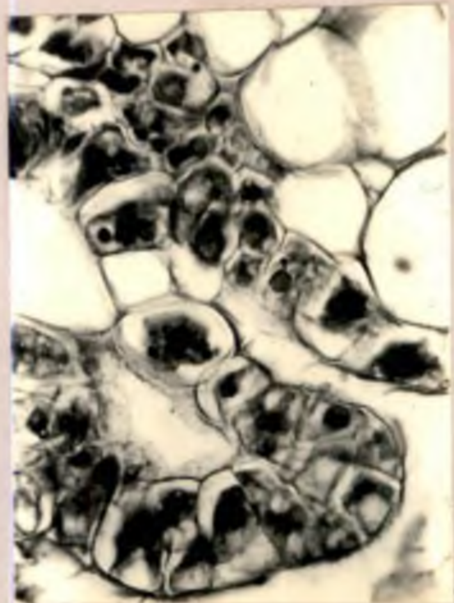
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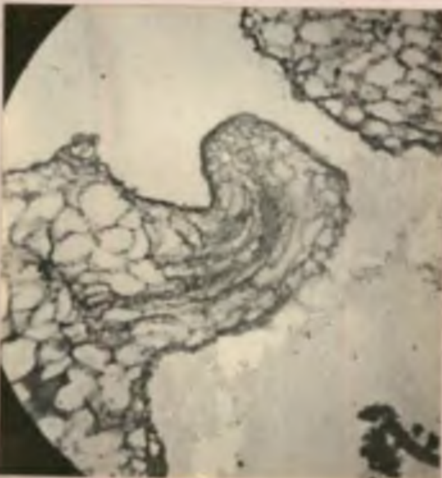
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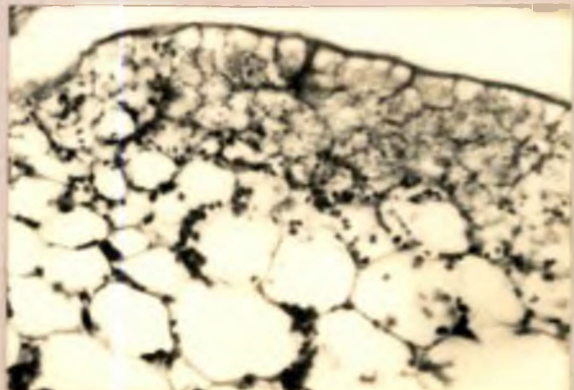
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cells are enlarged to various degree (Plate 10.4). At certain locations in the peripheral layers of the callus, groups of small cells differentiate into shoot apices. The leaf primordia differentiate laterally near the base of shoot, apex. Later the vascular strand differentiation takes place (Plate 10.5) acropetally in these structures of plantlets. In the meanwhile the roots with well differentiated vascular strands also develop near the basal regions of the shoot apices.

4.3.8 <u>Insoluble polysaccharides</u>

The peripheral cells of the callus have small sized starch grains except at the region of shoot apex differentiation(Plate 11.3). The cytoplasm in the cells at the periphery is PAS Positive. The enlarged cells at the interior part of the callus have more number of large sized starch grains (Plate 10.6). The cytoplasm is sparcly distributed and is PAS Positive. In the shoot primordium the cells are small and are free from starch accumulation. However, their cytoplasm is PAS Positive. The vascular strands of both root and shoot regions do not contain starch deposition.

4.3.9 <u>Insoluble proteins and RNA</u>

The cytoplasm in the peripheral cells is rich in protein and RNA (Plate 10.4 and 11.4). The cells of the shoot and root primordia have still higher concentrations of cytoplasmic protein and RNA (Plate 11.1).

PLATE No. 11

Callus differentiation and plantlet formation

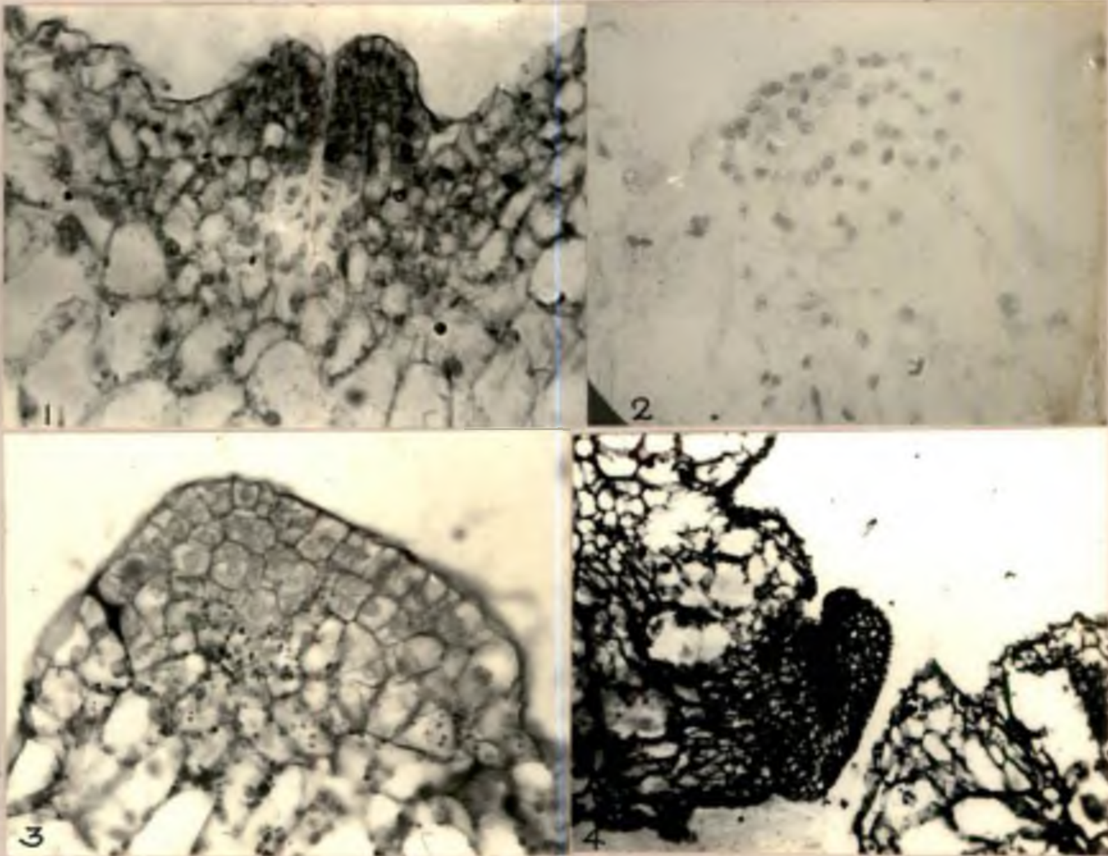
1. Cytoplasmic protein and RNA at the shoot primordia.

2. DNA content at the differentiating apical region

3. Distribution of insoluble polysaccharides at the growing apex.

4. Protein and RNA content at the differentiating zone of the callus.

PLATE-II



4.3. 10 DNA

The peripheral cells and the cells of shoot and root primordia in the stem callus have small spherical nuclei intensely stained by Schiffs' reagent (Plate 11.2). The large interior cells have proportionately enlarged nuclei with rich DNA content.

4.4 Chromosome instability in tissue culture

In the squash preparation of cultured cells of Dendrobium pierardii (2n =40) a large number of cells were observed with various abnormalities. The most common being the varying number of chromosomes in different cells followed by fragmentation of chromosomes and cellular and nuclear budding of various cells nuclei showing hair like projections were also seen.

The number of cells showing abnormal chromosome was very high (31 to 47 per cent) most of the cells were having fragmentation of chromosomes, the normal cells were not more than 40 per cent which showed chromosome numbers ranging from 2n = 27 to 48.

Anaphases were also not very regular most of them showed dicentric bridges and lagging chromosomes.

The number of abnormal cells however started decreasing when the tissue from differentiated calli were analysed and the abnormalities were not more than 10 to 17 per cent.

Table No. 38 Table showing the distribution of Chromosome numbers in callus cells, of *Dendrobium pierardii*

Sl. No.	No. of chromosomes	Other abnormalities or fragmentation
1	27	-
2	40	-
3	40	-
4	37	fragmentation
5	40	-
6	40	-
7	40	-
8	32	-
9	48	-
10	40	stickness
11	40	fragmentation
12	36	-
13	40	-
14	40	-
15	40	-
16	48	-
17	30	-
18	32	-
19	39	-
20	40	-
21	40	-
22	41	fragmentation
23	40	-
24	40	-
25	40	-

DISCUSSION

CHAPTER V

DISCUSSION

The results of the various experiments are discussed in this chapter.

5.1 Orchid seeds

The orchid seeds are dust or flour-like and vary in size, shape and colour in different species. The colour may be white (Epidendrum radicans), Cream (Phaius Wallechii), Blue green (Bletilla hyacinthina), brown (Spathoglottis plicata), Red and orange. Diversity of seed shape is considerable but five basic forms are recognised (Clifford and Smith, 1969; Woolhouse, 1976)(Fig. 16).

The seeds are unique in several respects, they are exceedingly small, produced in large numbers, the seeds have no endosperm (Savine ,1974), no cotyledons and no root initials.

Orchids embryos consist of undifferentiated mostly isodiametric cells with dense granulated cytoplasm and conspicuous nuclei. Cells of the posterior end are larger and vacuolated. A suspensor consisting of very large vacuolated cells is attached to the posterior end (Alvarez, 1969) and the anterior chalazal or meristematic portion of the embryo is composed of relatively smaller cells(Plate 10.3).

All the cells regardless of the position in the embryo are heavily packed with food reserves, most of which in the form of lipid bodies (Weismeyer and Hofsten, 1976). Protein bodies are also found but are restricted to upper two third of the embryo (Plate 10.2). No starch or other carbohydrates are found in dry seeds.

5.2 Standardization of media

Media:

After the discovery of Knudson C medium in 1921 a large number of media have been developed for the germination of different orchid species. Five different media(Knudson C, Murashige-Skoog, Vacin and Went, Burgeff N_3F and Modified Vacin and Went) were selected to study its effect on germination and growth of different orchid species. The effect of media on plant height, root number and root length were tabulated and represented graphically (Fig. 1, 2 and 3). Some media contains only the major nutrients whereas others contains minor elements also. Incorporation of micronutrients in the culture medium is often recommended as even if not beneficial they are not harmful (Arditti, 1967). All the five media behaved differently in different species in inducing germination and subsequent growth.

5.2.1 *Bletilla hyacinthina*

Better germination was recorded on Vacin and Went medium. The quick germination of the seed may be

FIG. 1. PLANT HEIGHT OF ORCHID SPECIES AS INFLUENCED BY DIFFERENT NUTRIENT MEDIA

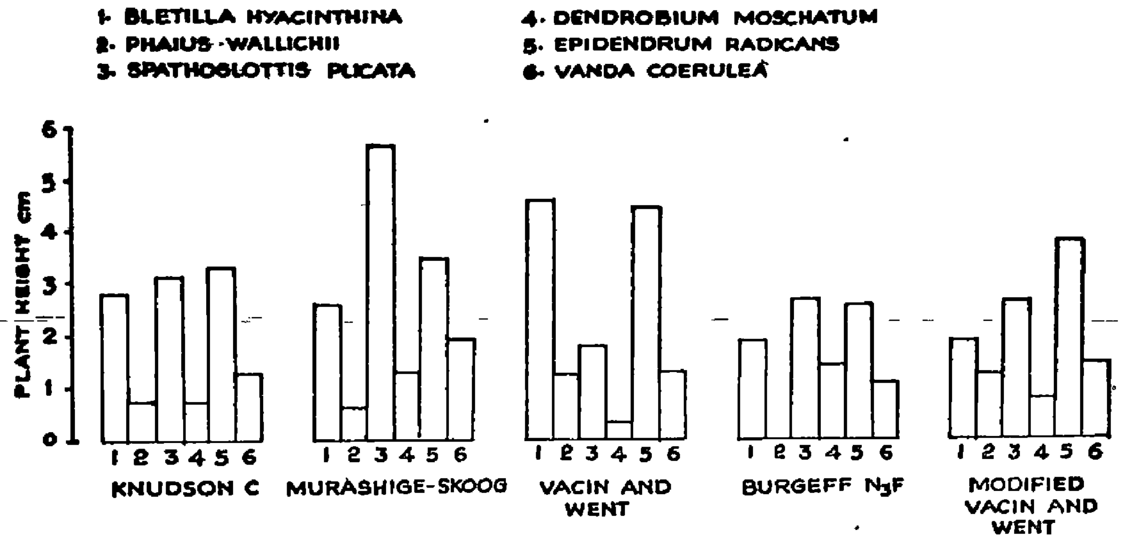
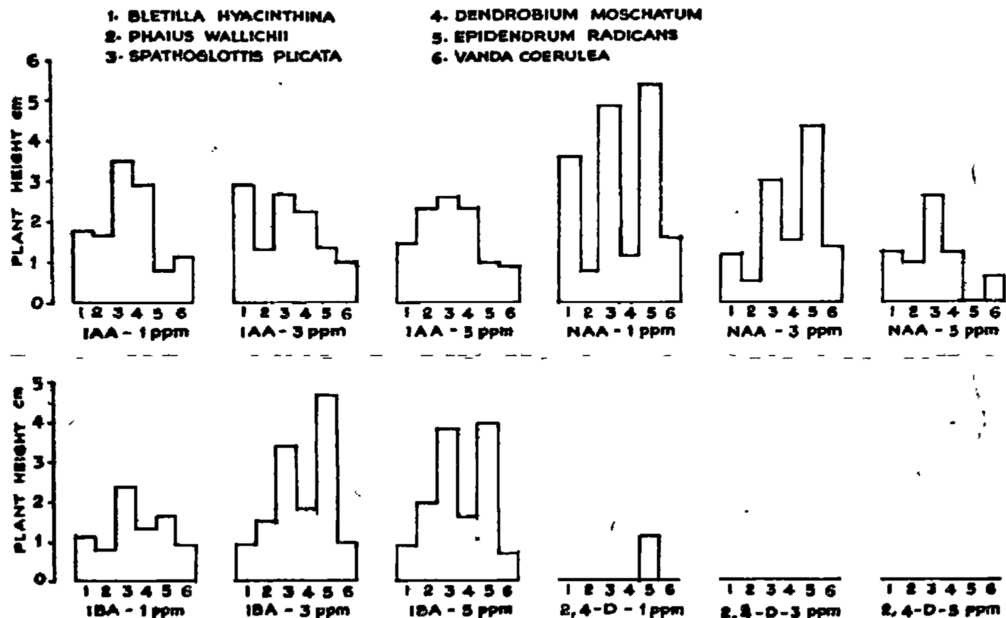


FIG.10. EFFECT OF AUXINS ON PLANT HEIGHT OF ORCHID SPECIES CULTURED ON DIFFERENT MEDIA



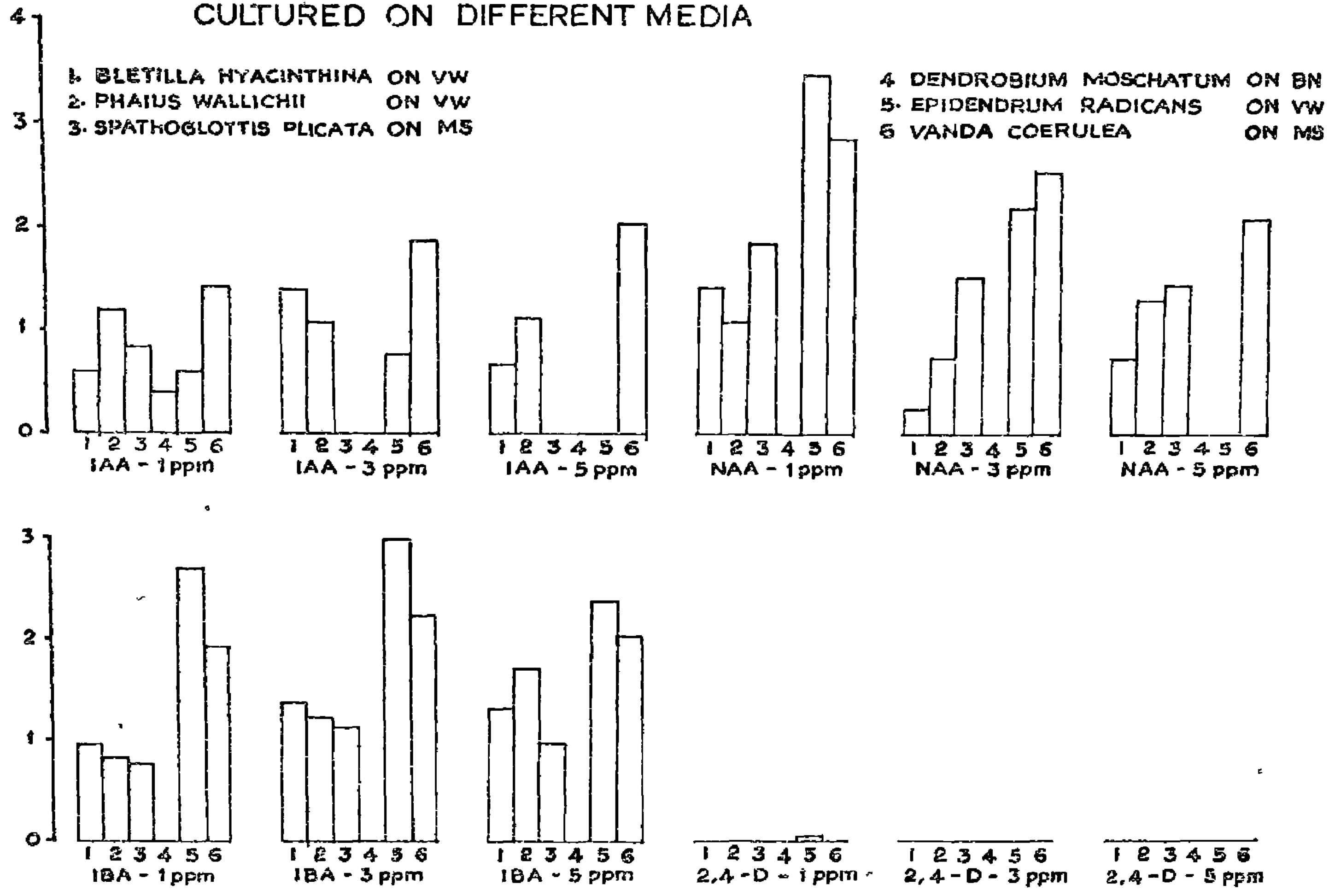
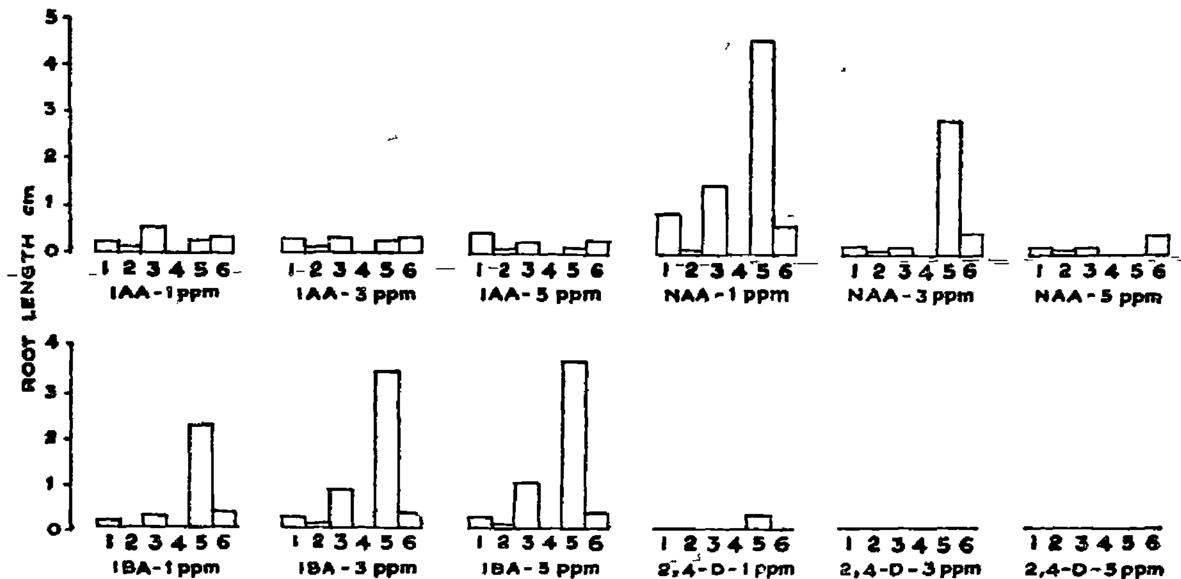


FIG. 11. EFFECT OF AUXINS ON ROOT NUMBER IN ORCHID SPECIES CULTURED ON DIFFERENT MEDIA

FIG. 12. EFFECT OF AUXINS ON ROOT LENGTH OF ORCHID SPECIES CULTURED ON DIFFERENT MEDIA

1- BLETILLA HYACINTHINA ON VW
2- PHAIUS WALLICHII ON VW
3- SPATHOGLOTTIS PLICATA ON MS
4- DENDROBIUM MOSCHATUM ON BN
5- EPIDENDRUM RADICANS ON VW
6- VANDA COERULEA ON MS



5.2.4 Spathoglottis plicata

This is a terrestrial orchid which responded well to Murashige-Skoog mediumfor germination and further growth. The high salt concentration seem to induce better germination, and growth because of its terrestrial nature. The germination and leaf development was faster on MS medium. Other growth measurements were also significantly superior to other media. However, Chennaveeraiah and Patil (1975) reported Whites medium as suitable for this species. Beechy (1970) reported Vacin and Went medium is suitable for germination and growth of Spathoglottis plicata (Fig.8).

5.2.5 Vanda coerulea

Among the five media used, maximum germination and growth was obtained with MS mediuminspite of thes high salt content and epiphytic nature of the species. Seedlings were healthy with dark green and broad leaves. Number of days for germination were almost similar in all the five media. However, MS medium and Vacin and Went medium took the shortest time. Whereas slow germination was noticed in Knudson C medium. The micronutrients present in the medium induced better growth of seedlings. The results agree with the report of Arditti (1967). (Fig.7).

5.2.6 Dendrobium moschatum

The Burgeff N_3F medium was proved superior for germination and growth of seedlings. Mowe (1973) however

reported that Knudson C medium is satisfactory for the growth of Dendrobium. In the present study Knudson C medium was inferior to Burgeff N_3F medium.

In general MS medium induced better growth of Spathoglottis plicata and Vanda Coerulea. The high salt concentration is due to presence of micro-elements which induced better growth. While Vacin and Went medium does not contain micro-nutrients B.hyacinthina, P. Wallechii and E.radicans were found to grow well in this medium. Comparative studies have revealed that the reduction of phosphate levels can increase germination. This may be due to the reason that orchid seeds are sensitive to phosphorus, further high phosphorus concentration may lead to iron deficiency.

5.3 Use of growth regulators in seed germination

Growth substances were applied individually at different concentrations to study their effect on growth of the orchid seedlings. The effect of auxins on plant height, root numbers and root length were tabulated and represented grapically (Fig.10, 11 and 12).

It has been reported that in most of the cases IAA, NAA or IBA enhanced the germination or growth of orchid seedlings. Some of the growth substances like, 2, 4-D have reported to have inhibitory effect. The absence of auxins causes death in Dendrobium ovaries (Isrel, 1963).

The role of cytokinin is well established in seed germination. The germinating seeds are sensitive to cytokinin concentration than the protocorms. The seeds have very low requirement of cytokinin and the seeds can produce the quantity required for its deielopment. The protocorm are capable of producing enough quantity of cytokinins.

5.3.1 Bletilla hyacinthina

Individual treatment of auxins (IAA, NAA, IBA and 2,4-D at 1,3 and 5 ppm) GA (0.1, 0.3 and 0.5 ppm) Kinetin and BA (1,3 and 5 ppm) and vitamins (nicotianic acid, pyridoxin and thiamin 0.5, and 3 ppm) were applied on Vacin and Went medium. IAA at all the three levels induced better growth. However, a concentration of 1 ppm NAA was found superior over others. The effect of NAA on plant growth has been well established (Boesman, 1962; Bose and Mukherjee, 1974, and Mathews and Rao, 1980). IBA and 2, 4-D at all the three levels were not satisfactory for growth. 2,4-D at all the three levels induced heavy callus and produced no seedlings. IAA at 3 ppm had slight increase in growth of plants. This agrees with the report of Boesman (1962).

5. . Vitamins such as nicotinic acid, thiamine and Pyrydroxin were used at different levels (0.5, 1 and 3 ppm). Thiamine has been proved to enhance the germination and growth of orchids (Withner, 1974). The pyramidine fractions of the vitamin also sometimes enhance the growth of plants.

However the results obtained with Bletilla hyacinthina were not encouraging. Nicotinic acid, Thiamin and pyridoxin at all the three levels proved inferior to control.

5.3.2 Phaius wallichii

Growth substances were used individually at different concentration(IAA, NAA, IBA and 2, 4-D at 1, 3 and 5 ppm, GA and BA at 0.5,1and 3 ppm) on Vacin and Went medium and the seeds were sub-cultured. IAA at all the three levels induced better differentiation and growth. IAA at 5 ppm produced better growth compared to all the other treatments. IAA induces better growth in Cattleya (Boesman, 1962). Hadley and Harvis (1968) reported that IAA is not satisfactory for growth of orchid seedlings. IBA at higher levels (3 and 5 ppm) induced better growth. A concentration of 5 ppm was proved superior. Plants with such treatments produced better root system. The increased fresh and dry weight were due to better root development and also the ultimate vegetative growth. The favourable effect of IBA on growth has not been reported earlier. The well developed root system was responsible for the increased growth and weight. No germination was obtained with different concentration of 2, 4-D, GA and BA.

5.3.3. Spathoglottis plicata

Results of the individual treatment of growth substances indicated that IAA at a lower level (1 ppm)

induced maximum plant height with more number of leaves and roots. Similar results were obtained with Cymbidium culture (Ueda and Torikata, 1968). Higher concentrations of NAA induced cellussing and the few plantlets produced thick roots. Matsui et al. (1970) reported that concentration below 1 ppm is good for shoot growth and upto a concentration of 1 ppm do not affect the growth of Cymbidium.

Higher levels of IBA (5ppm) induced better shoot growth and more number of leaves. The fresh and dry weight was considerably increased but the root number and length was less, compared to NAA treatments. IBA induced thickened roots, which increased the fresh and dry weight, IBA does not seem to improve plant growth. IAA at lower levels (1ppm) increased plant height and also the green and dry weight. Earlier reports of Hadley and Harvis (1966) showed that IAA does not seem to promote plant growth. IAA inhibit the germination and growth of Miltonia and Odontoglossum (Hays, 1969). However, the finding of Boesman (1962) is in support of the present finding. Fonnesbech (1972) reported that IAA alone had no effect on Cymbidium seed germination.

Treatment with 2,4-D at all the three levels produced only callus. Heavy callussing was obtained with 1 and 3 ppm level. Ueda and Torikata reported that very low concentration (0.01 ppm) promoted shoot growth. However a recent report of Fonnesbech (1972) states that

2,4-D induce high weight increase by abnormal growth of protocorm. In *Spathoglottis plicata* no seedlings were produced but only callus were formed.

GA and BA at all the three levels were not comparable to control. Higher level of BA induced callussing. Treatments with NAA, IBA and 2, 4-D did not give any satisfactory results. No germination was obtained with all the three levels of 2, 4-D.

5.3.4 *Dendrobium moschatum*

Individual treatment of growth substances were made along with Burgeff N_3F medium. GA and IAA at all the 3 levels had better plant height. The plant height has been influenced to a great extent by GA 1 ppm. IAA 1 ppm also produced more plant height. The effect of GA on germination and growth has been explained in detail by Miyazuki and Nagamatsu (1965). It is reported that lower concentration of GA_3 1 ppm induce plant growth and higher levels were found toxic to plants. Subsequently Bose and Mukherjee (1976) reported the GA induced leaf elongation. The increased plant height obtained in this study is in conformity with the above reports. The number of leaves were maximum with an individual treatment of IAA 1 ppm and GA 1 ppm.

5.3.5 *Epidendrum radicans*

The growth substances were applied individually on Vacin and Went medium (IAA, NAA, IBA and 2,4-D at 1,3 and

5 ppm, and GA and BA at 0.1, 1 and 3 ppm) for culturing. NAA at lower (1 and 3 ppm) levels and IBA at higher (3 and 5 ppm) levels were proved superior over other treatments. Maximum plant height and number of leaves were obtained with 1 ppm NAA. It was followed by a treatment of IBA (3 ppm). The growth promoting effect of NAA has been reported in many of the orchid species such as Cypripedium (Boesman, 1962) and Vanda (Mathew and Rao,1980). However, concentration above 1 ppm seems to stimulate production of less number of seedlings and more amount of callus tissue. Such tissues do not differentiate into plantlet at any stage of growth. The few seedlings produced at higher levels of NAA are abnormally tall and stout with thick roots. Bose and Mukherjee reported that lower concentration of NAA (1 ppm) induce callus and higher concentration induce only roots. IBA at 3 ppm had also produced increased plant height and leaf number. No reports are available to substantiate the finding.

Treatment with 2,4-D at lower levels (1ppm) produced few seedlings. At higher levels (3 and 5 ppm) heavy callussing was observed. Induction of growth at lower levels of 2,4-D had been reported by Ueda and Torikata (1969). Ichihashi and Kako (1973) reported that 0.1 ppm, 2,4-D promoted shoot growth in Cattleya. The results indicated that Epidendrum radicans can grow at lower levels of 2,4-D.

5.3.6 Vanda Coerulea

In this experiment the seeds were allowed to initiate callus in MS medium and later sub cultured on fresh MS medium with additives. The seedling development was very slow. Growth substances were applied individually (IAA, NAA, IBA and 2,4-D at 1, 3 and 5 ppm and GA and BA at 0.1, 1 and 3 ppm). Among all the treatments NAA at lower (1 and 3 ppm) levels and GA at higher (1 and 3 ppm) levels proved superior to other treatments. IAA at all the three levels were not superior to control. NAA at 1 ppm resulted in maximum plant height, leaf and root number, root length and fresh and dry weight. The findings agree with the report of Mathews and Rao (1980). GA (1ppm) also had exhibited better growth performance in plant height, leaf and root number, root length, fresh and dry weight. The favourable effect of lower concentration of GA has been reported by Miyazuki and Nagamatsu (1965) in Dendrobium. The statement holds good for Vanda.

All the other treatments with IBA, 2,4-D and BA were inferior to control. With 2,4-D at all levels no growth was obtained. The callus turned black in a short time.

5.4 Effect of complex organic additives on seed germination and growth

The substances other than growth regulators were also found to influence growth of orchid seedlings. This includes complex mixtures like coconut water, banana pulp,

tomato juice and other fruit juices. In the present study only coconut water and banana pulp were used. The effect of tomato juice on Bletilla hyacinthina was also studied.

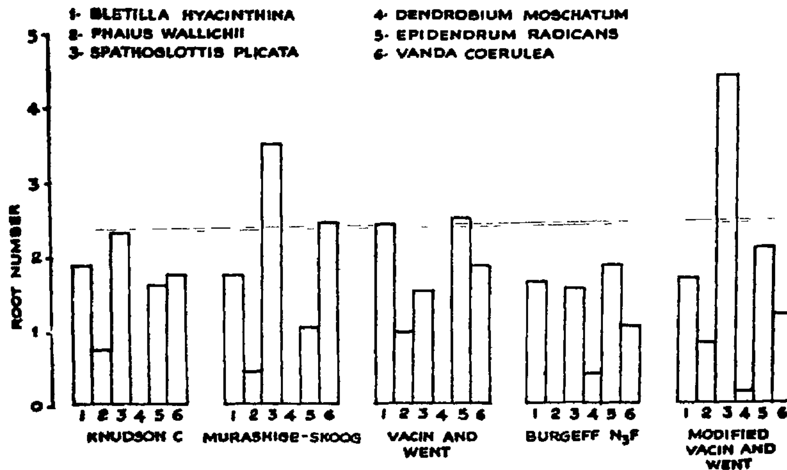
5.4.1 Bletilla hyacinthina

The different additives used were coconut water (10 and 15 per cent), banana pulp (10 and 15 per cent) and tomato juice (10 and 15 per cent) coconut water at both levels (10 and 15 per cent) induced better growth. However 15 percent coconut water produced satisfactory results. Banana pulp also induced better growth at 15 per cent. Effect of coconut water and banana pulp in growth has been well established. (Lawrence and Arditti, 1964., Arditti, 1967, Mc Intyre et al. 1974). Treatment with tomato juice did not produce any satisfactory results. Arditti (1966) reported that tomato juice is detrimental to the growth of Cattleya seedlings which is in support of the present study.

5.4.2 Phaius wallichii

The effect of coconut water and banana pulp on growth were satisfactory. However the results were not comparable with the auxin treatments. Banana pulp treatment was superior to coconut water treatment. Effects with coconut water were slightly superior to control. The findings agrees with the report of Arditti (1967) and Mc Intyre et al (1974) in Cattleya seedings. Coconut water

FIG. 2. ROOT NUMBER IN ORCHID SPECIES AS INFLUENCED BY DIFFERENT NUTRIENT MEDIA



does not seems to promote the growth of *Phaius wallichii* seedlings.

5.4.3 *Spathoglottis plicata*

Banana pulp and coconut water (15 per cent) induced better plant growth. The same results were obtained with some of the other species. The findings agree with the earlier reports of Arditti (1967), Anderson (1967), Arditti (1968), Mowe (1973) and Mac Intyre (1974).

5.4.4 *Dendrobium moschatum*

The individual treatment of banana and coconut water (15 per cent) produced better plant height. Treatment with banana pulp produced maximum number of leaves. The finding is in full agreement with the report of Mowe (1973) where he reported that banana pulp promote better vegetative growth. No treatments produced good roots. A slight root promoting activity was observed with banana pulp. Banana pulp also favoured the increment in green and dry weight.

Epidendrum radicans

Banana pulp and coconut water (15 per cent) each applied individually increased the plant height, leaf number, green and dry weight. The results agree with the findings of the other experiments and earlier reports.

Vanda Coerulea

Treatments with 15 per cent coconut water v/v and banana pulp were slightly superior over control. However, banana pulp exhibited superiority over coconut water. Other

growth characteristics were also not encouraging. Earlier reports states that coconut water and banana pulp increased germination percentage of Vanda to about 80 per cent (Lawrence and Arditti, 1964). In Cymbidium better growth was observed with coconut water (Rosa et al. 1977). However, the present study does not agree with the above reports.

Effect of growth substances in combination with complex organic additives

Different treatments were given combining the growth substances and other additives to study its combined effect on growth and development of orchid seedlings.

Bletilla hyacinthina

A combination of treatments with NAA 1 ppm, IAA 3 ppm, GA 0.3 ppm, coconut water 15 per cent and banana pulp 15 per cent were applied on Vacin and Went medium. A high rate of growth was noticed when banana pulp alone was used. Similar results were obtained with a combination of all the additives, but without coconut water. The results indicate that banana pulp alone is capable of increasing growth of Bletilla hyacinthina seedlings. No appreciable growth increase was observed when all the five additives were used together.

5.5.2 Phaius wallichii

A combination of auxins (IAA and IBA 5 ppm), coconut water and banana pulp (15 per cent) were applied along with medium. A combination of IAA, IBA and banana pulp

produced better results compared to other treatments. IAA at 5 ppm alone also produced better results but to a lesser degree. The effect of coconut water and banana pulp on growth of *Cattleya*, *Cymbidium* and *Dendrobium* has been emphasised (Lawrence and Arditti (1964), Rose *et al*.(1977) and Mowe (1973). Reason

5.5.3 *Spathoglottis plicata*

A combination of treatments (NAA and IAA 1 ppm, IBA 5 ppm, banana pulp and coconut water) were applied on MS medium for the culture of *Spathoglottis plicata*. Better plant height, root number, green and dry weight were recorded with a treatment of all the five additives. Treatment with banana pulp alone was slightly inferior to the treatment combination. This indicated that the growth promotion in treatment combination is mainly due to banana pulp. The number of leaves were maximum in a combination of coconut water along with all the three auxins and it was closely followed by a treatment with all the three auxins alone. Hence the increased leaf number can be due to the effect of interaction of auxins and the coconut water has a negligible role in increasing the number of leaves. However, Chennaveeraiah and Patil (1975) reported that 10 per cent coconut water with casecin hydrolysate increased the growth of *Spathoglottis* on Whites media. The beneficial effect of coconut water has been reported by Mac Intyre *et al*(1974). The results indicate that coconut water along with other additives such as auxin or casein hydrolysate accelerate the growth of *Spathoglottis plicata* seedlings. Treatment

with all the three auxins produced more fresh and dry weight.

5.5.4 Dendrobium moschatum

The combination of treatments were made with IAA 1 ppm and GA 1 ppm with and without coconut water and banana pulp (15 per cent). Maximum plant height, leaf number, root number, root length, green and dry weight were observed with a combination of IAA and GA 1 ppm and banana pulp. Combination of all the four additives were also superior but they were on par with the treatment of banana pulp alone. The superiority of banana pulp over other additives are further emphasised, in this experiment. The effect of coconut water was not promising. The seedlings produced on media with coconut water were pale and unhealthy.

5.5.5 Epidendrum radicans

The treatment combinations were fixed with NAA (1 ppm) and IBA (3 ppm) with and without coconut water and banana pulp. Maximum growth (plant height, leaf and root number root length green and dry weight) was observed in a combination of NAA 1 ppm, IBA 3 ppm and coconut water. Combination of all the four additives also had better growth. Treatment with coconut water alone was proved inferior to treatment with banana pulp alone. But along with NAA and IBA coconut water exhibited better growth performance. The increased growth promoting activity of coconut water has been described early by Lawrence and Arditti(1964), Arditti,(1967) Mac Intyre (1974) and Rosa et al (1977).

Vanda coerulea

The combination of treatments were fixed at 1 ppm of NAA and GA, 15 per cent coconut water v/v and banana pulp. A treatment with NAA, GA and coconut water was superior to other treatments. The growth invigouration was exhibited when coconut water was used along with NAA and GA. Combination of all the four additives were also superior in promoting the growth of protocorms and differentiation. The results obtained in a combination of NAA with coconut water and GA with coconut water were almost similar, whereas NAA and GA with banana pulp had very poor growth. The results indicate that in Vanda seed culture coconut water is more effective than banana pulp in the growth of seedlings. The results agree with the findings of Arditti (1967), Rosa et al (1977).

5.6 Tissue culture

5.6.1 Cymbidium meristem culture

Cymbidium meristem responds very well to VW medium(Liquid). The other media tried (KC, MS, BN and MVW) did not give very good results. Maximum fresh weight of 85 mg was obtained in VW medium followed by 67 mg in MS, BN and KC did not give any results and meristem remained without change and ultimately turned brown and died.

IAA alone had no effect. However in combination with NAA fresh weight was increased. Higher concentration

of NAA and 2, 4- D (5 ppm) were inhibitory and inhibited the synthesis of chlorophyll, similar results of higher concentration of NAA and 2,4 -D were also repeated earlier by Scully (1967). Further 2,4-D is least suitable than NAA as an auxin for in vitro propagation of Cymbidium as it disturbs the chlorophyll synthesis and also induces abnormalities, this was also observed earlier by Ueda and Torikata (1968).

5.6.2 Nodal culture

Dendrobium pierardii

Experiments conducted to standardise a suitable medium for Dendrobium pierardii nodal cuttings had shown that the Vacin and Went medium took the shortest time for initiation of growth. No growth initiation was observed on Burgeff N_3F medium. Knudson C and MS medium took almost the same time for initiation of growth. Knudson C medium produced callus from 2 nodal sections. None of the other media could produce callus. However maximum number of plantlets were produced in Vacin and Went medium. Number of multiple shoots were also maximum on Vacin and Went medium. Other growth characteristics such as plant height, number of leaves and mean number of roots were maximum on Vacin and Went medium. The results indicated that Knudson C medium is best suited for callus formation from lateral buds and Vacin and Went medium for plantlet formation. Suitability of media for callus formation, differentiation and growth has been reported by many investigators on different species of Dendrobium Knudson C medium (Valmayor, 1974), Marston medium (Marston, 1966), Vacin and Went medium

for Dendrobium Bali Dendrobium Claracoores, Dendrobium nobile, (Sagawa Valmayor, 1966 and Valmayor Sagawa (1967). Vacin and Went medium for Dendrobium "Jaquelyn Thomas" (Israel, 1963, Singh and Sagawa, 1972), Knudson B ' medium for Dendrobium "May neal," D. "Pompadour" (Vajrabhaya and Vajrabhaya 1974).

The callus formed on the basic medium were again subcultured into Knudson C liquid medium for proliferation of tissue. The effect of tissue proliferation on liquid media has been well established (Street, 1969 and Cheng, 1978). Callus production is further enhanced on shaking the media in a shaker (Heller, 1953 and White, 1963). The basic media was supplemented with 0.1 ppm 2,4-D and 15 per cent coconut water and its combinations. The callus tissue further proliferated with in a period of 60 days. It has been reported that 2,4-D at very low concentration induces abnormal growth of tissue (Fonnesbech, 1972). However, it is reported that in some other species 2,4-D at lower levels promoted shoot development (Ueda and Torikata, 1969 and Ichihashi and Kako, 1973). Coconut water stimulate growth in many epiphytic and terrestrial orchids (Mac Intyre, 1970, Rosa et al. 1977).

The callus bodies were again sub-cultured on solid Knudson C medium along with IAA (1ppm) BA(1ppm) coconut water 15 per cent and its combination. However, coconut water treatment alone had differentiated more number of plantlets within a short period. Number of leaves and roots produced and plant height were also more with coconut water treatment. The results of treatment combination with all

the 3 additives had almost identical results with NAA (1ppm), treatment. The effect of NAA on plant growth has been reported in Cattleya (Boesman, 1945, Lindemann, 1967, Ueda and Torikata, 1968, Matsui et al. 1970, Fonnesbech, 1972. Ichihashi and Kako, 1973, Bapat and Narayanaswami, 1977 and Kusumoto, 1979.

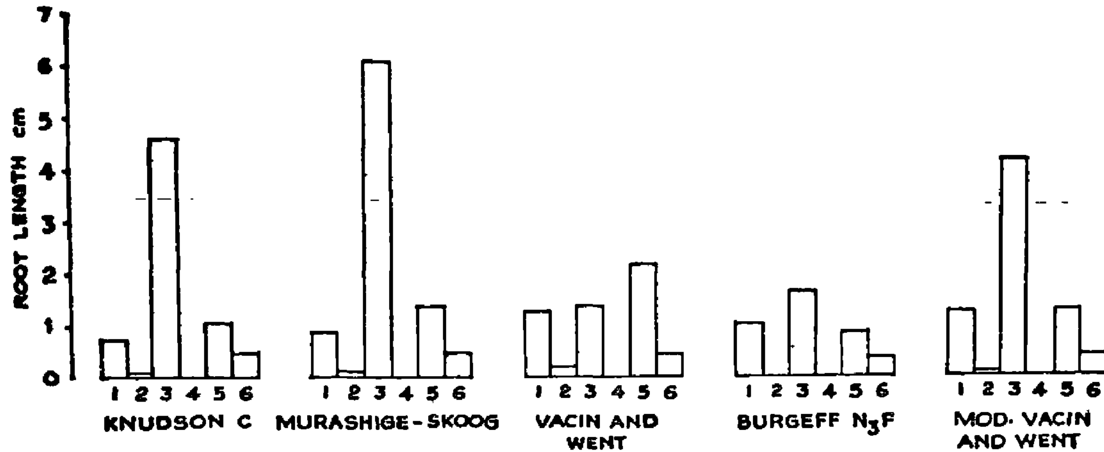
5.6.3 Epidendrum radicans

While comparing the selected five media for callus development and plantlet formation, it was observed that Knudson C solid mediumwas found to induce more number of callus bodies and plantlets. Vacin and Went mediumwas also found satisfactory in plantlet formation but only one callii was produced. Suitability of media for Epidendrum tissue culture has been studied in detail and various media are recommended. Murashige-Skoog and Knudson C media(Churchill, et al. 1970), Vacin and Went medium (Sagawa and Valmayor, 1966 and Valmayor and Sagawa, 1967), Heller and Knudson C. medium (Churchill et al. 1971 and 1973), Ojima and Fujiwara medium (Churchill et al, 1972). However, the present results also indicated Knudson C medium as the most suitable for callus formation.

Further proliferation of the callus was made by subculturing the tissue into solid Knudson C medium with 2,4-D (0.1 ppm) and coconut water (15 per cent) alone and in combinations. The callus proliferation was satisfactory

FIG.3. ROOT LENGTH IN ORCHID SPECIES AS INFLUENCED BY DIFFERENT NUTRIENT MEDIA

1. BLETILLA HYACINTHINA
2. PHAIUS WALLICHII
3. SPATHOGLOTTIS PLICATA
4. DENDROBIUM MOSCHATUM
5. EPIDENDRUM RADICANS
6. VANDA COERULEA



on treatment with coconut water alone. 2,4-D also induced callus formation but to a lesser degree. Although 2,4-D induce abnormal growth (Fonnes bech, 1972) no such results could be obtained with Epidendrum radicans. However, the reports of Chennaveeraiah and Patil (1975) on Spathoglottis plicata and Rosa et al.(1977) on Cymbidium were found to substantiate the results obtained in this study with coconut water.

The callus bodies were subcultured into the same fresh media with IAA (1ppm) BA (1ppm) and coconut water (15 per cent) individually and in combination for differentiation of shoot and root. Treatment with a combination of NAA (1 ppm) and coconut water, took minimum time for shoot initiation and produced healthy dark green plantlets. Treatment with coconut water alone also initiated shoots in a short time and produced healthy plants. However, NAA alone took more time for shoot initiation. This indicates that coconut water accelerated the growth when used with growth substances. The increased effect of the treatment may be possibly due to the effect of coconut water. The stimulatory effect of coconut water on protocorm differentiation and greater production of chlorophyll in the plants were already reported by Pollard (1961) and Vanstaden and Drewes (1975). The results of the present study also fully agree with their finding.

5.6.4 Vanda teres

Single node cuttings of Vanda teres were cultured on different media. Only three media (Vacin and Went, Burgeff N_3F and Modified Vacin and Went) could initiate growth of explants. Knudson C and Murashige-Skoog media did not produce any symptoms of growth. Maximum growth was observed with Vacin and Went medium followed by Modified Vacin and Went medium.

The development of explants and subsequent growth was very fast onVacin and Went medium. But the development of explants were very slow on Modified Vacin and Went and Burgeff N_3F media. However the subsequent growth on Modified Vacin and Went medium was little faster than in other media. No plants produced roots except on Burgeff N_3F medium which produced few roots. The growth promoting effect of different media with Vanda species has been reported early. The Vacin and Went medium has been reported suitable for Vanda. (Rao and Avadhani, 1963, 1964; Rao, 1963, 1964; Sagawa and Valmayor, 1966; Sagawa and Sehgal, 1967; Goh, 1971; Kunisaki et al. 1972; and Valmayor,1974). Whites medium has been reported by Goh (1970).

The lateral buds of the explants bulged slightly showing a symptom of bud initiation. Later they formed into semi spherical bodies. Kunisaki et al. (1972) reported the formation of such semi spherical bodies inVanda " Miss Jaoquim". These bodies were excised and sub-cultured on liquid Vacin and Went medium for further proliferation. Treatment

with NAA (1ppm) alone produced callus bodies.

The callus bodies sub-cultured on solid media with NAA 1 ppm and coconut water formed into plantlets. The results of the experiment are in full agreement with the reports of Kumisaki *et al.* (1972).

5.7 Root culture studies

The meristematic region of the aerial roots of *Dendrobium* were cultured on different media. All media initiated growth of the roots but limited to root extension only. No explant produced callus. The roots extended to varying degrees. The findings agree with the report of Churchill, Ball and Arditti (1972). They reported in *Epidendrum* root culture, all they produced was roots, albeit longer roots. Stewart and Button (1978) also reported that the media supported root tip growth for a limited time. However he could get very small callus in some roots of *Epidendrum*. The results of the present study did not show any sign of callus development. The roots only extended and later they dried.

5.8 Histochemical studies

5.8.1 Seed germination

The structure of orchid seed has already been described in detail (Arditti, 1967). The seeds of *Bletilla hyacinthina* contains relatively differentiated embryo with a rudimentary cotyledon. In addition to this the seed does not contain even a trace of endosperm tissue. The

spherical or oval shaped embryo has got a suspensor protruding at its one end. The remaining part of the embryo is made up of cells almost similar in their structure and histochemical contents such as insoluble lipids and proteins. Thus the seed does not seem to show any polarity so far as the distribution of lipids are concerned. The embryo of B.hyacinthina is free from starch deposition which is in conformity with the composition of the embryo of Cattleya (Harrison, 1977).

The seeds sown in the nutrient medium are ready for germination. The cells of the embryo adjacent to nutrient medium imbibe and absorb water and some nutrients from the medium. Such an absorption results in the swelling of the embryo. The membranous seed coat is ruptured and the embryo protrudes out. The subsequent increase in the size of the embryo is accompanied by the enlargement of its cells. The intake of the carbohydrates in the form of sugars from the medium seems to hasten the degradation of lipids through β-oxidation and to form starch. This observation is in conformity with the happenings in the germinating seeds of Cattleya (Harrison, 1977).

The enlargement of the embryo is followed by the formation of some absorptive hairs or rhizoids at its basal end. Such an embryo with absorptive hairs and stored food material in its prenchyonatous cells can be called protorm

like body (PLB). The nutrients absorbed by the rhizoids seem to move in the basal part of the PLB by a simple diffusion phenomenon as there is no evidence of the formation of vascular or provascular strands in that region. The PLB enlarges further by an increase in the size and number of its cells and becomes a top shaped body.

The cells at the basal end of the PLB are large while those at its apex are very small. A decreasing gradience is exhibited in the PLB from base to apex in the size of cells, size of nuclei and in the size and quantity of the stored starch grains. On the contrary and increasing gradience is observed in the RNA and protein contents from base to apex in the PLB. The enlarged cells near the basal end of PLB appear to be concerned with the active starch accumulation and subsequent degradation into simpler carbohydrates to be transported to the shoot apex, which is a region of active growth and differentiation.

The increased DNA content in the enlarged nuclei of the basal cells perhaps facilitates in governing their specific role in supplying the nutrients. The presence of rich cytoplasmic RNA along with the absence of storage carbohydrates in the small sized apical cells perhaps indicates their meristematic nature. In support of this, their proportionately small sized diploid nuclei are densely stained with feulgen reagent for their DNA content. The narrow group of cells in the upper middle part of the germinating seed,

connecting the apical meristematic zone with the basal storage region perhaps aid in translocation of nutrients from the latter to the former. Hence these group of cells lacking xylem and pholem differentiation can aptly be called as provascular strands. However the presence of rich cytoplasmic RNA and proteins in these cells suggests a high rate of metabolism.

Thus the provascular strands seems to be not merely a passive structure of translocation but its cells are also metabolically active. The increased activity of this group of cells may be perhaps concenred with the prevention of reversible reactions and/or with the transformation of some raw metabolites into other forms as needed by the meristematic cells of the shoot apex.

The greening phase of PLB is marked by the differentiation of chloroplasts in its cells exposed to light. In the meanwhile the stomata with well formed guard cells differentiate in the epidermis of that region. The chloroplasts take part in photosynthesis, stomata aid in exchange of gases and the rhizoids absorb water and nutrients thereby the PLB shows the signs of initiation of autotropism. With this crucial event further structural differentiation seems to get hastened.

The leaf primordia, which arise laterally near the primodium of the shoot apex, have horse shoe shaped structures made up of small cells rich in RNA and protein.

The storage polysachharides are completely absent in these cells. The differentiation of leaf tissues is gradual and acropetal. With the differentiation of the leaf, its cells enlarge and the cytoplasmic RNA and protein contents diminish. In the meanwhile the chloroplasts appear in the mesophyll cells and stomata differentiate in the epidermis of leaf.

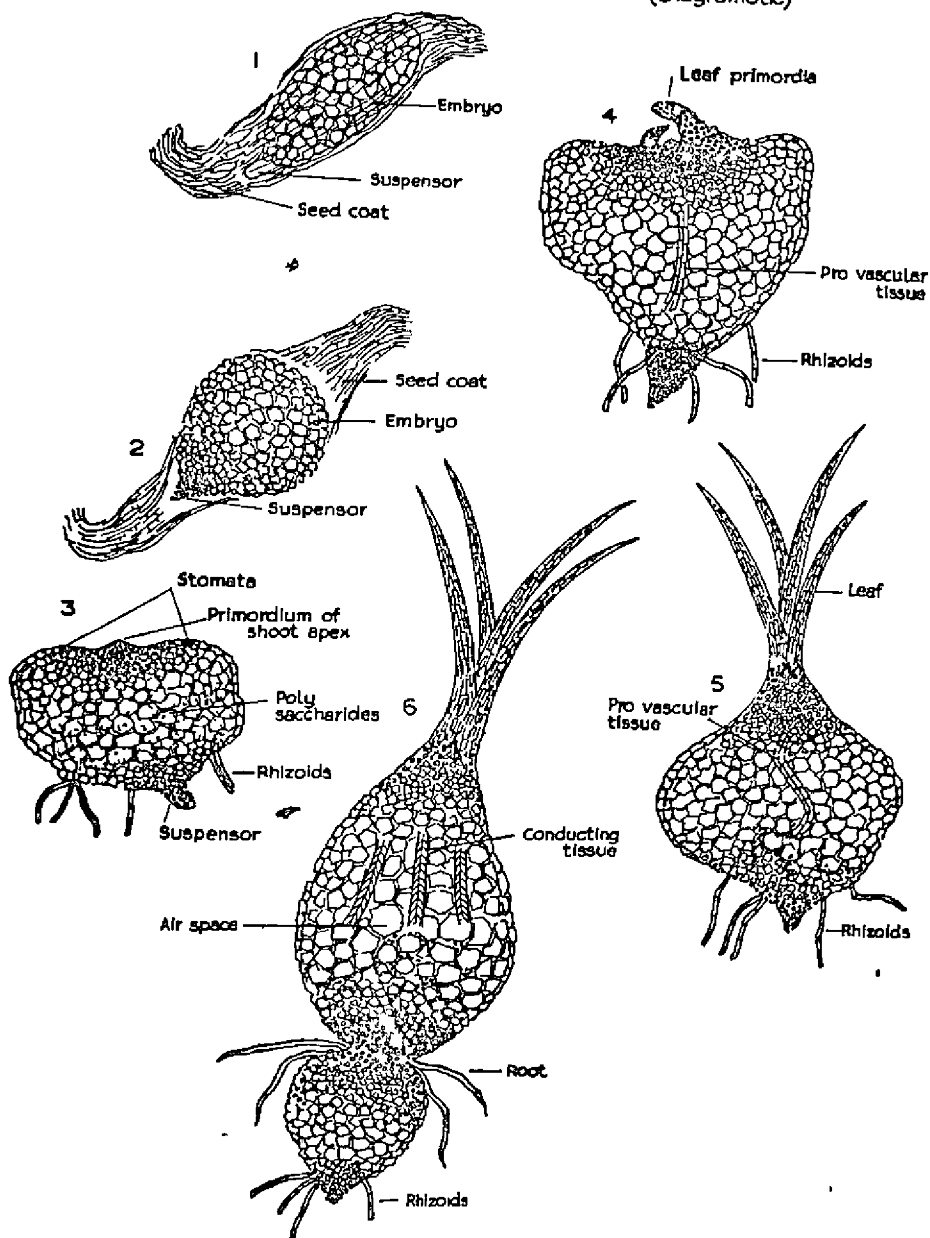
With the differentiation of leaves, the plantlet becomes photosynthetically more active. The surplus photosynthates flow back to the leaf base, then move to the base of the shoot and get converted to storage starch. The cells at the base of the shoot multiply and enlarge to form a bulbous structure known as pseudobulb. At the time of initiation of the pseudobulb all the cells are similar both in structure and chemical contents. Subsequently with the enlargement of the bulb some of the parenchymatous cells also enlarge enormously, while their surrounding cells remain as such with little structural alterations. Thus the well differentiated pseudobulb has got two kinds of parenchymatous cells which differ from each other both in structure and chemical contents. The large cells are isodiametric and they contain large nuclei rich in DNA, higher concentration of RNA, proteins and starch grains at various stages of degradation. The small cells appear narrow in longitudinal section and they contain small sized nuclei, low concentration of cytoplasmic RNA and protein and rich accumulation of storage starch. All these show that the large cells are metabolically more active than the small cells. The large cells perhaps play an important role

in supplying the nutrients to the shoots and roots, which differentiate near the base of tho pseudobulb. Hence these cells are comparable to the large basal cells of PLB of Vanda in their structure, chemical composition and function to a certain extent (Alvarez and Sagawa, 1965). Subsequently the large cells completely loose their cell contents and they may become air or water storing structures.

The vascular strands which get differentiated in the meanwhile connect the base of leaves to the base of pseudobulb. The well differentiated xylem and pholem of these strands seem to facilitate the movement of metabolites in this storage organ.

When an optimum level of concentration of storage substance is achieved some of the cells at the base of pseudobulb probably differentiate into lateral shoot primordia. These may develop into positively phototropic vegetative or reproductive shoots. In the meanwhile at a slightly lower level some of the cells at the base of the pseudobulb differentiate into root primordia which further develop into positively geotropic adventitious roots. The plantlet with its true roots capable of absorbing water and minerals and with its photosynthesising shoot system has become completely autotrophic. Even after this the PLB remains attached to the base of the pseudobulb for some more time acting as a vestigeal structure which degenerates in due course. The developmental stages during the germination of Bletilla

FIG 13. DEVELOPMENTAL STAGES DURING GERMINATION OF BLETILLA HYACINTHINA (Diagrametic)



hyacinthina seeds is represented diagramatically (Fig.13).

Carbohydrate mobilization pattern

The carbohydrates absorbed from the media through the rhizoids diffuse to the basal cells of the PLB from which they flow into other cells. The absorbed carbohydrates probably get converted to storage starch and later the stored starch grains degrade into specific type of sugars which move to the tip of PLB to help the differentiation of shoot apex. The mobilization of sugars in the PLB is through diffusion from cell to cell in its basal part, while it is through provascular strands in its upper part. All these indicates that the mobilization of carbohydrates is acropetal in the PLB.

In the autotrophic plantlet the leaves are photosynthetically active. After utilizationof some of the photosynthates for its own differentiation, the leaf allows the excess to flow into the base of the pseudobulbs. The photosynthates further diffuse from the basal cells to their contiguous cells, where they get converted into starch and accumulate. While the photosynthates more downward in the vascular strands, some sugars diffuse into the neighbouring cells of the vascular strands and accumulate as starch grains. Later, after attaining an optimum level of storage the starch near the base of the pseudobulb gets degraded into specific kinds of sugars which flow into differentiating and growing lateral shoots and roots. The mobilization pattern of

possibly due to the presence of partially differentiated embryo (Burgeff, 1936; Harley, 1951). The number of days for seed germination and leaf production are represented graphically. Although Bletilla hyacinthina is a ground orchid it responded well to a medium with low salt concentration. The plant height, root number, green dry weight were recorded maximum in this medium. Bletilla seedlings grow at a low salt concentration.(Fig.4).

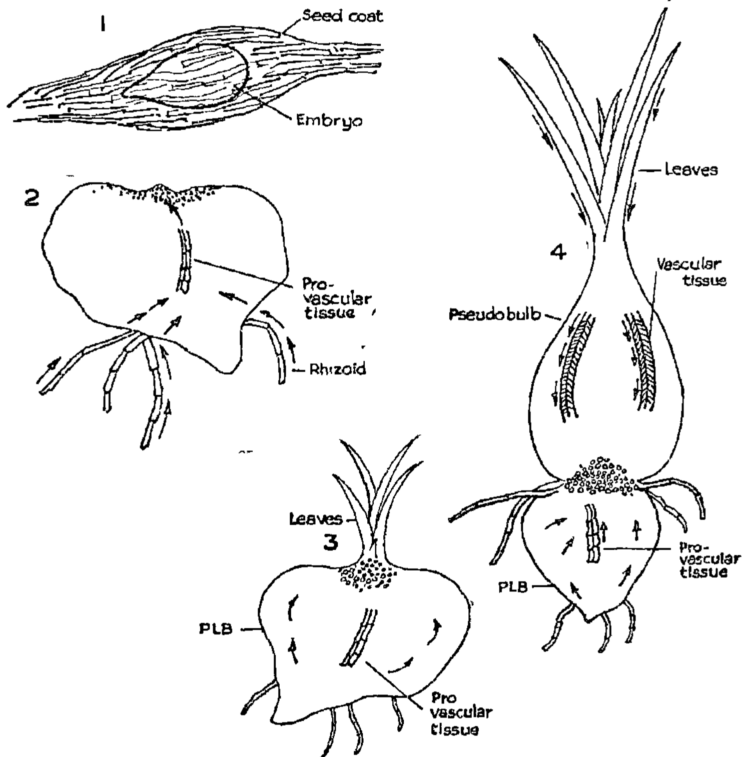
5.2.2 Phaius Walichii

Seed germination was very slow in all the media. No germination was noticed in Burgeff N_3F media. However Vacin and Went media proved superior over others. A rapid seed germination and growth was observed on Vacin and Went mediumalthough the mediumcontains only major elements. (Fig.5).

5.2.3 Epidendrum radicans

All the five nutrient media responded well in germination and further growth of seedlings. However, quick germination and growth was observed on Vacin and Went medium. Epidendrum is an epiphytic orchid, which requires low salt concentration in the medium. Knudson C also induced quick germination. The results agree with the report of Sagawa and Valmayor, 1966 and Valmayor and Sagawa, 1967. (Fig.6).

FIG.14. NUTRIENT MOBILIZATION PATTERN IN A DEVELOPING ORCHID SEEDLING (Diagramatic)



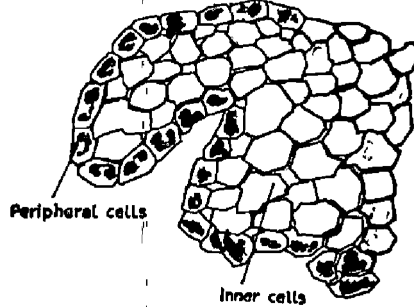
carbohydrate is represented diagramatically (Fig.14).

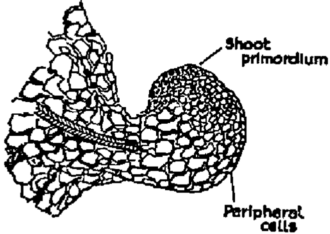
5.8.2 Tissue culture

In the first phase of callus formation uniformly sized peripheral cells are formed to contain rich cytoplasmic RNA and proteins, while the starch grains are few in number. These cells appear to be metabolically more active than the interior cells of the callus which are enlarged to various degrees and contain less of RNA and proteins but a rich accumulation of storage starch. In the second phase where the shoot apex differentiates from the peripheral cells, the meristematic zone is made up of small sized cells rich in RNA and proteins, while starch is completely absent from them. Perhaps the prescussors of RNA, proteins and the soluble sugars needed for shoot differentiation diffuse from the neighbouring peripheral cells and also the enlarged interior cells. Thus the peripheral cells together with the enlarged interior cells perhaps play the role of nutrient reserveirs to the differentiating shoot apex.

Near the lower part of the shoot apex the leaf primordia get differentiated, which develop into leaves subsequently. At this stage the plantlet takes part in photosynthesis and it has become autotrophic. In the meanwhile from the base of the shoot, the adventitious roots differentiate, which grow as positively geotropic structures. To facilitate the movement of water, minerals and metabolites the vascular strands also differentiate in the roots, leaves and shoot regions of the plant. The callus differentiation

FIG.15. CALLUS DIFFERENTIATIONS IN ORCHIDS
DENDROBIUM PIERARDII





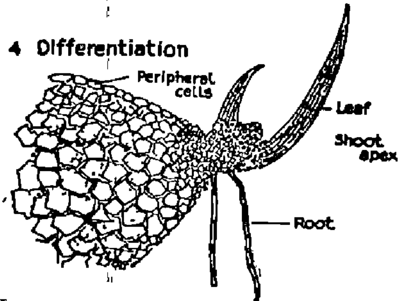
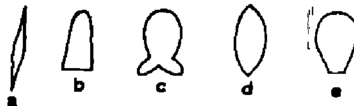


FIG.16 BASIC FORM OF ORCHID SEEDS



in orchid (Dendrobium pierardii) is represented diagramatically(Fig.15).

5.9 Chromosome instability in tissue culture

In the present study cell abnormalities with large scale chromosome variations in cultured cells were noticed. The cells which contain more than diploid number of chromosomes, cell fusion or chromosome doubling followed by elimination is the probable cause.

It is well known fact that cellular and nuclear budding are brought about by the presence by the surrounding cells which is at its optimum in callus cells, cytological and anatomical observations also suggests that stress may be an important factor in abnormal cell behaviour (Yeoman and Brown, 1971) further higher concnetration of auxins, hormones and other adjuncts in nutrient media also help in the formation of polyploid cells by arresting the spindle function. Demoise and Partanen (1969) on their study of Paeonia have given the possible explanation of myxploid cells in tissue culture on the assumption that the environmental condition of in vitro cultures encourages a process of endoreduplication among the diploid cells, stimulating a portion of it to become tetraploid, however if the tetraploid cells do not have the capacity of continued cell division and stopped dividing, the diploids took over in following cultures. This explains the higher percentage of normal cells in the present case when cells from differentiated callii were analysed.

SUMMARY

CHAPTER VI

SUMMARY

Investigations were made to standardise the most suitable media for seed and tissue culture of orchids. Studies were also made to know the individual and combined effect of growth substances and other additives in promoting the growth of plants. Cytological and histochemical investigations were also made to know the cytological variations in the cells of callus tissues and to know the histochemical changes associated with orchid seed development. The results are summarised in this chapter.

6.1 Seed culture studies

6.1.1 Standardization of media

Five different media (Knudson C, Murashige-Skoog Vacin and Went, Burgeff N_3F and modified Vacin and Went) were compared for the germination and growth of seeds of six different orchid species. Vacin and Went medium was superior in respect of germination, growth and development of *Bletilla hyacinthina*, *Phaius wallichii* and *Epidendrum radicans*.

Bletilla hyacinthina seeds had better germination (83 per cent) due to the presence of partially differentiated, embryo. *Phaius wallichii* took more number of days for germination and the percentage was poor and the rate of growth was slow compared to the growth of other

species on Vacin and Went medium. The germination and seedling growth of Epidendrum radicans was better on Vacin and Went medium.

Murashige-Skoog medium was found superior for culture of Spathoglottis plicata and Vanda coerulea. The germination of Spathoglottis plicata was better on MS medium. The plant growth was also excellent. Seedlings were tall, dark green and thick with a well developed root system. Although the germination percentage was low in Vanda coerulea, the seedlings were healthy with dark green foliage and thick and long aerial roots. The increased growth rate in these species may be due to the effect of higher amount of macro and micro nutrients present in the medium compared to other media.

The Burgeff N_3F medium proved superior for Dendrobiums only. The growth and development was quite satisfactory in this medium.

6.1.2 Effect of growth substances and other additives

Bletilla hyacinthina

Individual treatments with IAA at 1 ppm level proved superior to all the other treatments. Other treatments with IAA 3 ppm and GA 0.3 ppm also exhibited growth stimulation, but to a lesser extent. All the other treatments tried were either inferior or on par with the control.

Banana pulp and coconut water at 15 per cent had better growth promoting effect. A combination of treatment with IAA-1 ppm, NAA-1ppm, GA, 0.3 ppm, banana pulp and coconut water 15 per cent v/v resulted in excellent growth of seedlings.

Phaius wallichii

Only auxins exerted growth promoting effects. Individual treatment of IBA at 5 ppm and IAA at 5 ppm resulted maximum shoot and root growth. Coconut water at 15 per cent did not produce any promising results. Banana pulp (15 per cent) resulted in better plant growth and green and dry weight. No germination was obtained with 2,4-D, GA and BA at all concentrations. Treatment with IAA and IBA 5 ppm and banana pulp 15 per cent resulted in better shoot, leaf and root development. The green and dry weight were also higher.

Spathoglottis plicata

Individual treatments with NAA (1ppm), IAA (1ppm) and IBA (5 ppm) were found promising in increasing the growth of seedling. 2,4-D at all concentrations induced heavy callussing. Other treatments were not superior over control. Treatment with banana pulp produced better growth. But the results with coconut water were not encouraging. However, the production of root and green and dry weight were satisfactory. A combination of treatments with all the five additives (NAA 1 ppm, IAA 1 ppm, IBA 5 ppm, banana and coconut

water 15 per cent gave very promising results in respect of growth and development of seedlings.

Dendrobium moschatum

The results obtained with different additives were not encouraging, compared to the control. However, treatment with IAA (1 ppm), GA (1ppm), banana pulp (15 per cent) and coconut water (15 per cent) were superior to other treatments. Treatment with banana pulp and GA 1 ppm had induced better shoot and root growth and green and dry weight. A combination of treatments with IAA 1 ppm, GA 1 ppm and banana pulp 15 per cent resulted in significantly high rate of growth and dry matter content.

Epidendrum radicans

Individual treatments with NAA at 1 ppm and IBA 3 ppm produced better growth and development, compared to other treatments. Individual treatment of banana pulp 15 per cent and coconut water 15 per cent proved slightly inferior to treatment with auxins. 2,4-D at lower concentration induced plant growth. NAA at higher levels (5 ppm) produced heavy amount of callus and at lower levels healthy seedlings with stout and thick roots. A combination of treatments with NAA 1 ppm, IBA 3 ppm and coconut water 15 per cent favoured the development of very healthy seedlings with thick long roots and more green and dry weight.

Vanda coerulea

Better growth was obtained when NAA (1 ppm) and GA (1.0 ppm) was used individually. The results with the use of banana pulp at 15 percent and coconut water at 15 per cent were not encouraging and were proved inferior to treatments with growth substances. However, they brought about slight increase in growth rate compared to control. Out of the several combination of treatments, treatment with GA 1 ppm, NAA 1 ppm and coconut water 15 per cent resulted in maximum growth, including green and dry weight. A combination of all the four additives (NAA 1 ppm , GA 1 ppm, coconut water 15 per cent and banana pulp 15 per cent also caused better growth, but to a lesser degree.

6.2. Tissue culture studies

6.2.1 Cymbidium: meristem culture

Five different liquid media (Knudson C, Murashige-Skoog, Vacin and Went, Burgeff N_3F and Modified Vacin and Went) were tested for callus formation. Meristems cultured on Vacin and Went medium produced highest amount of callus (85 mg) followed by Murashige-Skoog medium (67 mg). Treatment with IAA alone had no effect, but a combination of NAA and IAA at 1 ppm increased fresh weight. Higher concentration of NAA (3 and 5 ppm) and 2,4-D(5 ppm) were found inhibitory.

FIG. 4. EFFECT OF MEDIA ON GERMINATION AND LEAF PRODUCTION IN BLETILLA HYACINTHINA

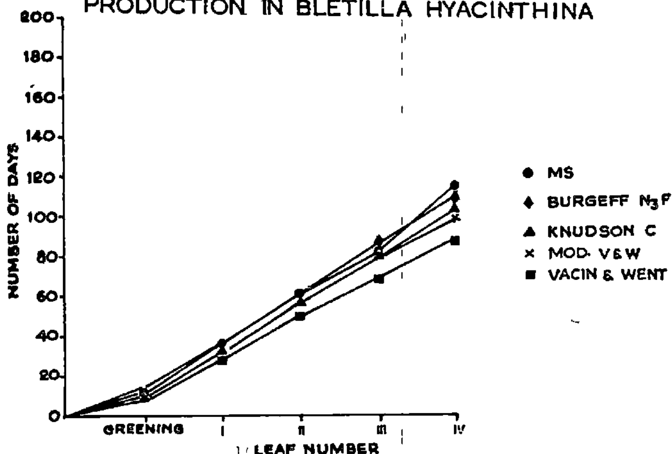
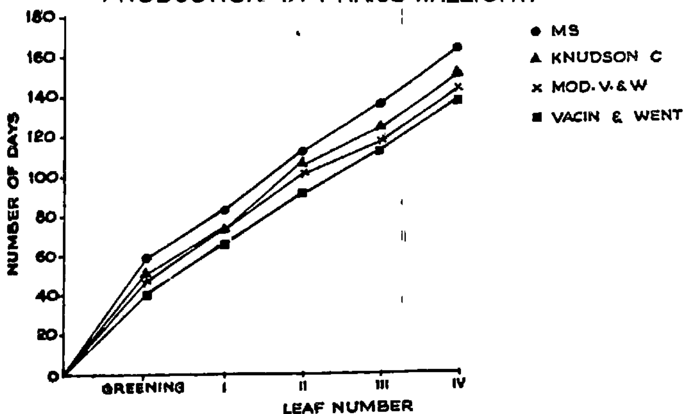


FIG. 5. EFFECT OF MEDIA ON GERMINATION AND LEAF PRODUCTION IN PHAIUS WALLICHII



The lateral buds along with a portionof the stem from Dendrobium pierardii were cultured on different nutrient media. Out of the five different media tested, Knudson C initiated more number of callus bodies, whereas no callus was initiated on Vacin and Went medium. Instead they produced maximum number of plantlets. Subculturing the ca-llus on Knudson C medium with coconut water (15 per cent) differentiated maximum number of plantlets with more number of leaves and roots. The time taken for development of tissue was also slow.

The nodal cuttings of Epidendrum radicans were also cultured on five different media, out of which Knudson C medium proved superior to all others in provuding highest number of callus bodies and plantlets. The callus bodies were sub-cultured into the same medium with auxins(IAA 1 ppm) and cytokinin (BA 1 ppm) along with coconut water (15 per cent). Very promising results were obtained when NAA (1 ppm) was used along with coconut water 15 per cent. Plant height leaf and root number were also increased by the above treatment.

The nodal section of Vanda teres were cultured on five different media. No media produced callus bodies. But a small semispherical body was formed at each node. These bodies formed into callus when sub-cultured on liquid medium. The callus bodies proliferated more and later again sub-culturod into a solid medium with the addition of auxins(NAA 1 ppm) and cytokinin (BA 1 ppm). More number of plantlets were formed due to a combination of treatments with NAA 1 ppm

and coconut water 15 per cent. Treatment with coconut water and NAA alone resulted only callus growth.

6.2.2. Root culture

Dendrobium moschatum aerial root tips cultured on different media did not produce any callus bodies. But the roots further extended for some more time and later dried.

6.3 Histochemical studies

The seeds of *Bletilla hyacinthina* contain considerable amount of stored lipids and proteins but no starch. No distinct polarity was exhibited in respect of the distribution of cell contents. Subsequently with the germination of seed, the sugars from the medium moving into the embryo seemed to aid hastening the degradation of lipids through β-oxidation to form starch. The protocorm thus formed exhibited a decreasing gradience in the size of its cells, its nuclei and in the size as well as quantity of the acumulated starch grains from the base to apex. Ribonucleic acid and proteins show an increasing gradience in the protocorm like bodies from its base to apex.

The basal enlarged cells took part in active degradation of stored starch and the degraded substances were transported to the shoot apex. The translocation of nutrients in the protocorm like bodies were by cell to cell diffúsion in its lower part and by a group of conducting cells, lacking xylem and phloem differentiation in its upper part. Conducting cells were rich in RNA and proteins

indicating their high rate of metabolic activity.

The protocorm like bodies developed chloroplasts and stomata which was the beginning of an autotrophic phase. Subsequently, after the development of photosynthetically active leaves, more of photosynthates flows into lower part of shoot and get converted into storage starch. The basal portion of the shoot enlarged to form a bulbous structure the pseudobulb. The lateral shoots and roots differentiated at the basal portion of the pseudobulb after an optimum level of storage substance was achieved.

The mobilisation pattern of carbohydrate was quite specific. Its movement in the protocorm like body is acropetal and in pseudobulb it was basipetal. A neutral zone formed in between the two [illegible] mobilisation.

The peripheral cells of the callus of *Dendrobium pierardii* contained rich cytoplasmic RNA and proteins and a few starch grains. The callus did not have specific conducting vessels and the movement seemed to be through diffusion. Basal cells were rich in carbohydrate reserves. A group of small peripheral cells with rich RNA and proteins differentiated to form shoot apex.

6.4 Chromosome instability in tissue culture

In the present study large amount of chromosome variations were observed in the cultured cells. In many of the cells containing more than diploid number of chromosomes the variation might possibly be due to cell fusion or chromosome doubling followed by elimination.

CHAPTER VII

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FIG. 6. EFFECT OF MEDIA ON GERMINATION AND LEAF PRODUCTION IN EPIDENDRUM RADICANS

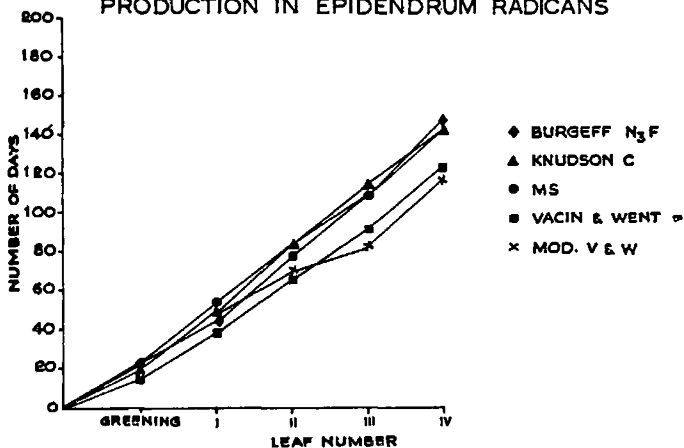

FIG. 7. EFFECT OF MEDIA ON GERMINATION AND LEAF PRODUCTION IN VANDA COERULEA

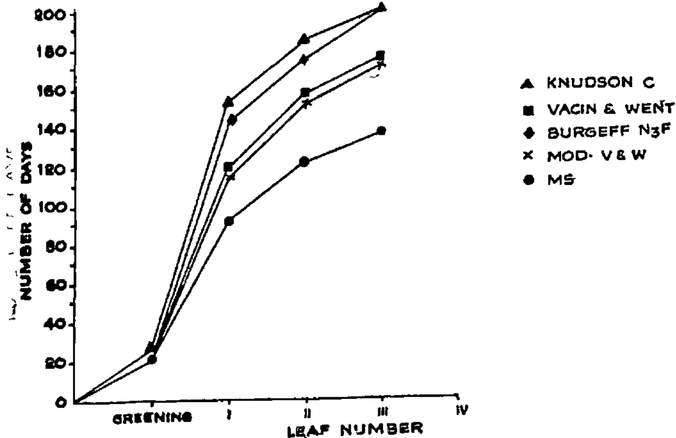

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FIG. 8. EFFECT OF MEDIA ON GERMINATION AND LEAF PRODUCTION IN SPATHOGLOTTIS PLICATA

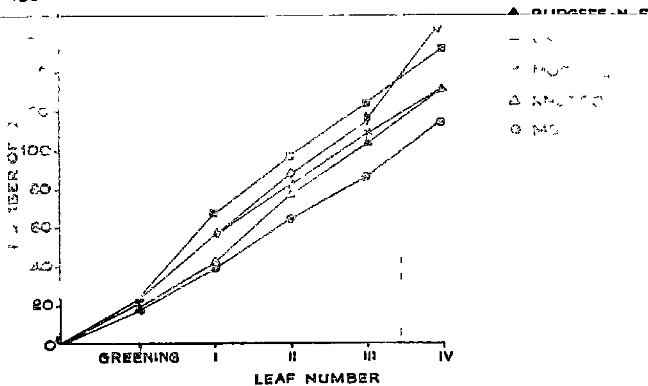
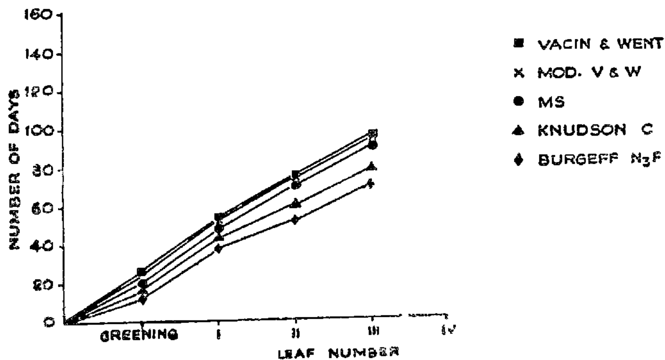


FIG. 9. EFFECT OF MEDIA ON GERMINATION AND LEAF PRODUCTION IN DENDROBIUM MOSCHATUM



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* Original not seen