STANDARDISATION OF *IN VITRO* TECHNIQUES FOR RAPID MULTIPLICATION OF *Holostemma annulare* K. Schum

By

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THESIS

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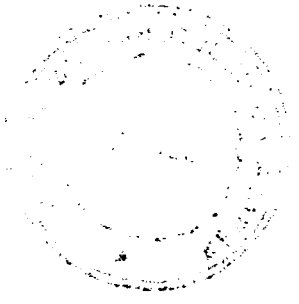
DECLARATION

I hereby declare that the thesis entitled "**Standardisation of *in vitro* techniques for the rapid multiplication of *Holostemma annulare* K. Schum.**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship, associateship or other similar title, of any other university or society.



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ABBREVIATIONS

BA	benzyl adenine
BAP	benzyl amino purine
°C	degree celsius
cm	centimeter
2,4-D	2,4-dichlorophenoxy acetic acid
EDTA	ethylene diamine tetra acetic acid
GA	gibberelic acid
h	hour(s)
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
2iP	2-isopentenyl adenine
KIN	kinetin, N^6 - furfuryl adenine
mg l^{-1}	milli gram(s) per litre
min	minute(s)
mM	milli molar
MS	Murashige and Skoog's (1962) medium
½ MS	Murashige and Skoog's (1962) medium with half the salt concentration
N	normality
NAA	naphthalene acetic acid
pH	hydrogen ion concentration
psi	pounds per square inch
Rm	relative mobility
rpm	revolutions per minute

s	second(s)
TDZ	N-phenyl-N'-1,2,3-thiadiazol - 5 yl urea
U-V	ultra violet
v/v	volume in volume
WP	wettable powder
WPM	woody plant medium (Lloyd and Mc Cown, 1980)
w/v	weight in volume
μM	micromolar

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Introduction

INTRODUCTION

Medicinal plants have a vital role to play in different traditional and modern systems of medicine. Due to the indiscriminate collection of these plants and deforestation, the forest ecosystem itself has changed and many of the medicinal plants have become extinct, endangered, rare or vulnerable. Inorder to provide regular and sustained supply of medicinal and aromatic plants, it is essential now to domesticate and systematically cultivate these plants for which availability of planting materials on a large scale has to be ensured.

Holostemma annulare K. Schum is a medicinal plant belonging to the family Asclepiadaceae which is widely used for the cure of different ailments of the eye and as a general tonic. The roots of this plant are reported to possess cooling, tonic and lactative properties. The root preparations are used for relief in gonorrhoea, diabetes, cough and stomach-ache as well as in ophthalmia (Chopra *et al.*, 1956). 'Jeevanthi' which is a medicine prepared from the roots of this plant is found to have curative action on the diseases affecting the retina of the eyes. The plant as such is highly valued and the entire demand is met through collection from wild sources. The plant is not domesticated and it can be considered as an endangered species as well. The availability of planting material is a major problem in the domestication of this crop. Standardisation of suitable *in vitro* techniques for rapid multiplication of this plant for the production of large amount of planting material is hence important as the first step in the domestication of this species.

The use of *in vitro* techniques for clonal propagation has become the most widely used application of tissue culture technology in horticulture in the recent

years (Thorpe, 1990). Micropropagation can be achieved by enhancing axillary bud break, production of adventitious buds directly or indirectly and somatic embryogenesis (Murashige, 1974). Following the successful rapid multiplication of orchids by shoot meristem culture (Morel, 1965), there has been, in recent years, an increasing interest in the application of micropropagation techniques as an alternate means of vegetative propagation of horticultural plants.

The *in vitro* derived plantlets have to be acclimatised to the entirely different environment prevailing *in vivo* as they are poorly adapted to resist the low relative humidity, higher light intensity and temperature met within the outside. A period of hardening is essential for the newly transferred plantlets to adapt to the outside environment during which the plantlets undergo morphological and physiological adaptation enabling them to ensure maximum survival when planted out (Sutter, 1985).

The major cost of producing *in vitro* plants is in the rooting and hardening stages. The shoots produced *in vitro* could be rooted *ex vitro* by treating with growth regulators and keeping under high humidity conditions in a mist chamber. Rooting under *ex vitro* conditions also facilitates the combining of the rooting stage with acclimatization.

Encapsulation of differentiating tissue in a biodegradable synthetic polymer is a method to facilitate mechanical handling of the propagules and also help in economising the time, space and cost involved in normal micropropagation procedures.

In the light of this background, the present study was conducted with the following objectives.

1. To standardise the method of rapid multiplication of *Holostemma annulare* K. Schum. through enhanced axillary bud breaking, production of adventitious buds directly or indirectly and somatic embryogenesis directly or indirectly.

2. To standardise the rooting and hardening of plants derived *in vitro*.

3. To develop a method of encapsulating shoot buds.

Review of Literature

REVIEW OF LITERATURE

Plants, as a source of medicine have a special importance in countries like India, Pakistan, Bangladesh and Sri Lanka which have well developed traditional systems of medicine called Ayurveda, Siddha and Unani, all of which derive more than 90 per cent of medicaments from higher plants. Several of these also constitute important ingredients of Allopathic medicines. Most of the requirements for the medicinal and aromatic plants are met from wild sources. The collection of these plants from forests cannot cope up with the ever increasing and changing demand from the pharmaceutical, perfumery and cosmetic industries (Pushpangadhan, 1992). In order to provide regular and sustained supply of medicinal and aromatic plants, it is essential now to domesticate and systematically cultivate these plants for which large scale availability of planting material has to be ensured. Nayar (1992) has reported that domestication of wild plants and its cultivation could be possible only after standardising the cultivation practices. Of late, plant tissue culture techniques have come to be used widely for large scale multiplication of various crops. The available literature on the plant and use of tissue culture techniques on other medicinal plants is presented in this chapter.

The various uses of the roots of *Holostemma annulare* have been listed by Chopra *et al.* (1956). Roots are beaten into a paste and applied to the eyes in ophthalmia, rubbed to a paste and given in cold milk in diabetics and as a remedy for scalding in gonorrhoea. The roots of this plant are reported to possess cooling, tonic and lactative properties (CSIR, 1959).

The sugars and the aminoacids present in the roots are reported to be responsible for the medicinal properties. Ramiah *et al.* (1981) have isolated and identified different sugars such as α amyrin, lupeol, ß sitoslerol from the benzene extract as well as six aminoacids such as alanine, glycine, serine, aspartic acid, threonine and valine from the ethanol extracts of *Holostemma annulare* roots.

The entire demand of the roots is met through collection from wild sources and the plant is not yet domesticated. The dried roots of the plant are also highly priced.

Studies conducted at the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara have revealed that this crop could be vegetatively propagated through use of vine and root cuttings and root suckers to a limited extent. Around 10-15 two-node cuttings and 10-12 numbers of root segments are available from a plant which could be used as propagules. However, the material available for multiplication is limited as the plant dries up in summer and also because the roots are used for medicinal purpose. Though sparse seed setting occurs, only around 50 per cent germination is obtained. Standardisation of suitable *in vitro* techniques for rapid multiplication of this plant will help in production of large amount of planting material especially of any superior types to be identified of this species which could be used for large scale cultivation of this rare crop. The use of tissue culture techniques for clonal propagation has become the most widely used application of tissue culture technology in horticulture in the recent years (Thorpe, 1990). Though plant tissue culture offers great potential for multiplication of many species, no work has been reported for *Holostemma annulare*.

The concept of totipotency which is inherent in the cell theory of Schleiden (1838) and Schwann (1839) is the foundation for plant tissue culture. The first person to isolate fully differentiated cells was Haberlandt (1902) and he postulated that the isolated cell is capable of resuming uninterrupted growth. Hanning (1904) excised nearly mature embryos of some crucifers and successfully grew them to maturity on mineral salts and sugar solution. Van Overbeek *et al.* (1941) for the first time demonstrated the stimulatory effect of coconut milk on embryo development and callus formation. The first successful report of continuously growing cultures of tomato root tips was made by White (1943).

Skoog and Miller (1957) put forth the concept of hormonal control of organ formation and showed that the differentiation of roots and shoots was a function of the auxin-cytokinin ratio and that organ differentiation could be regulated by changing the relative concentration of these two substances in the medium.

Morel (1960) applied the technique of shoot tip culture for rapid propagation of orchids and it became possible to produce large numbers of genetically identical plants from a single bud within a short time. Murashige (1977) developed methods for *in vitro* propagation of several species ranging from ferns to foliage, flower and fruit plants. He also developed the first completely defined nutrient medium along with Skoog in 1962. Wickson and Thimman (1958) showed that the growth of axillary buds which remained dormant in the presence of terminal buds can be initiated by the exogeneous application of cytokinin. Since then, plant tissue culture has worked as a powerful research tool in the fundamental and applied aspects of agriculture.

Several aspects of tissue culture are currently being applied to agriculture. The main commercial application so far has been in the production of clonal plants at a very rapid rate compared to the conventional methods through tissue culture techniques. These plants are reported to grow faster and mature earlier than seed propagated plants (Vasil and Vasil, 1980).

As per Murashige (1974) there are three possible routes available for *in vitro* propagule multiplication.

(a) Enhanced release of axillary buds
(b) Production of adventitious shoots through organogenesis
(c) Somatic embryogenesis

Shoot tip culture ensures genetic uniformity while organogenesis through a callus phase may be useful for recovery of useful variant lines. Somatic embryogenesis though limited to a few plant species is the most rapid mode of plant regeneration (Evans *et al.*, 1981).

2.1 Routes of *in vitro* propagation

2.1.1 Enhanced release of axillary buds

Micropropagation by axillary bud proliferation has proved to be the most reliable method for large scale production of many crop plants (Satyakala *et al.*, 1995).

Morel (1960) reported the application of shoot apex culture for rapid clonal multiplication of plants for the first time. The greatest success using this technique has been achieved in herbaceous horticultural plants. The success may be

due to the weak apical dominance and strong root regeneration capacity of the herbaceous plants (Hu and Wang, 1983). In most plants, each leaf has an axillary meristem which has the capacity to develop into shoot identical to the main shoot. But the axillary meristems are inhibited by apical dominance, and this can be overcome by the supply of cytokinins (Wickson and Thimann, 1958; Phillips, 1975).

All the shoots arising by this method, may not originate from axillary buds. Adventitious shoots often also arise either directly from cultured shoot material or indirectly from callus formed at the base of the explant, but precise origin can sometimes only be determined from a careful anatomical examination (George and Sherrington, 1984). Shoots of axillary origin will normally be genetically more stable than those regenerated from callus. In some circumstances, callus arising at the base of shoot tips may be semi-organised and therefore capable of producing genetically-stable plants. Single node culture is of possible value for propagating species that produce elongated shoots in culture (George and Sherrington, 1984). Following are some of the reports on axillary bud culture of a few medicinal and aromatic plants. Clonal propagation of *Dioscorea* spp. could be achieved through culture of shoot meristems and nodal segments. Chaturvedi (1975) propagated *D. floribunda in vitro* from single node stem segments in MS medium supplemented with growth regulators. Same technique was used by Lakshmi Sita *et al.* (1976) for *D. florbunda* propagation. Barve and Mehta (1993) could regenerate clonal plantlets of *Commiphora wightii* through forced axillary branching. Vincent *et al.* (1992) could obtain clonal plantlets of *Kaempferia galanga* through direct shoot proliferation when axillary buds from rhizomes were used as explant in MS medium supplemented with different concentrations and combinations of auxins and cytokinins. George *et al.* (1993) could produce clonal plantlets of *Gardenia*

jasminoides Ellis through axillary bud culture. Similar reports could be obtained in scented geranium (*Pellargonium* spp.) (Satyakala *et al.*, 1995), *Catharanthus roseus, Thymus vulgaris* (Bajaj *et al.*, 1988), *Glycirrhiza glabra* (Shah and Dalal, 1980), *Adhatoda vasica* (Jaiswal *et al.*, 1989), *Adhatoda beddomei* (Sudha and Seeni, 1994), *Passiflora* spp (Drew, 1991), *Tylophora indica* (Sharma and Chandel, 1992) and *Aristolochia indica* (Kavitha and Raju, 1995).

2.1.2 Organogenesis

Levels of plant growth regulating substances in the culture medium, particularly high auxin and low cytokinins, often lead to callus formation. On the other hand, if the auxin level is reduced in the medium, it may lead to the adventitious shoot formation (Skoog and Miller, 1957, Hussey, 1986).

Organogenesis may be direct or callus mediated (Evans *et al.*, 1981). Direct adventitious shoot initiation is a method of micropropagation, where adventitious shoots arise directly from the tissues of the explant and do not develop within previously formed callus. The induction of direct shoot regeneration depend on the plant organ from which the explant was derived and above all, on the plant species. Direct morphogenesis is unknown in many kinds of plants.

Though callus may be obtained from virtually any species, only in some can plants be regenerated. The reason for this inability may be due to the higher proportion of polyploid or aneuploid cells in those callus (Smith and Street, 1974). The main disadvantage in callus mediated organogenesis compared to clonal propagation, is the genetic variation developing in many of its component cells. However, the regenerated variants can be used to complement the existing natural

variability (Hussey, 1986). Shoots regenerated from callus seldom have vascular connections with callus-derived roots, and do not survive in soil unless new adventitious roots are initiated directly from their bases.

Plant regeneration through direct or indirect organogenesis has been reported in many medicinal and aromatic plants. Shoot regeneration could be obtained from callus derived from leaf and stem segments of *Gomphrena officinalis* in MS media supplemented with BAP and α-NAA. MS media containing IBA resulted in rooting of the regenerated shoots as reported by Mercier *et al.* (1992). Plant regeneration from callus cultures of *Piper longum* was achieved through organogenesis. IAA in combination with BA in MS medium was necessary for organogenesis as reported by Bhat *et al.* (1992). Young inflorescence segments pretreated in liquid MS medium supplemented with growth regulators for 2 h and cultured in MS medium supplemented with 2,4-D and kineten resulted in profuse callus formation in vetiver. The callus on culturing in MS medium supplemented with BAP alone or combined with NAA produced maximum number of shoots which could be rooted on subculture to media containing IBA as reported by Keshavachandran and Abdulkhader (1993). Similar reports on organogenesis are in *Gloriosa superba* (Somani *et al.*, 1989). *Eucalyptus* spp. (Lakshmi Sita, 1986), *Trigonella foenum-graecum* (Sen and Gupta, 1979), *Tylophora indica* (Mhatre *et al*, 1984), *Mentha piperita* (Cellarova *et al.*, 1984), *Papaver somniferum* (Jaiswal and Narayan, 1985). In aromatic grasses like Lemongrass, Citronella and Palmarosa, callus regeneration has been reported by Mathur *et al.* (1989b). They could obtain high yielding somaclonal variants through callus regeneration of leaf sheath explants.

2.1.3 Somatic embryogenesis

The first report of somatic embryogenesis was given by Reinert (1959) in carrot cultures. The positive results are limited to a few species, but is a more rapid mode of plant regeneration (Evans *et al.*, 1981). Cells, within or on the initial explant, or derived from them through proliferation in culture, progress through a series of developmental stages to form embryos and then complete plants. Thus there are two routes for somatic embryogenesis as described by Sharp *et al.* (1980), the first in which embryos are formed directly from the explant without the callus formation and the second in which first there is callus formation, and on the callus, embryos are formed. Level of growth regulators in the culture medium determines induction of embryogenesis. Particularly when the auxin level is lowered, there is the chance for embryo formation or somatic embryogenesis (Skoog and Miller, 1957; Hussey, 1986). The presence of auxin in the proliferation medium (embryo induction medium) seems essential for the tissue to develop embryos in the embryo development medium. But if the embryo development medium is also auxin rich, it is not desirable. Removal of auxin in embryo development medium exerts its influence by promoting more cell to cell contact in proembryogenic masses, as well as increased expression of polarity so that embryos develop to maturity.

The route of *in vitro* culture with greatest potential for producing largest number of plantlets is somatic embryogenesis. This is due to a phenomenon called recurrent or repetitive or secondary embryogenesis in which the primary somatic embryo instead of giving rise to plantlet gives rise to successive cycles of embryos. The value of repetitive embryogenesis to obtain stable transformants was originally demonstrated by Mc Granahan *et al.* (1988, 1990) in walnut. Subsequently,

repetitive embryogenesis in a liquid culture system has been used for stable transformation in other species also. Partial or complete removal of auxins from media results in embryos to initiate bipolar growth.

Reports on somatic embryogenesis have been published by Murashige (1978), Sharp *et al.* (1979), Vasil and Vasil (1980) in various crops. Somatic embryogenesis has been reported successful in various medicinal and aromatic plants.

Cheng and Raghavan (1985) could obtain somatic embryos whose pattern of development was similar to that of zygotic embryos. The somatic embryos were formed from friable calli produced from petiole and ovary explants in medium containing 2,4-D. Embryogenesis was initiated when the friable calli was cultured in a medium lacking auxin.

In *Nardostachys jatamansi*, it was found that embryogenesis could be initiated from callus upon subculture to a media containing lesser auxin (NAA) and more cytokinin (kintein) while the concentrations of NAA was to be more and KIN less for callus initiation as reported by Mathur (1993).

In *Cayratia japonica*, Zhou *et al.* (1994) have reported spontaneous somatic embryogenesis when embryogenic callus (formed in MS medium supplemented with 2,4-D alone or in combination with TDZ) was subcultured on the basal medium.

Embryogenesis was induced in suspension cultures raised from callus of androgenetic origin of Datura, using LS liquid medium supplemented with 2,4-D as reported by Sharma *et al.* (1993). In *Kaempferia galanga* L. embryoids were

produced which further developed into plants from rhizome explants in MS media supplemented with growth regulators as reported by Vincent *et al.* (1992). Induction of somatic embryogenesis by different growth regulators was observed in leaf disc cultures of *Nicotiana tabaccum* L. by Gill and Saxena (1993). Similar reports are in *Atropa belladona* (Thomas and Street, 1970), *Dioscorea* (Sinha and Chaturvedi, 1979 and Singh, 1978), *Panax ginseng* (Chang and Hsing, 1980), *Tylophora indica* (Mhatre *et al*, 1984), *Digitalis* spp. (Kuberski *et al.*, 1984), *Rauwolfia caffra* (Upadhyay *et al.*, 1992), *Aconitum* spp. (Giri *et al.*, 1993), *Plantago ovata* (Jasrai *et al.*, 1993).

Enhanced release of axillary buds, organogenesis and somatic embryogenesis are currently being exploited for commercial propagation (Lakshmi Sita, 1991) as described earlier. The first method also called as micropropagation and somatic embryogenesis results in the production of nonchimeric and true to type plants that comprise clonal populations. An important requirement for plants produced *in vitro* is that the plants must be genetically identical to the source plants. Plants regenerated via micropropagation or somatic embryogenesis are derived from characteristic organized meristems or meristematic cells. These cells are by nature genetically stable and less prone to mutational changes. There is also an increasing evidence to show that there is a strong selection in favour of genetically normal cells during somatic embryo development. Consequently, plants derived from micro-propagation as well as somatic embryogenesis, give rise to truly clonal populations. Both systems, therefore, are well suited for the mass production of plants (Vasil, 1994).

2.2 Use of suspension culture

Compared to the relatively rapid rates of propagation that are possible with shoot tip and nodal segment culture of some plants, propagation from morphogenetically competent callus can be slow initially. The rate at which propagation can proceed after that depends on the rate at which callus, can be grown and subdivided. A much faster rate of multiplication can be achieved if it is possible to initiate a suspension culture from the regenerative callus, in which plated cells or cell aggregates retain the capacity to reform callus colonies with the competence for morphogenesis (George and Sherrington, 1984). Cell suspension culture is a type of culture in which single cells or cell aggregates multiply while suspended in agitated liquid medium (De, 1992). The morphogenesis in cell suspension is not an easy operation, as growth regulators favouring the formation of a dispersed cell suspension can cause the cells to lose their regenerative capacity (George and Sherington, 1984). Biomass accumulation in *Glycyrrhiza glabra* cell suspension cultures was found to be affected by the density and age of the inoculum, vessel size and sucrose level as reported by Arias-Castro *et al.* (1993). In *Valeriana wallichii*, Mathur (1992), has reported that the callus upon dispersion in the liquid medium produced a suspension of free cells and cell aggregates which lateron produced bipolar structures reminiscent of somatic embryos in their later stages of development, but developing through organogenetic pathways. With constant medium replenishment exponential growth of suspension cultures of *Ephedra* spp. could be sustained indefinitely as reported by O'Dowd *et al.* (1993). The cells of *Coscinium fenustratrum* grew better in liquid culture than in static callus culture and better berberine production was also reported by Nair *et al.* (1992).

Differentiation of embryos and further maturation took place in liquid medium but further germination of these embryos into plantlets could be obtained only on semisolid MS medium without growth substances. This has been reported in *Datura innoxia* by Sharma *et al.* (1993).

In 2,4-D supplemented liquid LS medium a number of embryos of all stages (globular, heart and torpedo) could be observed after 1 week following the first subculture in fresh medium of same composition @ 200/ml of suspension culture.

Drew (1980) estimated that one litre of carrot suspension culture contained over 1.35 million somatic embryos. If larger vessels like bioreactors are made use of somatic embryo production could be further scaled up. Subbaiah and Minocha (1990) have reported that suspension cultures of *Eucalyptus terretecornis* could be maintained by subculture at two week intervals in light in presence of 2,4-D 0.5 mg l^{-1} but the cell clumps never differentiated into shoots in the presence of growth regulators. Most of the *Glycirrhiza* suspension cultures reported have been grown in the dark (Hayashi *et al.*, 1988; Kobayashi *et al.*, 1985; Ayabe *et al.*, 1986). However, Arias-castro *et al.* (1993) reported that cell suspension cultures of *G. glabra* did not show any significant difference in their growth in the light or dark. They also reported that two per cent and eight per cent sucrose gave better values for growth rates, biomass yield and productivity while if the concentration was measured further to 10 per cent there was lower value for growth parameters.

2.3 Multiplication rate

The rapid multiplication of medicinal and aromatic plants help in large scale multiplication of plants which are difficult to propagate by conventional methods or which are threatened with extinction. In a situation where large number of plants are to be regenerated in a short time somatic embryogenesis is promising as somatic embryos can be produced in very large numbers per gram of the callus.

In Palmarosa, from 20 mg of the calli initiated from nodal explant, whole plants could be regenerated either through organogenesis or embryogenesis. Somatic embryogenesis gave rise to 35-50 plantlets per culture tube. This could be continued for 18 subculturings in a period of two years so that nearly one lakh plants produced per year per tube assuming that atleast 4 g of callus is produced per tube. Through somatic organogenesis, 20-25 plantlets could be obtained per culture (Mathur *et al.*, 1989b).

The results of the study in *Glycirrhiza glabra* by Shah and Dalal (1980) indicated that from a single axillary bud, more than 200,000 plants can be obtained in the flasks in six months through enhanced release. Assuming that 50 per cent of the plants survive, it is possible to have a yield of 100,000 *G. glabra* plants within eight months, starting from a single bud. Chaturvedi *et al.* (1982) reported that aseptically established plant of *Dioscorea floribunda* from a single node stem segment, produced about 25,60,000 true to type plants in one year by rooting of single node leaf cutting obtained from such plants.

In *Gardenia jasminoides*, the multiplication observed was at the rate of 21 microshoots per explant on the 60th day using an agar-based medium, and two

subsequent transfers of 15 days duration in liquid medium which resulted in 400 ± 25 shoots per explant in a total period of 90 days (George *et al.*, 1993). In *Pelargonium graveolens* by enhanced axillary shoot multiplication there is enormous potential for mass multiplication, with a high rate of upto 50 to 60 shoots per subculture and about 95 per cent rooting has been reported (Sathyakala *et al.*, 1995).

The potential of shoot bud proliferation rate (40-50 multiple shoot buds multiplied 30-40 times in 10 weeks) remained unchanged over two year of 10 weekly subcultures. It was not difficult to obtain over a million plants in a year from a single explant excised from a six month old *Eucalyptus camaludensis* plant as reported by Gurung and Rajbhandari (1989). Kapoor and Chauhan (1992) have reported that 2,00000 tissue culture plants of *Eucalpytus* F_1 hybrid could be obtained from a single nodal segment in 1 year.

2.4 Factors influencing success of *in vitro* propagation

2.4.1 Explant source

Plant tissues differ in their ability to undergo morphogenesis. Takayama and Misawa (1979) examined the ablity of different explants of *Lilium auratum* and *L. speciosum* to produce bulbs *in vitro*. Fifty per cent of the peduncle explants, 75 per cent of the petal explants and 95 per cent of the bulb scale explants produced bulbs. Leaf explants and explants from stamens and anthers did not even survive the culture condition while the explants from bulbs, showed 100 per cent success. Dornelas and Vieira (1994) observed that organogenesis was clearly affected by the explant source used in *Passiflora edulis* var. *flavicarpa*, best results being obtained from cotyledons. This was also dependent on physical environment viz. shoot regeneration was observed in the dark from cotyledonary cultures only but in the

presence of light regeneration frequency was improved. It was found that at least 56 per cent of *Passiflora* cultures derived from cotyledon explants formed shoots.

Ovules collected 90-120 days after pollination was the best explant for organogenetic differentiation in *Aegle marmelos* as reported by Hossain *et al.* (1994). Among the primary explants used for starting the culture, the nodes proved the best source of callus in *Dioscorea deltoidea*. Callus of the hypocotyl origin grew as well as that of nodal origin, but growth ceased after ten passages as reported by Furmanowa and Guzewska (1988). Immature inflorescence proved to be the best explant source of oil palm for somatic embryogenesis as reported by Teixeira *et al.* (1994). Response of explants either produced *in vitro* or those taken from field grown mother plants were also compared by certain workers. Among the explants taken from field grown plants (leaves, nodal and internodal explants), only nodal explants showed response initiating maximum number of shoots. Among the *in vitro* explants used, adventitious shoots could be obtained from the cut ends of petioles, leaf lamina and nodal explants but there was no response when field grown petiole and leaf lamina were used as explants. However, the number of shoots initiated from lamina and petioles were less compared to nodal explants. This has been reported by Satyakala *et al.* (1995) in *Pelargonium graveolens*. The number and frequency of shoot multiplication between the nodal explants of field-grown plants and shoot cultures were somewhat similar although 25-30 per cent of the explants from the former were lost due to infection. This was reported in *Adhatoda beddomei* by Sudha and Seeni (1994).

Morphogenesis of cultures from single pollen grains of *Hyoscyamus niger* cultured in a liquid medium was reported by Raghavan and Nagmani (1983).

In the same crop embryoids could be differentiated though more or less same path-ways from callus originating from the hypocotyl, ovary and petiolar explants. The plants so regenerated were characterised by the presence of small thick leaves as reported by Cheng and Raghavan (1985). Foliar explants were found to be excellant material for clonal propagation of *Duboisia myoporoides* as reported by Kukreja *et al.* (1986) as direct organogenesis was observed.

Geier (1986) reported that large number of shoots were produced in apical and central segments of leaves of *Anthurium andreanum* (Araceae) cultivated *in vitro* suggesting that leaf maturation might be more advanced in basal segments. In *Valeriana wallichii*, Mathur and Ahuja (1991) have reported that petiole explants did not produce any callus when inoculated in MS basal media without any growth regulators. However when a portion of the leaf lamina remained attached to the petiole explant, roots were produced from the cut end even on the basal medium. Kumari and Saradhi (1992) have reported that cotyledonary explants proved to be the best source for compact and nodulated calli which showed shoot induction on further subculture in *Origanum vulgare* a herbaceous medicinal plant.

In *Asparagus cooperi*, there was typical morphological difference between calli produced by different explants. Calli derived from spear and node were soft and white, from the shoot tip they were soft and yellowish green and from root segments they were compact white and nodular (Ghosh and Sen, 1992).

Direct regeneration frequency and number of embryoids and shoot buds was greater from roots than from hypocotyl especially if the explants where split as reported in *Clitorea ternatea* by Lakshmanan and Dhanalakshmi (1990). It has been shown that a direct shoot bud formation (without callus) could be obtained from

subcultured roots using a basal medium without any growth regulator (Ahee and Duhoux, 1994). Bud formation from cultured roots has also been reported in *Citrus aurantifolia* (Bhat *et al.*, 1992). Highest yield of buds was obtained from intact roots on whole seedlings in *Citrus mitis* (Sim *et al.*, 1989).

The age of the tissue may influence whether an explant can be used to intiate a culture, or its direct or indirect morphogenetic potential. Callus derived from seedlings and inflorescence explants of various *Cymbopogon* species had a higher morphogenic capacity than that arising from seeds, culms, roots or rhizomes (Jagadish Chandra and Sreenath, 1987).

Explants taken from newly originated organs are also most likely to be capable of direct organogenesis. Pierik (1969) and Pierik and Steegman (1975) found that the rhizogenetic potential of *Rhododendron* stem segments decreased with the age of the shoot from which they were obtained. In the experiments of Thakayama and Misawa (1982), most segments derived from young leaves of Begonia produced buds and roots whereas those derived from mature leaves normally dried.

The youngest and less differentiated tissues are found in plant meristems and the culture of these tissues have been successful in a wide range of species (Hughes, 1981). During the maturation process of tissues several physiological changes occur which may influence the *in vitro* behaviour of the explants (David, 1982). In general, young tissues have a higher degree of morphogenic competence than older tissues. Rao and Lee (1986) and Lakshmi Sita *et al.* (1986) found that callus could be induced from young tissues of *Dalbergia latifolia* but not from the mature tissues.

Hossain *et al.* (1994) reported that in *Aegle marmelos* shoot differentiation efficiency varied remarkably when cotyledons from seedlings of different ages were cultured on medium supplemented with BA and IAA. Explants from 10 day old seedlings showed maximum regeneration of 90 per cent.

Retention of the explants with axillary shoot buds and shoots in the initiation medium led to growth of the shoots. However, no additional shoots were produced as reported by Krishnan and Seeni (1994) in *Woodfordia fruticosa*. The presence of shoots initially regenerated from the explants is reported to suppress further development (Douglas, 1990). In such cases more buds were induced to develop by removal of the existing shoots and reculture of the entire explant.

Tissues taken from field grown plants are not equally amenable to tissue culture conditions throughout the year. Maliarcikova (1981) found that for taking explants in strawberry, August month was more suitable than July to September. Bhaskar (1991) reported that the short tips collected during November to April gave least contamination rates in cultures of banana. Vegetative buds of *Commiphora wightii* were available on the trees only from March to October and those materials collected during April-June gave a good response whereas sprouting was reduced for the explants collected during July-August and was poor for explant collected during September-October as reported by Barve and Mehta (1993). Similar effects of season on bud sprouting and explant contamination have also been noticed in guava (Jaiswal and Amin, 1987), *Tectona grandis* (Gupta *et al.*, 1980) and *Gymnema sylvestre* (Anu, 1993).

Genotype of the explant chosen for propagation is also another important factor in the success of *in vitro* culture. Within a species, some genotypes respond easily, while others fail to respond. Genotype specific effects have been reported in Anthurium (Pierik and Steegman, 1976), Gladiolus (Hussey, 1977) and in Geranium (Pillai and Hildebrandt, 1968).

If the explants were retained in the BA rich medium for more than four weeks, they always became nonproductive, chlorotic and eventually died. To avoid this, the explant after culture on medium with growth regulators for 4 weeks was subcultured on to basal MS medium without any growth regulater as reported by Kantharajah and Dodd (1990) in *Passiflora edulis*.

Sustained growth of adult buds was not achieved until nodal buds were placed horizontally on the culture medium. Growth was influenced by endogeneous growth factors and hormones and the best growth occured with buds positioned 6-8 nodes from the apex in *Passiflora* spp. (Drew, 1991).

2.4.2 Surface sterilization

The purpose of surface sterilization is to remove all the microorganisms present on the explant with minimum damage to the plant tissue. Explants for surface sterilization are usually cut into a size larger than that of the final and after sterilization they are trimmed to smaller size and transferred to the medium (Hussey, 1979). To check bacterial and fungal contamination, antibiotics and fungicides are found used either as surface sterilant or medium additives. Several workers have reported the use of various fungicides in cultures for reducing fungal contamination (Brown *et al.*, 1982; Shields *et al.*, 1984). But it was found that most of the

systemic fungicides and some antibiotics inhibit growth of the explant cultures. Dodds and Roberts (1985) suggested to avoid the use of antibiotics for sterilization since they or their degradation products metabolised by plant tissue may cause unpredictable results. Consequent addition of systemic fungicide in the medium showed chlorophyll degradation and vitrification in leaves of cardamom (Reghunath, 1989).

The most commonly used surface sterilant is an aqueous solution of sodium hypochlorite. Dilution of 10 per cent (v/v) is normally effective for the purpose, particularly when it is mixed with a surfactant like teepol or similar liquid detergent. Sodium hypochlorite being toxic to plant cells, it is necessary to wash the treated tissue twice or thrice with sterile water (Hu and Wang, 1983). Concentrations ranging from 1.0 per cent (Minocha, 1980) to 10.0 per cent (Kuo and Tsay, 1977) have been reported for various plant species. In *Nardostachys jatamansi*, Mathur (1993) has reported that 2 per cent sodium hypochlorite treatment for 7 min was effective.

Kantharajah and Dodd (1990) have reported in *Passiflora edulis* that seeds could be surface sterilized for 10 min in 10 per cent hypochlorite solution (4% w/v of available chlorine as sodium hypochlorite).

Chlorine water alone or in combination with few drops of detergent also was reported to be effective. Kavitha and Raju (1995) has reported in *Aristolochia indica* that chlorine water with few drops of Triton X-100 treatment for 15 min was very effective.

Ethanol and mercuric chloride are the other popular surface sterilants. According to Maroti and Levi (1977) it was better to rinse first with ethanol (45 per cent) for three min followed by a 10 min bleach treatment (5.0 to 10.0 per cent) and finally three rinses with sterile water. Alcohol alone was used for surface sterilization (Bonga, 1982).

Lakshmi Sita *et al.* (1986) used 0.1 per cent mercuric chloride for 10 to 12 min for sterilization of seedling explants of *Dalbergia latifolia*. Mercuric chloride 0.10 to 0.15 per cent gave better sterilization of explants than sodium hypochlorite and absolute alcohol. In *Piper longum*, Bhat *et al.* (1992) reported that washing in water for 5-10 min followed by treatment with 0.1 per cent mercuric chloride for 20 min was an effective sterilization method. In *Adhatoda beddomei*, Sudha and Seeni (1994) reported that a treatment with 1 per cent Labolene for 6-8 min followed by tap water washing and treatment with 0.1 per cent mercuric chloride for 15 min could produce contamination free cultures. Teepol wash for 15 min followed by treatment with 0.1 per cent mercuric chloride 7 min was effective sterilization procedure for *Tylophora* spp. as reported by Sharma and Chandel (1992). In *Woodfordia fruticosa*, Krishnan and Seeni (1994) reported that treatment with 1 per cent Labolene reagent for 5-6 min followed by 0.1 per cent mercuric chloride for 5 min was effective.

Combination of ethyl alcohol and mercuric chloride was also effective as reported in *Duboisia myoporoides* (Kukreja *et al.*, 1986), *Coscinium fenustratum* (Nair *et al.*, 1992), *Vetiveria zizanioides* (Keshavachandran and Abdul Khader, 1993). Utilization of antibiotics as sterilizing agent in addition to hypochlorite solution increased survival rate of excised shoot tips in culture. The effectiveness of

antibiotics was significantly higher at high concentrations except for amoxicillin which was effective at even low concentrations. This was reported in *Zingiber cassumunar*.

In *Commiphora wightii*, the problem of contamination experienced when explants were derived from an adult wild tree was alleviated by treatment with nystatin and streptomycin in 70 per cent alcohol. It was found that the contamination rate was higher for the cultures initiated during July-August and September-October inspite of nystatin treatment (Barve and Mehta, 1993).

2.4.3 Basal Media

Recently, the complexity of *in vitro* nutrients has been considered in a more unified fashion. The earlier media were characterised by a low overall concentration of inorganic ions, especially those of potassium and nitrate, and by providing nitrogen solely in the form of nitrate. Later, the media developed for germination of orchid seeds were similar, except that they contained ammonium ions (Knudson, 1922, Vacin and Went, 1949). Further experiments resulted in the development of White's (1943) medium and Heller's (1953) medium. Since 1960, however, most researchers have been using MS (Murashige and Skoog, 1962), B5 (Gamborg *et al.*, 1968) or SH (Schenk and Hildebrandt, 1972) media. After 1980, the most popular media are WPM (Lloyd and McCown, 1980) and DCR (Gupta and Durzan, 1985) especially for woody plants. The B5 medium has been used for cell and protoplast culture (Gamborg and Shyluk, 1981). Another basal medium N6 (Chu, 1978) was specially developed for cereal anther culture.

In most of the reports on medicinal plants, the media used was Murashige and Skoog's (1962) medium. Rapid propagation of *Solanum xanthocarpum* was achieved by Rao and Narayanaswamy (1968) in White's media. Use of basal medium was reported in *Datura innoxia* (Engvild, 1973). Nitsch medium along with 15 per cent coconut water (Sopory and Maheshwari, 1976) was also found to be effective in *Datura innoxia*). Somatic embryogenesis was reported in *Hygoscyamus niger* in Bourgin and Nitsch's (1967) medium by Cheng and Raghavan (1985). Kumari and Saradhi (1992) have reported that indirect organogenesis of *Origanum vulgare* could be achieved in B5 semi solid (Gamborg *et al.*, 1968) medium. In *Adhatoda beddomei*, Sudha and Seeni (1994) have reported axillary bud release using nodal segments when inoculated in SH (Schenk and Hildebrandt, 1972) media. Subbaiah and Minocha (1990) obtained plantlet regeneration of *Eucalyptus tereticornis* in woody plant medium.

Murashige and Skoog (1962) medium is found to be the most popular one and has been successfully reported in *Aristolochia indica, Tylophora indica, Aegle marmelos, Gomphrena officinalis, Valeriana wallichii, Nardostachys jatamansi* and *Piper longum* tissue culture.

Nature of media also has been found to be a very important criterion for morphogenesis. The embryoids differentiated in suspension culture, required for its further germination and growth a semisolid, hormone free MS medium as reported by Sharma *et al.* (1993) in *Datura innoxia*. In large scale bioreactors also, liquid medium alone can be used. This has several advantages as cultures are always in contact with the medium which stimulates the uptake of nutrients and therefore the growth. Oxygen supply also is obtained well as cultures move in a bioreactor.

2.4.4 Growth regulators

Plant growth regulators are compounds that occur naturally within plant tissues which have a regulatory rather than a nutritional role in growth and development. There are several recognised classes of plant growth substances such as auxins, cytokinins, gibberellins, ethylene and abscissins. The most important factor in successful tissue culture is the addition of growth regulators (Krikorian, 1982). The growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium, and the growth substances produced endogeneously by cultured cells. Hempel (1979) concluded that in majority of cases callus growth was supported by auxin. Hasegawa (1980) also reported that high concentration of auxin may not only inhibit axillary bud breaking but also induce callus formation.

For axillary shoot proliferation, cytokinin has been utilized to overcome the apical dominance of shoots and to enhance the branching of lateral buds from the leaf axis (Murashige, 1974). He observed that a variety of auxins including IAA, NAA, IBA and 2,4-D were used either alone or in combination, but among those auxins, IAA was the weakest, but showed minimum harmful effect on explant tissue. 2,4-D was the most potent and it stimulated callus cultures. Among the various cytokinins like zeatin, kinetein and BAP the latter is more commonly used. Lo *et al.* (1980) reported that high cytokinin content was deleterious to the initiation and elongation of roots. In general, monocotyledonous species do not show a pronounced response to cytokinins and require high concentrations of potent auxins such as 2,4-D to achieve changes in the development of cultured tissues (Harms,

1982). Hu and Wang (1983) had described the young shoot apex as an active site for auxin biosynthesis.

Production of plants through multiple shoot formation is a advantageous for clonal propagation as the chances of somaclonal variation among the regenerated plants is highly minimised through complete removal of an intervening genetically unstable - dedifferentiation redifferentiation cycle and moreover it does not require long term culture cycle for the mass production of planting material (Kavitha and Raju, 1995). Satyakala *et al.* (1995) have also come to the same conclusion that induction of direct multiple shoots is a superior method for getting true to type plants compared to regeneration from callus cultures, where chances of variation are high.

Multiple shoot induction from nodal segments and shoot tips of *Commiphora wightii* have been reported by Barve and Mehta (1993). Axillary bud explants of *Kaempferia galanga* have the potential to induce multiple shoots as well as roots in the same medium containing two cytokinins viz. BA and KIN (Vincent *et al.*, 1992). In *Passiflora edulis* var. flavicarpa Dornelas and Vieira (1994) reported direct organogenesis in media supplemented with BA only as was observed in other species of Passiflora by Kantharajah and Dodd (1990).

Nodal explants of mature trees of *Eucalyptus grandis* produced multiple shoots on media containing BA alone (Lakshmi Sita and Shoba Rani, 1985). Multiple shoots in large numbers could be obtained from excised hypocotyls of young *Eucalyptus terretecornis* seedlings on media containing BA alone (Subbaiah and Minochia, 1990).

In *Aegle marmelos*, Hossain *et al.* (1994) reported that adventitious shoots could be developed in media supplemented with BA or with a combination of auxin of which IAA was the best. Shoots produced in BA medium were elongated but were thin compared to those induced in KIN medium.

Although cytokinin alone was effective in inducing adventitious buds, significant differences were observed on the effectiveness of various cytokinins. The number of shoot forming embryo explants and their subsequent development were affected by the concentration and duration of exposure of BA (Lu *et al.*, 1991). When a comparison was done between various cytokinins it was found that KIN was not as effective on bud induction in *Taxus* spp (Chee, 1995). In *Duboisia myoporoides* Kukreja *et al.* (1986) have compared the interaction effect of different cytokinins and auxins on the foliar explants. KIN and BAP at lower levels favoured only callusing when used in combination with IAA, IBA or NAA. But at higher levels of KIN and BAP in combination with lower levels of IAA extensive shoot bud regeneration was reported directly in the case of the former and indirectly when the latter cytokinin was used. Among auxins, IAA was superior Indirect regeneration could also be obtained with another cytokinin viz. Zeatin.

In *Commiphora wightii*, a higher frequency of shoot formation with multiple shoots was obtained when KIN and BA were used together than when either of the cytokinins was used separately and thus had a synergistic effect on the number of shoots per explant and almost additive effect on the frequency of bud burst (Barve and Mehta, 1993).

Nodal explants of *Pellargonium graveolens* induced more multiple shoots in a combination of BAP and IAA incorporated in the medium and as the concentration of BAP increased there was a marked increase in the number of multiple shoots per explant (Satyakala *et al.*, 1995).

Under appropriate conditions of auxin and cytokinin 3 month old callus showed the formation of a number of adventitious shoot buds in compact masses, the best frequency being obtained using a combination of BA, NAA and GA in *Aegle marmelos* (Hossain *et al.*, 1994).

The synergistic action of KIN and IAA at equal concentrations promoted vigorous growth of shoot buds within the explant and embryoids with bipolar organization also developed in *Clitorea ternatea* (Lakshmanan and Dhanalakshmi, 1990). In *Aristolochia indica* maximum multiple shoots could be obtained when a combination of BAP and NAA was incorporated in the medium (Kavitha and Raju, 1995). Medium containing BA plus NAA or Zeatin plus NAA or BA alone stimulated organogenesis in callus of *Artemisia annua* as reported by Paniego and Giulietti (1994).

Shoots of *Adhatoda beddomei* produced in the presence of BAP and NAA had shorter internodes (Sudha and Seeni, 1994) as also reported in *Cephaelis ipacacuanha* (Ideda *et al.*, 1988).

A combination of KIN and NAA could produce highest number of shoot buds and lateron roots from them, from dark green nodular callus masses induced in a high cytokinin media in *Valeriana wallichii* (Mathur and Ahuja, 1991).

In *Gardenia jasminoides*, George *et al.* (1993) reported that multiple shoot proliferation was maximum after two subcultures in high BA medium. To maintain high multiplication rate subculture to the same media was sufficient. However the concentration had to be reduced in further subcultures to produce healthy shoots.

In *Woodfordia fruticosa*, highest multiplication was recorded when callus initiation media was supplemented with a combination of BAP and NAA followed by subculture in media supplemented with BAP alone (Krishnan and Seeni, 1994). The same workers have made the following conclusions. The significant differences in shoot multiplication rate obtained as a result of the phytohormone regimes employed during culture initiation were indicative of changing endogeneous hormone levels and consequent differential sensitivity and the requirement of the tissues for optimal shoot production during subculture. Differentiation of shoots presumably through the adventitious route accounted for the enormity of shoot production. The number of shoots regenerated per explant increased concomittantly with the concentration of BAP. Reculture of the nodal explants added a higher propagation rate to shoot tip culture and together formed the basis of the rapid micropropagation protocol in the crop.

Initially high cytokinin levels stimulated axillary branching but continued exposure to high cytokinin levels inhibited further axillary bud development as reported by Barve and Mehta (1993) in *Commiphora wightii*. A combination of BAP and 2iP was essential to induce maximum axillary shoots in *Adhatoda beddomei* but necrosis of the cultures resulted after 4 weeks which could be alleviated by the inclusion of an auxin also in the medium which could help in sustained growth

(Sudha and Seeni, 1994). Hu and Wang (1983) reported that elongation of micro-propagated shoots is often inhibited by higher cytokinin levels. Further, cytokinin cause fasciation.

The role of a proper balance between the kind and concentration(s) of plant growth regulators at different stages of embryoid development, from the induction of somatic cells into an embryogenic pathway to the maturation of a somatic embryo (Rangaswamy, 1986). In general, auxins are known to stimulate the induction of embryogenically competent tissue (Fujimura and Komamine, 1980) and initiation of early stages of embryogenesis (Ammirato and Steward, 1971). Cytokinins, on the other hand are required for growth and maturation of differentiated embryoid (Kavathekar *et al.*, 1978; Krikorian and Kaun, 1981). Studies on *Nardostachys jatamansi* by Mathur (1993) has proved thus. Auxin alone or a high auxin: cytokinin ratio were responsible for continued inorganized callus proliferation or callus growth accompanied by rhizogenesis while high levels of cytokinin alone produced dark green nodular structures on callus surface with meristematic zones in them but failed to develop further when transferred in same medium but a combination of auxin and cytokinin (NAA-KIN) proved effective in differentiation of somatic embryos, thus conforming with the findings of earlier workers.

Bhat *et al.* (1992) has reported in *Piper longum* that a medium containing 2,4-D gave soft, watery callus while that containing BA gave nodular white callus with green margins.

Ammirato (1983) has reported that the callus growth is induced in an auxin enriched medium and somatic embryogenesis can take place only upon transfer

of callus to a medium free of the auxin. The presence of auxin in the induction medium seems essential for the tissue to develop embryos in the emrbyo development medium. Anatomical studies on *Hyoscyamus niger* have shown that friable callus cells giving rise to embryoids are loose and are not organized into proembryoids in a medium containing 2,4-D and that proembryoids are initiated only upon transfer of the callus to a medium lacking auxin. It appears that removal of 2,4-D from the medium provides the signal for the callus cells to initiate organized growth.

2.4.4.1 Effect of Thidiazuron

2.4.4.1.1 Stimulatory effect

Thidiazuron was developed originally by Schering AG for utilization as a defoliant for *Gossypium hirsuitum* L. (Arndt *et al.*, 1976). Mok *et al.* (1982) found that cytokinin dependant *Phaseolus lunatus* callus was stimulated to grow when low concentrations of TDZ were added to an *in vitro* medium. Extremely low concentrations of TDZ stimulate axillary shoot proliferation in many woody species including *Juglans nigra* (Driver and Kumyuki, 1984). Effect of TDZ has been reported in *Brassaia actinophylla* by Badzian *et al.* (1989).

A range of TDZ concentrations from 0.1 to 20 μM has been used to stimulate shoot organogenesis from leaves of woody species including *Malus domestica* (Fasolo *et al.*, 1988). Shoots of *Miscanthus xogiformis* transferred from medium with BA to TDZ formed significantly more axillary shoots than shoots grown continuously on either medium or transferred from TDZ to BA (Nielsen *et al.*, 1995).

There are reports of somatic embryogenesis using TDZ as in *Vitis vinifera* (Matsuta and Hirabayashi, 1989). Within a species, TDZ can stimulate the production of somatic embryo and adventitious shoots (Bates *et al.*, 1992). Other reports are in *Nicotiana tabaccum* (Gill and Saxena, 1993) and *Cayratia japonica*, a medicinal plant (Zhou *et al.*, 1994). For the induction of embryogenic callus and somatic embryogenesis of *Cayratia japonica* both cytokinin and auxin are required in the medium and the cytokinin activity of TDZ is much stronger than that of KIN even when the concentration of TDZ used was only 4 per cent of KIN. Shoot proliferation and elongation could be increased when a low concentration of auxin or a cytokinin is added to the proliferating TDZ containing medium as reported in cultures of *Robinia pseudoacacia* (Chalupa, 1987).

2.4.4.1.2 Inhibitory action

High concentration of TDZ tend to stimulate callus formation in many woody species often at the expense of axillary shoot proliferation (Huetteman and Preece, 1993). Capelle *et al.* (1983) showed that *Phaseolus lunatus* L. callus became cytokinin autonomous (habituated) when cultured on media containing various concentrations of TDZ. Rooting of excised microshoots were found difficult because of a 'carry over' effect from TDZ in the shoot proliferation medium (Huetteman and Preece, 1993). Increasing the concentration of TDZ can increase the incidence of vitrification, as was shown with 'Barbara grape' (Gribaudo and Fronde, 1991).

2.4.5 Vitamins

Vitamins are the accessory food factors required by plant cells in very small quantities to perform certain essential roles in metabolism. The vitamins most

frequenty used in plant tissue culture are thiamine, nicotinic acid, pyridoxine, biotin, riboflavin and folic acid. Among these, thiamine is very essential and most frequently added in plant tissue culture media at levels of 0.1 mg l^{-1} to 1.0 mg l^{-1}. Linsmaier and Skoog (1965) demonstrated that, for tobacco, most vitamins were not essential for callus growth. Pyridoxine, nicotinic acid and biotin could be deleted from the medium without loss of growth. Letham (1966) found that myo-inositol interacted with cytokinin to promote cell division in carrot phloem explants. Thorpe and Patel (1984) have reported that thiamine is the most often added vitamin, followed by nicotinic acid and pyridoxine. Addition of biotin at 1.0 mg l^{-1} level was found to have a complementary effect on the growth and development of leaves in cardamom (Reghunath, 1989). Influence of arginine on *in vitro* rooting of dwarf apple rootstock was reported by Orlikowska (1992).

In *Pelargonium graveolens,* Satyakala *et al.* (1995) have reported that the shoot multiplication was unaffected by the addition of vitamins like folic acid, biotin, pantothenic acid, or complex organic substances like casein hydrolysate, yeast extract, malt extract or coconut water. However, increased myoinositol (1.0 g l^{-1}) was found to enhance the proliferation along with auxins and cytokinins.

2.4.6 Carbon sources

Normally for the culture of cells, tissues or organ, it is necessary to incorporate a carbon energy source into the medium. Sucrose is the main carbon energy source in most tissue culture media. The concentration of sucrose varied from 2 to 30 g l^{-1} (Oka and Ohyama, 1982). Many other carbon sources are found used, instead of sucrose. First work in this line was reported by Gautheret (1945). The use of alternative carbon sources like glucose, maltose, raffinose, fructose and

galactose were found to be less effective and mannose and lactose being the least effective.

Thakayama and Misawa (1979) reported that organogenesis in Liliaceae was inhibited at high sucrose levels. In *Episcia*, root formation was greatly enhanced by adding sucrose at 30 g l^{-1} to the medium (Pearson, 1979). In *Lilium* sucrose at 90 g l^{-1} increased root dry weight (Thakayama and Misawa, 1979). Favourable effect of maltose on embryo formation of *Digitalis lanata* was reported by Reinbothe *et al.* (1990). In *Aegle marmelos*, the shoot induction media contained a higher sucrose (40 g l^{-1}) level than the root induction media (20 g l^{-1}) as reported by Hossain *et al.* (1994).

2.4.7 Other organic compounds

Many undefined supplements are reported to be incorporated in tissue culture media. For successful growth of tissues and organs, addition of complex organic compounds to the basal medium was reported by Conger (1981). Some of these are casein hydrolysate, coconut water, yeast extract, malt extract, orange and tomato juices. The drawback of these are that being undefined in chemical composition, they have little control over the experiment and has been therefore recommended to avoid their use as far as possible (Gamborg and Shyluk, 1981).

In cases where nutritional requirements have not been established, mixtures of amino acids such as casein hydrolysate may be added between 0.05 and 0.1 per cent (Huang and Murashige, 1977). In *Eucalyptus camaludensis*, addition of casein hydrolysate was found to be superior than any other growth regulators (Kumar, 1993).

Adenine sulphate when added to the medium, often can enhance growth and shoot formation (Skoog and Tsui, 1948). Multiple shoot induction in *Dioscorea floribunda* was reported by Sinha and Chaturvedi (1979) in a medium supplemented with BAP, adenine sulphate and NAA. BA in combination with adenine sulphate, coconut milk etc. accelerated morphogenic potential in *Eucalyptus* as reported by Kumar (1993).

The discovery of Pollard *et al.* (1961) that myo inositol was present in coconut water and had growth promoting activity, led to the inclusion of inositol in plant tissue culture media. Coconut water is reported to be promoting growth and differentiation in a wide variety of excised plant tissues including *Datura* embryos (Van Overbeek *et al.*, 1941) and tobacco pith (Jablonski and Skoog, 1954). Hawker *et al.* (1973) observed that replacement of casein by coconut water could double the growth rate of grape berry callus. Illahi (1983) reported the use of coconut water along with 2,4-D and KIN, on successful *in vitro* propagation of *Papaver somniferum*. An enhancing effect on axillary shoot production was reported by Bhaskar (1991) in banana. Ilahi and Akram (1987) reported formation of roots in *Rauwolfia serpentina* by adding 100 mg l^{-1} coconut water in the culture medium. Addition of coconut water at 15 per cent level was found to be beneficial on shoot proliferation in Orchid, *Dendrobium* (Lakshmidevi, 1992). In *Eucalyptus tereticornis*, Subbaiah and Minocha (1990) reported the favourable effect of coconut water (10% v/v) in combination with BA on shoot induction from callus which would not occur if BA alone was used.

Coconut water had an important role on the morphogenic capacity of *Passiflora* root explants. Regeneration was noted after 56 days of culture and a large

number of buds was observed in MS media supplemented with 10 per cent coconut water in combination with BA as reported by Dornelas and Vieira (1994).

Addition of activated charcoal to plant tissue culture media may have either beneficial or harmful effects. Growth, rooting, organogenesis and embryogenesis are reported to be stimulated in a variety of species and tissues including ginger shoot tips, palm embryos (Wang and Huang, 1976); onion (Fridborg and Eriksson, 1975); carrot (Fridborg *et al.*, 1978); ivy (Banks and Hackett, 1978) and in banana (Bhaskar, 1991).

Activated charcoal is reported to inhibit growth of soybean (Fridborg and Eriksson, 1975). It is also reported as preventing browning in cultured tissues of date palm (Tisserat, 1979). Charcoal was found to bind hormones and other metabolites (Weatherhead *et al.*, 1978). Activated charcoal, added to liquid MS medium at concentrations ranging from 0.1 per cent to 5 per cent, reduced IAA and IBA concentrations by more than ninety seven per cent (Scott and Ellen, 1990).

The addition of phloroglucinol to the medium promoted the culture growth in *Cinchona ledgeriana* (Hunter, 1979) and in *Theobroma cacao* (Mallika *et al.*, 1992).

2.4.8 Ascorbic acid

In *Tylophora indica*, Sharma and Chandel (1992) have reported that addition of ascorbic acid to the hormone supplemented medium was essential for initial bud break and further shoot multiplication. The increase in shoot number in the presence of ascorbic acid has also been reported in tobacco cultures (Richard *et al.*, 1988). Mechanism of action of ascorbic acid, a common antioxidant and

antibrowning agent, is presently not known. Ascorbic acid or some products of its oxidation may possibly be increasing shoot number through ascorbate protection of endogeneous phytohormones as implicated for Pinus, Picea and Tobacco (Berlyn and Beck, 1980; Rumary and Thorpe, 1984; Richard *et al.*, 1988).

2.4.9 Culture conditions

The culture conditions have a major role in the success of tissue culture. The physical forms of the medium, pH, light, temperature and relative humidity play an important role in the growth and differentiation.

Light requirement involves a combination of several components viz, intensity, quality and duration. An optimum combination of these are necessary for certain photomorphogenic events. According to Murashige (1977), the optimum daylight period is 16 h for a wide range of plants. Wang *et al.* (1989) reported the favourable effect of increasing the illumination period on bud production of *Petunia hybrida*. Maintenance of cultures in a 16 h light/8 h dark cycle was reported by Mumtaz *et al.* (1990) in *Catharanthus roseus*. Subbaiah and Minocha (1990) reported that healthy callus could be formed from leaf and stem explants of *Eucalyptus* in the dark while those kept in light generally turned brown and callus formation was poor.

The rate of callus induction from different explants was significantly high in full darkness rather than 16/8 h light/dark period. The number of shoots was significantly higher when calli for organogenesis were incubated in a 16/8 h light/dark cycle than in complete darkness when calli induced under a 16/8 h photo-period were used for shoot induction under similar photoperiod, rate of shoot

induction was much lower. The shoot induction rate was nearly 20 per cent (± 2.5) higher when calli induced under complete darkness were used for shoot induction under a 16/8 h light/dark period. This was the response in *Asparagus cooperi* irrespective of the explant type from which calli were derived. It was also found that NAA was found to be more effective in dark conditions for callusing rather than in light conditions.

Nakayama (1966) used dark incubation to promote growth of adventitious buds with *Passiflora caerulea*. It has been suggested that dark incubation has association with IAA component since IAA breaks down rapidly in tissue culture media during light incubation (Nissen and Sutter, 1990). In *Passiflora edulis*, shoot regeneration was observed in the dark only in cotyledonary explants while in all others it was favoured in light as reported by Dornelas and Vieira (1994).

In *Clitoria ternatea*, cultures were maintained under complete darkness for 48 h and then under 6 h direct fluorescent light (4000 lux) from cool white fluorescent tubes.

Yeoman (1986) reported that the usual environment temperature of a species concerned should be taken into account for its better performance under *in vitro* conditions. However, most of *in vitro* cultures are grown successfully at temperatures around 25 ± 2°C. In general, tropical species need higher temperature. Durahvila *et al.* (1992) reported the effect of incubation temperature on morphogenesis, callus culture and plantlet culture of sweet orange. Bud culture and callus induction were optimal at incubation temperature between 21°C and 30°C. The optimum temperature for root formation was 27°C. Incubation in the dark improved the root formation in many species.

Hu and Wang (1983) reported that air humidity is not often controlled and when it is controlled 70 per cent has been found to be the most frequent setting. Relative humidity became an important factor in hardening and planting out of *in vitro* raised cultures.

2.5 Root induction

2.5.1 Rooting of shoots *in vitro*

In vitro plants must have a strong and functional root system. The shoots can be rooted under *in vitro* conditions and the rooted plantlets transferred to soil or the propagules could be treated like microcuttings and rooted *ex vitro* in a nonsterile, highly humid, low light environment. Hu and Wang (1983) observed that three phases are involved in rhizogenesis viz. induction, initiation and elongation.

Among the auxins, IBA and NAA have been most effective for root induction (Ancora *et al.*, 1981). All cytokinins inhibit root induction and BA which is widely used for shoot multiplication does so particularly strongly that roots are delayed even after transferring to cytokinin free medium (Yeoman, 1986). The root elongation phase is very sensitive to auxin concentration. High concentrations of auxin inhibited root elongation (Thimman, 1977). Thidiazuron also inhibits rooting in some reports.

Several researchers have shown that *in vitro* rooting can successfully be achieved by reducing salt concentrations in the media, particularly in MS, B5 and LS which contain high salt concentrations. Abundant rooting was observed when the salt concentration in the medium was reduced to one-half, one-third or one-fourth of the standard strength (Lane, 1979; Skirvin and Chu, 1979). Lowering of nitrogen

content in the culture media promoted root formation in *Digitalis purpurea*, Rucker (1982).

Rhizogenesis in *Plumbago rosea* was reported by Satheeshkumar and Bhavanandan (1989) by adding IBA in the culture medium. Sometimes a combination of growth regulators are used for rooting in cultures. Mumtaz *et al.* (1990) reported the use of GA and BAP in rooting medium of *Catharanthus roseus*. Maximum root length was obtained in MS basal medium with half the salt concentration, but the roots so produced were very slender as reported in *Gardenia jasminoides* by George *et al.* (1993). In *Pelargonium graveolens*. Satyakala *et al.* (1995) reported that maximum rooting could be obtained in half strength MS medium supplemented with IBA within 10-12 days after transfer to the media and the roots developed directly from the shoots without callus phase. Efficient rooting in *Clitorea ternatea* could be obtained in media (BM) supplemented with 0.5 mg l^{-1} of IBA.

Shah and Dalal (1980) reported in *Glycirrhiza glabra* that if NAA was incorporated in MS medium used for rooting, short, thick roots were produced.

In *Duboisia myoporoides*, NAA was necessary for rooting of shoots (Kukreja *et al.*, 1986) while IAA or IBA could not substitute NAA. NAA levels greater that 1.0 mg l^{-1} failed to elicit root induction. In *Passiflora edulis* only NAA could induce roots and IBA proved non effective (Kantharajah and Dodd, 1990). IBA when incorporated @ 0.5 mg l^{-1} into the medium produced normal roots in *Eucalyptus terreticornis* (Subbaiah and Minocha, 1990). In *Gomphrena officinalis*, IBA resulted in earlier rooting than NAA and MS basal medium was (without growth regulators) totally ineffective for rooting.

Kukreja *et al.* (1986) have stressed the importance of static liquid medium for root induction of shoots in *Duboisia*. Liquid medium helps in maintaining an optimum O_2:Co_2 balance and or regulates the availability and uptake of nutrients and hormones more effectively (Biondi and Thorpe, 1981).

Muralidharan and Mascarenhas (1989) have reported the need for a brief liquid culture exposure with low auxin levels before rooting of *Eucalyptus*. Rooting of shoots in half strength B5 liquid medium was reported by Kumari and Saradhi (1992) in *Origanum vulgare*.

In *Adhatoda beddomei* it has been found that stationery liquid nutrient medium (SH) supplemented with IAA or IBA (0.2 mg l^{-1}) could produce 10-15 thick long roots as compared to agar gelled medium which produced 6-8 roots only. The enhanced rooting response was attributed to possible dilution of the inhibitory substances released from the cut ends. This was corroborated by the observation that supplementation of the agar medium with 500 mg l^{-1} activated charcoal which could adsorb inhibitory substances, was effective in inducing the formation of 8-14 healthy roots (Sudha and Seeni, 1994).

Kapoor and Chauhan (1992) have reported that rooting of tissue culture shoot derived from F_1 hybrid of *Eucalyptus* could be successfully rooted in 1/5th MS media supplemented with IBA. Complete darkness during root induction phase was found to promote the rooting frequency in *Commiphora wightii* (Barve and Mehta, 1993). In *Aegle marmelos* rooting could be obtained only when IBA and NAA (0.5 mg l^{-1}) were used in combination. However, rooting was poor with a maximum of 25 per cent of cultures showing root formation.

Exposure to Root Inducing Medium (RIM) which was a combination of IAA and IBA in half strength MS medium for 24 h followed by transfer of shoots to rooting medium which was half strength basal MS medium, resulted in high frequency root induction in *Commiphora wightii* as reported by Barve and Mehta (1993). Similarly transfer to auxin free medium for rooting was necessary in many woody species (Ravishankar and Jagadish, 1989).

2.5.2 Rooting of shoots *ex vitro*

The cost of production of *in vitro* plantlets could be reduced by changing the rooting stage from an *in vitro* step to an *ex vitro* one. The major cost of producing *in vitro* plants lies in the rooting and hardening stages (Rajeevan and Pandey, 1986). For rooting under *ex vitro* conditions, the shoots for rooting could be handled as microcuttings without using aseptic conditions. By adopting such a technique, the sterile tissue culture phase would end with proliferation of shoots and rooting could be considered as conventional propagation. Rooting under *ex vitro* conditions also facilitates the combining of the rooting stage with acclimatization which is an essential part of the micropropagation procedure (George and Sherrington, 1984).

The formation of adventitious roots on a microcutting is a crucial step in commercial micropropagation (Mc Clelland *et al.*, 1990). The type of root system formed depends on the physical characteristic of the rooting environment (Nemeth, 1986), as well as the species and quality of the microcutting (George and Sherrington, 1984).

2.5.2.1 Methods of *ex vitro* rooting

2.5.2.1.1 Two step process

Micropropagated shoots of *Pinus radiata* were pretreated for rooting by inserting them into water agar medium containing auxins for five days after which they were moved to a potting mix of peat and purmice for rooting under high humidity (Aitken Christie and Thorpe, 1984).

Maene and Deberg (1985) described a simpler technique of adding a layer of liquid medium over the agar surface in proliferating cultures and *in vitro* proliferated shoots of *Magnolia soulangeana* were successfully rooted *ex vitro* by this method. They further reported that addition of water, auxin solution, sucrose soltuion or a combination of auxin and sucrose helped in forming roots in several herbaceous species.

Kavitha and Raju (1995) reported in *Aristolochia indica* that the shoots produced *in vitro* were separated and transferred to MS basal media without any hormones before the rooting. The shoots were planted in autoclaved wet soilrite mixed with IBA 0.1 mg l^{-1} and covered with polythene sheet to provide high humidity.

2.5.2.1.2 One step process

The *in vitro* produced shoots were inserted into rooting media such as peat, perlite, vermiculite or a mixture of these compounds. The micro cuttings were then placed in a high humidity environment for rooting (George and Sherrington, 1984).

Treating the cut basal ends of micropropagated shoots with an auxin carried on talc powder before inserting them into a rooting medium and then placing them under mist or high humidity conditions were successful with black berry and blue berry (Zimmerman and Broome, 1980) and apple (Zimmerman and Broome, 1980; Simmonds, 1983).

2.6 Hardening and planting out

Hardening of rooted plants was a prerequisite for the successful establishment of plants.

2.6.1 Standardisation of environmental conditions

Wainwright (1988) observed that the environment in a tissue culture container is that of very high humdiity, low light level and usually a constant temperature. Leaves on shoots or plantlets leaving this environment are as a result, very poorly adapted to resist the low relative humidity, higher light levels and more variable temperature found *in vivo*. Langford and Wainwright (1987) observed that physiologically, the leaves grown *in vitro* are incapable of significant photosynthesis. The stomata are unable to close and as cuticular wax on the leaf surface is minimal, are unable to control water loss. Improper development of vascular connections between the shoot and the roots may also cause poor establishment of the plantlets.

Light, temperature and relative humidity are the three major factors to be controlled during acclimatization. Hu and Wang (1983) suggested a period of humidity acclimatization for the newly transferred plantlets. Three methods of

controlling relative humidity are the polythene tent, misting and fogging. In polythene tent, the aerial weaning environment is closed, it is possible to take advantage of carbondioxide enrichment during hardening (Lakso *et al.*, 1986).

Standardisation of rhizosphere environment is also necessary for getting better growth of plantlets (Zimmerman and Fordham, 1985). Kyte and Briggs (1979) found that a porous potting mixture of peat, perlite and composted bark (1:1:1) was the best for rooting *in vitro* cultured Rhododendrons.

In *Adhatoda beddomei*, Sudha and Seeni (1994) observed that more than 70 per cent of 120 plantlets transferred directly to the nursery were lost within 5-8 days. The plants hardened for atleast 4 weeks in the humidity chamber showed 95 per cent rate of subsequent establishment in pots.

2.6.2 Nutrition

Nutrition of the micropropagules during rooting and hardening has been shown to be species dependant and Scott (1987) has shown that inclusion of fertilizer during hardening can be detrimental for *Kalmia*, improve plant quality of *Rhododendron* and is essential for quality *Magnolia*. Wong (1986) recommended addition of 3 g of nutricate (14:14:14 NPK) to each pot one week after transplanting to get healthier *in vitro* banana plantlets for planting in the main field. Mathur *et al.* (1988) initially irrigated *in vitro* derived Java citronella plantlets with Hoagland and Arnon (1950) nutrient solution for one week. Keshavachandran (1991) obtained cent per cent establishment of Vetiver plantlets with the application of half strength MS nutrients as well as an NPK fertilizer soltuion (10:5:10 g l^{-1}) at weekly intervals. However, better vigour of the plantlets was observed by application of the latter

solution. Ramesh *et al.* (1993) found that application of half strength MS basal medium was found to be the best for maximum survival of jack plantlets.

Prabha (1993) has reported that application of a nutrient starter solution of NPK fertilizer solution once a week or one-fourth strength basal MS salts was found to be sufficient to induce healthy growth of transplanted pineapple plantlets in early stages of growth. In later stages application of NPK fertilizer solution twice a week or Hoagland's solution once a week was found to be better.

2.6.3 Pre-treatments

Kar and Sen (1985) have described a procedure for hardening *Asparagus racemosus* plantlets. Prior to transfer to the potted soil, all plantlets were maintained in half strength liquid MS basal medium with 1 per cent sucrose. After two to three weeks the plants were transferred to the same basal medium without sucrose. Two weeks later plants were washed with water, planted into pots containing sandy soil and humus (3:1) and kept in growth chamber (temperature 25°C, humidity 55 per cent, light 30 x 10^8 μM s^{-1} m^{-2} for 16/8 h light/dark photoperiod). After 4 weeks the pots were transferred to the field and 70 per cent plants survived.

In *Pellargonium graveolens*, Satyakala *et al.* (1995) reported that the rooted plantlets should be transferred to half strength MS mineral solution for about a week and then planted in autoclaved soilrite and kept in a growth chamber at 80 per cent relative humidity and 25°C. After 25 days, the hardened plants were gradually transferred to pots. In pineapple, Prabha (1993) has reported the need for immersing the roots of the tissue culture derived plantlets for 18 h in sterile water prior to transplanting to give hundred per cent survival of the plantlets.

Hardening treatments for effective planting out of the *in vitro* plantlets of vetiver were standardised by Keshavachandran (1993). Immersing the roots of the plantlets in sterile water for 12 hr followed by transfer into pots and keeping in the mist chamber or coating the leaves with paraffin oil and keeping in the mist chamber recorded the highest percentage of establishment as well as the greatest length and area of the longest leaves, the largest diameter of the bush and the highest number of tillers per plant.

2.6.4 Effect of media

Elongated shoots of *Eucalyptus camaludensis* when planted in non sterile sand beds produced roots in 70 per cent of the shoots as reported by Gurung and Rajbhandari (1989). *In vitro* produced plantlets of *Tylophora* showed 90-100 per cent survival in soilrite. After 6-8 weeks they could be successfully transferred to the field as reported by Sharma and Chandel (1992). Ramesh *et al.* (1993) found that sand supported 53.3 per cent survival of *in vitro* produced jack plantlets. Vermiculite, peat and sand + soil mixture were found to record 40 per cent survival. Regenerated plantlets of *Aegle marmelos* were transferred to pots containing soil mixed with compost which was the best media as reported by Hossain *et al.* (1994).

Prabha (1993) observed that potting mixes such as cocopeat, soilrite, biofibe and vermiculite were better in inducing vigorous growth of tissue cultured pineapple plantlets. Plantlets grown in plastic pots with cocopeat or plastic bags with soilrite mix, in general grew more vigorously. Keshavachandran (1993) found that vetiver plants when transferred into earthern pots filled with equal quantities of

soilrite and ordinary potting mixure, induced maximum length and area of the leaves and the highest number of tillers.

2.6.5 Effect of containers

Prabha (1993) found that tissue cultured pineapple plantlets grown in plastic pots showed maximum vigour with respect to the number of leaves, height and width of the largest leaf, followed by those in mud pots and polybags. The maximum percentage increase in these parameters were observed for the plantlets in protrays.

2.7 Encapsulation studies

The micropropagation studies is greately constrained due to the high cost involved in terms of culture space and labour. In addition, inadequate standardisation of hardening procedures during transfer of the plants from laboratory to the field generally results in high mortality rates that further lower the efficacy of results achieved *in vitro*. So efforts were made to encapsulate somatic embryos (which usually lack seedcoat and cotyledons unlike other seeds and hence are prone to dessication) in biodegradable synthetic polymer protein which acts as artificial seedcoats. Redenbaugh *et al.* (1984) discovered that hydrogels such as sodium alginate could be used to produce single embryoid artifical seeds. The concept has changed now to include other tissues or organs which could give rise to a plantlet by itself. According to Kamada (1985), a synthetic seed is a capsule containing an actively growing part of a tissue or an organ capable of giving rise to a complete plant.

2.7.1 Production of synthetic seeds

Redenbaugh *et al.* (1986) encapsulated somatic embryos of alfalfa, celery and cauliflower as single beads to produce individual somatic artifical seeds. The somatic embryos were mixed in 3.2 per cent (w/v) sodium alginate, dropped individually into a 50 mM solution of calcium chloride and allowed to undergo complexation for 30 min to form an alginate bead.

Kitto and Janick (1985) produced synthetic seeds by encapsulating somating embryos of carrot. Synthetic seed coats were applied to asexual embryos of carrot, by mixing equal volumes of embryo suspension and a 5 per cent (w/v) solution of polyethylene oxide (Polyox WSR-N 750) and dispensing 0.2 ml drops of this mixture into teflon sheets. The drops got dried to form detachable wafers consisting of embryo suspension embedded in polyox. Muralidharan and Mascarenhas (1989) produced synthetic seeds of *Eucalyptus citriodora* somatic embryos. Keshavachandran (1993) reported that differentiating calli of vetiver could be successfully encapsulated into beads using sodium alginate at 2.5 per cent and calcium chloride at 75 mM. This resulted in formation of firm and round beads.

Mathur *et al.* (1989a) made an assessment of various concentrations of sodium alginate and calcium chloride for the formation of beads in *Valeriana wallichii* and indicated that a 6 per cent solution of sodium alginate upon complexation with 75 mM of $CaCl_2.2H_2O$, solution gave optimal firm and round beads within an ion exchange complexation duration of 30 min.

Prabha (1993) has reported that the globular bodies of pineapple could be encapsulated using 2.5 per cent sodium alginate and 75 mM calcium chloride with a

complexation time of 30 min. Senaratna (1992) reported that the quality of artificial seed depends on the temporal, quantitative and qualitative supply of growth regulator and nutrients along with optimal physical environment. Dessication of somatic embryo provided a quiscent phase analogous to true seeds, facilitating the convenience of year round production, storage and distribution.

Onishi *et al.* (1994) produced novel self breaking gel beads and sustained release microcapsule as an artifical endosperm developed for the encapsulation of carrot embryos. Also a production system for synthetic seed was developed with which 80 thousand beads could be produced per day. Dupius *et al.* (1994) found that synthetic seed could be given a coating similar to a pharmaceutical type capsule. Capsule body was covered on its innersurface by a water tight film, composed of polyvinyl chloride (PVC), Polyvinyl acetate (PVA) and bentone as a thickener, to control the nutrient supply and subsequent development of somatic embryos. The germination medium and the embryo were then placed within this improved capsule. The carrot somatic embryos were able to sustain conversion rates of 90 per cent in such coatings. Artificial seed production through shoot bud encapsulation has been reported in a range of medicinal plants like *Hyoscyamus muticus, Picrorrhiza kurroa, Atropa belladona, Dioscorea floribunda, Rauvolfia serpentina* and *Mentha arvensis* (Ahuja *et al.*, 1989).

2.7.2 Storage and plantlet conversion of synthetic seeds

Bapat *et al.* (1987) encapsulated axillary buds of mulberry in alginate and agar to produce individual beads. The beads could be stored at 4 °C for 45 days without loss of viability. Encapsulated beads regenerated to complete plantlets on an appropriate medium. Bapat and Rao (1988) encapsulated somatic embryos of

sandalwood in an alginate matrix. Encapsulated single embryos germinated to form plants with roots and shoots. Embryogenic cell suspensions encapsulated and stored at 4°C for 45 days produced embryos when recultured as suspensions.

Mathur *et al.* (1989a) encapsulated apical and axillary shoot buds of *Valeriana wallichii* in calcium alginate beads and plantlet development was observed under both *in vitro* (98%) and *in vivo* (64%) conditions in pots with vermiculite under glasshouse conditions. Keshavachandran (1993) could successfully store encapsulated beads of vetiver at 4°C on cotton wool moistened with MS basal salts for 45 days without losing their regeneration capacity.

The frequency of conversion of fresh encapsulated somatic embryos to plants was 34 per cent which was affected by the concentration of calcium chloride, commercial sources of sodium alginate and the nutrient medium. The conversion frequency of artificial seeds to seedling plants was 8.3 per cent after storage for 90 days at 2°C. The self breaking gel beads produced with carrot somatic embryos by Onishi *et al.* (1994) showed 52 per cent conversion frequency after sowing on humid soil in greenhouse.

Lulsdorf (1993) reported that the addition of 0.5 per cent (w/v) of activated charcoal to the alginate capsule could significantly enhance root development and germination of somatic embryos of Spruce (*Picea* spp.). Ahuja *et al.* (1989) has reported that for better response of synthetic seeds under glasshouse conditions it was better to incorporate antibiotics like Ampicillin, Chloramphenicol, Tetracycline and fungicides like Bavistin.

2.8 Biochemical studies

Isozymes have been defined as multiple molecular form of an enzyme with similar or identical substrate specificities within the same organism (Markert and Muller, 1959). Murashige and Li (1985) observed that the changes that occur in different enzyme systems during cell or callus cultures could be studied to distinguish the organogenic from the non-organogenic tissue. Kocchar *et al.* (1984) noted characteristic taxonomic markers for six varieties in betel vine by peroxidase pattern study. They had also observed that different types of same cultivar possessed the same band pattern in most of the cases.

Rao *et al.* (1990) reported that two isoperoxidases 3 and 5 (Rm 0.11 and 0.14) observed in embryogenic calli of maize; were absent in non-embryogenic calli indicating a possible association of specific isozyme with embryogenesis. In vetiver, protein and peroxidase isozyme gel electrophoresis revealed that the banding pattern for tisisues from *in vitro* regenerated plantlets and *in vivo* plants were not similar which indicate the different genetical make up of the regenerants (Keshavachandran, 1993).

Material and Methods

MATERIALS AND METHODS

The present study on standardisation of *in vitro* propagation techniques in *Holostemma annulare* K. Schum. was carried out in the Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara, Thrissur, during 1993 to 1995.

3.1 MATERIALS

The major and minor elements required for the preparation of media were of analytical grade and procured from M/s.British Drug House (BDH) Laboratories, Sisco Research Laboratories (SRL) and Merck. The amino acids, vitamins and plant growth regulators were obtained from M/s.Merck, SRL and Sigma Chemicals, USA.

3.1.1 Glasswares

Borosilicate glasswares of Corning/Borosil brand were used for the study. They were cleaned by initially soaking in potassium dichromate solution in sulphuric acid for 12 hours, followed by thorough washing with jets of tap water in order to completely remove all traces of dichromate solution. They were then soaked in detergent solution (Teepol 0.1%) overnight, thoroughly washed with tap water and rinsed twice with double distilled water. The glasswares were then dried in a hot air oven at 100°C for 24 hrs. They were then stored in cupboards away from contaminants and dust until used.

3.1.2 Culture media

3.1.2.1 Composition of media

Murashige and Skoog (MS) medium (1962) and Woody Plant Medium (WPM) (Lloyd and McCown, 1980) were used in the present study. The nutrient media included inorganic salts, vitamins, growth regulators, organic supplements and a carbon source. The composition of the media are given in Table 1.

3.1.2.2 Preparation of media

The stock solutions for micronutrients, vitamins, Fe-EDTA and growth regulators were prepared with sterile distilled water as given in Table 2.

To make up one litre of medium, the required quantity of each stock solution was added into a glass beaker. A known quantity of double distilled water was added into the beaker and the required quantity of sucrose was weighed, added as solids and dissolved fully. The pH of the solution was adjusted using an electronic pH meter to 5-7 using 0.1N HCl or 0.1N NaOH. The volume was made upto one litre. Agar (0.7%) was weighed out, added to the medium and melted by keeping the solution in a waterbath maintained at 90-95 $^{\circ}$C. The medium was distributed to test tubes (150 mm x 25 mm) at the rate of 15 ml each or to 100 ml Erlenmeyer flasks at the rate of 25 ml each. The test tubes or flasks were plugged with non-absorbent cotton and autoclaved at 121 $^{\circ}$C and 15 psi (1.06 kg cm^{-2}) for 20 min (Dodds and Roberts, 1982). The medium was allowed to cool to room temperature and stored in the culture room until used. For static liquid medium, filter paper bridge was used to hold the explant in position.

Table 1. Composition of various basal media tried for *in vitro* culture of *H. annulare*

Ingredients mg^{-1}	MS	WPM
Inorganic constituents		
$(NH_4)NO_3$	1,650	400
KNO_3	1,900	
K_2SO_4		990
KH_2PO_4	170	170
$Ca(NO_3)_2.4H_2O$		556
$CaCl_2.7H_2O$	370	370
$FeSO_4.7H_2O$	27.8	27.8
Na_2EDTA	37.3	37.3
$MnSO_4.4H_2O$	22.3	22.3
$ZnSO_4.7H_2O$	8.6	8.6
H_3BO_3	6.2	6.2
KI	0.83	
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.25
$CaCl_2.6H_2O$	0.025	
Organic constituents		
Myo-inositol	100	100
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Thiamine HCl	0.1	1.0
Glycine	2.0	2.0
Sucrose	30,000	30,000

Table 2. Preparation of stock solutions of plant growth regulators

Sl. No.	Growth regulator	Comon abbreviation	Quantity for 50 ml stock (mg)	Preparation	Concentration range (mg l^{-1})
1	Auxins				
	a. α-Napthalene acetic acid	NAA	50	Dissolved in 2-5 ml of 1N NaOH with slight heating and gradually diluted to 50 ml with distilled water	1
	b. Indole-3-butyric acid	IBA	50		
2	Cytokinins				
	a. 6-Benzyl adenine	BA	50	Dissolved in 2-5 ml of 1N NaOH with slight heating and gradually diluted to 50 ml with distilled water	1
	b. Kinetin	KIN	50		
	c. 6 Γ.Γ. Dimethyl allyl amino purine	2iP	50		
	d. N-phenyl-N'-1,2,3-thiadiazol - 5 ml urea	TDZ	--	1 ml of 1N KoH solubilize technical grade TDZ for the preparation of 100 ml of a 1 mM stock solution (Huetteman and Preece, 1993)	

3.1.2.3 Preparation of antibiotic media

As the antibiotics are thermolabile, they were added after filter sterilization. All equipments viz. syringe, filter, holder with filter and beaker were heat sterilized. The culture medium ingredients except the antibiotic was taken in a flask and sterilized in the autoclave. After the period of sterilization, the contents of the flask was allowed to cool to about 50°C and the antibiotic solution was passed through the filter assembly directly into the medium.The medium was shaken well and poured into the previously sterilized culture tubes under aseptic conditions.

3.1.3 Growth regulators

Auxins (2,4-D, NAA, IBA, IAA), Cytokinins (BA, 2iP, KIN, TDZ) etc. were incorporated in the media at various stages of culture for direct and indirect morphogenesis and axillary bud break (Table 2).

3.1.4 Organic supplements

Coconut water, activated charcoal, phloroglucinol adenine sulphate and ascorbic acid were tried for their effect on axillary bud break and morphogenesis.

3.1.5 Carbon sources

Sucrose was the main source of carbon used for the study.

3.1.6 Transfer area and aseptic manipulations

All the aseptic manipulations such as surface sterilization of explants, preparation and inoculation of explants and subsequent subculturing were carried out

under the hood of a clean air laminar flow cabinet (Klenzaids). The working table of the laminar air flow cabinet was first surface sterilized with absolute alcohol and then by putting on the U.V. light for 30 min. The petridishes as well as the instruments used for inoculation were first steam sterilized in an autoclave at 15 psi at 121°C for 30 min and then flame sterilized before each inoculation. The hands were also scrubbed with alcohol before each inoculation.

3.1.7 Culture room

The cultures were incubated at 30° ± 1°C in an air conditioned culture room with a 16 h photoperiod of around 2000 lux supplied by cool day light fluorescent tubes.

3.1.8 Source of explants

The explants were taken from the stock plants maintained under controlled conditions in the glasshouse. The mother stock was collected from two localities viz., Vellanikkara and Palghat. The young root suckers brought were planted in previously fumigated potting mixture (1:1:1 of sand, earth and farmyard manure) and kept in glasshouse giving daily irrigation and frequent fertilisation. Plants were regularly sprayed with systemic fungicide, Bavistin 50 WP (Carbendazim) and a contact fungicide Dithane M-45 (Mancozeb) each at one per cent concentration at fortnightly intervals. To study the mode of establishment of field grown plants under *in vitro* conditions, a few explants were collected from mature plants grown in the medicinal plant garden attached to the All India Co-ordinated Research Project on Medicinal and Aromatic Plants at Vellanikkara.

3.2 METHODS

3.2.1 Preparation of explants

Vine cuttings of 30-40 cm length with 5-6 nodes were either taken from glasshouse or from field for the study. Explants of 3-4 months and 10-14 months age were tried. Cuttings were defoliated leaving a portion of the petiole intact and washed free of dust. The leaves, stem and root segments were then immersed in teepol solution for ten min. Leaf segments, petiole segments, nodal segments, stem and root segments were used as explants for the study. Mature explants and root explants were given pretreatments with Cetrimide and fungicides like Bavistin to reduce the rate of contamination. The explants were then subjected to surface sterilization treatments.

3.2.2 Standardisation of surface sterilization

The different types of explants were subjected to the surface sterilants at different concentrations and for different durations as detailed in Table 3.

The observations on percentage of cultures contaminated and explant survival were recorded three weeks after inoculation.

3.2.2.1 Effect of season of explant collection

The explants were collected month wise from January to December in all months in which they were available. The explants were subjected to surface sterilization by treating with 0.1 per cent mercuric chloride for five min. The surface sterilized explants were thoroughly washed with sterile distilled water and inoculated on MS establishment medium with BA at 1 mg l^{-1}.

Table 3. Concentration and duration of treatment of surface sterilants on 2-3 month old explants of *H. annulare** (from glasshouse)

Treatment No.	Treatment sterilant	Concentration (%)	Duration of treatment
1	Alcohol	50	30 s
2	Alcohol	50	60 s
3	Alcohol	100	30 s
4	Alcohol	100	60 s
5	Mercuric chloride	0.05	5 min
6	Mercuric chloride	0.05	10 min
7	Mercuric chloride	0.1	5 min
8	Mercuric chloride	0.1	10 min
9	Alcohol + Mercuric chloride	100 0.05	30 s 5 min
10	Alcohol + Mercuric chloride	100 0.05	30 s 10 min
11	Alcohol + Mercuric chloride	100 0.1	30 s 5 min
12	Alcohol + Mercuric chloride	100 0.1	30 s 10 min
13	Alcohol + Mercuric chloride	100 0.05	60 s 5 min
14	Alcohol + Mercuric chloride	100 0.05	60 s 10 min
15	Alcohol + Mercuric chloride	100 0.1	60 s 5 min
16	Alcohol + Mercuric chloride	100 0.1	60 s 10 min
17	Alcohol + Mercuric chloride	100 0.1	60 s 13 min
18	Chlorine water (saturated)		5 min
19	Chlorine water (saturated)		7 min
20	Control (No sterilant)		

* Explants include nodal segments, shoot tips and leaf segments

Observations on the percentage of cultures contaminated and the explant survival were recorded three weeks after inoculation.

3.2.3 Culture establishment

3.2.3.1 Effect of different growth regulators

Three to four month old nodal segments and shoot tip explants isolated from *Holostemma annulare* K. Schum were cultured in MS medium supplemented with the following growth regulators.

1. BA 0.25 mg l^{-1}
2. BA 0.5 mg l^{-1}
3. BA 1.0 mg l^{-1} (Both in full and half strength MS medium)
4. BA 2.0 mg l^{-1}
5. BA 2.5 mg l^{-1}
6. BA 4.0 mg l^{-1}
7. BA 5.0 mg l^{-1}
8. BA 10.0 mg l^{-1}
9. KIN 0.25 mg l^{-1}
10. KIN 0.5 mg l^{-1}
11. KIN 1.0 mg l^{-1} (in full and half strength MS medium)
12. KIN 2.0 mg l^{-1}
13. KIN 2.5 mg l^{-1}
14. KIN 4.0 mg l^{-1}
15. KIN 5.0 mg l^{-1}
16. 2iP 0.25 mg l^{-1}
17. 2iP 0.5 mg l^{-1}

18. 2iP 1.0 mg l^{-1}
19. 2iP 2.0 mg l^{-1}
20. 2iP 2.5 mg l^{-1}
21. 2iP 4.0 mg l^{-1}
22. 2iP 5.0 mg l^{-1}
23. BA 0.25 + NAA 0.5 mg l^{-1}
24. BA 0.25 + NAA 1.0 mg l^{-1}
25. BA 0.25 + IAA 0.5 mg l^{-1}
26. BA 0.25 + IAA 1.0 mg l^{-1}
27. BA 1.0 + IAA 1.0 mg l^{-1}
28. BA 0.5 mg l^{-1} + 0.1 per cent Streptomycin
29. Full and half strength basal medium

Observations on the percentage of cultures established, number of days taken for first bud burst, number of shoots and buds, length of the longest shoot and average length of a shoot, number of nodes per culture and average weight of callus (fresh) formed were recorded three weeks after initial culture.

3.2.3.2 Effect of media

The explants were cultured in the following media for standardisation.

1. Full strength MS
2. Full strength MS + BA 1 mg l^{-1}
3. Full strength WPM + BA 1 mg l^{-1}
4. Half strength MS

Explants were also cultured in solid and stationary liquid media.

3.2.3.3 Effect of additives

The following additives were incorporated into full or half strength MS medium with or without agar to find out their effect on establishment..ls1

1. Activated charcoal (0.1%)
2. Silver nitrate (5 mg^{-1}) with or without BA 1.0 mg l^{-1}
3. Ascorbic acid (100 mg l^{-1})
4. Coconut water (15%)
5. Adenine sulphate (2 mg l^{-1})
6. MS basal (full or half strength)

The same observations as were taken to find the effect of growth regulators were also taken for the above mentioned experiments.

3.2.4 Proliferation

3.2.4.1 Standardisation of growth regulators

The fully developed shoots were cut into single nodal segments, the shoot buds were separated with a part of the explant intact and these were cultured into fresh MS media, with 0.7 per cent agar and 3 per cent sucrose and supplemented with any of the following growth regulators for further proliferation.

1. BA 0.25 mg l^{-1}
2. BA 0.5 mg l^{-1}
3. BA 1.0 mg l^{-1} (full and half strength)
4. BA 2.5 mg l^{-1}
5. BA 5.0 mg l^{-1}

6. BA 10.0 mg l^{-1}
7. BA 20.0 mg l^{-1}
8. KIN 0.25 mg l^{-1}
9. KIN 0.5 mg l^{-1}
10. KIN 1.0 mg l^{-1} (full and half strength MS)
11. KIN 2.0 mg l^{-1}
12. KIN 2.5 mg l^{-1}
13. KIN 5.0 mg l^{-1}
14. 2iP 0.25 mg l^{-1}
15. 2iP 0.5 mg l^{-1}
16. 2iP 1.0 mg l^{-1}
17. 2iP 2.5 mg l^{-1}
18. 2iP 5.0 mg l^{-1}
19. 2iP 10.0 mg l^{-1}
20. NAA 1.0 mg l^{-1}
21. BA 0.25 + IAA 0.5 mg l^{-1}
22. BA 0.5 + IAA 1.0 mg l^{-1}
23. BA 0.5 + IAA 2.0 mg l^{-1}
24. BA 1.0 + IAA 1.0 mg l^{-1}
25. BA 2.6 + IAA 1.0 mg l^{-1}
26. BA 0.25 + NAA 0.5 mg l^{-1}
27. BA 0.5 + NAA 1.0 mg l^{-1}
28. BA 0.5 + NAA 2.0 mg l^{-1}
29. KIN 1.0 + IAA 1.0 mg l^{-1}
30. KIN 2.5 + IAA 1.0 mg l^{-1}

31. Solid Basal MS (full or half strength)

32. Liquid Basal MS

There were nine replications per treatment. Observations on the number of shoots and buds produced per culture, length of the longest shoot, number of nodes per culture and the weight of the callus (fresh) were recorded three weeks after transfer.

3.2.4.2 Standardisation of media

Following media were tried to standardise the best one for proliferation of cultures.

1. Full strength solid MS basal
2. Half strength solid MS basal
3. Full strength stationary liquid MS
4. WPM basal (full strength)

To find out the effect of physical nature of media on proliferation of cultures, the following treatments were tried

1. Solid MS basal
2. Stationary liquid basal MS media
3. Solid MS supplemented with BA 1 mg l^{-1} and silver nitrate (5 mg l^{-1})
4. Stationary liquid MS ,,

To find out the effect of reducing the salt concentration of MS media to half, the following treatments were tried.

1. Full and half strength MS medium
2. ,, supplemented with activated charcoal (0.1%)
3. ,, ,, BA 1.0 mg l^{-1}
4. ,, ,, KIN 1.0 mg l^{-1}
5. ,, ,, adenine sulphate 2 mg l^{-1})

Observations as mentioned in the earlier experiment (3.2.4.1) were recorded for these also.

3.2.4.3 Effect of additives

The effect of the following additives on proliferation of cultures were observed.

1. Activated charcoal 0.1% (to half and full strength of MS)
2. Silver nitrate (5 mg l^{-1}) supplemented with BA 1 mg l^{-1} in solid or liquid media
3. Coconut water (15%)
4. Adenine sulphate 2 mgl^{-1} (in full and half strength liquid MS)
5. Solid and liquid MS basal
6. Half strength MS basal

Observations were recorded after four weeks. Number of shoots, buds and nodes per culture, length of the shoot and weight of the callus (fresh) were observed.

3.2.4.4 Effect of growth regulators and additives on each subculture

Effect of different growth regulators and additives on first, second, third, fourth and fifth subculture were studied to find out the best for proliferation of cultures in each subculture. BA, KIN, 2iP at concentrations ranging from 0.25 to 10 mg l^{-1} and combinations with certain auxins were used. Observations like

number of shoots, buds, nodes and length of the shoot were observed. In addition, maximum number of nodes that could be obtained at the end of each subculture were also determined out so that the medium which gives the maximum multiplication rate could be standardised.

3.2.5 Induction of rooting

3.2.5.1 *In vitro* rooting

The individual shoots regenerated and having attained 4-9 cm length were separated and put in different rooting treatments.

The percentage of root initiation, days to root initiation, number and length of the root and nature of roots were recorded among the different cultures subjected to different auxin treatments. The treatments were as follows:

1. Full MS + NAA 0.25 mg l^{-1}
2. Full MS + NAA 0.5 mg l^{-1}
3. Full MS + NAA 1.0 mg l^{-1}
4. Full MS + IBA 0.25 mgl^{-1}
5. Full MS + IBA 0.5 mg l^{-1}
6. Full MS + IBA 1.0 mg l^{-1}
7. MS basal
8. MS + activated charcoal
9. Half strength MS + charcoal
10. IBA 1000 mg l^{-1} plus treatment → inoculation in MS basal/MS + charcoal

The influence of physical nature of media was also determined.

There were 10 replications per treatment.

3.2.5.2 *Ex vitro* rooting

The shoots formed in the proliferation stage were used for the study. Shoots of size 12-14 cm were treated with the following concentrations of growth regulators.

1. IBA 1000 mg l^{-1}
2. IBA 500 mg l^{-1}
3. NAA 1000 mg l^{-1}
4. NAA 500 mg l^{-1}
5. Growroot
6. Control

The growth regulator solutions were prepared in ethanol. The shoots were dipped in the solution for a period of 60 s and then planted in plastic pots filled with different media like sand, potting mixture or vermiculite. For treatment with Grow root powder, the shoots were first dipped in distilled water and then in the powder before planting in different media. The pots were then placed in the mist chamber. In another trial in order to standardise the type of containers and the potting mixes to be used, the following potting mixes and containers were used for *ex vitro* rooting of shoots produced *in vitro* after giving a pretreatment with 1000 mg l^{-1} IBA for 60 seconds.

Potting mixes	Container
1. Sand	1. Plastic pot (7.0 cm diameter)
2. Potting mixture	2. Mud pot (7.0 cm diameter)
3. Vermiculite	3. Polybag (11.0 x 8.5 cm)
4. Vermicompost	4. Pot tray (with 64 wells)
5. Soilrite*	
6. Cocofibe**	

* Soilrite is a mixture of peat moss, vermiculite and perlite marketed by M/s. KEL Perlite, Bangalore

** Cocofibe is a proprietory compound containing decomposed peat and coir dust produced by M/s.Langalee Ltd., Bangalore

Observations on the days for rooting, number of roots, length of longest root and thickness of thickest root were made 30 days and 60 days after rooting. There were three replications per treatment.

3.2.6 Hardening

Fungicidal drenching with 0.1 per cent Bavistin were given two days after transplanting and subsequently at fortnightly intervals. Spraying was also done. The plantlets were watered on alternate days with tap water. A nutrient solution was prepared by dissolving 5 g l^{-1} of a fertilizer (N:P:K - 10:5:20) mixture and applied once a week to the plantlets, at the rate of 10 ml per plantlet in mudpots, plastic pots and polybags and 3 ml per plantlet in protrays.

3.2.7 Indirect morphogenesis

3.2.7.1 Induction of callus

3.2.7.1.1 Standardisation of explants

Explants such as leaf segments, stem segments, petiole segments and young leaves of mature vines were used for callus induction.

3.2.7.1.2 Standardisation of growth regulators

3.2.7.1.2.1 *In vitro* explants

The following are the different concentrations of growth regulators tried for callus induction.

1. 2,4-D 0.5 mg l^{-1}
2. 2,4-D 1.0 mg l^{-1}
3. 2,4-D 2.5 mg l^{-1}
4. 2,4-D 5.0 mg l^{-1}
5. NAA 0.5 mg l^{-1}
6. NAA 1.0 mg l^{-1}
7. NAA 2.5 mg l^{-1}
8. NAA 5.0 mg l^{-1}

Observations like percentage of cultures initiating callus, number of days taken for callus initiation, callus index at 7th and 20th day after inoculation and morphology of callus were recorded. Callus index (CI) was worked out as CI = P x G where P is the percentage of callus initiation, G is the growth score. Scoring was made based on the spread of the calli and a maximum score of four was given for

those that have occupied the whole surface of the media within three weeks of culture period in culture tubes.

3.2.7.1.2.2 *In vitro* explants

In another experiment with explants taken directly from mother stock (in the glasshouse), the following growth regulators were used to find the effect on morphogenesis and callus initiation. Cultures were exposed to light.

1. 2,4-D 0.5 mg l^{-1}
2. 2,4-D 1.0 mg l^{-1}
3. NAA 1.0 mg l^{-1}
4. 2iP 0.5 mg l^{-1}
5. 2iP 2.0 mg l^{-1}
6. BA 2.0 mg l^{-1}
7. BA 4.0 mg l^{-1}
8. BA 10.0 mg l^{-1}

Observations as recorded earlier (3.2.7.1.2.1) were taken.

Thus, different data on effect of auxins, effect of explants, effect of cytokinins, effect of *in vitro* and glasshouse explants, effect of light and effect of orientation of explants were obtained.

3.2.7.2 Callus proliferation and regeneration (embryogenesis/organogenesis)

3.2.7.2.1 Solid medium

The relative performance of different explants for callus induction and proliferati was observed for those proliferated in solid medium. Subculturing was done at 25

days interval each time inoculating uniform bits of callus (0.01 g) to the fresh medium. Following concentrations of auxins were tried for first subculture.

2,4-D/NAA

0.5 mg l^{-1}

1.0 mg l^{-1}

2.5 mg l^{-1}

5.0 mg l^{-1}

For the second subculture only 1.0 mg l^{-1} of 2,4-D or NAA were tried.

3.2.7.2.1.1 Embryogenesis

The treatments discussed above were the same for embryogenesis studies also. Observations on number of embryos produced and the stage of embryo were taken. The effect of growth regulators in callus induction medium, effect of explants and the effect of position of the explants on further embryogenesis was also studied.

3.2.7.2.1.2 Organogenesis

Uniform bits of calli (0.02 g) were subcultured into media containing different growth regulators to initiate organoids. Following were the growth regulators tried

1. BA 2.5 mg l^{-1}
2. BA 5.0 mg l^{-1}
3. BA 10.0 mg l^{-1}
4. BA 20.0 mg l^{-1}
5. KIN 0.25 mg l^{-1}

6. KIN 0.5 mg l^{-1}
7. KIN 2.5 mg l^{-1}
8. KIN 5.0 mg l^{-1}
9. 2iP 0.25 mg l^{-1}
10. 2iP 0.5 mg l^{-1}
11. 2iP 1.0 mg l^{-1}
12. 2iP 2.5 mg l^{-1}
13. BA 0.5 + IAA 1 mg l^{-1}
14. BA 0.5 + IAA 2.0 mg l^{-1}
15. BA 1.0 + IAA 1.0 mg l^{-1}
16. BA 0.25 + NAA 0.5 mg l^{-1}
17. BA 0.5 + NAA 1.0 mg l^{-1}
18. BA 0.5 + NAA 2.0 mg l^{-1}
19. Half strength MS + KIN 1 mg l^{-1}
20. TDZ 0.05 mg l^{-1}

Observations on callus index at 20th and 30th day, percentage establishment of cultures, morphology of callus and number of organoids (if any) were made.

3.2.7.2.2 Liquid medium

Calli was also proliferated in liquid medium as suspension culture in conical flasks and transferred to orbital shaker, maintained at a speed of 80-90 rpm and with the same culture conditions as in culture room. The conical flasks of 100 ml capacity were filled with 30 ml liquid media and the cultures were agitated continuously. These were subcultured after three weeks.

3.2.7.2.2.1 Subculture

The batch cultures were taken from the orbital shaker. The supernatant medium was poured off to a sterilized beaker or flask taking care to see that the embryoids and cells were retained in the original culture flask and 20-25 ml of the fresh medium was added to the flask and replaced in the shaker.

3.2.7.2.2.2 Determination of packed cell volume (PCV)

The suspension culture was shaken thoroughly, 5 ml of the culture was pipetted out to a graduated centrifuge tube. The samples were placed into the receiver of the centrifuge and spun at 200 x G for 5 min. The volume of the sedimented mass was recorded. The results were expressed as ml pellet per ml culture (Bhojwani and Razdan, 1983).

3.2.7.2.2.3 Proliferation and regeneration (organogenesis/embryogenesis)

The growth regulators tried for callus proliferation and embryogenesis/organogenesis in liquid MS medium were as given below:

1. 2,4-D 0.5 mg l^{-1}
2. 2,4-D 5.0 mg l^{-1}
3. 2,4-D 0.5 + BA 5.0 mg l^{-1}
4. 2,4-D 5.0 + BA 5.0 mg l^{-1}
5. 2,4-D 5.0 + BA 0.5 mg l^{-1}
6. MS basel

The embryoids were kept for germination in MS basal medium.

3.2.8 Direct morphogenesis

Direct organogenesis was tried by transferring different axenic explants like leaf and stem bits into MS media supplemented with cytokinins like BA, KIN and TDZ at different concentrations.

3.2.9 Encapsulation studies

The shoots formed in MS medium supplemented with growth regulators were cut into nodal segments and used for encapsulation studies in order to form beads which could be stored without loss of the ability to differentiate.

3.2.9.1 Encapsulation in alginate

Gels of sodium alginate (Sigma) were prepared in MS basal salt solution at concentrations of 2.0, 2.5, 3.0 per cent. The shoots were cut into tiny nodal segments and mixed thoroughly with the alginate gel. Calcium chloride solutions were prepared at concentrations of 50, 75 and 100 mM. Sterilised glass tubes of 3 mm diameter were used to pick up the nodal segments along with the sodium alginate solution. Sodium alginate drops were allowed to fall into $CaCl_2$ solution at the rate of 30-40 drops per minute. The formation for round firm beads were noted. The resultant beads (0.5 to 0.7 cm in diameter) were left in the $CaCl_2$ solutions for 30 min on a rotary shaker (80 rpm for complexation) beads were removed, washed three times with sterile distilled water and put in regeneration media or subjected to storage studies.

3.2.9.2 Storage and plantlet conversion of alginate beads

The alginate beads formed were put into 100 ml flasks on cotton wool, moistened with MS basal salts in solid MS basal medium and stored at room temperature at 26 ± 1 °C and at 4 °C. The beads were removed at intervals of 7 days and put on MS regeneration medium to find out the period for which the beads could be stored without losing the regeneration capacity.

3.2.10 Biochemical studies

3.2.10.1 Plant and tissue material

Fresh samples of *in vitro* leaves and roots and *in vivo* leaves and roots were collected and used for the biochemical studies.

3.2.10.2 Enzyme extraction

3.2.10.2.1 Assay of peroxidase activity

The samples (0.5 g) were homogenized in 2 ml of ice cold 0.1 M Tris hydrochloride (pH 7.6) centrifuged at a temperature below 4 °C at a speed of 15000 rpm for 20 min and the supernatant was used for the assay of peroxidase. Gel concentration of 7.5 per cent acrylamide was found best for the peroxidase enzyme separation.

3.2.10.3 Polyacrylamide gel electrophoresis (PAGE)

3.2.10.3.1 Preparation of the gel

The following stock solutions were prepared first.

Solution A

Tris 36.6 g

TEMED 0.23 ml

1N HCl 48 ml

Volume made upto 100 ml in distilled water

pH 8.9

Solution B

Acrylamide 28.0 g

N'N' methyline bis acrylamide 0.735 g

Volume made upto 100 ml with distilled water

Solution C

Ammonium per sulphate - 0.14 g

Volume made upto 125 ml with distilled water

Solution D

Acrylamide 18.0 g

Bisacrylamide 0.47 g

Made up the volume to 100 ml with distilled water

Working solution

Working solution was prepared by mixing stock solutions A, B and C in the following quantities to get required gel concentrations.

	7.5% acrylamide	8.5% acrylamide
A (ml)	2	2
B (ml)	4.285	4.860
C (ml)	9.715	9.150

Preparation of stacking gel

Stacking gel contained solution A (2 ml), solution C (10 ml) and solution D (4 ml).

Preparation of electrode buffer

Stock solution

Tris - 6 g

Glycine - 28.8 g

Volume was made upto one litre with distilled water keeping the pH at 8.3. The stock buffer was diluted with sterile distilled water (1:9) before use.

3.2.10.4 Electrophoresis

The working solution was gently poured in between the glass plates kept in polymerisation stand. Polymerisation was achieved within one hour. Stacking gel was poured to a width of 1-1.5 cm for better resolution of bands. After polymerisation, the gels along with glass plates were removed to electrophoretic apparatus. The upper and lower trays were filled with electrode buffer and each was connected to cathode and anode respectively. Electrophoresis was carried out at 5°C. A constant current of 25 mA per slab was maintained throughout the run.

3.2.10.5 Staining of gels for peroxidase enzyme

Fresh stain was prepared by mixing 0.2 M Acetate buffer (pH 5.6) with benzidine (0.1 g), heated to boil, cooled, filtered and then hydrogen peroxide was added to the mixture. The gels were immersed in staining solution for about one hour and destained in 7 per cent acetic acid.

3.2.11 Statistical analysis

The data generated from the various experiments were subjected to statistical analysis as per Panse and Sukhatme (1985). The experiments were of completely randomised design and factorial design. The results were analysed by analysis of variance. Angular as well as $\sqrt{x}$ transformation were made wherever necessary before analysis.

Results

RESULTS

The results of the studies on standardisation of *in vitro* propagation techniques in *Holostemma annulare* K. Schum conducted during 1993-'95 at the Plant Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara are presented in this chapter.

4.1 Standardisation of surface sterilization of explants

4.1.1 Surface sterilization of explants taken from glasshouse

The results of the trial for surface sterilization of 2-3 month old explants taken from glasshouse are presented in Table 4a.

Among the different treatments tried, the least contamination rate (0%) was observed in cultures receiving treatments T_7 (0.1% mercuric chloride for 5 min), T_8 (0.1% mercuric chloride for 10 min) and T_{19} (chlorine water for 7 min). However, only the first two treatments showed better growth rate (90%). Treatments involving chlorine water or alcohol resulted in high percentage of bleached cultures. Most of the bleached cultures did not show further growth and resulted in drying up of cultures and the percentage of establishment was only 60 per cent. Explants when cultured in appropriate media but without any sterilization treatment resulted in cent per cent contamination.

Among the various fungicide/antibiotic and mercuric chloride combinations tried for sterilization of 10-14 month old glasshouse explants, treatment T_2 (0.1% Bavistin for 30 min followed by 1.0 per cent Cetrimide for 10 min followed by 0.1 per cent mercuric chloride for 15 min) was the best and gave

Table 4a. Effect of surface sterilants on the establishment of explants*** of *H. annulare* (2-3 month old from glasshouse)

Treatment No.	Treatments			Observations**					
	Sterilant	Concentration (%)	Duration	Contamination (%)	Bacterial contamination(%)	Fungal contamination (%)	Establishment (%)	Cultures bleached (%)	**** Cultures showing growth (%)
1	Alcohol	50	30 s	77.770 (1.086)	61.110 (0.898)	16.660 (0.420)	22.220 (0.491)	0.000 (0.226)	27.700 (0.554)
2	Alcohol	50	60 s	66.660 (0.955)	66.660 (0.955)	1.783 (0.102)	32.300 (0.604)	0.000 (0.226)	38.880 (0.673)
3	Alcohol	100	30 s	75.000 (1.048)	70.000 (0.991)	5.000 (0.235)	25.000 (0.524)	0.000 (0.226)	10.000 (0.321)
4	Alcohol	100	60 s	55.000 (0.836)	30.000).580)	20.000).462)	40.000 (0.685)	0.000 (0.226)	20.000 (0.462)
5	Mercuric chloride	0.05	5 min	11.517 (0.346)	3.367 (0.161)	10.000 (0.321)	80.000 (1.108)	0.000 (0.226)	80.000 (1.116)
6	Mercuric chloride	0.05	10 min	12.500 (0.361)	10.000 (0.320)	2.500 (0.158)	83.850 (1.158)	0.000 (0.226)	80.170 (1.112)
7	Mercuric chloride	0.1	5 min	0.000 (0.226)	0.000 (0.226)	0.000 (0.226)	100.00 (1.406)	0.000 (0.226)	89.770 (1.246)
8	Mercuric chloride	0.1	10 min	0.000 (0.226)	0.000 (0.226	0.000 (0.226)	100.00 (1.406)	0.000 (0.226)	90.167 (1.253)
9	Alcohol + Mercuric chloride	100 0.05	30 s 5 min	30.00 (0.579)	12.00 (0.354)	18.00 (0.438)	60.000 (0.886)	28.000 (0.557)	50.000 (0.785)
10	Alcohol + Mercuric chloride	100 0.05	30 s 10 min	12.500 (0.360)	0.000 (0.226)	12.500 (0.361)	70.000 (0.991)	25.000 (0.524)	69.233 (0.984)
11	Alcohol + Mercuric chloride	100 0.1	30 s 5 min	10.000 (0.320)	10.000 (0.320)	0.000 (0.226)	90.000 (1.251)	30.000 (0.578)	80.250 (1.110)
12	Alcohol + Mercuric chloride	100 0.1	30 s 10 min	15.000 (0.397)	10.000 (0.320)	0.000 (0.226)	85.000 (1.174)	30.000 (0.579)	33.000 (0.612)
13	Alcohol + Mercuric chloride	100 0.05	60 s 5 min	50.000 (0.785)	13.330 (0.374)	36.670 (0.650)	50.000 (0.785)	30.000 (0.579)	30.000 (0.580)
14	Alcohol + Mercuric chloride	100 0.05	60 s 10 min	40.000 (0.685)	27.200 (0.549)	12.800 (0.366)	60.000 (0.886)	36.360 (0.647)	40.000 (0.685)

Table 4a. Continued

1	2	3	4	5	6	7	8	9	10
15	Alcohol + Mercuric chloride	100 0.1	60 s 5 min	20.000 (0.462)	20.000 (0.463)	1.867 (0.109)	40.000 (0.684)	30.000 (0.579)	20.000 (0.462)
16	Alcohol + Mercuric chloride	100 0.1	60 s 10 min	20.000 (0.463)	1.783 (0.102)	20.000 (0.463)	50.000 (0.785)	44.440 (0.730)	50.230 (0.788)
17	Alcohol + Mercuric chloride	100 0.1	60 s 13 min	10.000 (0.320)	10.000 (0.322)	3.500 (0.174)	30.000 (0.579)	50.267 (0.788)	30.167 (0.581)
18	Chlorine water		5 min	25.00 (0.523)	8.300 (0.292)	16.700 (0.421)	50.000 (0.785)	66.660 (0.955)	50.000 (0.785)
19	Chlorine water		7 min	0.000 (0.226)	0.000 (0.226)	0.000 (0.226)	60.000 (0.890)	77.770 (1.080)	63.000 (0.918)
20	Control (No sterilant)			100.00 (1.345)	5.000 (0.236)	95.000 (1.351)	0.000 (0.226)	0.000 (0.226)	0.000 (0.226)
	CD			0.052	0.074	0.074	0.090	0.052	0.074
	SE±			0.018	0.026	0.026	0.031	0.018	0.026

* Values in paranthesis represent arc sine transformed ones
** Observations recorded three weeks after inoculation
*** Explants include nodal segments, shoot tips and leaf segments
**** Also include those cultures showing growth but contaminated

Table 4b. Effect of surface sterilants on the establishment of explants*** of *H. annulare* (10-14 month old from glasshouse)

Treatment No.	Treatments: Sterilants	Concentration (%)	Duration	Observations**: Contamination (%)	Bacterial contamination (%)	Fungal contamination (%)	Establishment (%)	***** Cultures showing growth (%)
1	Bavistin + Cetrimide + Mercuric chloride	0.1 1.0 0.1	20 min 5 min 14 min	70.180 (0.994)*	56.140 (0.847)	14.040 (0.384)	29.820 (0.578)	49.120 (0.777)
2	Bavistin + Cetrimide + Mercuric chloride	0.1 1.0 0.1	30 min 10 min 15 min	50.000 (0.785)	39.657 (0.681)	10.347 (0.326)	50.000 (0.785)	32.750 (0.609)
3	Emisan + Cetrimide + Alcohol wipe + Alcohol + Mercuric chloride	0.1 1.0 70 100 0.1	30 min 10 min 30 s 12 min	70.000 (0.991)	30.000 (0.579)	40.000 (0.685)	30.000 (0.580)	20.000 (0.463)
4	Bavistin + Mercuric chloride ****	0.1 0.1	55 min 15 min	77.780 (1.080)	25.930 (0.534)	51.950 (0.805)	22.220 (0.491)	22.220 (0.491)
5	Control (No sterilant)			100.00 (1.345)	25.000 (0.524)	95.467 (1.331)	0.000 (0.226)	0.000 (0.226)
	CD			0.058	0.000	0.115	0.000	0.058
	SEm±			0.018	0.000	0.037	0.000	0.018

* Values in paranthesis represent arc sine transformed ones
** Observations recorded three weeks after inoculation
*** Explants include nodal segments, shoot tips and leaf segments
**** Cultures were inoculated in MS medium incorporated with 0.1% streptomycin
*****Also include those cultures showing growth but contaminated

50 per cent establishment of cultures (Table 4b). The extent of bacterial contamination (39.66%) was much higher than fungal contamination (10%). Explants when inoculated in a medium containing Streptomycin (T_4), the percentage of bacterial contamination decreased (25.93%) but at the same time establishment percentage also showed a decline. None of the cultures were bleached.

4.1.2 Surface sterilization of explants taken from the field

The data on the effect of surface sterilization of explants taken from the field are presented in Table 4c. Among the various treatments tried, T_7 Emisan 0.1% for 30 min → Cetrimide 1.0% for 10 min → Alcohol 70% wipe → dip in absolute alcohol 100% for 60 s → mercuric chloride 0.1% for 13 min, T_8 Bavistin 0.1% for 30 min → Cetrimide 1.0% for 10 min → mercuric chloride 0.1% for 20 min), T_9 Bavistin 0.1% for 30 min → Cetrimide 1.0% for 10 min → Norfloxacin 0.1% for 30 min → Alcohol 70% wipe → mercuric chloride 0.1% for 13 min etc. were on par with respect to the establishment per cent 48-50%. However, treatment T_9 gave more cultures showing growth 76.7%. It was also observed that the percentage establishment of field explants was less compared to those taken from the glasshouse. A series of sterilization treatments were needed for establishment of explants taken from the field.

4.1.3 Seasonal variation in the rate of establishment of explants

The data on seasonal influence on culture establishment and sustained growth of explants is presented in Table 5a and Fig.1. When the sterilization treatment with 0.1 per cent mercuric chloride for 5 min was repeated at monthly intervals it was found that better establishment was obtained during the period from

Table 4c. Effect of surface sterilants on the establishment of explants*** of *H. annulare* (explants from field)

Treatment No.	Treatment: Sterilants	Concentration (%)	Duration	Observations**: Contamination (%)	Bacterial contamination (%)	Fungal contamination (%)	Establishment (%)	**** Cultures showing growth (%)
1	Cetrimide	1.0	5 min	86.500 (1.195)*	35.300 (0.636)	54.533 (0.831)	13.500 (0.374)	21.180 (0.478)
2	Cetrimide + Alcohol	1.0 100	5 min 60 s	86.960 (1.201)	37.690 (0.661)	50.000 (0.785)	13.040 (0.369)	10.140 (0.324)
3	Bavistin + Cetrimide	0.1 1.0	30 min 5 min	81.250 (1.123)	12.500 (0.361)	68.750 (0.978)	18.750 (0.447)	43.750 (0.723)
4	Bavistin + Cetrimide + Alcohol + Mercuric chloride	0.1 1.0 100 0.1	5 min 20 min 60 s 14 min	80.960 (1.119)	14.300 (0.388)	66.667 (0.956)	19.040 (0.452)	33.330 (0.615)
5	Bavistin + Cetrimide + Alcohol wipe + Mercuric chloride + Norfloxacin	0.1 1.0 70 0.1 0.1	40 min 20 min 13 min 30 min	69.700 (0.989)	35.700 (0.640)	34.000 (0.622)	30.300 (0.582)	16.070 (0.412)
6	Emisan + Cetrimide + Norfloxacin + Alcohol wipe + Mercuric chloride	0.1 1.0 0.1 70 0.1	30 min 30 min 30 min 13 min	67.600 (0.966)	18.960 (0.451)	48.640 (0.772)	32.400 (0.605)	27.020 (0.546)
7	Emisan + Cetrimide + Alcohol wipe + Alcohol + Mercuric chloride	0.1 1.0 70 100 0.1	30 min 10 min 30 s 13 min	52.077 (0.806)	22.817 (0.496)	49.270 (0.778)	49.600 (0.781)	64.000 (0.927)
8	Bavistin + Cetrimide + Mercuric chloride	0.1 1.0 0.1	30 min 10 min 20 min	55.433 (0.840)	22.667 (0.494)	30.000 (0.579)	46.667 (0.752)	52.767 (0.813)
9	Bavistin + Cetrimide + Norfloxacin + Alcohol wipe + Mercuric chloride	0.1 1.0 0.1 70 0.1	30 min 10 min 30 min 13 min	52.213 (0.808)	25.500 (0.529)	33.380 (0.616)	47.777 (0.763)	76.660 (1.067)

Contd.

Table 4c. Continued

1	2	3	4	5	6	7	8	9
10	Mercuric chloride	0.1	5 min	91.250 (1.291)	32.500 (0.606)	58.750 (0.873)	8.750 (0.300)	15.760 (0.408)
11	Mercuric chloride	0.1	10 min	88.683 (1.233)	27.850 (0.566)	60.833 (0.895)	12.983 (0.368)	13.800 (0.381)
12	Control (No sterilant)			95.000 (1.345)	87.200 (1.232)	96.600 (1.406)	5.000 (0.226)	3.133 (0.173)
	CD			0.107	0.107	0.075	0.075	0.000
	SEm±			0.037	0.037	0.026	0.026	0.000

* Values in paranthesis represent arc sine transformed ones
** Observations recorded three weeks after inoculation
*** Explants include nodal segment, shoot tips and leaf segments
**** Also include those cultures showing growth but contaminated

Table 5a. Effect of season of collection and age on establishment of *H. annulare* explants***

Treatment No.	Treatment		**Establishment
	Month	Age of the explants (months)	
1	January	2	96.600 (1.410)*
2	February	3	98.000 (1.434)
3	March	4	70.000 (0.991)
4	April	5	86.360 (1.193)
5	May	6	62.900 (0.916)
6	June	7	68.000 (0.970)
7	July	8	30.000 (0.580)
8	August	9	27.000 (0.546)
9	September	10	29.000 (0.567)
10	October	11	20.000 (0.455)
11	November	12	15.500 (0.405)
12	December	13	15.017 (0.398)
CD			0.092
SEm ±			0.032

* Values in paranthesis represent arc sine transformed ones
** Observations recorded three weeks after inoculation
*** Explants include nodal segment, shoot tip and leaf segment

Fig.1. Effect of season of collection and age on the establishment of *H. annulare* explants

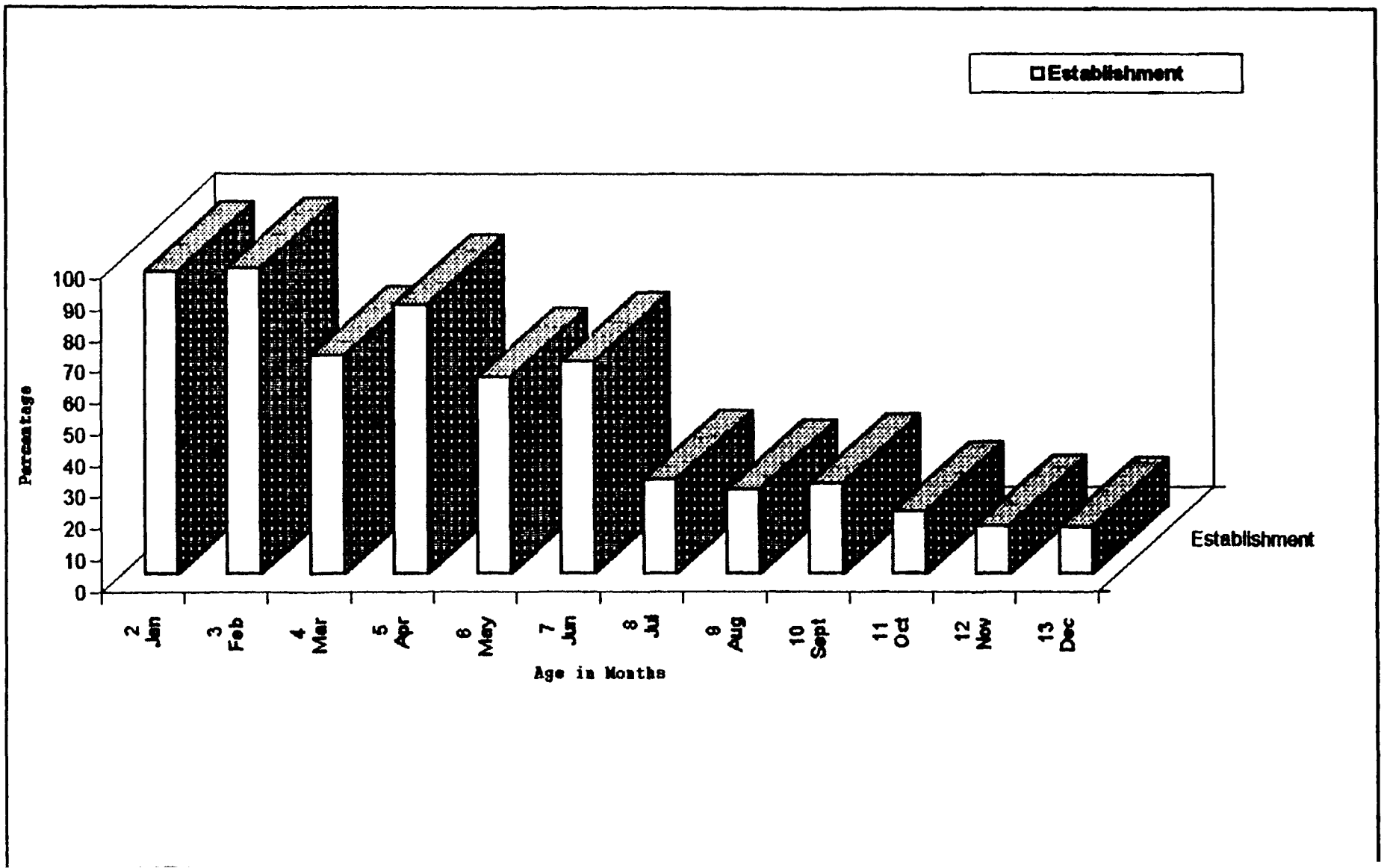

Table 5b. Effect of combinations of surface sterilant treatments in different seasons on explants*** of *H. annulare*

Treatment No.	Treatment	Concentration (%)	Duration	Month ****	Establishment (%) **
1	Bavistin +	0.1	40 min	May	46.500
	Cetrimide +	1.0	10 min		(0.750)*
	Mercuric chloride	0.1	30 min		
2	Bavistin +	0.1	30 min	June	61.543
	Norfloxacin +	0.1	30 min		(0.902)
	Alcohol wipe +	70			
	Mercuric chloride	0.1	13 min		
3	Bavistin +	0.1	30 min	July	46.637
	Norfloxacin +	0.1	30 min		(0.752)
	Alcohol wipe +	70			
	Mercuric chloride	0.1	13 min		
4	Emisan	0.1	30 min	August	50.000
	Cetrimide	1.0	10 min		(0.785)
	Alcohol wipe	70			
	Alcohol	100	30 s		
	Mercuric chloride	0.1	12 min		
5	Emisan	0.1	30 min	September	52.300
	Cetrimide	1.0	10 min		(0.808)
	Alcohol wipe	70			
	Alcohol	100	30 s		
	Mercuric chloride	0.1	12 min		
6	Bavistin	0.1	30 min	October	30.120
	Cetrimide	1.0	10 min		(0.581)
	Mercuric chloride	0.1	30 min		
CD					0.097
SEm±					0.045

* Values in paranthesis represent arc sine transformed ones
** Observations recorded three weeks after inoculation
*** Explants include nodal segments, shoot tips and leaf segments
**** Experiments done in the year 1995

January to April maximum in February (98%) followed by January (96.6%) while the rate of contamination was higher during the other months of the year. Maturity of the explants also had an influence on the establishment rate, better establishment rate being obtained for younger explants. A set of combination treatments was also tried with mature explants in the rainy months and T_2 Bavistin 0.1% for 30 min → Norfloxacin 0.1% for 30 min → alcohol 70% wipe → mercuric chloride 0.1% for 13 min when tried in sequence gave better establishment rates (Table 5b).

All the sterilization treatments tried for nodal segment, shoot tip and leaf segments were tried for sterilization of roots, but none of the cultures were free of contamination. Two per cent of the cultures initiated 6-7 shoot buds from root explants but were contaminated and did not grow further.

4.2 Establishment of explants

4.2.1 Establishment and growth of nodal segments

4.2.1.1 Standardisation of growth regulators

The data on the effect of growth regulators such as cytokinins and auxins and their interaction on the establishment and growth of nodal segment explants are presented in Tables 6a and 6b, Fig.2 and Plates 1 and 2. Maximum establishment (98%) was observed with treatment T_{11} (KIN 1.0 mg l^{-1}). However T_{21} (2iP 4.0 mg l^{-1}) and T_{25} [combination of BA (0.25 mg l^{-1}) and NAA (0.5 mg l^{-1})] were on par (96%) with T_{11}. Subsequent growth of cultures was better in the establishment medium supplemented with BA. The number of days for the first bud to burst was minimum when BA was used at concentrations ranging from 0.25 to 4.0 mg l^{-1}. However longer shoots (17 cm), more multiple shoot buds (4.5), more axillary branches (2.5) and more number of nodes (11.0) were obtained in MS medium

Table 6a. Effect of different growth regulators on establishment of nodal segments of *H. annulare*

Treatment No.	Treatment			Establishment (%)
	Media	Growth regulator ($mg\ l^{-1}$)	Concentration (%)	
1	2	3	4	5
1	MS	BA	0.25	90.000* (1.258)
2	MS	BA	0.5	80.000 (1.109)
3	MS	BA	1.0	90.333 (1.273)
4	MS	BA	2.0	59.233 (0.880)
5	MS	BA	2.5	50.667 (0.792)
6	MS	BA	4.0	72.667 (1.027)
7	MS	BA	5.0	90.667 (1.278)
8	MS	BA	10.0	80.333 (1.136)
9	MS	KIN	0.25	50.667 (0.792)
10	MS	KIN	0.5	81.000 (1.121)
11	MS	KIN	1.0	98.000 (1.432)
12	MS	KIN	2.0	91.333 (1.290)

Contd.

Table 6a. Continued

1	2	3	4	5
13	MS	KIN	2.5	92.667 (1.300)
14	MS	KIN	4.0	90.400 (1.275)
15	MS	KIN	5.0	91.333 (1.281)
16	MS	2iP	0.25	61.666 (0.903)
17	MS	2iP	0.5	91.667 (1.293)
18	MS	2iP	1.0	82.667 (1.142)
19	MS	2iP	2.0	65.000 (0.938)
20	MS	2iP	2.5	63.000 (0.917)
21	MS	2iP	4.0	96.000 (1.373)
22	MS	2iP	5.0	80.667 (1.116)
23	½MS	BA	1.0	86.200 (1.192)
24	½MS	KIN	1.0	82.800 (1.149)
25	MS	BA + NAA	0.25 + 0.5	96.667 (1.390)
26	MS	BA + NAA	0.25 + 1.0	82.000 (1.133)

Contd.

Table 6a. Continued

1	2	3	4	5
27	MS	BA + IAA	0.25 + 0.5	81.333 (1.129)
28	MS	BA + IAA	0.25 + 1.0	70.667 (0.999)
29	MS	BA + IAA	1.0 + 1.0	85.000 (1.196)
30	MS with streptomycin (0.1%)	BA	0.5	81.333 (1.125)
31	MS	(Control)		83.667 (1.158)
32	½ MS	(Control)		79.700 (1.106)
CD				0.146
SEm±				0.052

* Values in paranthesis represent arc sine transformed ones

Table 6b. Effect of different growth regulators on growth of nodal segments of *H. annulare*

Treatment No.	Treatment: Media	Treatment: Cytokinin	Treatment: Concentration (mg l^{-1})	Days to bud release	No. of shoots	No. of shoot buds	Length of the longest shoot (cm)	Average length of a shoot (cm)	No. of nodes	Weight of callus (g)
1	2	3	4	5	6	7	8	9	10	11
1	MS	BA	0.25	3.667 (1.911)*	1.797 (1.325)	0.667 (0.998)	10.283 (3.159)	4.650 (2.155)	5.667 (2.373)	2.450 (1.552)
2	MS	BA	0.5	3.000 (1.732)	1.933 (1.378)	3.267 (1.883)	12.507 (3.528)	6.233 (2.468)	7.667 (2.768)	0.930 (0.960)
3	MS	BA	1.0	3.333 (1.821)	2.453 (1.563)	4.500 (2.234)	17.020 (4.125)	6.733 (2.595)	10.600 (3.253)	2.100 (1.446)
4	MS	BA	2.0	3.667 (1.900)	1.833 (1.343)	5.667 (2.461)	4.400 (2.075)	2.100 (1.445)	5.333 (2.308)	0.750 (0.866)
5	MS	BA	2.5	4.000 (1.989)	1.907 (1.374)	3.000 (1.858)	11.917 (3.446)	5.050 (2.233)	7.000 (2.641)	1.450 (1.203)
6	MS	BA	4.0	3.667 (1.911)	1.117 (1.056)	0.667 (1.052)	6.000 (2.444)	2.533 (1.591)	4.333 (2.079)	0.633 (0.793)
7	MS	BA	5.0	4.333 (2.079)	2.167 (1.470)	4.000 (2.112)	6.183 (2.330)	2.167 (1.445)	4.332 (2.076)	1.901 (1.378)
8	MS	BA	10.0	6.333 (2.515)	1.077 (1.037)	5.000 (2.339)	3,250 (1.787)	0.750 (0.858)	3.000 (1.715)	2.250 (1.488)
9	MS	KIN	0.25	5.333 (2.307)	1.340 (1.141)	0.000 (0.707)	6.250 (2.493)	4.350 (2.068)	3.333 (1.794)	0.190 (0.396)
10	MS	KIN	0.5	5.667 (2.307)	1.343 (1.143)	0.000 (0.707)	8.133 (2.848)	4.167 (2.032)	3.667 (1.911)	0.300 (0.546)
11	MS	KIN	1.0	6.000 (2.378)	1.000 (1.000)	0.333 (0.880)	7.767 (2.783)	3.867 (1.938)	5.000 (2.229)	0.137 (0.363)
12	MS	KIN	2.0	5.333 (2.449)	1.410 (1.171)	0.333 (0.880)	7.333 (2.703)	4.417 (2.091)	4.650 (2.154)	0.533 (0.722)
13	MS	KIN	2.5	5.333 (2.307)	1.000 (1.000)	0.000 (0.707)	5.800 (2.407)	3.600 (1.890)	4.000 (1.989)	0.060 (0.234)

Contd.

Table 6b. Continued

1	2	3	4	5	6	7	8	9	10	11
14	MS	KIN	4.0	7.667 (2.768)	1.417 (1.73)	0.333 (0.880)	6.667 (2.580)	4.833 (2.172)	4.333 (2.079)	0.947 (0.966)
15	MS	KIN	5.0	8.333 (2.886)	1.667 (1.276)	0.667 (0.998)	6.533 (2.554)	5.550 (2.332)	6.000 (2.402)	0.933 (0.906)
16	MS	2iP	0.25	6.333 (2.515)	1.000 (1.000)	0.000 (0.707)	0.067 (2.463)	4.800 (2.189)	4.000 (1.989)	1.620 (1.272)
17	MS	2iP	0.5	5.667 (2.365)	1.000 (1.000)	0.000 (0.707)	5.067 (2.235)	4.500 (2.105)	4.000 (1.989)	0.583 (0.763)
18	MS	2iP	1.0	4.667 (2.157)	1.167 (1.080)	0.000 (0.707)	6.067 (2.461)	4.067 (2.014)	4.600 (2.143)	1.450 (1.203)
19	MS	2iP	2.0	5.667 (2.378)	1.787 (1.318)	1.000 (1.171)	7.500 (2.736)	5.300 (2.280)	5.000 (2.235)	1.367 (1.168)
20	MS	2iP	2.5	6.667 (2.580)	1.777 (1.321)	0.333 (0.880)	10.000 (3.152)	5.800 (2.408)	5.367 (2.310)	1.500 (1.224)
21	MS	2iP	4.0	5.000 (2.229)	1.533 (1.225)	0.667 (0.998)	7.333 (2.688)	4.433 (2.096)	3.867 (1.961)	1.900 (1.378)
22	MS	2iP	5.0	4.333 (2.077)	1.183 (1.083)	0.333 (0.880)	5.667 (2.378)	4.667 (2.139)	3.667 (1.911)	0.867 (0.929)
23	½MS	BA	1.0	6.183 (2.475)	1.167 (1.075)	0.330 (0.880)	11.967 (3.456)	6.133 (2.475)	3.700 (1.910)	1.817 (1.334)
24	½MS	KIN	1.0	10.583 (3.252)	0.973 (0.985)	0.000 (0.707)	3.550 (2.355)	3.600 (1.895)	5.033 (2.241)	0.540 (0.641)
25	MS	BA + NAA	0.25 0.5	9.000 (2.997)	1.047 (1.021)	0.000 (0.707)	4.683 (2.162)	2.600 (1.606)	2.500 (1.592)	1.817 (1.348)
26	MS	BA + NAA	0.25 1.0	9.667 (3.108)	0.973 (0.985)	0.333 (0.880)	6.800 (2.607)	5.633 (2.335)	3.793 (1.945)	1.900 (1.378)
27	MS	BA + IAA	0.25 0.5	4.667 (2.157)	1.283 (1.132)	0.667 (1.052)	9.583 (3.075)	5.250 (2.263)	4.113 (2.018)	1.250 (1.093)
28	MS	BA + IAA	0.25 1.0	4.333 (2.079)	1.470 (1.178)	0.333 (0.880)	10.367 (3.218)	6.667 (2.562)	6.567 (2.561)	1.000 (0.995)

Contd.

Table 6b. Continued

1	2	3	4	5	6	7	8	9	10	11
29	MS	BA + IAA	1.0 1.0	5.333 (2.307)	1.983 (1.408)	1.667 (1.386)	13.000 (3.604)	10.450 (3.200)	7.600 (2.753)	1.700 (1.303)
30	MS + Streptomycin 0.1%	BA	0.5	7.333 (2.707)	1.333 (1.138)	0.333 (0.880)	10.000 (3.160)	8.960 (2.968)	4.333 (2.079)	0.933 (0.964)
31	MS (control)			5.667 (2.378)	1.063 (1.029)	0.333 (0.880)	9.667 (3.098)	7.600 (2.734)	3.333 (1.794)	0.027 (0.155)
32	½MS (control)			5.667 (2.378)	1.133 (1.064)	0.000 (0.707)	7.000 (2.641)	5.033 (2.240)	3.667 (1.883)	0.013 (0.114)
	CD			0.258	0.278	0.506	0.509	0.514	0.393	0.263
	SEm±			0.091	0.098	0.179	0.180	0.182	0.139	0.093

* Values in paranthesis represent $\sqrt{x}$ transformed ones

Fig.2. Effect of different growth regulators on establishment of nodal segments of *H. annulare*

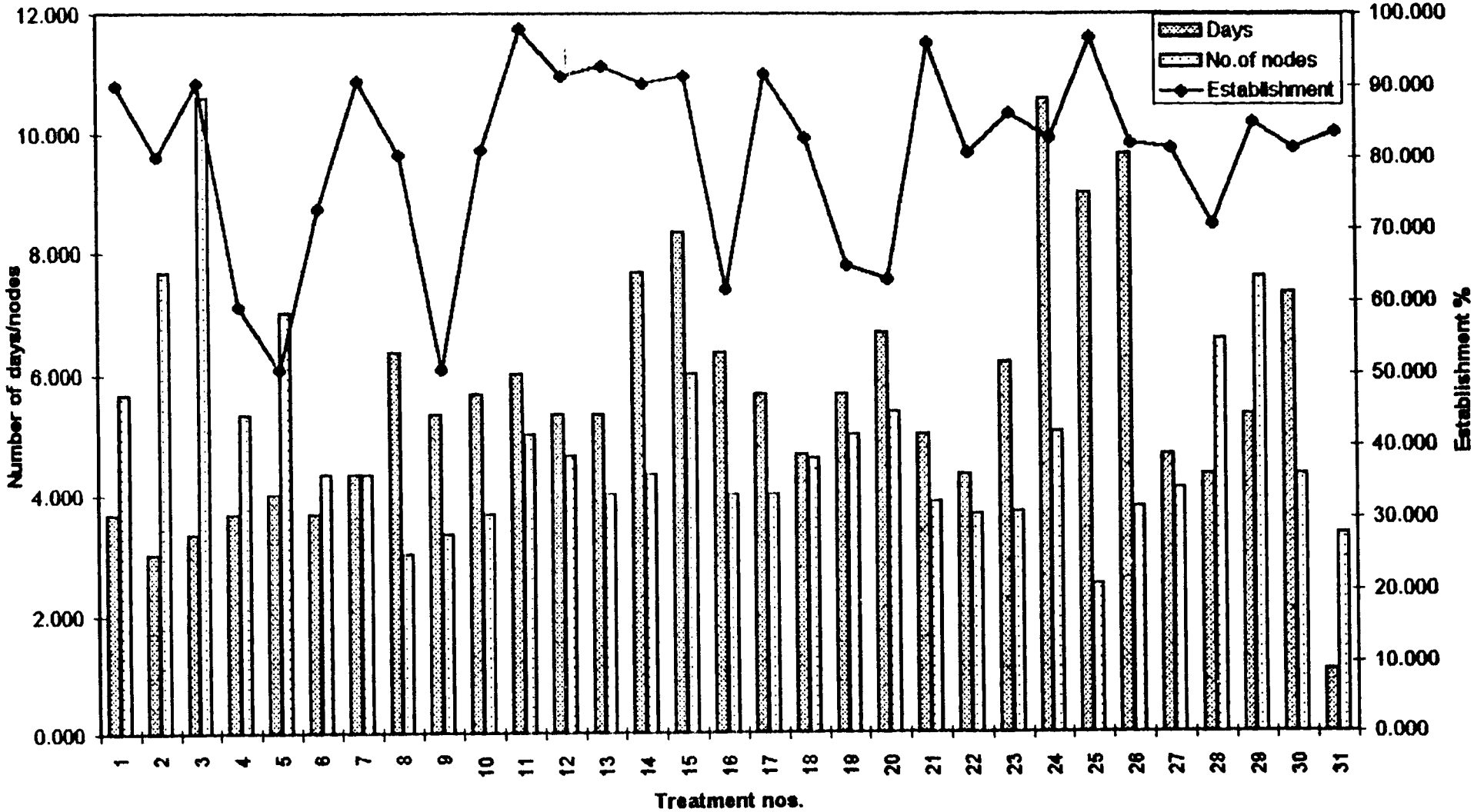

Plate 1. Axillary bud release from nodal segment

Plate 2. Nature of shoots formed in media containing different cytokinins

Plate 1

Plate 2

supplemented with 1.0 mg l^{-1} of BA. At higher concentrations of BA (5 mg l^{-1}, 10 mg l^{-1}) the number of multiple shoot buds were higher but no further elongation was observed if maintained in the same media. Moreover the shoots were thinner, lighter in colour and with weak nodes. When such single nodal segments were subcultured, healthy shoots were not produced. However, when shoots and buds were together subcultured into media containing very low levels of BA (0.5 mg l^{-1}) or in basal media they became stronger and produced stronger nodes which were potential shoots. Callus formation was observed at the base of the explants irrespective of the cytokinin used, however their intensity was minimum when KIN was incorporated (0.4 g on an average). When TDZ was incorporated in MS medium at a concentration of 0.1 mg l^{-1}, an average of 7.65 nodes were obtained while at a concentration of 0.2 mg l^{-1} around 8.05 nodes were obtained.

4.2.1.2 Standardisation of media

The data on the effect of media on the establishment and growth of nodal explant are presented in Tables 7a and 7b. All the media tried were comparable with respect to the establishment percentage but MS medium at full strength supplemented with or without growth regulator proved better than woody plant medium for better growth of the explants. Multiple shoot production was more but at the same time profuse callus formation also was there.

The number of buds formed, multiple shoots developed and the number of nodes were much higher in solid media than in stationary liquid media, while at the same time the number of days taken for bud break and the intensity of callus formation were more in solid media (Table 8).

Table 7a. Effect of media combination on establishment of nodal segments of *H. annulare*

Treatment No.	Treatment			Establishment (%)
	Media	Growth regulator	Concentration (mg l^{-1})	
1	MS	-	-	83.667 (1.158)*
2	MS	BA	1.0	90.333 (1.273)
3	WPM	BA	1.0	75.333 (1.052)
4	½MS	-	-	79.700 (1.06)
CD				0.168
SEm±				0.052

* Values in paranthesis represent arc sine transformed ones

Table 7b. Effect of media combinations on growth of nodal segments of *H. annulare*

Treatment No.	Treatment: Media	Treatment: Growth regulator	Treatment: Concentration (mg l^{-1})	Days to bud release	No. of shoots	No. of shoot buds	Length of the longest shoot (cm)	Average length of a shoot (cm)	No. of nodes	Weight of callus (g)
1	MS	-	-	5.667 (2.378)*	1.063 (1.029)	0.333 (0.880)	9.667 (3.098)	7.600 (2.734)	3.333 (1.794)	0.027 (0.155)
2	MS	BA	1.0	3.333 (1.821)	2.453 (1.563)	4.500 (2.234)	17.020 (4.125)	6.733 (2.595)	10.600 (3.253)	2.100 (1.446)
3	WPM	BA	1.0	7.667 (2.768)	1.170 (1.080)	0.000 (0.707)	6.667 (2.570)	5.000 (2.215)	3.667 (1.911)	0.243 (0.491)
4	½MS	-	-	5.661 (2.378)	1.133 (1.064)	0.000 (0.707)	7.000 (2.641)	5.033 (2.240)	3.667 (1.883)	0.013 (0.114)
	CD			0.238	0.158	0.298	0.465	0.555	0.595	0.146
	SEm±			0.073	0.048	0.091	0.143	0.170	0.183	0.045

* Values in paranthesis represent $\sqrt{x}$ transformed ones

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Table 8. Effect of physical nature of media* on establishment and growth of nodal segments of *H. annulare*

Treatment No.	Treatment	Establishment (%)	Days to bud release	No. of shoots	No. of shoot buds	Length of the longest shoot (cm)	Average length of a shoot (cm)	No. of nodes	Callus weight (g)
1	Solid	80.66	8.682	1.051	0.333	9.376	6.160	4.233	1.317
2	Liquid	76.92	6.667	1.3	0	8.4	6.210	3.500	0.004

* Media used was MS + Adenine sulphate 2 mg l^{-1}

4.2.1.3 Effect of additives

Additives like silver nitrate, ascorbic acid, adenine sulphate, activated charcoal and coconut water were incorporated into MS medium to determine the effect on establishment per cent, growth and the extent of callus formation in the cultures. The data are presented in Tables 9a and 9b.

None of the additives when incorporated in MS medium gave an establishment per cent as high as control ie. MS basal medium. Among the additives used, adenine sulphate induced better establishment percentage (80.7%) but the rate of growth of the explants was better in media containing coconut water (15%) which gave more number of axillary shoots, maximum length and number of nodes. Shoots had a pinkish coloration in media containing coconut water. However, callusing was high with both these additives. The extent of callusing was reduced when activated charcoal was added into the medium followed by addition of silver nitrate and ascorbic acid. The effect of silver nitrate in reducing callusing was more pronounced in solid media than in liquid media. When additives were added into MS media to reduce callus formation, along with reduced callus production, shoot growth also was reduced.

4.2.2 Establishment and growth of shoot tip explants

4.2.2.1 Standardisation of growth regulators

The results on the effect of growth regulators on establishment and growth of shoot tip explants are presented in Tables 10a and 10b. KIN at 0.5 mg l^{-1} gave maximum establishment of cultures foilowed by lower concentrations of BA and combination of BA with IAA or NAA, while the lowest percentage of

Table 9a. Effect of different additives on establishment of nodal segments of *H. annulare*

Treatment No.	Treatment			Establishment (%)
	Media	Additives	Concentration	
1	MS	Activated charcoal	0.1%	78.167 (1.085)*
2	½MS	Activated charcoal	0.1%	74.500 (1.042)
3	MS	Silver nitrate	5 mg l^{-1}	49.667 (0.782)
4	MS	Ascorbic acid	100 mg l^{-1}	66.177 (0.950)
5	MS	Coconut water	15%	71.333 (1.006)
6	MS	Adenine sulphate	2 mg l^{-1}	80.667 (1.116)
7	Liquid MS + 1 mg l^{-1} BA	Silvernitrate	5 mg l^{-1}	59.333 (0.880)
8	MS	(Control)		83.667 (1.158)
9	½MS	(Control		79.700 (1.106)
	CD			0.077
	SEm±			0.026

* Values in paranthesis indicate arc sine transformed ones

Table 9b. Effect of different additives on growth of nodal segments of *H. annulare*

Treatment No.	Treatments: Media	Additive	Concentration	Days to bud release	No. of shoots	Length of the longest shoot (cm)	Average length of a shoot (cm)	No. of nodes	Weight of callus (g)	No. of shoot buds **
1	MS	Charcoal	0.1%	10.000 (3.152)*	1.030 (1.002)	5.000 (2.229)	4.500 (2.111)	3.000 (1.715)	0.000 (0.707)	0.000
2	½MS	Charcoal	0.1%	6.333 (2.515)	0.673 (0.774)	4.000 (1.989)	4.000 (1.989)	3.000 (1.751)	0.047 (0.739)	0.000
3	MS	Silver nitrate	5 mg l^{-1}	11.000 (3.314)	0.917 (0.952)	2.667 (1.626)	2.667 (1.626)	1.667 (1.276)	0.600 (1.048)	0.000
4	MS	Ascorbic acid	100 mg l^{-1}	7.333 (2.707)	1.117 (1.056)	8.000 (2.825)	8.000 (2.825)	3.333 (1.821)	0.500 (0.999)	0.000
5	MS	Coconut water	15%	10.267 (3.204)	1.217 (1.102)	9.850 (3.138)	7.827 (2.788)	6.217 (2.447)	1.253 (1.324)	0.000
6	MS	Adenine sulphate	2 mg l^{-1}	8.683 (2.944)	1.050 (1.025)	9.367 (3.059)	6.160 (2.476)	4.233 (2.045)	1.317 (1.345)	0.333
7	MS (Liquid supplemented with 1 mg $^{-1}$ BA	Silver nitrate	5 mg l^{-1}	6.667 (2.580)	1.117 (1.056)	2.000 (1.407)	2.000 (1.407)	2.067 (1.414)	0.427 (0.962)	0.000
8	MS	Control (no additive)		5.667 (2.378)	1.063 (1.029)	9.667 (3.098)	7.600 (2.734)	3.333 (1.794)	0.027 (0.726)	0.333
CD				0.274	0.257	0.355	0.448	0.597	0.095	
SEm±				0.091	0.086	0.118	0.149	0.199	0.032	

* Values in paranthesis represents $\sqrt{x}$ transformed ones

** Not statistically analysed

Table 10a. Effect of different growth regulators on establishment of shoot tip explants of *H. annulare*

Treatment No.	Treatment			Establishment (%)
	Media	Growth regulators	Concentration (mg l^{-1})	
1	2	3	4	5
1	MS	BA	0.25	89.667 (1.250)*
2	MS	BA	0.5	82.667 (1.142)
3	MS	BA	1.0	90.000 (1.256)
4	MS	BA	2.0	80.333 (1.113)
5	MS	BA	2.5	42.333 (0.708)
6	MS	BA	4.0	57.133 (0.857)
7	MS	BA	5.0	51.683 (0.802)
8	MS	BA	10.0	52.833 (0.814)
9	MS	KIN	0.25	81.000 (1.120)
10	MS	KIN	0.5	94.000 (1.324)
11	MS	KIN	1.0	66.000 (0.948)
12	MS	KIN	2.0	61.667 (0.903)
13	MS	KIN	2.5	48.333 (0.769)

Contd.

Table 10a. Continued

1	2	3	4	5
14	MS	KIN	4.0	66.950 (0.959)
15	MS	KIN	5.0	70.333 (0.995)
16	MS	2iP	0.25	50.000 (0.785)
17	MS	2iP	0.5	36.667 (0.650)
18	MS	2iP	1.0	71.667 (1.010)
19	MS	2iP	2.0	65.333 (0.942)
20	MS	2iP	2.5	62.667 (0.914)
21	MS	2iP	4.0	44.000 (0.725)
22	MS	2iP	5.0	50.000 (0.785)
23	½MS	BA	1.0	74.600 (1.045)
24	½MS	KIN	1.0	78.900 (1.094)
25	MS	BA + NAA	0.25 + 0.5	90.000 (1.259)
26	MS	BA + NAA	0.25 + 1.0	80.000 (1.109)
27	MS	BA + IAA	0.25 + 0.5	80.000 (1.108)

Contd.

Table 10a. Continued

1	2	3	4	5
28	MS	BA + IAA	0.25 + 1.0	90.000 (1.255)
29	MS	BA + IAA	1.0 + 1.0	85.033 (1.175)
30	MS supplemented with 0.1% streptomycin	BA	0.5	73.233 (1.029)
31	MS	(Control)	-	64.133 (0.930)
32	½MS	(Control)	-	70.433 (0.996)
CD				0.089
SEm±				0.032

* Values in paranthesis represent arc sine transformed ones

Table 10b. Effect of different growth regulators on growth of shoot tip explants of *H. annulare*

Treatment No.	Treatment: Media	Treatment: Growth regulator	Treatment: Concentration (mg l-1)	Days to bud release	No. of shoots	No. of shoot bud	Length of the longest shoot (cm)	Average length of shoot (cm)	No. of nodes	Weight of callus (g)
1	2	3	4	5	6	7	8	9	10	11
1	MS	BA	0.25	21.000 (4.582)*	1.400 (1.166)	0.667 (1.052)	7.917 (2.808)	6.167 (2.455)	4.667 (2.899)	2.417 (1.701)
2	MS	BA	0.5	17.000 (4.118)	1.217 (1.102)	0.333 (0.880)	5.000 (2.250)	4.233 (2.012)	3.667 (1.865)	1.250 (1.301)
3	MS	BA	1.0	15.667 (3.936)	1.933 (1.375)	3.333 (1.932)	12.667 (3.557)	8.333 (2.885)	8.667 (2.868)	1.917 (1.551)
4	MS	BA	2.0	20.433 (4.520)	1.767 (1.327)	2.933 (1.842)	10.213 (3.182)	6.200 (2.466)	5.680 (2.330)	2.167 (1.632)
5	MS	BA	2.5	23.667 (4.863)	1.883 (1.361)	6.000 (2.544)	6.667 (2.580)	6.650 (2.560)	3.867 (1.965)	2.333 (1.683)
6	MS	BA	4.0	21.250 (4.609)	1.943 (1.389)	5.483 (2.440)	4.600 (2.143)	2.440 (1.546)	6.383 (1.814)	3.500 (1.992)
7	MS	BA	5.0	22.433 (4.735)	2.017 (1.418)	6.833 (2.706)	3.817 (1.944)	2.020 (1.391)	4.067 (1.974)	3.667 (2.034)
8	MS	BA	10.0	24.150 (4.906)	1.140 (1.039)	5.317 (2.387)	2.867 (1.691)	0.877 (0.929)	3.450 (1.836)	3.890 (2.086)
9	MS	KIN	0.25	25.000 (4.999)	0.937 (0.967)	0.000 (0.707)	7.000 (2.698)	6.333 (2.407)	3.000 (1.656)	0.433 (0.962)
10	MS	KIN	0.5	20.000 (4.471)	1.017 (1.005)	0.000 (0.707)	5.667 (2.320)	5.667 (2.320)	4.333 (2.033)	0.753 (1.082)
11	MS	KIN	1.0	23.667 (4.864)	0.910 (0.930)	0.000 (0.707)	5.250 (2.074)	4.833 (1.995)	2.000 (1.382)	1.367 (1.335)
12	MS	KIN	2.0	22.500 (4.739)	1.407 (1.183)	0.000 (0.707)	6.500 (2.541)	5.333 (2.294)	4.300 (2.060)	0.330 (0.908)

Contd.

Table 10b. Continued

1	2	3	4	5	6	7	8	9	10	11
13	MS	KIN	2.5	26.33 (5.130)	0.967 (0.965)	0.667 (0.998)	5.167 (2.227)	5.167 (2.227)	4.667 (2.099)	1.233 (1.295)
14	MS	KIN	4.0	26.633 (5.159)	1.213 (1.098)	1.067 (1.197)	7.317 (2.701)	6.033 (2.454)	4.933 (2.206)	0.223 (0.842)
15	MS	KIN	5.0	28.000 (5.289)	1.103 (1.050)	2.000 (1.470)	7.500 (2.682)	5.500 (2.325)	5.667 (2.365)	0.760 (1.087)
16	MS	2iP	0.25	25.000 (4.999)	0.870 (0.930)	0.000 (0.707)	7.500 (2.618)	7.500 (2.618)	4.000 (1.955)	0.827 (1.113)
17	MS	2iP	0.5	25.000 (5.000)	1.833 (1.343)	0.000 (0.707)	3.333 (1.802)	2.417 (1.544)	2.333 (1.520)	1.520 (1.389)
18	MS	2iP	1.0	25.000 (4.999)	1.600 (1.256)	0.000 (0.707)	3.167 (1.777)	2.667 (1.625)	3.000 (1.715)	0.917 (1.183)
19	MS	2iP	2.0	22.667 (4.754)	1.283 (1.131)	0.000 (0.707)	4.333 (2.078)	3.167 (1.777)	3.250 (1.755)	0.850 (1.131)
20	MS	2iP	2.5	23.667 (4.863)	1.310 (1.125)	0.000 (0.707)	3.550 (1.880)	3.417 (1.839)	3.100 (1.732)	2.060 (1.506)
21	MS	2iP	4.0	27.333 (5.226)	1.117 (1.056)	0.000 (0.707)	2.917 (1.658)	2.917 (1.658)	3.000 (1.715)	1.767 (1.490)
22	MS	2iP	5.0	22.000 (4.690)	1.333 (1.138)	0.333 (0.880)	2.333 (1.429)	1.433 (1.115)	5.333 (2.294)	1.450 (1.323)
23	½MS	BA	1.0	10.333 (3.214)	1.333 (1.138)	0.333 (0.880)	9.333 (2.968)	4.167 (2.030)	3.000 (1.688)	1.900 (1.530)
24	½MS	KIN	1.0	14.333 (3.785)	1.333 (1.138)	0.333 (0.880)	6.667 (2.580)	5.417 (2.323)	4.000 (1.955)	2.750 (1.801)
25	MS	BA+NAA	0.25+0.5	14.000 (3.740)	1.000 (1.000)	0.333 (0.880)	15.417 (3.924)	5.117 (2.261)	5.333 (2.276)	1.577 (1.361)
26	MS	BA+NAA	0.25+1.0	12.667 (3.557)	1.333 (1.138)	1.667 (1.351)	11.417 (3.368)	5.417 (2.323)	4.333 (2.076)	1.483 (1.400)
27	MS	BA+IAA	0.25+0.5	14.367 (3.783)	1.000 (1.000)	0.333 (0.880)	4.083 (2.019)	3.500 (1.868)	2.667 (1.626)	2.017 (1.583)

Contd.

Table 10b. Continued

1	2	3	4	5	6	7	8	9	10	11
28	MS	BA+IAA	0.25+1.0	24.750 (4.975)	1.167 (1.075)	0.333 (0.880)	7.083 (2.661)	3.867 (1.965)	2.867 (1.691)	1.383 (1.366)
29	MS	BA+IAA	1.0+1.0	17.600 (4.183)	1.067 (1.033)	0.333 (0.880)	8.833 (2.971)	6.000 (2.449)	3.333 (1.817)	2.090 (1.600)
30	MS Supplemented with 0.1% Streptomycin	BA	0.5	24.233 (4.920)	0.620 (0.784)	0.000 (0.707)	4.683 (2.162)	3.050 (1.746)	3.000 (1.715)	0.190 (0.821)
31	MS (Control)			28.333 (5.323)	1.000 (1.000)	0.000 (0.707)	3.583 (1.877)	1.750 (1.321)	1.000 (1.000)	0.000 (0.707)
32	½MS (Control	)		26.533 (5.150)	1.000 (1.000)	0.000 (0.707)	3.683 (1.908)	1.750 (1.321)	1.333 (1.138)	0.000 (0.707)
	CD									
	SEm±									

* Values in paranthesis represent $\sqrt{x}$ transformed ones

establishment was obtained with treatment containing 2iP. BA produced longer shoots when incorporated at concentrations ranging from 0.25 to 2.5 mg l^{-1}, but at higher concentrations, the shoot growth was stunted. In general, BA produced long, light green shoots with longer internodes while those produced by KIN were short, thick and robust with large dark leaves with slight purple coloration. The shoots produced in medium supplemented with 2iP were also short and robust. More number of nodes and multiple shoots were produced when MS medium was supplemented with BA, the latter being at higher concentrations of BA. The intensity of callus formation from the explants varied with the different cytokinins. Minimum callus was observed in medium supplemented with KIN (0.6 g on an average) followed by treatments containing 2iP while maximum callusing was observed in medium supplemented with BA (2.6 g on an average).

At half the concentration of MS, with BA at 1 mg l^{-1} earlier bud burst was observed, but at the same time the length of the shoot, the multiple shoots produced and the number of nodes formed were significantly reduced.

4.2.2.2 Effect of media

Among the various treatments tried, MS with BA at 1.0 mg l^{-1} proved best with respect to the establishment percentage early release of buds, length of shoot and number of nodes (Tables 11a and 11b). Solid media proved better than stationary liquid media for better establishment and formation of longer shoots with more number of nodes. However callus production was also more in solid media (Table 12).

Table 11a. Effect of different media on establishment of shoot tip explants of *H. annulare*

Treatment No.	Treatment			Establishment (%)
	Media	Growth regulator	Concentration (mg l^{-1})	
1	MS	-	-	64.133 (0.930)*
2	MS	BA	1.0	90.000 (1.256)
3	WPM	BA	1.0	71.417 (1.007)
4	½ MS	-	-	70.433 (0.996)
CD				0.103
SEm±				0.032

* Values in paranthesis represent arc sine transformed ones

Table 11b. Effect of different media on growth of shoot tip explants on *H. annulare*

Treatment No.	Treatment: Media	Treatment: Growth regulator	Treatment: Concentration (mg l^{-1})	Days to bud release	No. of shoots	No. of shoot buds	Length of the longest shoot (cm)	Average length of a shoot (cm)	No. of nodes	Weight of callus (g)
1	MS	-	-	28.333 (2.307)*	1.000 (1.000)	0.000 (0.707)	3.583 (1.877)	1.750 (1.321)	1.000 (1.000)	0.000 (0.707)
2	Ms	BA	1.0	15.667 (1.981)	1.933 (1.375)	3.333 (1.932)	8.667 (2.900)	5.333 (2.307)	5.667 (2.298)	1.917 (1.551)
3	WPM	BA	1.0	21.800 (2.158)	0.917 (0.955)	0.000 (0.707)	4.617 (2.141)	2.550 (1.587)	3.300 (1.801)	0.967 (1.208)
4	½ MS	-	-	26.533 (2.269)	1.000 (1.000)	0.000 (.707)	3.683 (1.908)	1.750 (1.321)	1.333 (1.138)	0.000 (0.707)
	CD			0.146	0.246	0.362	0.719	0.266	0.799	0.158
	SEm±			0.045	0.075	0.111	0.221	0.082	0.245	0.048

* Values in paranthesis represent $\sqrt{x}$ transformed ones

Table 12. Effect of physical nature of media on establishment and growth of shoot tip explants c *H. annulare*

Treatment No.	Nature of media*	Establish-ment (%)	Days to release buds	No. of shoots	No. of of buds	Length of longest shoot(cm)	Average length of a shoot (cm)	No.of nodes	Callus weight (g)
1	Solid	77.667	16.900	1.070	0.333	8.450	5.800	4.400	1.350
2	Liquid	64.633	15.533	1.000	0.000	2.833	2.000	2.000	0.000

* Media used was MS supplemented with Adenine sulphate (2 mg l^{-1})

4.2.2.3 Effect of additives

The data on the effect of additives on establishment and growth of shoot tips are given in Tables 13a and 13b.

Among the various additives tried, maximum establishment of cultures (77.7%) and the best growth was obtained in MS medium supplemented with adenine sulphate. Early release of buds (16.9 days), maximum shoot length (8.45 cm) and larger number of nodes (4.4) were obtained in this medium but callus formation was also higher. Multiple shoots could be produced only in this medium. Medium supplemented with silver nitrate was found inhibitory to shoot growth.

4.2.3 Effect of culture conditions on establishment and growth of nodal and shoot tip explants

Both nodal and shoot tip explants performed better at higher culture temperature (30 $\pm$ 1 $^{\circ}$C) than, at lower temperature (26 $\pm$ 1 $^{\circ}$C). The early release of buds and growth of axillary shoots were achieved in light. If exposed to dark it resulted in etiolated shoots without chlorophyll pigment and resulted in early drying up of the shoots.

Thus, it could be observed that shoot tips take more time for the bud break (15-16 days) than nodal segments (3-4 days only). The number of shoots and shoot buds were higher (2.5 and 4.5 respectively) than shoot tips (2 and 3 respectively). The length of shoots and the number of nodes also were more when nodal segments were used (17 cm, 11) than shoot tip explants (13 cm, 9). However, the callus formation was comparatively lower for shoot tip explants than nodal

Table 13a. Effect of different additives on establishment of shoot tip explants of *H. annulare*

Treatment No.	Treatment			Establishment (%)
	Media	Additive	Concentration	
1	MS	Activated charcoal	0.1%	60.233 (0.889)*
2	½MS	Activated charcoal	0.1%	49.150 (0.777)
3	MS	Silver nitrate	5 mg l^{-1}	41.250 (0.697)
4	MS	Ascorbid acid	100 mg l^{-1}	57.033 (0.856)
5	MS	Coconut water	15 %	65.733 (0.945)
6	MS	Adenine sulphate	2 mg l^{-1}	77.667 (1.079)
7	MS liquid supplemented with 1 mg l^{-1} BA	Silver nitrate	5 mg l^{-1}	61.700 (0.904)
8	MS	(Control)		64.133 (0.930)
CD				0.055
SEm±				

* Values in paranthesis represents arc sine transformed ones

Table 13b. Effect of different additives on growth of shoot tip explants of *H. annulare*

Treatment No.	Treatment: Media	Treatment: Additive	Treatment: Concentration	Days to bud releast	No. of shoots	Length of the longest shoot (cm)	Average length of a shoot (cm)	No. of nodes	Weight of callus (g)	No. of** shoot buds
1	MS	Activated charcoal	0.1%	28.817 (5.368)*	0.917 (0.955)	3.700 (1.918)	2.533 (1.585)	2.750 (1.657)	0.003 (0.709)	0.000
2	½MS	Activated charcoal	0.1%	27.667 (5.256)	1.000 (1.000)	3.000 (1.728)	2.417 (1.538)	2.000 (1.414)	0.007 (0.712)	0.000
3	MS	Silver nitrate	5 mg l^{-1}	29.500 (5.431)	1.000 (1.000)	2.250 (1.486)	2.083 (1.432)	2.667 (1.609)	0.003 (0.709)	0.000
4	MS	Ascorbic ACID	100 mg l^{-1}	23.083 (4.801)	1.000 (1.000)	4.000 (1.989)	3.500 (1.862)	3.333 (1.794)	0.423 (0.946)	0.000
5	MS	Coconut water	15%	25.933 (5.089)	1.000 (1.000)	5.000 (2.229)	3.917 (1.958)	4.000 (1.989)	0.700 (1.093)	0.000
6	MS	Adenine sulphate	2 mg l^{-1}	16.900 (4.105)	1.070 (1.033)	8.450 (2.904)	5.800 (2.406)	4.400 (2.095)	1.350 (1.360)	0.330
7	MS Liquid medium with 1 mgl^{-1} BA	Silver nitrate	5 mg l^{-1}	22.067 (4.686)	1.000 (1.000)	7.167 (2.675)	3.750 (1.927)	4.000 (1.989)	2.000 (1.580)	0.000
8	MS (control)			28.333 (5.323)	1.000 (1.000)	3.583 (1.877)	1.750 (1.321)	1.000 (1.000)	0.000 (0.707)	0.000
	CD			0.391	0.055	0.371	0.391	0.402	0.145	
	SEm±			0.130	0.018	0.124	0.130	0.134	0.048	

* Values in paranthesis represent $\sqrt{x}$ transformed ones

** Not statistically analysed

segments. The number of nodal segment explants that could be obtained from a mother plant was much higher than shoot tips.

4.3 Proliferation

4.3.1 Standardisation of media and growth regulators

4.3.1.1 Standardisation of growth regulators

The results of the experiment on the effect of different concentrations of cytokinins such as BA, 2iP and KIN and combinations of BA with different auxins like NAA and IAA on proliferation of the explants are presented in the Table 14 and Fig.3.

The number of shoots produced increased with the increasing concentration of BA in the medium. Thus the number of shoots and buds were maximum when BA at a concentration of 20 mg l^{-1} was used (16 shoots). The morphology of shoots that were produced at this concentration were typical. Cent per cent of the shoots produced were very short (2.3 cm), swollen and stunted. They were light green in colour and very weak (Plate 3). Larger number of nodes (17-18) were observed but there was also profuse callus formation. There were tiny green shoot buds at the base which could be subcultured as a clump either to MS medium with lower concentrations of BA or to the basal medium for further elongation (Plates 6a, 6b, 6c and 6d). When the concentration of BA was lowered, longer shoots were produced (Plate 4). A range of concentration of BA 0.25-2.5 mg l^{-1} thus produced longer shoots (11 cm) with medium number of nodes (8). In general, incorporation of BA resulted in profuse callus production. BA when incorporated at 1 mg l^{-1} into the media produced more shoots and buds and with low callus production.

Table 14. Effect of different growth regulators on proliferation of nodal segments of *H. annulare*

Treatment No.	Treatments			No. of shoots	No. of shoot buds	Length of the longest shoot (cm)	No. of nodes	Weight of callus
	Media	Growth regulator	Concentration (mg l^{-1})					
1	2	3	4	5	6	7	8	9
1	MS	BA	0.25	3.000 (1.732)*	0.000 (0.000)	7.000 (2.642)	4.500 (2.120)	3.000 (1.731)
2	MS	BA	0.5	3.440 (1.854)	6.150 (2.480)	11.040 (3.314)	10.050 (3.169)	1.857 (1.362)
3	MS	BA	1.0	4.037 (2.009)	7.033 (2.650)	15.660 (3.942)	10.680 (3.260)	1.550 (1.244)
4	½MS	BA	1.0	1.450 (1.204)	2.250 (1.500)	13.773 (3.710)	5.550 (2.355)	0.573 (0.757)
5	MS	BA	2.5	3.293 (1.815)	7.583 (2.753)	8.827 (2.971)	8.377 (2.894)	2.833 (1.683)
6	MS	BA	5.0	6.760 (2.600)	6.077 (2.465)	6.363 (2.522)	13.910 (3.730)	2.707 (1.645)
7	MS	BA	10.0	9.770 (3.126)	14.410 (3.790)	4.500 (2.121)	9.960 (3.151)	3.707 (1.922)
8	MS	BA	20.0	16.000 (4.000)	12.500 (3.533)	2.293 (1.514)	17.500 (4.180)	2.023 (1.380)
9	MS	KIN	0.25	1.200 (1.047)	2.050 (1.432)	7.250 (2.693)	8.900 (2.983)	0.020 (0.138)
10	MS	KIN	0.5	1.807 (1.344)	2.517 (1.586)	7.467 (2.733)	6.717 (2.591)	0.700 (0.837)
11	MS	KIN	1.0	1.100 (1.048)	0.000 (0.000)	7.850 (2.800)	5.600 (2.366)	0.813 (0.902)
12	½MS	KIN	1.0	1.250 (1.118)	0.000 (0.000)	6.677 (2.583)	4.783 (2.187)	0.223 (0.473)

Contd.

Table 14. Continued

1	2	3	4	5	6	7	8	9
13	MS	KIN	2.0	2.500 (1.581)	0.000 (0.000)	7.227 (2.688)	11.500 (3.391)	0.750 (0.864)
14	MS	KIN	2.5	2.500 (1.581)	0.000 (0.000)	9.877 (3.143)	6.750 (2.597)	1.377 (1.173)
15	MS	KIN	5.0	1.667 (1.291)	1.500 (1.223)	8.330 (2.886)	6.330 (2.515)	0.207 (0.454)
16	MS	2iP	0.25	2.300 (1.515)	3.500 (1.871)	8.660 (2.943)	5.657 (2.377)	2.000 (1.375)
17	MS	2ip	0.5	1.000 (0.999)	0.000 (0.000)	9.000 (2.996)	4.243 (2.059)	1.500 (1.223)
18	MS	2iP	1.0	4.000 (2.000)	5.000 (2.235)	12.000 (3.463)	7.000 (2.645)	1.000 (0.999)
19	MS	2iP	2.5	1.220 (1.104)	0.660 (0.807)	8.333 (2.887)	6.333 (2.516)	1.807 (1.344)
20	MS	2iP	5.0	2.400 (1.548)	1.500 (1.222)	5.250 (2.291)	5.000 (2.231)	1.303 (1.142)
21	MS	2iP	10.0	2.020 (1.421)	0.500 (0.690)	9.083 (3.014)	5.000 (2.230)	2.490 (1.578)
22	MS	NAA	1.0	2.000 (1.405)	0.000 (0.000)	7.560 (2.748)	9.000 (2.999)	3.887 (1.971)
23	MS	BA+ IAA	0.25+ 0.5	6.800 (2.607)	5.000 (2.232)	6.500 (2.549)	14.500 (3.807)	0.917 (0.957)
24	MS	BA+ IAA	0.5+ 1.0	7.050 (2.655)	0.000 (0.000)	10.000 (3.157)	12.220 (3.492)	1.460 (1.208)
25	MS	BA+ IAA	0.5+ 2.0	5.250 (2.291)	2.250 (1.500)	5.580 (2.362)	10.850 (3.294)	2.027 (1.423)
26	MS	BA+ IAA	1.0+ 1.0	3.690 (1.918)	2.167 (1.472)	11.870 (3.442)	8.667 (2.944)	0.987 (0.993)
27	MS	BA+ IAA	2.5+ 1.0	2.000 (1.412)	4.590 (2.141)	8.830 (2.971)	3.887 (1.971)	0.657 (0.810)

Contd.

Table 14. Continued

1	2	3	4	5	6	7	8	9
28	MS	BA+ NAA	0.25+ 0.5	2.000 (1.410)	0.000 (0.000)	9.000 (2.999)	7.500 (2.736)	1.750 (1.323)
29	MS	BA + NAA	0.5 + 1.0	2.500 (1.581)	1.750 (1.322)	5.500 (2.345)	5.627 (2.372)	2.190 (1.479)
30	MS	BA + NAA	0.5 + 2.0	1.660 (1.288)	0.000 (0.000)	1.660 (1.282)	2.330 (1.524)	2.000 (1.410)
31	MS	KIN+ IAA	1.0+ 1.0	3.350 (1.830)	0.917 (0.950)	9.750 (3.122)	9.910 (3.147)	2.070 (1.437)
32	MS	KIN+ IAA	2.5+ 1.0	2.740 (1.655)	0.627 (0.789)	9.763 (3.124)	5.527 (2.351)	1.127 (1.058)
33	½MS	-	-	2.000 (1.414)	0.000 (0.000)	15.000 (3.871)	4.000 (2.000)	2.500 (1.581)
34	MS	-	-	1.687 (1.298)	2.127 (1.458)	7.627 (2.762)	5.860 (2.421)	0.660 (0.812)
35	MS liquid	-	-	1.237 (1.105)	0.000 (0.000)	9.760 (3.124)	6.230 (2.494)	0.007 (0.100)
	CD			0.155	0.146	0.207	0.186	0.186
	SEm±			0.055	0.052	0.073	0.066	0.066

* Values in paranthesis represent $\sqrt{x}$ transformed ones

Fig.3. Effect of different growth regulators on proliferation of nodal segments of *H. annulare*

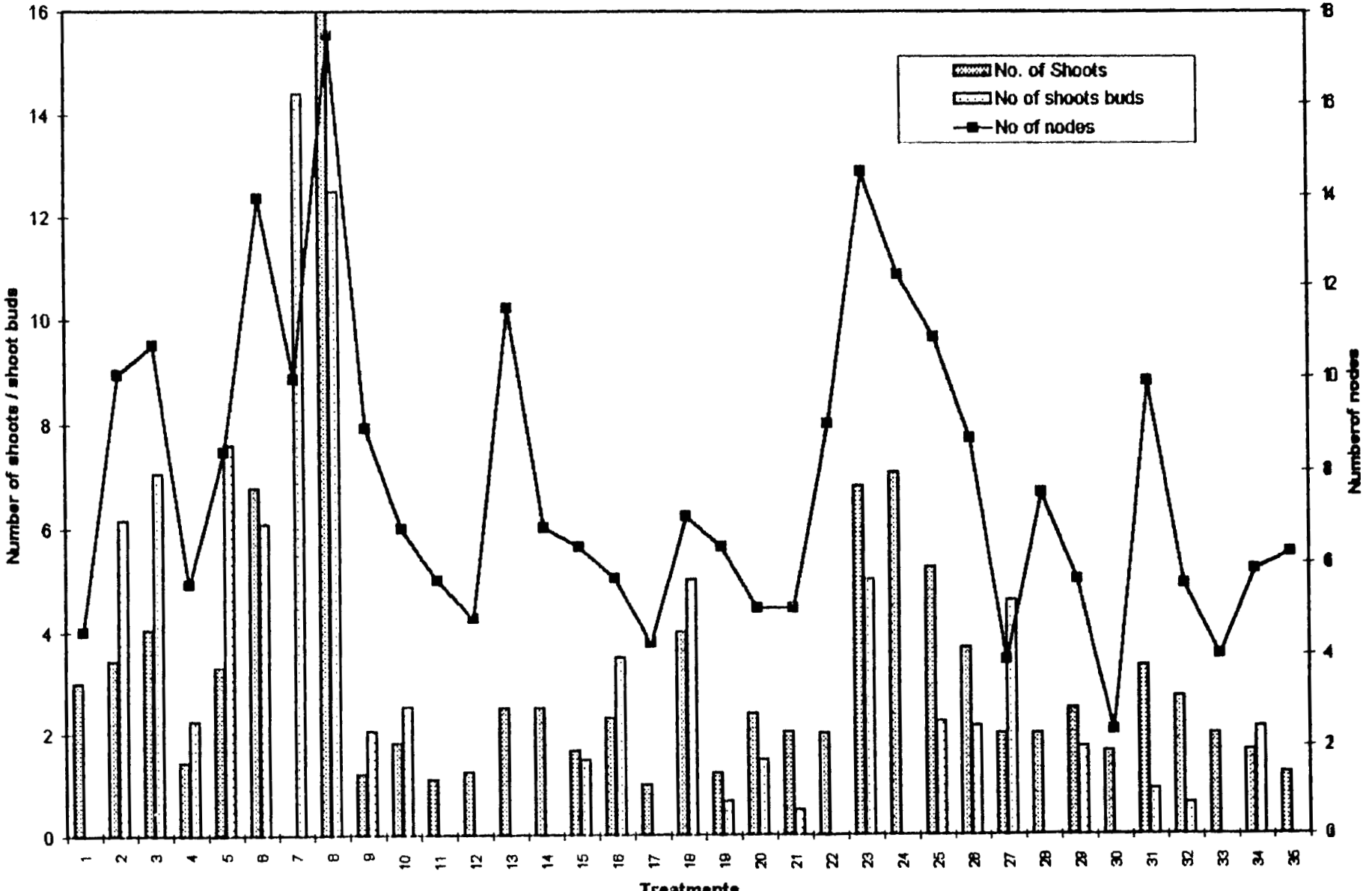


Plate 3. Proliferation in MS medium + BA at high concentration

Plate 4. Proliferation in MS medium + BA at low concentration

Plate 5. Proliferation in MS medium + TDZ

Plate 5

Plate 3

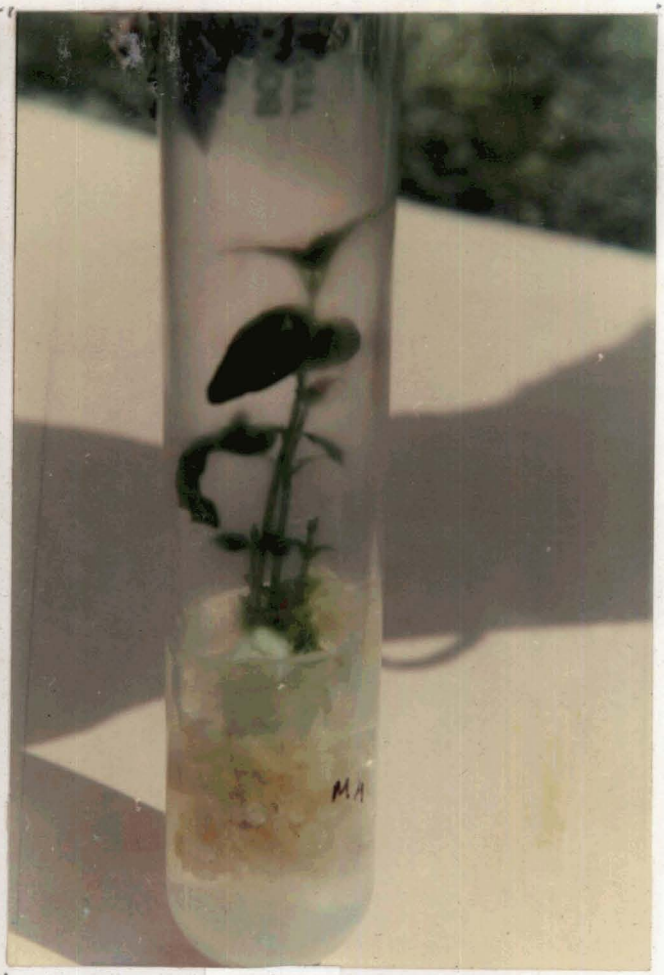


Plate 4

Plate 6a. Multiple shoot buds at the base of the explant

Plate 6b. Multiple shoot buds subcultured as a clump

Plate 6a

Plate 6b

Plate 6c. Elongation of shoots at low concentrations of BA

Plate 6d. Further elongation of shoots in basal MS medium

Plate 6c

Plate 6d

KIN when incorporated into MS media produced medium sized shoots (8 cm) with moderate number of nodes and low callus (0.6 g). But the number of shoots and buds produced were very much reduced. As the concentration of KIN was increased, the shoot bud production was decreased drastically. Among the different concentrations of KIN tried, KIN at 2 mg l^{-1} gave better performance. Among the different concentrations of 2iP tried, 2iP at 1.0 mg l^{-1} produced more number of nodes and buds. The morphological features of shoots produced in medium containing 2iP were almost similar to that produced in medium containing KIN as the shoots produced were short, robust and with smaller number of nodes and buds than that produced in the medium containing BA as the cytokinin. However, the shoots were darker coloured in medium containing KIN.

Among the cytokinin - auxin combinations tried, BA at 0.25 mg l^{-1} along with IAA at 0.5 mg l^{-1} proved better than others with respect to larger number of nodes and buds. When IAA was also incorporated with KIN, the length of the shoots produced was increased and with more number of nodes but the shoot bud production was very less (0.92). However, it was better than that without IAA. NAA alone when incorporated at 1 mg l^{-1} concentration resulted in shoots of 7.56 cm length and number of nodes (9) but the callus production was very high.

Experiments were conducted by subculturing shoots produced in MS media supplemented with 0.1 or 0.2 mg l^{-1} of TDZ to MS media containing a lower concentration (0.05 mg l^{-1}) of TDZ (Plate 5). It was found that when 0.1 mg l^{-1} TDZ was used, it resulted in the production of 1.75 shoots on an average having an average length of 9.66 cm and with 6.67 nodes while when shoots from

0.2 mg l^{-1} TDZ was subcultured to 0.05 mg l^{-1} TDZ, shoots of around 10.54 cm length and with 6.9 nodes were produced. Callus production was around 2.5 g.

4.3.1.2 Standardisation of media

The data with respect to the study on standardisation of different media on proliferation of cultures are presented in Table 15.

Longest shoots could be obtained in half strength MS medium (15 cm) but the internodal length was higher than other treatments tried. The effective number of nodes was lesser (4) in this case. Full strength MS medium with or without agar proved to be better with respect to the number of nodes. The shoot bud formation was maximum in full strength MS medium with the solidifying agent added (2.127) while it was totally nil in stationery liquid medium and half strength MS medium. Woody plant medium showed an average performance with respect to the parameters studied.

Multiple shoot buds and axillary shoots were mostly produced in solid media. In fact no multiple shoot buds were produced in stationery liquid media (Table 16).

A comparison between the solid and stationary liquid MS medium supplemented with silver nitrate and BA at 1 mg l^{-1} showed that liquid media proved better with respect to the number of nodes and multiple shoot production. Callus production was almost comparable though more callus was formed in solid media.

Table 15. Effect of different media on proliferation of nodal segments of *H. annulare*

Treatment No.	Treatment	No. of shoots	No. of shoot buds	Length of shoot (cm)	No. of nodes	Weight of callus (g)
1	MS basal	1.685	2.127	7.632	5.86	0.660
2	½MS basal	2.000	0.000	15.000	4.000	2.500
3	MS basal (liquid)	1.236	0.000	9.760	6.230	0.007
4	WPM basal	2.000	1.000	8.750	4.250	1.500

Table 16. Effect of physical nature of media on proliferation of nodal segments of *H. annulare*

Treatment No.	Treatment	No. of shoots	No. of shoot buds	Length of the longest shoot (cm)	No. of nodes	Weight of callus (g)
1	MS (liquid) + BA (1 mg l^{-1}) + Silver nitrate (5 mg l^{-1})	1.710 (1.143)*	2.000 (1.566)	7.973 (2.824)	6.550 (1.600)	1.240 (1.107)
2	MS (solid) + BA (1 mg l^{-1}) + Silver nitrate (5 mg l^{-1})	0.733 (0.878)	0.000 (0.707)	8.450 (2.907)	5.800 (1.552)	1.300 (1.140)
3	MS basal (solid)	1.687 (1.139)	2.127 (1.621)	7.630 (2.762)	5.860 (1.556)	0.660 (0.812)
4	MS basal (liquid)	1.237 (1.049)	0.000 (0.707)	9.760 (3.124)	6.230 (1.579)	0.010 (0.100)
	CD	0.223	0.253	0.060	0.000	0.133
	SEm±	0.068	0.078	0.018	0.000	0.041

* Values in paranthesis represent $\sqrt{x}$ transformed ones

When the salt concentration of MS media was reduced to half, the internodal length was increased and there was no shoot bud production. With full and half strength MS but with incorporation of adenine sulphate, the results obtained were similar. With activated charcoal, the half strength media was superior to full strength, with respect to the number of nodes and buds produced (Table 17).

4.3.2 Effect of additives

The data on the effect of additives on proliferation are summarised in Table 18. It was found that MS medium supplemented with coconut water (15%) resulted in longer shoots (10 cm) and more number of nodes (7.8). Other treatments were comparable with respect to the number of nodes. Coconut water incorporated medium did not produce any shoot buds. Maximum shoot bud production was observed in MS solid medium without any additives.

Morphological characters of cultures were also different. The shoots and leaves produced in medium containing coconut water were dark purplish green in colour.

4.3.3 Effect of growth regulators and additives on serial subculturing

4.3.3.1 Effect of growth regulators and additives on first subculture

The data on the effect of growth regulators on the first subculture are presented in Table 19a.

Treatments T_2 (BA 1 mg l^{-1}), T_5 (BA 5 mg l^{-1}) and T_{14} (BA 0.25 mg l^{-1} + IAA 0.5 mg l^{-1}) were comparable with respect to the number of nodes (18-21). However, maximum number of nodes (21) could be obtained when T_{14}

Table 17. Effect of varying media concentration on proliferation of nodal segments of *H. annulare*

Treatment No.	Treatment	No. of shoots	No. of shoot buds	Length of the longest shoot (cm)	No. of nodes	Weight of callus (g)
1	MS basal	2.000 (1.414)*	0.000 (0.707)	15.000 (3.871)	4.000 (2.000)	2.500 (1.732)
2	½MS basal	1.687 (1.298)	2.127 (1.621)	7.630 (2.762)	5.860 (2.421)	0.660 (1.077)
3	MS + Activated charcoal 0.1%	1.000 (1.000)	0.000 (0.707)	8.250 (2.866)	4.750 (2.177)	0.007 (0.712)
4	½MS + Activated charcoal 0.1%	1.313 (1.145)	0.500 (0.999)	7.693 (2.774)	6.647 (2.577)	0.423 (0.961)
5	MS + BA 1 mg l^{-1}	4.037 (2.009)	7.033 (2.743)	15.660 (3.942)	10.680 (3.260)	1.550 (1.431)
6	½MS + BA 1 mg l^{-1}	1.450 (1.204)	2.250 (1.658)	13.773 (3.710)	5.550 (2.355)	0.573 (1.036)
7	MS + KIN 1 mg l^{-1}	1.100 (1.048)	0.000 (0.707)	7.850 (2.800)	5.600 (2.366)	0.813 (1.146)
8	½MS + KIN 1 mg l^{-1}	1.250 (1.118)	0.000 (0.707)	6.677 (2.583)	4.783 (2.187)	0.223 (0.850)
9	MS (liquid) + Adenine sulphate 2 mg l^{-1}	1.063 (1.024)	0.500 (0.993)	9.637 (3.104)	6.027 (2.455)	0.013 (0.716)
10	½MS (liquid) + Adenine sulphate 2 mg l^{-1}	1.000 (0.999)	0.000 (0.707)	7.750 (2.783)	4.250 (2.052)	0.000 (0.707)
CD		0.108	0.108	0.295	0.229	0.054
SEm±		0.037	0.037	0.1	0.078	0.018

* Values in paranthesis represent $\sqrt{x}$ transformed ones

Table 18. Effect of different additives on proliferation of nodal segments of *H. annulare*

Treatment No.	Treatment			No. of shoots	No. of shoot buds	Length of the shoot (cm)	No. of nodes	Weight of callus (g)
	Media	Additive	Concentration					
1	½MS	Activated charcoal	0.1%	1.313 (1.145)	0.500 (0.999)	7.693 (2.774)	6.647 (2.577)	0.423 (0.961)
2	MS	Activated charcoal	0.1% (1.000)	1.000 (0.707)	0.000 (2.866)	8.250 (2.177)	4.750 (0.887)	0.343
3	MS+ BA 1 mg l^{-1}	Silver nitrate	5 mg l^{-1}	1.100 (1.047)	0.000 (0.707)	8.450 (2.907)	5.800 (2.408)	1.300 (1.342)
4	MS (liquid) BA 1 mg l^{-1}	Silver nitrate	5 mg l^{-1}	1.710 (1.307)	2.000 (1.566)	7.973 (2.824)	6.550 (2.559)	1.240 (1.315)
5	MS	Coconut water	15%	1.247 (1.116)	0.000 (0.707)	10.000 (3.162)	7.837 (2.790)	1.290 (1.338)
6	MS	Adenine sulphate	2 mg l^{-1}	1.063 (1.024)	0.500 (0.993)	9.637 (3.104)	6.027 (2.455)	0.013 (0.716)
7	½MS (liquid)	Adenine sulphate	2 mg l^{-1}	1.000 (0.999)	0.000 (0.707)	7.750 (2.783)	4.250 (2.052)	0.000 (0.707)
8	MS basal			1.687 (1.298)	2.127 (1.621)	7.630 (2.762)	5.860 (2.421)	0.660 (1.077)
9	MS basal (liquid)			1.237 (1.105)	0.000 (0.707)	9.760 (3.124)	6.230 (2.494)	0.010 (0.714)
10	½MS basal			2.000 (1.414)	0.000 (0.707)	15.000 (3.871)	4.000 (2.000)	2.500 (1.732)
CD				0.143	0.170	0.170	0.229	0.170
SEm ±				0.048	0.058	0.058	0.078	0.058

* Values in paranthesis represent $\sqrt{x}$ transformed ones

Table 19a. Effect of growth regulators on serial subculturing (first subculture) for proliferation of nodal segments of *H. annulare*

Treatment No.	Treatment: Media	Treatment: Growth regulator	Treatment: Concentration	No. of shoots	No. of shoot buds	Length of the longest shoot (cm)	No. of nodes	Weight of callus (g)
1	MS	BA	0.25	3.200 (1.771)*	0.000 (0.707)	3.950 (1.986)	6.400 (2.523)	1.267 (1.101)
2	MS	BA	1.0	6.370 (2.517)	7.500 (2.741)	14.000 (3.739)	20.000 (4.444)	3.033 (1.742)
3	½MS	BA	1.0	1.560 (1.229)	0.333 (0.880)	15.00 (3.865)	5.500 (2.328)	0.020 (0.138)
4	MS	BA	2.5	2.303 (1.516)	1.000 (1.221)	14.300 (3.778)	6.700 (2.585)	1.300 (1.131)
5	MS	BA	5.0	11.140 (3.338)	9.000 (3.077)	5.250 (2.284)	18.140 (4.258)	2.200 (1.470)
6	MS	BA	10.0	4.500 (2.091)	10.200 (3.269)	4.000 (1.994)	3.000 (1.729)	3.400 (1.844)
7	MS	KIN	1.0	1.000 (0.923)	0.000 (0.707)	7.750 (2.776)	3.000 (1.723)	0.750 (0.864)
8	MS	KIN	2.5	1.000 (0.986)	0.000 (0.707)	8.250 (2.860)	3.500 (1.862)	1.127 (1.061)
9	MS	KIN	5.0	1.250 (1.118)	0.000 (0.707)	3.660 (1.902)	5.000 (2.204)	0.017 (0.128)
10	MS	2iP	0.5	1.000 (0.947)	0.000 (0.707)	9.000 (2.979)	4.000 (1.991)	1.500 (1.224)
11	MS	2iP	2.5	1.660 (1.233)	2.000 (1.576)	8.000 (2.822)	8.000 (2.820)	2.000 (1.404)
12	MS	2iP	10.0	1.000 (0.930)	0.000 (0.707)	7.000 (2.635)	4.040 (2.010)	3.000 (1.722)
13	MS	BA + NAA	0.25 + 0.5	2.000 (1.311)	0.000 (0.707)	10.000 (3.158)	7.000 (2.638)	1.750 (1.322)
14	MS	BA + IAA	0.25 + 0.5	11.600 (3.405)	5.300 (2.407)	8.000 (2.819)	21.00 (4.564)	1.107 (1.019)
15	MS	BA + IAA	0.5 + 1.0	9.500 (3.078)	0.333 (0.880)	12.000 (3.456)	14.500 (3.807)	1.700 (1.301)
16	MS	BA + IAA	1.0 + 1.0	8.250 (2.839)	0.667 (0.998)	14.480 (3.804)	12.00 (3.463)	1.500 (1.223)
17	MS	BA + IAA	2.5 + 1.0	9.000 (2.972)	12.000 (3.531)	7.000 (2.641)	8.700 (2.949)	1.500 (1.222)
18	MS	KIN + IAA	1.0 + 1.0	5.000 (2.229)	1.000 (1.224)	10.000 (3.155)	11.300 (3.356)	3.160 (1.777)
19	MS	KIN + IAA	2.5 + 1.0	3.500 (1.870)	0.000 (0.707)	4.750 (2.178)	4.000 (1.948)	1.500 (1.222)
20	½MS basal			2.010 (1.415)	0.000 (0.707)	15.000 (3.863)	4.560 (2.131)	2.500 (1.580)
CD				0.564	0.427	0.414	0.492	0.239
SE				0.197	0.149	0.145	0.172	0.084

* Values in paranthesis represent $\sqrt{x}$ transformed ones

was used. Lowest number of nodes were obtained when either T_7 (KIN 1 mg l^{-1}) or T_6 (BA 10 mg l^{-1}) was incorporated.

Maximum number of well formed shoots were produced in treatments T_{14} (BA 0.25 + IAA 0.5 mg l^{-1}) and T_5 (BA 5 mg l^{-1}) ie. 11-12 and the minimum in treatments T_7, T_8 (KIN 1.0 and 2.5 mg l^{-1} respectively), T_{10} and T_{12} (2iP 0.5 and 10 mg l^{-1} respectively) all of them giving only a single shoot per culture tube on an average. Maximum number of shoot forming buds were produced in the treatment T_{17} (BA 2.5 + IAA 1 mg l^{-1}) ie. 12 followed by T_6 (BA 10 mg l^{-1}) giving 10 buds and T_5 (BA 5.0 mg l^{-1}) 9 buds. When the number of shoots and shoot forming buds were taken together, the maximum number was observed in T_{17} (21) closely followed by T_5 (20).

The length of the shoot was maximum for treatment T_3 and T_{20} (half strength MS supplemented with 1 mg l^{-1}, BA and half strength basal MS respectively ie. 15 cm) and minimum for treatment T_9 (KIN 5 mg l^{-1}).

When the concentration of BA was very high, it produced very short and thin shoots. When treatments T_{16} (BA and IAA each at 1 mg l^{-1}) was compared with T_{18} (KIN and IAA each at 1 mg l^{-1}), it was observed that the number of shoots, buds nodes and the length of the shoot were much higher in medium containing BA. MS medium with half the salt concentration produced very lanky shoots with longer internodes and a few number of nodes. No shoot bud production was observed.

The data on the effect of additives on the first subculture is presented in Table 19b. Silver nitrate did not act as an inhibitor when added to stationery liquid

Table 19b. Effect of additives on serial subculturing (first subculture) for proliferation of nodal segments of *H. annulare*

Treatment No.	Treatment			No. of shoots	No. of shoot buds	Length of the longest shoot (cm)	No. of nodes	Weight of callus (g)
	Media	Additive	Concentration					
1	½MS	Activated charcoal	0.1%	1.000	0.000	9.000	5.000	0.020
2	MS (liquid) + BA 1 mg l^{-1}	Silver nitrate	5 mg l^{-1}	2.000	1.500	9.830	8.000	1.330
3	½MS basal			2.010	0.000	15.001	4.56	2.500

MS medium supplemented with BA 1 mg l^{-1}. The action of the cytokinin was prominent in producing long shoots, large number of nodes and shoot buds. Without any additive, MS with half the concentration of salts produced lanky shoots (15 cm). When activated charcoal was also incorporated into this medium, shoots were shorter (9 cm) and robust and callus formation was greately reduced (0.02 g). However, the number of shoots produced also was reduced to half.

4.3.3.2 Effect of growth regulators and additives on second subculture

The data on the effect of growth regulators on second subculture are presented in Table 20a. Among the different treatments tried T_5 (BA 10 mg l^{-1}) was found superior to others for obtaining more number of nodes (25). Minimum nodes were obtained from cultures receiving T_6 (Half strength MS supplemented with BA 1 mg l^{-1}) T_8 (KIN 1 mg l^{-1}) and T_{18} (BA 0.5 mg l^{-1} + NAA 1 mg l^{-1}) which were comparable with respect to the number of nodes (4-5). Maximum number of shoots and buds were also obtained when BA was incorporated at a concentration of 10 mg l^{-1}. But the length of individual shoots was less and they were thin and weak. However, the number of nodes that could be obtained per culture was high (25) and the buds also (16) could be subcultured for further proliferation. BA-IAA/NAA combinations expressed moderate performance. BA when incorporated to full strength MS at 1 mg l^{-1} concentration produced more number of longer shoots and more of shoot buds as well as more number of nodes but when salt concentration was decreased to half but with BA at 1 mg l^{-1}, the number of shoots decreased drastically and multiple shoot buds were not observed. However the shoots were very lanky. When the salt concentration was reduced to half but with KIN at 1 mg l^{-1}, the shoot length was reduced drastically.

Table 20a. Effect of growth regulators on serial subculturing (second subculture) for proliferation of nodal segments of *H. annulare*

Treatment No.	Treatment: Media	Treatment: Growth regulator	Treatment: Concentration	No. of shoots	No. of shoot buds	Length of the longest shoot (cm)	No. of nodes	Weight of callus (g)
1	MS	BA	0.5	1.800 (1.341)*	2.000 (1.576)	13.300 (3.647)	7.800 (2.792)	1.310 (1.144)
2	MS	BA	1.0	5.800 (2.408)	5.600 (2.470)	19.000 (4.358)	15.000 (3.923)	2.400 (1.549)
3	MS	BA	2.5	2.750 (1.656)	4.250 (2.179)	9.000 (2.999)	7.000 (2.645)	2.377 (1.541)
4	MS	BA	5.0	5.400 (2.320)	4.300 (2.191)	8.200 (2.860)	11.00 (3.309)	2.800 (1.670)
5	MS	BA	10.0	14.520 (3.803)	16.000 (4.062)	2.500 (1.571)	25.000 (4.998)	3.750 (1.934)
6	½MS	BA	1.0	0.935 (0.974)	0.000 (0.707)	16.00 (3.999)	4.670 (2.160)	0.283 (0.531)
7	MS	KIN	0.5	1.800 (1.331)	3.000 (1.869)	10.467 (3.231)	8.400 (2.897)	0.443 (0.642)
8	MS	KIN	1.0	1.200 (1.094)	0.000 (0.707)	10.000 (3.132)	5.000 (2.197)	0.013 (0.114)
9	MS	KIN	2.5	1.000 (0.995)	0.000 (0.707)	11.900 (3.444)	7.700 (2.775)	0.500 (0.698)
10	MS	KIN	5.0	2.000 (1.409)	1.250 (1.322)	13.330 (3.650)	10.000 (3.140)	0.580 (0.759)
11	½MS	KIN	1.0	1.000 (0.991)	0.000 (0.707)	6.010 (2.448)	4.670 (2.159)	0.283 (0.532)
12	MS	2iP	1.0	4.000 (1.995)	5.000 (2.342)	12.000 (3.464)	7.000 (2.644)	1.000 (0.999)
13	MS	2iP	2.5	1.000 (0.973)	0.000 (0.707)	8.500 (2.914)	6.000 (2.447)	1.417 (1.189)
14	MS	2iP	10.0	2.660 (1.628)	0.000 (0.707)	15.017 (3.872)	6.000 (2.449)	3.160 (1.776)
15	MS	BA + IAA	0.25 + 0.5	2.000 (1.307)	0.000 (0.707)	5.000 (2.234)	8.033 (2.831)	1.020 (1.005)
16	MS	BA + IAA	0.5 + 1.0	4.600 (2.119)	0.000 (0.707)	8.000 (2.828)	10.000 (3.158)	1.420 (1.190)
17	MS	BA + IAA	0.5 + 2.0	6.590 (2.567)	4.500 (2.236)	7.660 (2.766)	12.717 (3.566)	2.300 (1.515)
18	MS	BA + NAA	0.5 + 1.0	3.500 (1.867)	2.000 (1.577)	4.500 (2.121)	4.250 (2.061)	1.867 (1.352)
19	MS	KIN + IAA	1.0 + 1.0 1.0	4.003 (1.996)	0.000 (0.707)	13.000 (3.604)	12.000 (3.455)	2.000 (1.276)
20	MS basal (liquid)			1.110 (1.052)	0.000 (0.707)	13.330 (3.650)	7.660 (2.766)	1.010 (0.100)
CD				0.373	0.104	0.295	0.338	0.322
SEm ±				0.131	0.036	0.103	0.118	0.113

* Values in paranthesis represents $\sqrt{x}$ transformed ones

Among the various additives added to full and half the salt concentration of MS, activated charcoal was found to produce more shoots and buds. The length was not that high, but the number of nodes was higher. When adenine sulphate in liquid MS at full and half the salt concentration was used the callus growth rate was found to be very much reduced (Table 20b).

4.3.3.3 Effect of growth regulators and additives on third subculture

The data on the effect of growth regulators and additives on third subculture are presented in Tables and 21a and 21b respectively.

Among the different cytokinins tried, maximum shoot and bud production and higher number of nodes were obtained when treatments involving BA was incorporated into MS media. Treatments T_5 (10 mg l^{-1} of BA) produced maximum shoots and buds but they were highly reduced in size that the effective number of nodes obtained was less. However these could be cut and subcultured as such into a fresh media with reduced cytokinin or to a basal medium. This treatment was followed by T_6 (20 mg l^{-1} of BA) for maximum shoot and bud production. But more number of well formed nodes were obtained when treatment T_4 was given (BA 5 mg l^{-1}) with moderate shoot and bud production. However, in all the above mentioned treatments, the callus production was very profuse. Callusing was lesser when KIN was used as cytokinin.

Silver nitrate when added to stationary liquid MS medium supplemented with BA at 1 mg l^{-1} proved better than others with respect to the length of the shoots and number of nodes. But in solid MS medium with the same additive, an inhibitory

Table 20b. Effect of additives on serial subculturing (second subculture) for proliferation of nodal segments of *H. annulare*

Treatment No.	Treatment			No. of shoots	No. of shoot buds	Length of the longest shoot (cm)	No. of nodes	Weight of callus (g)
	Media	Additive	Concentration					
1	½MS	Activated charcoal	0.1%	2.250 (1.500)*	2.000 (1.579)	9.250 (3.038)	13.250 (3.638)	1.377 (1.369)
2	MS (liquid)+ BA 1 mg l^{-1}	Silver nitrate	5 mg l^{-1}	1.660 (1.282)	2.000 (1.578)	8.660 (2.942)	7.250 (2.691)	1.500 (1.412)
3	MS (liquid)	Adenine sulphate	2 mg l^{-1}	1.127 (1.061)	1.000 (1.223)	8.710 (2.951)	6.220 (2.494)	0.003 (0.709)
4	½MS (liquid)	Adenine sulphate	2 mg l^{-1}	1.000 (0.876)	0.000 (0.707)	6.000 (2.443)	5.000 (2.236)	0.000 (0.707)
5	MS	Coconut water	15%	1.000 (0.976)	0.000 (0.707)	10.990 (3.314)	5.670 (2.380)	1.330 (1.353)
6	MS (liquid) basal			1.110 (1.052)	0.000 (0.707)	13.330 (3.650)	7.660 (2.766)	0.007 (0.712)
CD				0.487	0.126	0.239	0.169	0.079
SEm±				0.158	0.041	0.078	0.055	0.026

* Values in paranthesis represents $\sqrt{x}$ transformed ones

Table 21a. Effect of growth regulators on serial subculturing (third subculture) for the proliferation of nodal segments of *H. annulare*

Treatment No.	Treatment: Media	Treatment: Growth regulator	Treatment: Concentration	No. of shoots	No. of shoot buds	Length of the lonegest shoot (cm)	No. of nodes	Weight of the callus (g)
1	MS	BA	0.25	3.000 (1.708)	0.000 (0.707)	7.000 (2.641)	4.500 (2.088)	2.840 (1.643)
2	MS	BA	0.5	5.080 (2.254)	10.300 (3.275)	8.980 (2.996)	12.327 (3.498)	2.400 (1.507)
3	MS	BA	2.5	3.127 (1.763)	6.500 (2.644)	9.000 (2.992)	9.800 (3.120)	2.660 (1.576)
4	MS	BA	5.0	5.500 (2.345)	6.000 (2.548)	7.000 (2.634)	14.500 (3.801)	2.820 (1.669)
5	MS	BA	10.0	12.660 (3.556)	28.600 (5.389)	1.660 (1.287)	5.267 (2.294)	3.830 (1.933)
6	MS	BA	20.0	16.000 (3.999)	10.000 (3.223)	7.000 (2.643)	10.000 (3.154)	2.000 (1.311)
7	½MS	BA	1.0	1.860 (1.360)	1.000 (1.200)	16.640 (4.077)	5.140 (2.267)	1.230 (1.053)
8	MS	KIN	0.25	3.000 (1.706)	0.000 (0.707)	7.250 (2.692)	8.000 (2.804)	0.027 (0.161)
9	MS	KIN	0.5	1.377 (1.173)	4.300 (2.191)	5.187 (2.276)	5.500 (2.345)	1.140 (1.067)
10	MS	KIN	1.0	1.807 (1.344)	2.517 (1.737)	7.200 (2.678)	6.717 (2.591)	0.700 (0.837)
11	½MS	KIN	1.0	1.000 (0.991)	0.000 (0.707)	7.377 (2.697)	6.000 (2.401)	0.630 (0.794)
12	MS	KIN	2.0	2.500 (1.580)	0.000 (0.707)	7.000 (2.616)	11.500 (3.388)	0.750 (0.861)
13	MS	KIN	2.5	4.000 (1.9720	0.000 (0.707)	11.500 (3.388)	10.000 (3.080)	2.500 (1.580)
14	MS	KIN	5.0	2.000 (1.399)	1.500 (1.413)	8.000 (2.812)	4.000 (1.937)	0.020 (0.138)
15	MS	2iP	0.25	2.300 (1.479)	3.500 (1.994)	8.660 (2.939)	5.660 (2.379)	2.000 (1.408)
16	MS	2iP	2.5	1.000 (0.930)	0.000 (0.707)	8.500 (2.911)	5.000 (2.197)	2.000 (1.408)
17	MS	2iP	5.0	2.400 1.549)	1.500 (1.413)	5.250 (2.282)	5.000 (2.136)	1.423 (1.190)
18	MS	BA + NAA	0.25 + 0.5	2.000 (1.414)	0.000 (0.707)	8.000 (2.818)	8.000 (2.824)	1.750 (1.314)
19	MS	BA + NAA	0.5 + 1.0	1.500 (1.223)	1.500 (1.414)	6.500 (2.500)	7.000 (2.593)	3.400 (1.816)
20	MS	BA + NAA	0.5 + 2.0	1.693 (1.301)	0.000 (0.707)	1.660 (1.288)	2.330 (1.524)	2.003 (1.412)
21	MS	BA + IAA	0.5 + 2.0	4.000 (1.992)	0.000 (0.707)	3.500 (1.863)	8.950 (2.957)	1.750 (1.322)
22	MS	KIN + IAA	2.5 + 1.0	2.200 (1.403)	1.000 (1.221)	23.250 (4.821)	6.400 (2.530)	1.010 (1.005)
23	MS basal			1.750 (1.322)	0.000 (0.707)	7.253 (2.693)	7.250 (2.688)	0.003 (0.033)
CD				0.350	0.250	0.427	0.694	0.455
SE½				0.123	0.070	0.150	0.243	0.159

* Values in paranthesis represent $\sqrt{x}$ transformed ones

Table 21b. Effect of additives on serial subculturing (third subculture) for proliferation of nodal segments of *H. annulare*

Treatment No.	Treatment			No. of shoots	Length of the longest shoot (cm)	No. of nodes	Weight of callus (g)
	Media	Additive	Concent-ration**				
1	MS	Activated charcoal	0.1%	0.967 (0.980)*	9.500 (3.061)	5.000 (2.234)	0.000 (0.707)
2	½MS	Activated charcoal	0.1%	1.000 (0.967)	7.200 (2.683)	5.000 (2.233)	0.300 (0.894)
3	MS (liquid) + BA 1.0 mg l^{-1}	Silver nitrate	5 mg l^{-1}	2.587 (1.607)	9.900 (3.121)	5.600 (2.324)	1.600 (1.449)
4	MS + BA 1.0 mg l^{-1}	Silver nitrate	5 mg l^{-1}	1.000 (1.059)	2.000 (1.403)	4.660 (1.408)	1.000 (1.221)
5	½MS (liquid)	Adenine sulphate	2 mg l^{-1}	1.000 (0.983)	5.330 (2.309)	4.660 (2.099)	0.000 (0.707)
6	MS basal			1.750 (1.322)	7.253 (2.693)	7.250 (2.688)	0.003 (0.709)
CD				0.386	0.503	0.639	0.080
SEm±				0.125	0.163	0.207	0.026

* Values in paranthesis represent $\sqrt{x}$ transformed ones
** None of the treatments produced multiple shoot buds

effect was observed in that the length of the shoots and number of nodes were very much reduced. Activated charcoal added to full MS did not produce any callus at all.

4.3.3.4 Effect of growth regulators and additives on fourth subculture

The data on the effect of growth regulators and additives on fourth subculture are presented in Tables 22a and 22b respectively.

Treatments involving BA as the cytokinin produced maximum number of shoots, buds and nodes per culture. With increased concentration of BA, there was an increase in shoot bud and node production but the length of the shoots was very much reduced. At very low concentrations (BA 1 mg l^{-1}), the shoots were very lanky with longer internodes. This effect was similar to that observed in basal MS medium without any growth regulators. The callus production was practically nil in the basal medium.

Among the additives used, coconut water proved better than others with respect to the number of nodes. The leaves of the shoots were purplish green. When activated charcoal was added to the medium, callus growth was very little.

4.3.3.5 Effect of growth regulators and additives on fifth subculture

The data on the effect of growth regulators and additives on fifth subculture are presented in Tables 23a and 23b respectively.

Of the various treatments, those with BA at high concentrations of 10 mg l^{-1} resulted in more number of nodes (11.75) than other treatments. But the average length of shoot was very less (1.8 cm). When a combination of BA and IAA

Table 22a. Effect of growth regulators on serial subculturing (fourth subculture) for proliferation of nodal segments of *H. annulare*

Treatment No.	Treatment			No. of shoot	No. of shoot buds	Length of the longest shoot (cm)	No. of nodes	Weight of callus (g)
	Media	Growth regulator	Concentration (mg l^{-1})					
1	MS	BA	1.0	2.000 (1.180)*	0.000 (0.707)	12.877 (3.588)	4.493 (2.116)	0.013 (0.716)
2	MS	BA	2.5	5.000 (1.494)	12.000 (3.517)	3.000 (1.730)	10.000 (3.156)	5.000 (2.338)
3	MS	BA	5.0	5.000 (1.485)	5.000 (2.340)	5.000 (2.233)	12.030 (3.461)	3.000 (1.870)
4	MS	BA	20.0	16.000 (1.990)	15.000 (3.919)	2.000 (1.407)	25.000 (4.999)	2.000 (1.560)
5	MS	KIN	1.0	1.500 (1.106)	0.000 (0.707)	5.750 (2.396)	6.500 (2.549)	2.000 (1.574)
6	MS	KIN	1.0	2.000 (1.183)	0.000 (0.707)	3.000 (1.726)	3.000 (1.731)	0.050 (0.742)
7	MS	NAA	1.0	2.000 (1.187)	0.000 (0.707)	7.560 (2.749)	9.000 (2.997)	0.000 (0.707)
8	MS	BA+ IAA	1.0+ 1.0	2.000 (1.185)	0.000 (0.707)	10.500 (3.232)	4.000 (1.990)	0.000 (0.707)
9	MS	BA+ IAA	2.5+ 1.0	1.000 (0.968)	8.500 (3.000)	8.750 (2.958)	4.000 (1.999)	0.837 (1.156)
10	MS	KIN+ IAA	2.5+ 1.0	2.000 (1.175)	0.000 (0.707)	4.750 (2.179)	4.500 (2.119)	1.500 (1.414)
11	MS basal			1.000 (0.963)	0.000 (0.707)	12.330 (3.511)	5.033 (2.243)	0.000 (0.707)
12	MS basal (liquid)			1.600 (1.122)	0.000 (0.707)	7.093 (2.663)	5.690 (2.384)	0.000 (0.707)
CD				0.192	0.329	0.213	0.267	0.213
SEm±				0.066	0.113	0.073	0.092	0.073

* Values in paranthesis represent $\sqrt{x}$ transformed ones

Table 22b. Effect of additives on serial subculturing (fourth subculture) for proliferation of nodal segments of *H. annulare*

Treatment No.	Treatment**			No. of shoots	Length of the longest shoot (cm)	No. of nodes	Weight of callus (g)
	Media	Additive	Concentration				
1	MS	Activated charcoal	0.1%	1.000 (0.970)*	7.000 (2.643)	4.500 (2.121)	0.010 (0.714)
2	½MS	Activated charcoal	0.1%	1.000 (0.983)	5.330 (2.287)	3.330 (1.823)	0.000 (0.707)
3	MS (liquid) + BA 1.0 mg l^{-1}	Silver nitrate	5 mg l^{-1}	1.143 (1.066)	9.5000 (3.082)	6.127 (2.475)	0.740 (1.113)
4	MS (liquid)	Adenine sulphate	2 mg l^{-1}	1.000 (1.000)	10.500 (3.239)	5.830 (2.386)	0.017 (0.719)
5	½MS (liquid)	Adenine sulphate	2 mg l^{-1}	1.000 (0.992)	10.660 (3.232)	4.330 (2.080)	0.000 (0.707)
6	MS	Coconut water	15%	1.500 (1.219)	8.993 (2.998)	10.000 (3.128)	1.250 (1.322)
7	MS basal			1.000 (0.952)	12.330 (3.511)	5.033 (2.243)	0.000 (0.707)
8	MS basal (liquid)			1.600 (1.261)	6.960 (2.638)	5.690 (2.384)	0.000 (0.707)
CD				0.363	0.441	0.458	0.000
SEm±				0.121	0.147	0.153	0.000

* Values in paranthesis represent $\sqrt{x}$ transformed ones
** None of the treatments produced multiple shoot buds

Table 23a. Effect of growth regulators on serial subculturing (fifth subculture) for proliferation of nodal segments of *H. annulare*

Treatment No.	Treatment			No. of shoots	No. of shoot buds	Length of the longest shoot (cm)	No. of nodes	Weight of callus (g)
	Media	Growth regulator	Concentration (mg l^{-1})					
1	MS	BA	1.0	5.300 (1.816)*	0.000 (0.707)	16.330 (4.041)	6.000 (2.448)	1.167 (1.291)
2	½MS	BA	1.0	1.000 (0.976)	0.000 (0.707)	10.000 (3.135)	4.250 (2.061)	0.263 (.874)
3	MS	BA+ IAA	1.0+ 1.0	3.000 (1.730)	0.000 (0.707)	10.000 (3.161)	10.000 (3.140)	1.000 (1.200)
4	MS	BA+ IAA	2.5+ 1.0	2.000 (1.408)	0.660 (1.077)	9.083 (3.004)	3.660 (1.911)	0.080 (0.762)
5	MS	BA	10.0	8.500 (2.915)	8.750 (3.041)	1.800 (1.342)	11.750 (3.426)	3.750 (2.059)
6	MS	KIN	0.5	2.250 (1.500)	0.250 (0.866)	6.750 (2.597)	6.250 (2.500)	0.760 (1.122)
7	MS	KIN	1.0	1.000 (0.988)	0.000 (0.707)	6.900 (2.625)	7.500 (2.739)	0.750 (1.118)
8	½MS	KIN	1.0	1.250 (1.084)	0.000 (0.707)	9.500 (3.075)	5.250 (2.284)	0.140 (0.800)
9	MS	KIN+ IAA	1.0+ 1.0	1.400 (1.182)	0.000 (0.707)	10.250 (3.198)	8.000 (2.825)	0.360 (0.927)
10	MS basal			1.330 (1.153)	0.000 (0.707)	6.570 (2.563)	5.860 (2.418)	0.170 (0.818)
11	MS basal			1.000 (1.995)	0.000 (0.707)	9.000 (2.998)	5.330 (2.309)	0.003 (0.709)
CD				0.268	0.000	0.359	0.303	0.435
SEm±				0.091	0.000	0.122	0.103	0.148

* Values in paranthesis represent $\sqrt{x}$ transformed ones

Table 23b. Effect of additives on serial subculturing (fifth subculture) for proliferation of nodal segments of *H. annulare*

Treatment No.	Treatment			No. of shoots	Length of the longest shoot (cm)	No of nodes	Weight of callus (g)
	Media	Additive	Concentration				
1	MS + BA 1 mg l^{-1}	Silver nitrate	5 mg l^{-1}	1.000 (0.983)*	7.000 (2.646)	6.000 (2.449)	1.000 (1.218)
2	MS (liquid) + BA 1 mg l^{-1}	Silver nitrate	5 mg l^{-1}	1.750 (1.322)	9.877 (3.142)	9.627 (3.103)	1.627 (1.450)
3	½MS (liquid)	Adenine sulphate	2 mg l^{-1}	1.000 (0.939)	9.150 (3.025)	3.000 (1.731)	0.000 (0.707)
4	½MS	Activated charcoal	0.1%	2.000 (1.412)	8.000 (2.862)	10.000 (3.145)	0.173 (0.821)
5	MS basal			1.330 (1.153)	6.570 (2.563)	5.860 (2.418)	0.187 (0.828)
6	MS basal (liquid)			1.000 (0.995)	9.000 (2.998)	5.330 (2.309)	0.003 (0.709)
CD				0.369	0.159	0.313	0.126
SEm±				0.120	0.052	0.102	0.041

* Values in paranthesis indicates $\sqrt{x}$ transformed ones

each at 1 mg l^{-1} was used, long shoots (10 cm) with more number of nodes (10) were obtained. The number of shoots and shoot buds were lesser in the cultures receiving this treatment but higher number of shoots and buds were observed in treatments containing BA at higher concentration of 10 mg l^{-1} but the callus formation was also higher. When the MS salt concentration was reduced to half and with BA at 1 mg l^{-1}, only a single shoot was released with reduced number of nodes. When 1 mg l^{-1} of KIN was incorporated into half the salt concentration of MS, it produced more lanky shoots. The best combination of growth regulator involving KIN was that with IAA each at a concentration of 1 mg l^{-1}.

Stationary liquid MS medium with silver nitrate and BA at 1 mg l^{-1} produced better shoot growth than other treatments.

If the best treatment selected in each subculture is given to cultures in sequence, a maximum of 2,37,03,423 nodes could be obtained in 225 days starting from a single nodal segment.

The maximum number of nodes that could be obtained in various media from a single nodal explant at the end of the first subculture was (148) in treatment 6 followed by treatment 4. The number of nodes was minimum in media incorporated with silver nitrate (treatment T_8, 10 nodes) (Table 24a).

At the end of second subculture, among various treatments tried, T_{26} was superior giving an average of 1600 nodes. Least responsive was treatment T_{16} (Table 24b).

Treatment T_8 proved better (27,766) than others for maximum node production at the end of third subculture. Minimum number (340) was obtained in

Table 24a. Effect of serial subculturing (first subculture) on number of nodes produced from *H. annulare* in different media

Treatment No.	Treatment	No. of nodes
1	2	3
1	M_{48} M_{56}	32.200 (5.617)*
2	M_{11} M_{79}	26.200 (5.109)
3	M_4 M_2	42.720 (6.536)
4	M_4 M_4	113.393 (10.645)
5	M_{11} M_{11}	27.000 (5.189)
6	M_4 M_8	148.560 (12.126)
7	M_4 M_{47}	48.060 (6.888)
8	M_{75} M_{79}	10.910 (3.303)
9	M_{47} M_{63}	69.340 (8.322)
10	M_{47} M_{12}	35.830 (5.976)
11	M_{47} M_4	60.450 (7.768)
12	M_4 M_{58}	53.000 (7.279)
13	M_{13} M_{15}	25.320 (5.026)

Contd.

Table 24a. Continued

1	2	3
14	M_8 M_{52}	104.133 (10.186)
15	M_8 M_{50}	52.500 (7.241)
16	M_4 M_{55}	113.953 (10.667)
17	M_4 M_7	89.447 (9.448)
18	M_{46} M_{10}	18.670 (4.270)
19	M_9 M_{70}	25.620 (5.057)
20	M_{47} M_{13}	22.663 (4.612)
21	M_{13} M_3	23.443 (4.839)
22	M_{13} M_{74}	26.500 (5.136)
23	M_{13} M_{79}	26.200 (5.103)
24	M_{10} M_{87}	43.330 (6.581)
25	M_{10} M_{96}	49.540 (7.032)
26	M_2 M_{96}	36.260 (6.011)
27	M_{10} M_{94}	27.650 (5.229)
28	M_{10} M_{93}	19.430 (4.384)

Contd.

Table 24a. Continued

1	2	3
29	M_{10} M_4	53.400 (7.298)
30	M_{13} M_{79}	26.200 (5.093)
31	M_{79} M_{95}	12.930 (3.587)
32	M_{13} M_{91}	24.100 (4.898)
33	M_{76} M_{79}	21.830 (4.632)
CD		0.991
SEm±		0.351

* Values in paranthesis represent $\sqrt{x}$ transformed ones

Table 24b. Effect of serial subculturing (second subculture) on number of nodes produced from nodal segments of *H. annulare* in different media

Treatment No.	Treatment	No. of nodes
1	2	3
1	M_4 M_4 M_4	1218.19 (34.854)*
2	M_4 M_7 M_6	898.920 (29.855)
3	M_4 M_7 M_{12}	566.190 (23.775)
4	M_4 M_7 M_{63}	1095.70 (33.083)
5	M_4 M_7 M_{64}	970.480 (31.132)
6	M_4 M_4 M_8	1586.61 (39.520)
7	M_{11} M_{11} M_{92}	168.210 (12.967)
8	M_{11} M_{79} M_{95}	145.360 (12.054)
9	M_{11} M_{79} M_{56}	260.960 (16.127)
10	M_{11} M_{79} M_{90}	125.340 (11.189)
11	M_4 M_{47} M_{56}	478.680 (21.874)
12	M_4 M_{47} M_8	668.030 (25.887)
13	M_4 M_{47} M_{14}	336.420 (18.320)
14	M_{10} M_{71} M_{74}	157.820 (12.558)

Contd.

Table 24b. Continued

1	2	3
15	M_{75} M_{79} M_{56}	108.690 (10.421)
16	M_{75} M_{79} M_{90}	51.533 (7.174)
17	M_{75} M_{79} M_{95}	60.540 (7.780)
18	M_4 M_4 M_{11}	758.430 (27.538)
19	M_4 M_4 M_{63}	1376.41 (37.057)
20	M_4 M_4 M_{12}	711.240 (26.668)
21	M_{47} M_{12} M_{13}	143.310 (11.961)
22	M_4 M_{58} M_{52}	768.500 (27.721)
23	M_8 M_{52} M_7	850.060 (29.155)
24	M_4 M_4 M_4	1191.02 (34.510)
25	M_4 M_8 M_7	1234.86 (35.139)
26	M_4 M_8 M_{64}	1599.79 (39.970)
27	M_4 M_8 M_{15}	933.330 (30.545)
28	M_4 M_8 M_{30}	829.390 (28.799)
29	M_4 M_7 M_{64}	970.480 (31.150)

Contd.

Table 24b. Continued

1	2	3
30	M_4 M_7 M_{15}	566.200 (23.784)
31	M_4 M_7 M_{30}	503.130 (22.429)
32	M_4 M_7 M_{63}	1095.87 (33.103)
33	M_{53} M_{74} M_{10}	235.660 (15.335)
34	M_{53} M_{74} M_{91}	253.550 (15.919)
35	M_9 M_{56} M_{83}	154.840 (12.437)
36	M_{13} M_{79} M_{90}	125.340 (11.194)
37	M_{10} M_{87} M_{96}	429.240 (20.717)
38	M_4 M_{47} M_{79}	312.440 (17.675)
39	M_{76} M_{79} M_{95}	121.120 (11.005)
CD		2.343
SEm±		0.831

* Values in paranthesis represent $\sqrt{x}$ transformed ones

cultures receiving treatment T_{49} (Table 24c). The data with respect to the maximum number of nodes obtained at the end of fourth subculture are presented in Table 24d. Among the twenty six treatments tried, maximum number of nodes were obtained in treatment T_1 (4,85,899). Least response was in T_{25} (3,750).

At the end of fifth subculture, T_8 proved to be the best (33,56,744) among the various treatments tried. Minimum number was observed for treatment T_{25} (32,016) (Table 24e).

4.4 Induction of rooting

4.4.1 *In vitro* rooting

The data relating to the experiment on induction of rooting are presented in Table 25a, 25b, 26 and 27.

4.4.1.1 Percentage of rooting

The percentage of root initiation differed among the treatments tried as represented in Table 25a.

Of the different concentrations of NAA and IBA and treatments involving activated charcoal tried, T_7 (full strength MS medium without any growth regulator) proved far superior to others with respect to the percentage of rooting of cultures (90%). This was followed by T_2 (NAA 0.5 mg l^{-1}), T_5 (IBA 0.5 mg l^{-1}) and T_{10} (pulse treatment with IBA 1000 mg l^{-1} followed by inoculation in basal MS medium) giving seventy per cent rooting of cultures. Minimum percentage of rooting was observed in culture receiving treatments T_8, T_9 (Full and half strength MS incorporated with activated charcoal) and T_{11} (pulse treatment with 1000 mg l^{-1}

Table 24c. Effect of serial subculturing (third subculture) on number of nodes produced from nodal segments of *H. annulare* in different media

Treatment No.	Treatment	No. of nodes
1	2	3
1	M_4 M_2 M_{58} M_9	1434.67 (37.863)*
2	M_4 M_2 M_{58} M_{48}	1708.80 (41.334)
3	M_4 M_2 M_{58} M_8	2971.20 (54.327)
4	M_4 M_4 M_8 M_6	15945.4 (126.253)
5	M_4 M_4 M_8 M_{64}	17214.7 (131.202)
6	M_4 M_4 M_8 M_7	13287.8 (115.272)
7	M_4 M_4 M_8 M_{11}	10709.6 (103.451)
8	M_4 M_4 M_8 M_{65}	27765.6 (166.616)
9	M_4 M_4 M_8 M_{30}	8924.67 (94.470)
10	M_4 M_4 M_8 M_{62}	3696.79 (60.785)
11	M_4 M_4 M_8 M_{48}	12692.9 (112.658)
12	M_4 M_4 M_8 M_{47}	7169.74 (84.559)
13	M_4 M_4 M_8 M_9	10630.3 (103.078)
14	M_4 M_4 M_8 M_{12}	10043.2 (100.213)

Contd.

Table 24c. Continued

1	2	3
15	$M_4\ M_4\ M_4\ M_{11}$	8222.78 (90.679)
16	$M_4\ M_4\ M_4\ M_7$	10202.3 (101.001)
17	$M_4\ M_4\ M_4\ M_{50}$	9136.40 (95.584)
18	$M_4\ M_4\ M_4\ M_9$	8182.07 (90.455)
19	$M_4\ M_4\ M_4\ M_{12}$	7711.20 (87.752)
20	$M_4\ M_4\ M_4\ M_{16}$	14009.1 (118.360)
21	$M_4\ M_4\ M_4\ M_{71}$	5786.39 (76.068)
22	$M_4\ M_4\ M_4\ M_{56}$	12133.2 (110.150)
23	$M_4\ M_4\ M_4\ M_{49}$	6894.92 (83.032)
24	$M_4\ M_4\ M_4\ M_{64}$	13145.7 (114.651)
25	$M_4\ M_4\ M_4\ M_{15}$	7711.14 (87.809)
26	$M_4\ M_4\ M_4\ M_7$	10202.3 (101.005)
27	$M_4\ M_4\ M_4\ M_6$	12242.8 (110.647)
28	$M_4\ M_4\ M_4\ M_{62}$	2838.39 (53.267)
29	$M_4\ M_7\ M_6\ M_{10}$	5033.96 (70.950)

Contd.

Table 24c. Continued

1	2	3
30	$M_4\ M_7\ M_6\ M_{15}$	5690.18 (75.432)
31	$M_4\ M_7\ M_6\ M_{16}$	4494.65 (67.042)
32	$M_4\ M_7\ M_6\ M_{70}$	6292.45 (79.323)
33	$M_4\ M_7\ M_6\ M_{92}$	5600.28 (74.834)
34	$M_4\ M_7\ M_{62}\ M_{79}$	3708.52 (60.887)
35	$M_4\ M_7\ M_{12}\ M_3$	3319.86 (57.599)
36	$M_4\ M_7\ M_9\ M_{71}$	2686.97 (51.792)
37	$M_4\ M_7\ M_{64}\ M_{71}$	4628.77 (68.029)
38	$M_4\ M_7\ M_9\ M_{71}$	2853.64 (53.387)
39	$M_4\ M_7\ M_{63}\ M_3$	6420.81 (80.128)
40	$M_4\ M_7\ M_{63}\ M_7$	9176.68 (95.795)
41	$M_{11}\ M_{11}\ M_{92}\ M_{10}$	971.980 (31.176)
42	$M_{11}\ M_{11}\ M_{92}\ M_{78}$	975.620 (31.232)
43	$M_{11}\ M_{79}\ M_{95}\ M_{10}$	814.020 (28.528)
44	$M_4\ M_{47}\ M_{56}\ M_8$	6658.41 (81.567)

Contd.

Table 24c. Continued

1	2	3
45	$M_4\ M_{47}\ M_{56}M_3$	2805.05 (52.798)
46	$M_4\ M_{47}\ M_{56}\ M_{91}$	2884.03 (53.701)
47	$M_{10}\ M_{71}\ M_{74}\ M_{95}$	875.580 (29.587)
48	$M_{10}\ M_{71}\ M_{74}\ M_{56}$	1571.87 (39.646)
49	$M_{75}\ M_{79}\ M_{95}\ M_{10}$	339.030 (18.399)
50	$M_4\ M_4\ M_{11}\ M_8$	10549.8 (102.712)
51	$M_4\ M_4\ M_{12}\ M_{74}$	4726.18 (68.746)
52	$M_4\ M_4\ M_4\ M_{16}$	5955.08 (77.152)
53	$M_4\ M_4\ M_4\ M_9$	7999.58 (89.440)
54	$M_4\ M_4\ M_4\ M_{62}$	2775.07 (52.528)
55	$M_4\ M_4\ M_4\ M_7$	9974.76 (99.872)
56	$M_4\ M_8\ M_{30}\ M_{11}$	5598.34 (74.793)
57	$M_4\ M_8\ M_{30}\ M_{16}$	4146.92 (64.396)
58	$M_{53}\ M_{74}\ M_{91}\ M_{95}$	1406.70 (37.505)

Contd.

Table 24c. Continued

1	2	3
59	M_{13} M_{79} M_{95} M_{10}	814.000 (28.530)
60	M_4 M_{47} M_{79} M_{19}	1494.69 (38.661)
61	M_{13} M_{91} M_{95} M_{10}	748.760 (27.363)
62	M_{76} M_{79} M_{95} M_{10}	678.270 (26.042)
CD		2.932
SEM $\pm$		1.048

* Values in paranthesis represent $\sqrt{x}$ transformed ones

Table 24d. Effect of serial subculturing (fourth subculture) on number of nodes produced from nodal segments of *H. annulare* in different media

Treatment No.	Treatment	No.of nodes
1	2	3
1	M_4 M_4 M_8 M_{65} M_{65}	485899.0 (697.021)*
2	M_4 M_4 M_8 M_9 M_{70}	74596.3 (273.117)
3	M_4 M_4 M_8 M_9 M_{79}	69628.3 (263.863)
4	M_4 M_4 M_8 M_9 M_{74}	70638.1 (265.761)
5	M_4 M_4 M_4 M_{56} M_8	168772.0 (410.800)
6	M_4 M_4 M_4 M_{49} M_{71}	32751.0 (180.845)
7	M_4 M_4 M_4 M_{64} M_7	110695.0 (332.488)
8	M_4 M_4 M_4 M_7 M_7	85444.6 (292.309)
9	M_4 M_4 M_4 M_6 M_8	170297.0 (412.472)
10	M_4 M_7 M_{12} M_3 M_{10}	18580.0 (136.296)
11	M_4 M_4 M_4 M_6 M_{56}	121938.0 (348.974)
12	M_4 M_4 M_4 M_6 M_3	285243.0 (460.123)
13	M_4 M_4 M_4 M_{11} M_{10}	46047.6 (214.587)
14	M_4 M_7 M_{12} M_{79} M_{87}	32134.4 (179.243)

Contd.

Table 24d.Continued

1	2	3
15	M_4 M_7 M_{12} M_{79} M_{96}	36742.2 (191.277)
16	M_4 M_7 M_{12} M_{79} M_{87}	32134.4 (179.242)
17	M_4 M_7 M_9 M_{71} M_{96}	17193.2 (131.089)
18	M_{11} M_{11} M_{92} $M_{78}M_4$	10419.6 (102.075)
19	M_{11} M_{11} M_{92} M_{78} M_{87}	8453.73 (91.941)
20	M_{11} M_{11} M_{92} M_{78} M_3	5717.12 (75.579)
21	M_4 M_{47} M_{56} M_3 M_{95}	15562.4 (124.743)
22	M_{10} M_{71} M_{74} M_{95} M_{10}	4903.24 (69.961)
23	M_4 M_4 M_{12} M_{74} M_{91}	28475.3 (168.729)
24	M_{53} M_{74} M_{91} M_{95} M_{93}	5466.39 (73.934)
25	M_9 M_{56} M_{92} M_{83} M_{93}	3748.56 (61.224)
26	M_{47} M_{13} M_{74} M_{82} M_{94}	7696.72 (87.728)
CD		106.9426
SEm±		37.629

* Values in paranthesis represent $\sqrt{x}$ transformed ones

Table 24e. Effect of serial subculturing (fifth subculture) on number of nodes produced from nodal segments of *H. annulare* in different media

Treatment No.	Treatment	No.of nodes
1	2	3
1	M_4 M_4 M_8 M_9 M_{70} M_{92}	464734.0 (681.520)*
2	M_4 M_4 M_8 M_{12} M_{74} M_9	448247.0 (669.168)
3	M_4 M_4 M_8 M_{12} M_{92} M_{93}	243144.0 (496.367)
4	M_4 M_4 M_8 M_{12} M_{92} M_3	366656.0 (601.317)
5	M_4 M_4 M_8 M_{12} M_{92} M_{94}	345695.0 (587.404)
6	M_4 M_4 M_4 M_{12} M_{71} M_{82}	338808.0 (580.991)
7	M_4 M_4 M_4 M_{12} M_{71} M_{90}	175228.0 (417.976)
8	M_4 M_4 M_4 M_{12} M_{71} M_{96}	3356744.0 (1830.86)
9	M_4 M_4 M_4 M_{49} M_{92} M_{83}	182454.0 (427.122)
10	M_4 M_4 M_4 M_6 M_8 M_{71}	808913.0 (898.801)
11	M_4 M_4 M_4 M_6 M_3 M_{95}	398029.0 (629.253)
12	M_4 M_7 M_6 M_{70} M_{92} M_{83}	166510.0 (407.342)
13	M_4 M_7 M_{12} M_{79} M_{87} M_{10}	179952.0 (423.714)
14	M_4 M_7 M_{12} M_{79} M_{96} M_{10}	205756.0 (453.543)

Contd.

Table 24e. Continued

1	2	3
15	M_4 M_7 M_{12} M_{79} M_{96} M_{87}	318371.0 (564.097)
16	M_4 M_7 M_{12} M_{79} M_{87} M_{10}	179952.0 (424.017)
17	M_4 M_7 M_{12} M_3 M_{92} M_{78}	119887.0 (345.801)
18	M_4 M_7 M_{12} M_3 M_{92} M_{82}	191200.0 (437.216)
19	M_4 M_7 M_{12} M_3 M_{92} M_{79}	135390.0 (367.839)
20	M_4 M_7 M_{12} M_3 M_{92} M_{90}	98886.4 (314.223)
21	M_4 M_7 M_{12} M_3 M_{92} M_{56}	205876.0 (453.151)
22	M_4 M_7 M_{12} M_3 M_{92} M_{95}	114678.0 (338.559)
23	M_4 M_7 M_9 M_{13} M_{92} M_{10}	83838.1 (289.505)
24	M_4 M_7 M_9 M_{71} M_{91} M_{95}	95938.0 (309.731)
25	M_{11} M_{11} M_{92} M_{78} M_3 M_{10}	32015.9 (178.910)
26	M_{11} M_{11} M_{92} M_{78} M_4 M_{10}	58349.8 (241.241)
27	M_4 M_4 M_{12} M_{74} M_{91} M_{95}	157981.0 (396.893)
CD		53.704
SEm±		18.917

* Values in paranthesis represent $\sqrt{x}$ transformed ones

Table 25a. Effect of different auxins on *in vitro* rooting in *H. annulare*

Treatment No.	Treatment Media Auxin/additive	% rooting
1	MS + NAA 0.25 mg l^{-1}	60
2	MS + NAA 0.5 mg l^{-1}	70
3	MS + NAA 1.0 mg l^{-1}	50
4	MS + IBA 0.25 mg l^{-1}	60
5	MS + IBA 0.5 mg l^{-1}	70
6	MS + IBA 1.0 mg l^{-1}	60
7	MS basal	90
8	MS + Activated charcoal	30
9	Half strength MS + Activated charcoal	30
10	IBA 1000 mg l^{-1} pulse → inoculation in MS + Activated charcoal	70
11	IBA 1000 mg l^{-1} pulse → inoculation in MS + Activated charcoal	30

Table 25b. Effect of different auxins on *in vitro* root growth in *H. annulare*

Treatment No.	Treatment		Days to root	No. of roots	Length of the longest root (cm)	Average length of a root (cm)	Weight of callus (g)
	Auxin	Concentration (mg l^{-1})					
1	NAA	0.25	17.773 (4.228)*	2.497 (1.610)	3.833 (2.008)	3.543 (1.916)	0.997 (1.220)
2	NAA	0.5	11.333 (3.025)	4.167 (1.943)	2.917 (1.708)	2.333 (1.566)	1.500 (1.409)
3	NAA	1.0	7.333 (2.494)	6.333 (2.310)	1.333 (1.290)	0.750 (1.085)	0.557 (1.000)
4	IBA	0.25	12.000 (3.530)	3.667 (1.907)	1.667 (1.462)	1.833 (1.525)	0.633 (1.041)
5	IBA	0.5	19.333 (4.449)	1.333 (1.503)	5.600 (2.429)	5.333 (2.350)	0.960 (1.168)
6	IBA	1.0	16.000 (4.046)	4.500 (2.185)	5.333 (2.325)	2.227 (2.074)	1.480 (1.361)
7	IBA (Quick dip and inoculated) in MS basal medium	1000	11.167 (3.410)	4.833 (2.169)	2.333 (1.678)	1.960 (1.568)	0.490 (0.979)
8	IBA (Quick dip and inoculated) in MS medium + activated charcoal	1000	10.833 (3.366)	4.110 (2.090)	2.333 (1.678)	1.570 (1.427)	0.633 (1.040)
9	Control		6.333 (2.609)	9.833 (3.209)	7.833 (2.871)	6.627 (2.660)	0.000 (0.707)
CD			1.561	1.490	0.292	0.900	0.464
SEm±			0.525	0.502	0.313	0.303	0.156

* Values in paranthesis represents $\sqrt{x}$ transformed ones

Table 26. Effect of different additives on *in vitro* root growth in *H. annulare*

Treatment No.	Treatment		Days to root	No. of roots	Length of of the longest root (cm)	Average length of a root (cm)	Weight of callus (g)
	Media	Additive					
1	MS	Activated charcoal 0.1%	12.833 (2.553)*	1.500 (1.217)	0.127 (0.784)	0.060 0.746)	0.003 (0.709)
2	½MS	Activated charcoal 0.1%	6.800 (2.35)	0.833 (1.049)	0.167 (0.805)	0.083 (0.760)	0.000 (0.707)
3	MS basal		6.333 (2.609)	9.833 (3.209)	7.833 (2.871)	6.627 2.660)	0.000 (0.707)
CD			4.403	1.256	0.498	0.346	0.000
SEm±			1.273	0.363	0.144	0.1	0.000

* Values in paranthesis represent $\sqrt{x}$ transformed ones

of IBA) followed by inoculation in charcoal incorporated medium giving only thirty per cent rooting of cultures.

4.4.1.2 Days to root initiation

Though treatments T_2, T_3, T_4, T_6, T_7, T_8 and T_9 were statistically comparable with respect to the number of days to rooting, it was found that T_9 (MS basal) resulted in very early rooting (only 6 days) while maximum number of days was taken when T_5, IBA 0.5 mg l^{-1} was used (19 days). Among the additives, minimum number of days was taken when no additive was used (Tables 25b and 26).

4.4.1.3 Number and length of roots

Maximum number of roots was obtained in cultures put in treatment T_9 (full strength MS basal medium) and minimum was obtained in cultures receiving treatment T_5 (IBA 0.5 mg l^{-1}). Among the additives, maximum number of roots were obtained when no additive was added into the media.

Longest roots were formed in basal MS medium (7.8 cm) while the shortest ones (1.3 cm) were produced in treatment T_3 (NAA 1 mg l^{-1}). Additives like charcoal resulted in very short roots and among treatments tried (Tables 25b and 26) longest roots were produced in T_3 (control).

4.4.1.4 Nature of roots

NAA in general produced thick and short roots in clusters which were cream in colour and there was intervening callus in 90 per cent of the cultures but a

Table 27. Effect of physical nature of media on *in vitro* root growth in *H. annulare*

Treatment No.	Nature of media*	Days to root	No. of roots	Length of the longest root (cm)	Average length of a root (cm)	Weight of callus (g)
1	Solid	6.333 (2.511)**	9.833 (3.130)	7.833 (2.781)	6.627 (2.563)	0.000 (1.099)
2	Liquid	15.330 (3.901)	2.667 (1.624)	6.167 (2.235)	6.000 (2.425)	0.010 (1.102)
CD		0.727	0.507	2.212	0.820	0.000
SEm±		0.185	0.129	0.564	0.209	0.000

* MS basal medium was used

** Values in paranthesis represent $\sqrt{x}$ transformed ones

few roots originated from the stem directly. IBA produced thin roots which were white coloured and sometimes with rootlets. Basal MS mostly produced thin, long, unbranched, coiled roots which were mostly light green in colour. Roots produced in medium containing charcoal were white and thin (Plates 7a, 7b, 7c and 7d).

Basal part of the shoot was preferable to the top portion of the shoot for better roo ing per cent and number of roots.

4.4.1.5 Physical nature of media

Data on the effect of physical nature of media on rooting are presented in the Table 27. The physical state of the media had a significant effect on the number of days taken for rooting. Irrespective of the other treatments, considering the physical nature of the media alone, rooting in solid media was found to be faster. Solid media proved far better with respect to earlier root induction (6.3), number of roots (9.8), length of roots (7.8) and lower callus (0) than stationery liquid media (15.3, 2.67, 6.17, 0.01 respectively).

4.4.2 *Ex vitro* rooting

4.4.2.1 Effect of different auxins

Formation of strong roots through *ex vitro* rooting was successful in *H. annulare* (Plates 8, 9a and 9b).

4.4.2.1.1 Rooting

The data on the effect of auxins on percentage of shoots rooted are presented in Table 28a. IBA was found to be the best at a concentration of 1000 mg l^{-1} (T_1) dip for one min (quick dip) followed by planting in suitable media

Plate 7a. *In vitro* root formation in MS basal medium (solid medium)

Plate 7b. *In vitro* root formation in MS basal medium (stationary liquid medium)

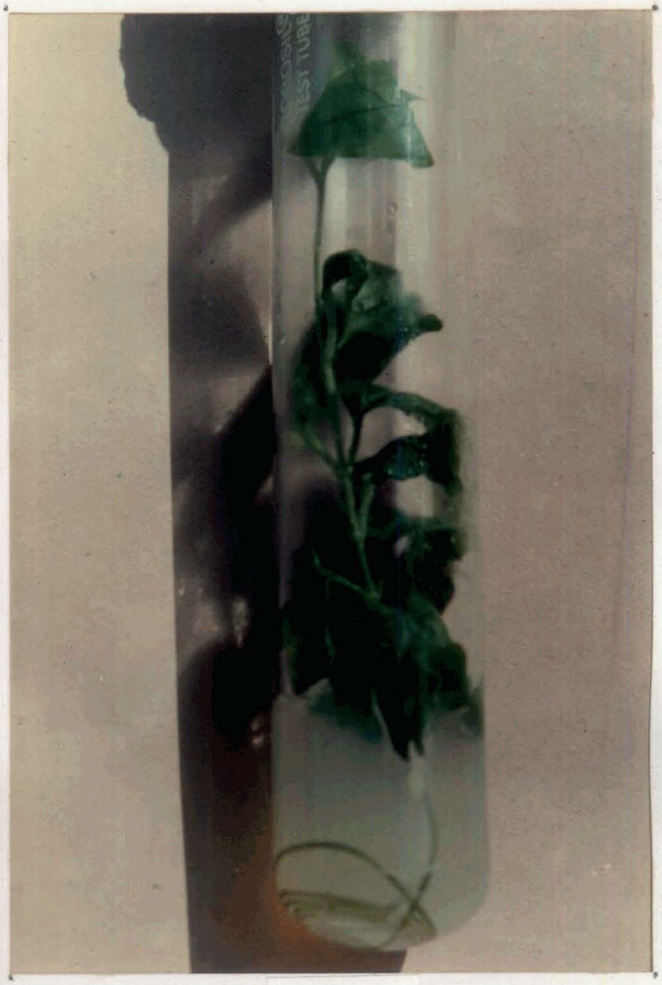


Plate 7a

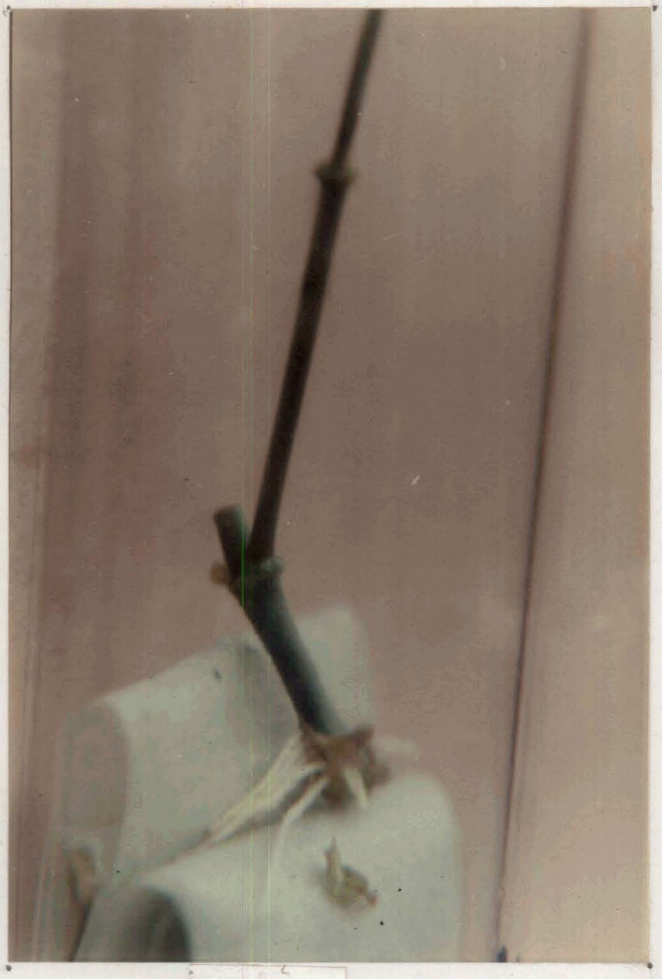


Plate 7b

Plate 7c. *In vitro* root formation in MS medium + NAA

Plate 7d. *In vitro* root formation in MS medium + activated charcoal

Plate 7c

Plate 7d

Plate 8. Shoots kept for *ex vitro* rooting

Plate 9a. Rooted plant

Plate 9b. *Ex vitro* root formation in shoots of *H. annulare*

Plate 8

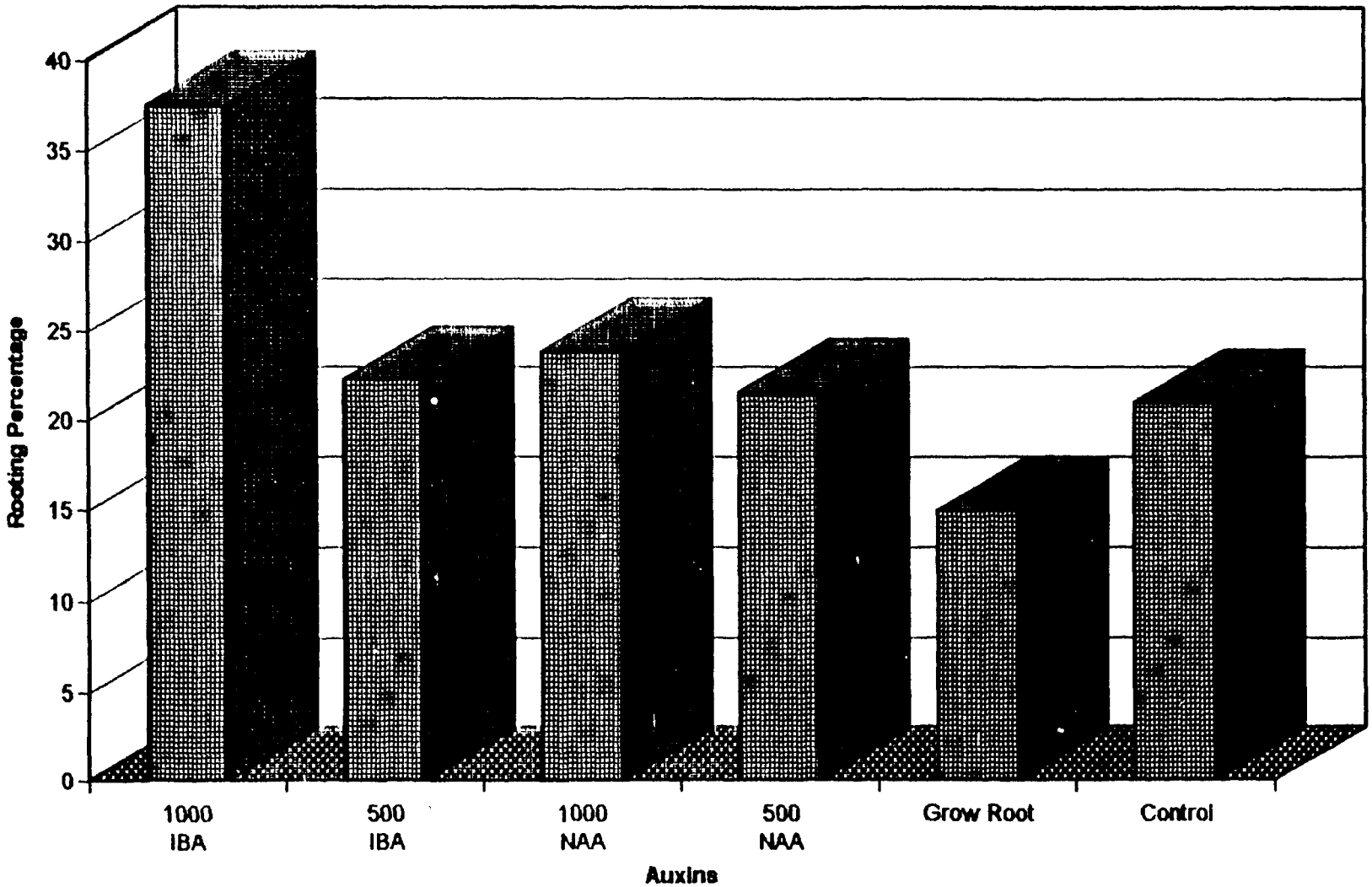
Plate 9a

Table 28a. Effect of different auxins on *ex vitro* rooting in *H. annulare*

Treatment No.	Treatment		Rooting (%)
	Auxin	Concentration ($mg\ l^{-1}$)	
1	IBA	1000	37.508 (0.644)*
2	IBA	500	22.228 (0.419)
3	NAA	1000	23.859 (0.500)
4	NAA	500	21.517 (0.432)
5	Growroot		14.969 (0.345)
6	Control		20.934 (0.474)
CD			0.096
SEm±			0.033

* Values in paranthesis represent arc sine transformed ones

Fig.4. Effect of different auxins on ex vitro rooting in *H.annulare*



giving 37.5 per cent rooting while the minimum percentage of rooting (15%) was observed when a dust formulation (Growroot) was used. A treatment without any growth regulator (T_6) gave 20.9 per cent rooting.

The result of the effect of different auxins on *ex vitro* root growth during different stages of growth (30 and 60 days after rooting) is presented in Tables 28b, 28c and Fig. 5a, 5b.

Rooting was earlier (10.5 days) in shoots receiving the three treatments T_1 (IBA 1000 mg l^{-1}), T_4 (NAA 500 mg l^{-1}) and T_6 (control) while it took 12-13 days in others.

The number of roots was maximum when treatments T_1 and T_4 were given which were on par (33) while treatments T_2 (IBA 500 mg l^{-1}) produced the least number of roots (24) when observed 30 days after rooting. By sixty days maximum number of roots (72) was produced in T_1 (IBA 1000 mg l^{-1}), the minimum number of roots (52.3) were produced for cultures receiving treatment T_5 - (Growroot). However, T_2 (IBA 500 mg l^{-1}) and T_3 (NAA 1000 mg l^{-1}) were on par with T_5.

The length of the longest root was maximum for cultures receiving the treatment T_1 - IBA 1000 mg l^{-1} (15.31 and 24.16 cm) during both stages of growth (30 and 60 days after rooting). However, T_1 was on par with that of T_3 (NAA 1000 mg l^{-1}) and T_5 (Growroot) with respect to this character when observed 30 days after rooting and the shortest was produced in shoots which did not receive any treatment T_6 (9.7 cm). While 60 days after rooting, the shortest roots were observed in shoots receiving T_4 (NAA 500 mg l^{-1}) treatment (17.6 cm). All the other treatments excepting T_1 were on par with one another.

Table 28b. Effect of different auxins on *ex vitro* root growth in *H. annulare* (30 days after rooting)

Treatment No.	Treatment		Days to root	No. of roots	Length of the longest root (cm)	Average length of a root (cm)	Thickness of the thickest root (cm)
	Auxin	Concentration (mg l^{-1})					
1	IBA	1000	10.500 (3.225)*	32.778 (5.570)	15.306 (3.881)	12.572 (3.501)	0.570 (0.396)
2	IBA	500	12.722 (3.562)	24.089 (4.650)	11.170 (3.333)	6.513 (2.466)	0.501 (0.356)
3	NAA	1000	12.333 (3.503)	26.222 (4.883)	13.172 (3.546)	10.628 (3.160)	0.497 (0.319)
4	NAA	500	10.583 (3.246)	33.167 (5.699)	10.317 (3.150)	7.861 (2.751)	0.453 (0.247)
5	Growroot		12.289 (3.501)	27.211 (5.115)	13.622 (3.662)	9.883 (2.980)	0.596 (0.371)
6	Control		10.500 (3.226)	26.833 (5.132)	9.661 (2.896)	7.650 (2.505)	0.557 (0.356)
CD			0.135	0.393	0.417	0.406	0
SEm±			0.047	0.137	0.145	0.141	0

* Values in paranthesis represents $\sqrt{x}$ transformed ones

Table 28c. Effect of different auxins on *ex vitro* root growth in *H. annulare* (60 days after rooting)

Treatment No.	Treatment		Days to root	No. of roots	Length of the longest root (cm)	Average length of a root (cm)	Thickness of the thickest root (cm)
	Auxin	Concentration ($mg\ l^{-1}$)					
1	IBA	1000	10.500 (3.225)*	72.000 (8.293)	24.161 (4.907)	17.000 (4.094)	1.333 (1.032)
2	IBA	500	12.722 (3.562)	54.611 (7.355)	19.456 (4.379)	15.804 (3.952)	1.096 (1.009)
3	NAA	1000	13.333 (3.503)	57.456 (7.426)	18.333 (4.142)	15.830 (3.916)	0.836 (0.829)
4	NAA	500	10.588 (3.246)	62.886 (7.893)	17.611 (4.173)	14.894 (3.850)	0.748 (0.792)
5	Growroot		12.289 (3.501)	52.333 (7.201)	19.749 (4.398)	14.650 (3.791)	1.007 (0.985)
6	Control		10.500 (3.226)	65.217 (7.908)	18.783 (4.291)	16.217 (3.929)	0.664 (0.742)
CD			0.135	0.606	0.590	0.370	0.135
SEm ±			0.047	0.211	0.205	0.129	0.047

* Values in paranthesis represent $\sqrt{x}$ transformed ones

Fig.5a. Effect of different auxins on ex.vitro root growth in *H.annulare* (30 days after rooting)

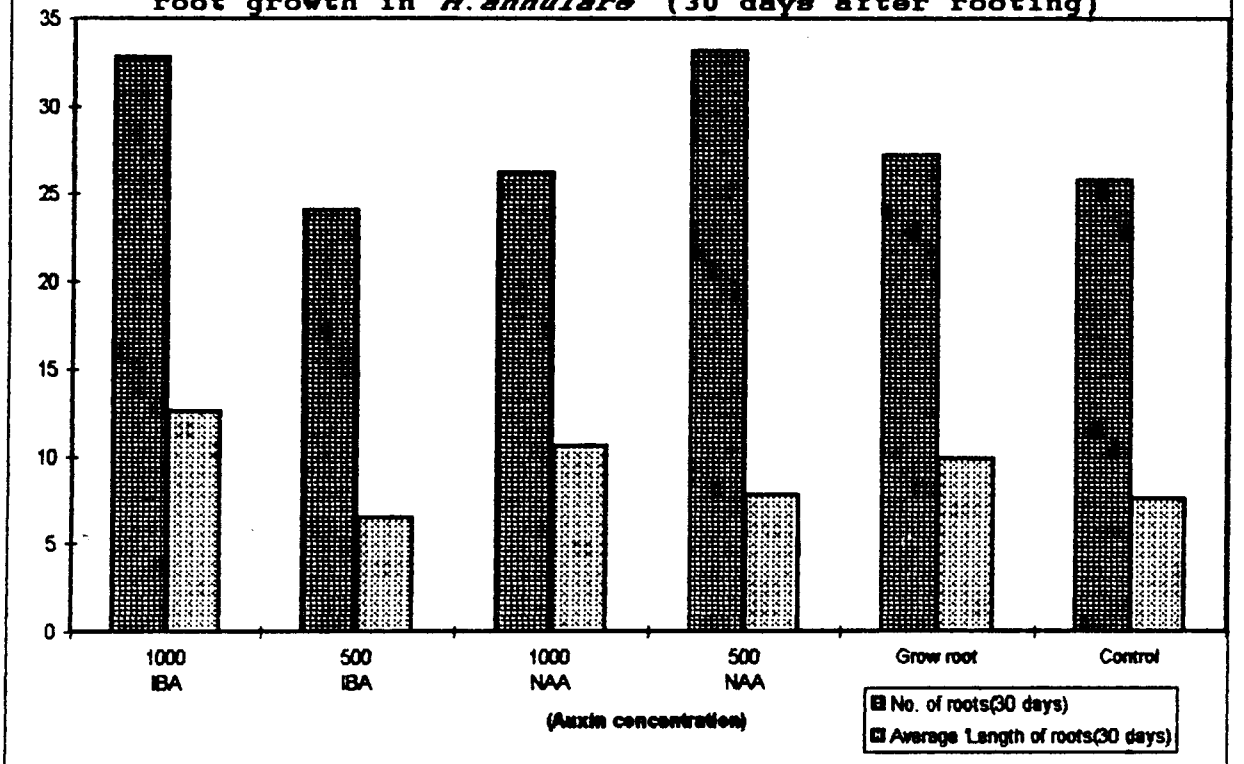
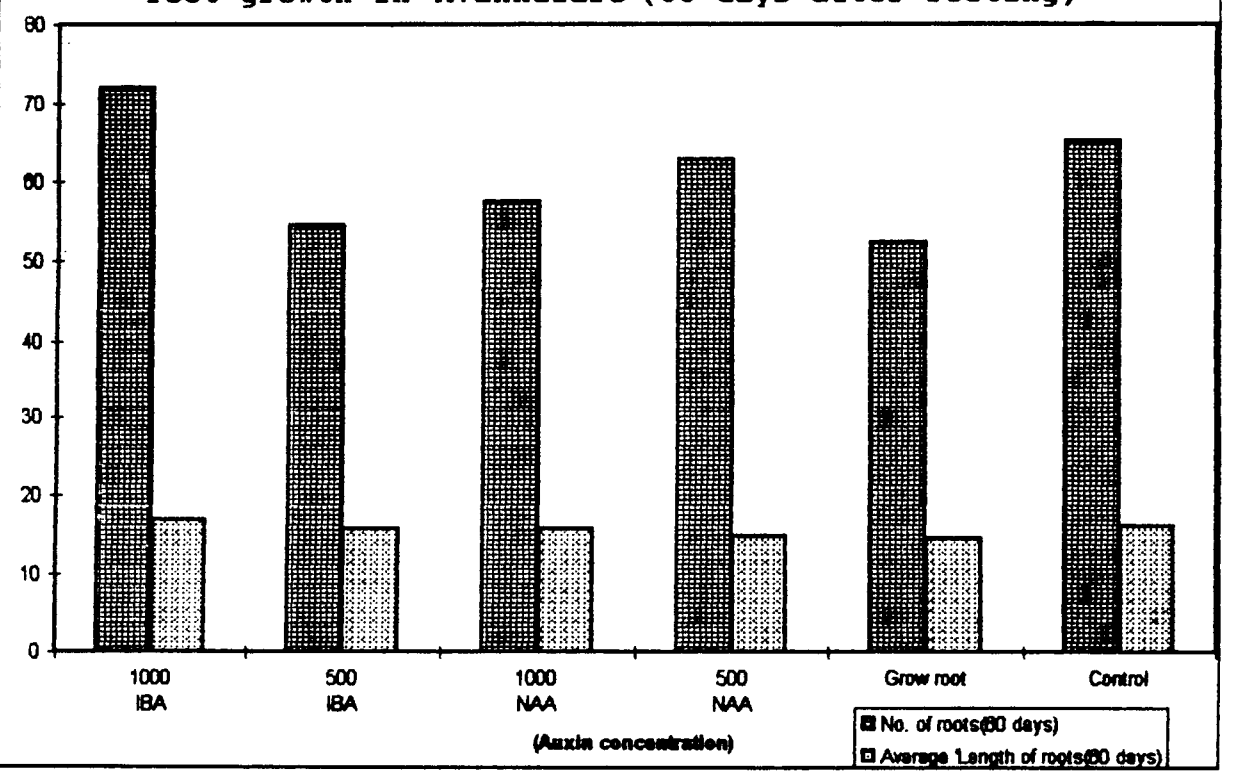


Fig.5b. Effect of different auxins on ex.vitro root growth in *H.annulare* (60 days after rooting)



The thickness of the thickest root was maximum (0.60 cm) for shoots receiving treatment T_5 - (Growroot) in 30 days while it was minimum for those treated with NAA 500 mg l^{-1} (0.45 cm). By 60 days, maximum thickness was obtained for shoots treated with IBA 1000 mg l^{-1} - T_1 (1.33 cm) followed by T_2 - IBA 500 mg l^{-1} (1.09 cm) while the thinnest roots were produced by shoots receiving treatment T_6 - control (0.66 cm).

Morphology of roots varied depending on the growth regulator used. NAA produced cream colour less branched roots while those produced by IBA induced ones were white coloured and highly branched.

4.4.2.1.2 Effect of different media

The result of the effect of media on percentage of rooting and root characters are presented in Tables 29a, 29b and 29c. Maximum rooting was obtained when sand was used (T_1) as the media (38.9%) followed by vermiculite - T_3 (17.9%), the minimum being for potting mixture - T_2 (13.7%).

Early rooting was observed when the shoots were planted in sand (9.7 days) while the other two treatments were on par (12 days).

Maximum number of roots could be obtained in sand (39.3) followed by potting mixture (28) at 30 days while by 60 days it look a reverse pattern. Higher number of roots could be obtained in medium containing potting mixture (71.7) while in sand a mean number of 67 roots were produced. Vermiculite produced less number of roots (18).

Table 29a. Effect of different media on *ex vitro* rooting in *H. annulare*

Treatment No.	Media	Rooting (%)
1	Sand	38.888 (0.669)*
2	Potting mixture	13.697 (0.335)
3	Vermiculite	17.923 (0.403)
CD		0.067
SEm ±		0.024

* Values in paranthesis represents arc sine transformed ones

Table 29b. Effect of different media on *ex vitro* root growth in *H. annulare* (30 days after rooting)

Treatment No.	Media	Days to root	No. of roots	Length of the longest root (cm)	Average length (cm)	Thickness of thickest root (cm)
1	Sand	9.681 (3.102)*	39.250 (6.214)	10.161 (3.125)	7.853 (2.720)	0.528 (0.287)
2	Potting mixture	12.478 (3.528)	27.994 (5.201)	14.142 (3.729)	10.569 (3.167)	0.802 (0.653)
3	Vermiculite	12.306 (3.502)	17.906 (4.109)	12.346 (3.359)	9.132 (2.794)	0.257 (0.082)
CD		0.096	0.278	0.295	0.287	0
SEm±		0.033	0.097	0.103	0.100	0

* Values in paranthesis represent $\sqrt{x}$ transformed ones

Table 29c. Effect of different media on *ex vitro* root growth in *H. annulare* (60 days after rooting)

Treatment No.	Media	Days to root	No. of roots	Length of the longest root (cm)	Average length of a root (cm)	Thickness of the thickest root (cm)
1	Sand	9.681 (3.102)*	66.996 (8.085)	17.283 (4.116)	12.637 (3.525)	0.649 (0.784)
2	Potting mixture	12.478 (3.528)	71.722 (8.385)	22.647 (4.731)	18.278 (4.232)	1.794 (1.327)
3	Vermiculite	12.306 (3.502)	43.533 (6.568)	19.117 (4.299)	16.283 (4.009)	0.398 (0.584)
CD		0.096	0.428	0.417	0.262	0.096
SEm±		0.033	0.149	0.145	0.091	0.033

* Values in paranthesis represents $\sqrt{x}$ transformed ones

The length of the longest root was maximum when potting mixture was used as the medium at all stages of growth of the plant. It was 14.1 cm in 30 days which increased to 22.6 cm in 60 days while the other treatments were on par. However, the roots in sand media were shorter (10 cm).

The thickness of the thickest root produced showed difference among the media tried, maximum being in potting mixture during the two stages (0.8 and 1.8 cm respectively) followed by sand (0.5 and 0.6 cm respectively) while the thinnest ones were that produced in vermiculite media (0.3 and 0.4 cm).

4.4.2.1.3 Effect of auxins and media

Considering the effect of auxins and media together, the performance of cultures were as detailed below (Tables 30a, 30b and 30c).

Maximum percentage of cultures were rooted when dipped in 1000 mg l^{-1} IBA for one min followed by planting in sand medium - T_1 (73.9%) followed by the treatment T_2 (IBA 500 mg l^{-1} for one min and planting in sand). Minimum percentage could be obtained when shoots were dipped in dust formulation viz. Growroot followed by planting in potting mixture medium T_{11} (1.6%).

Early rooting could be obtained when cultures were given a quick dip in IBA 1000 mg l^{-1} followed by planting in sand - T_1 (8 days). However, treatments T_4 (NAA 500 mg l^{-1} and plant in sand medium) and T_6(plant in sand medium without any auxin treatment) were on par with the first (8-9 days).

Table 30a. Effect of different auxins and media on *ex vitro* rooting in *H. annulare*

Treatment No.	Treatment			Rooting (%)
	Auxin	Concentration ($mg\ l^{-1}$)	Media	
1	IBA	1000	Sand	73.887 (1.037)*
2	IBA	500	Sand	47.777 (0.762)
3	NAA	1000	Sand	35.000 (0.627)
4	NAA	500	Sand	25.000 (0.523)
5	Growroot		Sand	26.663 (0.541)
6	Control		Sand	25.000 (0.524)
7	IBA	1000	Potting mixture	25.000 (0.524)
8	IBA	500	Potting mixture	16.657 (0.408)
9	NAA	1000	Potting mixture	19.917 (0.461)
10	NAA	500	Potting mixture	2.050 (0.117)
11	Growroot		Potting mixture	1.583 (0.073)
12	Control		Potting mixture	16.973 (0.425)
13	IBA	1000	Vermiculite	13.637 (0.372)
14	IBA	500	Vermiculite	2.250 (0.088)
15	NAA	1000	Vermiculite	16.660 (0.412)
16	NAA	500	Vermiculite	37.500 (0.656)
17	Growroot		Vermiculite	16.660 (0.412)
18	Control		Vermiculite	20.830 (0.473)
CD				0.166
SEm ±				0.058

* Values in paranthesis represent arc sine transformed ones

Table 30b. Effect of different auxins and media on *ex vitro* root growth in *H. annulare* (30 days after rooting)

Treatment No.	Treatment			Days to root	No. of roots	Length of longest root (cm)	Average length of a root (cm)	Thickness of the thickest root (cm)
	Auxin	Concentration ($mg\ l^{-1}$)	Media					
1	IBA	1000	Sand	8.000 (2.825)*	43.333 (6.733)	13.083 (3.614)	11.683 (3.417)	0.300 (0.547)
2	IBA	500	Sand	11.333 (3.366)	45.333 (6.732)	12.600 (3.549)	9.200 (3.031)	0.250 (0.494)
3	NAA	1000	Sand	10.500 (3.240)	40.667 (6.345)	6.500 (2.518)	4.417 (2.076)	0.303 (0.550)
4	NAA	500	Sand	9.083 (3.013)	44.667 (6.677)	12.200 (3.488)	10.233 (3.196)	0.150 (0.384)
5	Growroot		Sand	11.000 (3.314)	38.000 (6.164)	12.100 (3.469)	8.917 (2.973)	0.323 (0.568)
6	Control		Sand	8.167 (2.854)	21.500 (4.635)	4.488 (2.110)	2.667 (1.630)	0.393 (0.627)
7	IBA	1000	Potting mixture	11.000 (3.314)	39.000 (6.238)	13.833 (3.711)	11.583 (3.83)	0.820 (0.905)
8	IBA	500	Potting mixture	13.333 (3.648)	18.000 (4.240)	11.333 (3.366)	7.917 (2.810)	0.803 (0.896)
9	NAA	1000	Potting mixture	13.167 (3.620)	29.000 (5.350)	17.017 (4.122)	13.633 (3.687)	0.630 (0.792)
10	NAA	500	Potting mixture	12.333 (3.511)	30.333 (5.499)	12.750 (3.568)	9.133 (3.020)	0.527 (0.724)
11	Growroot		Potting mixture	12.700 (3.563)	14.633 (3.796)	9.917 (3.148)	3.900 (1.956)	0.537 (0.721)
12	Control		Potting Mixture	12.333 (3.511)	37.000 (6.082)	20.000 (4.4641)	17.250 (4.146)	0.600 (0.773)
13	IBA	1000	Vermiculite	12.500 (3.525)	14.000 (3.739)	19.000 (4.317)	14.450 (3.705)	0.067 (0,257)
14	IBA	500	Vermiculite	13.500 (3.673)	8.933 (2.977)	9.577 (3.083)	2.423 (1.557)	0.013 (0.114)
15	NAA	1000	Vermiculite	13.333 (3.651)	9.00 (2.954)	16.000 (3.998)	13.833 (3.717)	0.023 (0.149)
16	NAA	500	Vermiculite	10.333 (3.214)	24.500 (4.920)	6.00 (2.394)	4.217 (2.036)	0.063 (0.251)
17	Growroot		Vermiculite	13.167 (3.627)	29.000 (5.384)	19.000 (4.248)	16.833 (4.013)	0.253 (0.499)
18	Control		Vermiculite	11.000 (3.314)	22.000 (4.679)	4.500 (2.116)	3.033 (1.739)	0.073 (0.271)
CD				0.234	0.683	3.033	0.073	
SEm ±				0.082	0.238	0.252	0.245	0

*Values in paranthesis represent $\sqrt{x}$ transformed ones

Table 30c. Effect of different auxins and media on *ex vitro* root growth in *H. annulare* (60 days after rooting)

Treatment No.	Treatment			Days to root	No. of roots	Length of the longest root (cm)	Average length of a root (cm)	Thickness of the thickest root (cm)
	Auxin	Concentration ($mg\ l^{-1}$)	Media					
1	IBA	1000	Sand	8.000 (2.825)*	112.000 (10.500)	22.133 (4.704)	12.500 (3.526)	0.950 (0.987)
2	IBA	500	Sand	11.333 (3.366)	62.000 (7.845)	19.000 (4.328)	15.730 (3.954)	0.937 (0.965)
3	NAA	1000	Sand	10.500 (3.240)	58.000 (7.596)	12.000 (3.425)	10.490 (3.216)	0.750 (0.866)
4	NAA	500	Sand	9.083 (3.013)	62.990 (7.918)	15.767 (3.965)	14.750 (3.834)	0.317 (0.560)
5	Growroot	Sand	11.000	61.000 (3.314)	15.797 (7.809)	12.500 (3.962)	0.737 (3.533)	(0.858)
6	Control		Sand	8.167 (2.854)	45.983 (6.765)	19.000 (4.312)	9.850 (3.090)	0.503 (0.707)
7	IBA	1000	Potting mixture	11.000 (3.314)	64.000 (7.976)	28.017 (5.292)	20.500 (4.516)	2.850 (1.688)
8	IBA	500	Potting mixture	13.333 (3.648)	57.000 (7.526)	23.000 (4.773)	19.083 (4.363)	1.817 (1.348)
9	NAA	1000	Potting mixture	13.167 (3.620)	83.000 (9.094)	22.000 (4.683)	18.000 (4.211)	1.623 (1.273)
10	NAA	500	Potting mixture	12.333 (3.511)	73.667 (8.577)	21.867 (4.657)	15.433 (3.910)	1.627 (1.269)
11	Growroot		Potting mixture	12.700 (3.563)	51.000 (7.087)	17.650 (4.157)	11.350 (3.367)	1.483 (1.214)
12	Control		Potting mixture	12.333 (3.511)	101.667 (10.049)	23.350 (4.822)	25.300 (5.025)	1.367 (1.168)
13	IBA	1000	Vermiculite	12.500 (3.535)	40.000 (6.323)	22.333 (4.726)	18.000 (4.241)	0.500 (0.661)
14	IBA	500	Vermiculite	13.500 (3.673)	44.833 (6.695)	16.367 (4.037)	12.600 (3.539)	0.533 (0.714)
15	NAA	1000	Vermiculite	13.300 (3.651)	31.367 (5.588)	21.000 (4.319)	19.000 (4.321)	0.133 (0.347)
16	NAA	500	Vermiculite	10.333 (3.214)	52.000 (7.184)	15.200 (3.898)	14.500 (3.806)	0.300 (0.546)
17	Growroot		Vermiculite	13.167 (3.627)	45.000 (6.708)	25.800 (5.075)	20.100 (4.472)	0.800 (0.883)
18	Control		Vermiculite	11.000 (3.314)	48.000 (6.909)	14.000 (3.740)	13.500 (3.673)	0.123 (0.350)
CD				0.234	1.048	1.022	0.642	0.234
SEm±				0.082	0.365	0.356	0.224	0.082

* Values in paranthesis indicates $\sqrt{x}$ transformed ones

Maximum number of roots in 30 days could be obtained when shoots were given a quick dip either in 1000 mg l^{-1} or 500 mg l^{-1} of IBA followed by planting in sand media (45.3 ie. T_1 and T_2) though T_3 (NAA 1000 mg l^{-1} - sand), T_4 (NAA 500 mg l^{-1} - sand), T_5 (Grow root - sand) and T_7 (IBA 1000 mg l^{-1} - potting mixture) were on par with the earlier treatments with respect to the character studied. By 60 days, treatment T_1 (IBA 1000 mg l^{-1} quick dip and planting in sand) and T_{12} (planting in potting mixture without auxin pretreatment) were on par with respect to the number of roots (112 and 101.7 respectively). Minimum number of roots were obtained when given 1000 mg l^{-1} IBA dip and planted in vermiculite (T_{13}) while in 60 days it was NAA 1000 mg l^{-1} followed by planting in vermiculite media.

Treatments T_{12} (potting mixture without pretreatment), T_{13} (IBA 1000 mg l^{-1} - vermiculite), T_{15} (NAA 1000 mg l^{-1} - vermiculite), T_{17} (Growroot - vermiculite) and T_9 (NAA 1000 mg l^{-1} - potting mixture) were on par statistically with respect to the length of longest root in 30 days after rooting though longest root was obtained when planted in potting mixture without any pretreatment (T_{12}) giving 20 cm but by 60 days this treatment did not have much effect in the rate of elongation of roots while T_7 (IBA 1000 mg l^{-1} dip and planting in potting mixture) produced longest root (28 cm) followed by the dip in grow root and planting in vermiculite (25.8 cm). Shortest were those produced by T_3 (NAA 1000 mg l^{-1} - sand) ie. 12 cm.

Thickest roots were obtained for treatment T_7 (IBA 1000 mg l^{-1} and potting mixture 0.82 cm) closely followed by T_8 (IBA 500 mg l^{-1} and potting mixture) ie. 0.8 cm in 30 days. In same pattern was observed in 60 days too. T_7

gave 2.9 cm thick roots followed by T_8 giving 1.8 cm. Thinnest roots were produced in T_{14} (IBA 500 mg l^{-1} - vermiculite) 0.01 cm in 30 days and T_8 - planting in vermiculite without pretreatment (0.12 cm) in 60 days.

4.4.2.2 Standardisation of potting mixes and containers

4.4.2.2.1 Effect of potting mixes

The effect of different potting mixes on percentage of shoots rooted and the root characters are presented in Tables 31a and 31b.

Among the six different potting mixes tried, it was found that 30 days after planting cocofibe media gave maximum rooting percentage (65.3%) followed by vermicompost (37.6%). Least per cent of rooting was for those in potting mixture (14.3%) though T_3 (vermiculite) and T_5 (soilrite) were on par with this statistically (15 and 17.5 respectively).

The effect of potting mixes on the days to rooting irrespective of the container used was also studied. Earliest rooting was observed in shoots kept in sand (T_1 - 8.2 days) irrespective of the container used followed by cocofibe T_6 (9.1 days). Maximum number of days was observed with soilrite T_5 (10.9 days) though vermiculite T_3 (10.6) was also on par with this. Shoots kept in cocofibe media gave the maximum number of roots (33.9) though treatment T_4 (vermicompost media) was also on par with this (31.95) statistically. Lowest root number was observed in potting mixture media among the treatments tried (20.7). Maximum root length was observed in treatment T_5 - soilrite (18.4 cm) which was statistically on par with that of T_6 (cocofibe) 17.03 cm. Minimum length was observed in vermiculite media (8.1 cm). Thickest roots were more produced in cocofibe (0.47 cm) which was

Table 31a. Effect of different potting mixes on *ex vitro* rooting in *H. annulare*

Treatment No.	Treatment Potting mixture	Rooting (%)
1	Sand	25.096 (0.461)*
2	Potting mixture	14.303 (0.316)
3	Vermiculite	15.140 (0.366)
4	Vermicompost	37.583 (0.631)
5	Soilrite	17.479 (0.394)
6	Cocofibe	65.300 (0.977)
CD		0.082
SEm±		0.029

* Values in paranthesis represents arc sine transformed ones

Table 31b. Effect of different potting mixes on *ex vitro* root growth in *H. annulare* (30 days after rooting)

Treatment No.	Treatment Potting mixture	Days to root	No. of roots	Length of the longest root (cm)	Average length of a root (cm)	Thickness of the thickest root (cm)
1	Sand	8.191 (2.846)*	32.366 (5.518)	12.273 (3.493)	8.204 (2.797)	0.381 (0.608)
2	Potting mixture	9.588 (3.079)	28.688 (5.154)	11.785 (3.421)	7.863 (2.763)	0.305 (0.542)
3	Vermiculite	10.588 (3.250)	31.628 (5.491)	8.090 (2.815)	5.429 (2.301)	0.296 (0.542)
4	Vermicompost	9.423 (3.038)	31.950 (5.554)	13.566 (3.664)	9.929 (3.097)	0.443 (0.656)
5	Soilrite	10.933 (3.288)	30.873 (5.444)	18.396 (4.257)	12.354 (3.474)	0.365 (0.594)
6	Cocofibe	9.145 (3.0313)	33.850 (5.722)	17.025 (4.076)	12.900 (3.526)	0.468 (0.679)
CD		0.183	0.183	0.232	0.273	0.082
SEm±		0.065	0.065	0.082	0.096	0.029

* Values in paranthesis represents $\sqrt{x}$ transformed ones

statistically on par with vermicompost (0.44 cm). The thinnest ones were produced in shoots kept in vermiculite media (0.29 cm).

4.4.2.2.2 Effect of container

The data on the effect of container on rooting irrespective of potting mixes used are presented in Tables 32a and 32b. Maximum percentage of shoots were rooted when kept in plastic pot (45%) followed by polybags (33.4%) while the lowest rooting percentage was obtained in protrays (18.2%).

Earliest rooting was observed for those plants kept in protrays (7.4 days) while it look 11.1 days for rooting when kept in mudpots.

Maximum number of roots could be obtained when shoots were kept in plastic pots (42.8) followed by mudpots (37.665) and the least in protrays (12.24).

Length of the longest root produced was maximum in plastic pot (15.56 cm) though it was statistically on par with that in mudpot (14.22 cm).

Thickness was maximum in mudpot (0.42 cm). But statistically T_1 (plastic pot), T_2 (mud pot) and T_3 (polybag) were on par and thinnest roots were obtained in protrays (0.31 cm).

4.4.2.2.3 Effect of potting mixes and containers

The data on the effect of potting mixes and containers taken together are presented in Tables 33a, 33b and Fig 6. Maximum rooting percentage was obtained for shoots which received the treatment T_{22} - mudpots filled with cocofibe (97.3%) followed by polybag filled with cocofibe T_{23} (81.6%) and least in protrays filled with potting mixture T_8 (1.7%).

Table 32a. Effect of different containers on *ex vitro* rooting in *H. annulare*

Treatment No.	Treatment container	Rooting (%)
1	Plastic pot	45.022 (0.726)*
2	Mud pot	20.058 (0.406)
3	Polybag	33.352 (0.582)
4	Pro-tray	18.169 (0.383)
CD		0.067
SEm±		0.024

* Values in paranthesis represents arc sine transformed ones

Table 32b. Effect of different containers on *ex vitro* root growth in *H. annulare* (30 days after rooting)

Treatment No.	Treatment Container	Days to root	No.of roots	Length of the longest root(cm)	Average length of a root (cm)	Thickness of the thickest root (cm)
1	Plastic pot	9.710 (3.105)*	42.847 (6.541)	15.559 (3.866)	11.739 (3.320)	0.396 (0.619)
2	Mudpot	11.111 (3.327)	37.665 (6.132)	14.217 (3.72)	10.825 (3.231)	0.422 (0.640)
3	Polybag	10.361 (3.205)	33.483 (5.775)	13.642 (3.643)	9.853 (3.120)	0.378 (0.605)
4	Pro-tray	7.396 (2.707)	12.240 (3.474)	10.672 (3.255)	5.369 (2.301)	0.310 (0.550)
CD		0.151	0.151	0.189	0.222	0.068
SEm±		0.053	0.053	0.067	0.078	0.024

* Values in paranthesis represents $\sqrt{x}$ transformed ones

Table 33a. Effect of different potting mixes and containers on *ex vitro* rooting in *H. annulare*

Treatment No.	Treatments		Rooting (%)
	Potting mixture	Container	
1	2	3	4
1	Sand	Plastic pot	73.887 (1.037)*
2	Sand	Mudpot	2.580 (0.161)
3	Sand	Poly bag	20.000 (0.460)
4	Sand	Pro-tray	3.917 (0.186)
5	Potting mixture	Plastic pot	47.777 (0.762)
6	Potting mixture	Mud pot	5.733 (0.237)
7	Potting mixture	Polybag	2.000 (0.137)
8	Potting mixture	Pro-tray	1.700 (0.128)
9	Vermiculite	Plastic pot	24.500 (0.479)
10	Vermiculite	Mudpot	2.283 (0.150)
11	Vermiculite	Polybag	16.110 (0.412)
12	Vermiculite	Pro-tray	17.667 (0.425)
13	Vermicompost	Plastic pot	21.667 (0.484)
14	Vermicompost	Mudpot	6.667 (0.247)

Contd.

Table 33a. Continued

1	2	3	4
15	Vermicompost	Polybag	60.500 (0.891)
16	Vermicompost	Pro-tray	61.500 (0.901)
17	Soilrite	Plastic pot	40.000 (0.684)
18	Soilrite	Mudpot	5.767 (0.231)
19	Soilrite	Polybag	19.917 (0.462)
20	Soilrite	Pro-tray	4.233 (0.196)
21	Cocofibe	Plastic pot	62.300 (0.910)
22	Cocofibe	Mudpot	97.317 (1.407)
23	Cocofibe	Polybag	81.583 (1.129)
24	Cocofibe	Pro-tray	20.000 (0.464)
CD			0.164
SEm±			0.058

* Values in paranthesis represent arc sine transformed ones

Table 33b. Effect of potting mixes and containers on *ex vitro* root growth in *H. annulare* (30 days after rooting)

Treatment No.	Treatment		Days to root	No. of roots	Length of the root (cm)	Average length of a root (cm)	Thickness of the thickest root (cm)
	Potting mixes	Containers					
1	2	3	4	5	6	7	8
1	Sand	Plastic pot	8.000 (2.825)*	45.333 (6.733)	13.530 (3.656)	9.350 (2.899)	0.350 (0.587)
2	Sand	Mudpot	9.450 (3.078)	38.900 (6.233)	12.700 (3.561)	10.000 (3.131)	0.400 (0.622)
3	Sand	Polybg	8.850 (2.948)	35.000 (5.909)	12.360 (3.515)	8.017 (2.825)	0.503 (0.704)
4	Sand	Protray	6.463 (2.538)	10.230 (3.198)	10.500 (3.240)	5.450 (2.334)	0.270 (0.520)
5	Potting mixture	Plastic pot	11.333 (3.366)	45.150 (6.718)	12.773 (3.574)	9.200 (3.031)	0.250 (0.494)
6	Potting mixture	Mudpot	10.283 (3.200)	33.700 (5.804)	12.600 (3.548)	9.567 (3.090)	0.350 (0.590)
7	Potting mixture	Polybag	10.033 (3.164)	27.900 (5.281)	13.067 (3.613)	8.817 (2.966)	0.410 (0.629)
8	Potting mixture	Protray	6.700 (2.587)	8.000 (2.814)	8.700 (2.950)	3.867 (1.963)	0.210 (0.456)
9	Vermiculite	Plastic pot	10.500 (3.240)	43.300 (6.576)	6.500 (2.518)	4.417 (2.076)	0.303 (0.550)
10	Vermiculite	Mudpot	11.150 (3.339)	39.700 (6.298)	7.600 (2.755)	4.933 (2.025)	0.270 (0.516)
11	Vermiculite	Polybag	11.300 (3.360)	31.000 (5.551)	6.760 (2.600)	7.550 (2.739)	0.310 (0.556)
12	Vermiculite	Protray	9.400 (3.063)	12.510 (3.537)	11.500 (3.388)	4.817 (2.183)	0.300 (0.547)

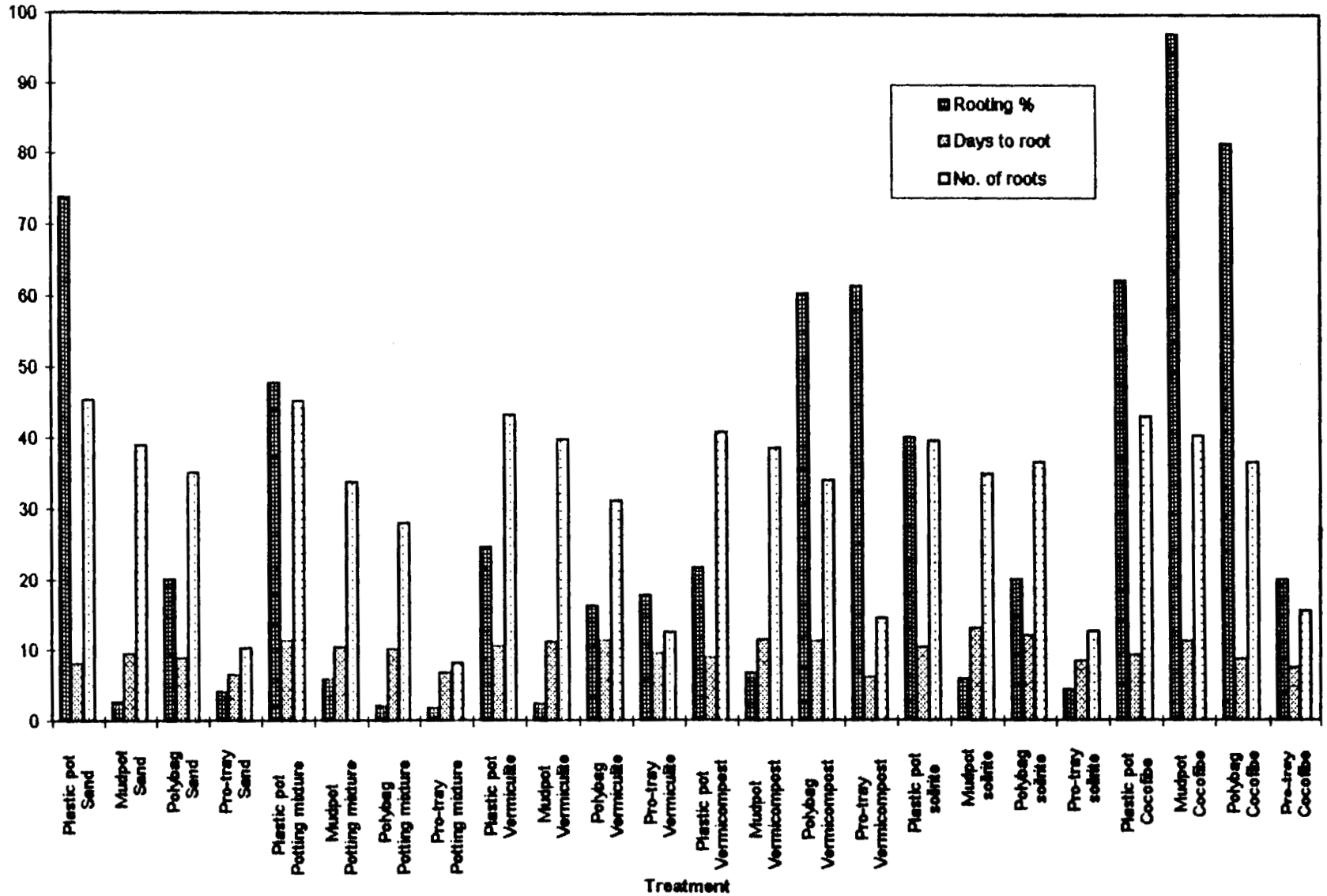
Contd.

Table 33b. Continued

1	2	3	4	5	6	7	7
13	Vermi-compost	Plastic pot	8.843 (2.971)	40.800 (6.380)	17.00 (4.120)	13.867 (3.720)	0.570 (0.752)
14	Vermi-compost	Mudpot	11.483 (3.384)	38.500 (6.204)	12.600 (3.549)	10.083 (3.175)	0.533 (0.726)
15	Vermi-composit	Polybag	11.267 (3.342)	34.000 (5.827)	13.830 (3.716)	10.950 (3.303)	0.267 (0.516)
16	Vermi-composit	Protray	6.100 (2.457)	14.500 (3.807)	10.833 (3.270)	4.817 (2.191)	0.400 (0.628)
17	Soilrite	Plastic pot	10.350 (3.192)	39.500 (6.285)	21.750 (4.661)	16.117 (4.013)	0.400 (0.626)
18	Soilrite	Mudpot	13.017 (3.608)	34.890 (5.907)	20.000 (4.470)	14.117 (3.752)	0.500 (0.698)
19	Soilrite	Polybag	12.067 (3.474)	36.500 (6.039)	19.333 (4.366)	12.167 (3.482)	0.300 (0.546)
20	Soilrite	Protray	8.297 (2.878)	12.600 (3.543)	12.500 (3.533)	7.017 (2.648)	0.260 (0.505)
21	Cocofibe	Plastic pot	9.233 (3.037)	43.000 (6.555)	21.800 (4.669)	17.483 (4.180)	0.500 (0.706)
22	Cocofibe	Mudpot	11.283 (3.358)	40.300 (6.348)	19.800 (4.439)	16.250 (4.029)	0.477 (0.687)
23	Cocofibe	Polybag	8.647 (2.940)	36.500 (6.041)	16.500 (4.048)	11.617 (3.404)	0.477 (0.687)
24	Cocofibe	Protray	7.417 (2.719)	15.600 (3.946)	10.000 (3.148)	6.250 (2.490)	0.420 (0.647)
CD			0.367	0.367	0.463	0.543	0.164
SEm±			0.129	0.129	0.163	0.191	0.058

* Values in paranthesis represents $\sqrt{x}$ transformed ones

Fig.6. Effect of potting mixes and containers on ex vitro rooting and root growth in *H. annulare* (30 days after rooting)



The effect of potting mixes and containers on character of the roots also differed among treatments as is shown in Table 33b. Early rooting was obtained in T_{16} protrays filled with vermicompost (6.1 days). But this was statistically on par with T_4 (sand protray) T_8 (potting mixture protray) and T_{24} (cocofibe protray) (6-7 days). Maximum days to root was observed for T_{18} mudpots filled with soilrite (13.02 days).

The maximum number of roots were observed in shoots receiving treatment T_1 sand in plastic pot (45.3) but this was on par with T_5 (potting mixture plastic pot) T_9 (vermiculite-plastic pot), T_{13} (vermicompost-plastic pot) and T_{21} (cocofibe-plastic pot) ie. (41-45). The minimum number was observed in T_8 protray with potting mixture (8).

The maximum length of the longest root was observed in T_{21} - plastic pot filled with cocofibe (21.0 cm) closely followed by T_{17} - plastic pot filled with soilrite (21.75 cm). These were almost on par with T_{18}, T_{19} (soilrite filled in mudpot and polybag respectively) and T_{22} (cocofibe in mudpot). The shortest roots were observed in plastic pots with vermiculite (T_9) and polybag with vermiculite (T_{11}) ie. 6.5 - 6.8 cm.

The thickest roots were produced in plastic pots with vermicompost (0.57 cm) and the thinnest in protrays filled with potting mixture (0.21 cm).

Effect of potting mixes on rooting of shoots which were not given treatment with growth regulator and planted in mudpots was also studied and the data are presented in Tables 34a and 34b. Maximum rooting was obtained in

Table 34a. Effect of different potting mixes on *ex vitro* rooting in *H. annulare* (30 days after rooting)***

Treatment No.	Treatment ** Potting mixes	Rooting (%)
1	Sand	17.700 (0.434)*
2	Potting mix	16.660 (0.420)
3	Vermiculite	20.830 (0.474)
4	Vermicompost	11.083 (0.338)
5	Cocofibe	50.000 (0.783)
6	Soilrite	5.950 (0.246)
	CD	0.169
	SEm±	0.055

* Values in paranthesis represents arc sine transformed ones
** Mudpots were used as containers
***Shoots were not treated with any auxin

Table 34b. Effect of different potting mixes on *ex vitro* root growth in *H. annulare* (30 days after rooting)***

Treatment No.	Treatment** Potting mix	Days to root	No. of roots	Length of longest root (cm)	Average length of a root (cm)	Thickness of the thickest root (cm)
1	Sand	10.363 (3.219)*	23.867 (4.88))	6.833 (2.614)	3.913 (1.978)	0.020 (0.138)
2	Potting mix	14.283 (3.778)	30.200 (5.494)	14.450 (3.766)	12.567 (3.499)	0.150 (0.384)
3	Vermiculite	13.100 (3.616)	23.467 (4.844)	4.783 (2.176)	2.500 (1.571)	0.040 (0.193)
4	Vermi compost	13.783 (3.711)	21.017 (4.582)	7.700 (2.770)	4.503 (2.114)	0.073 (0.236)
5	Cocofibe	13.317 (3.648)	28.587 (5.342)	14.617 (3.799)	6.233 (2.496)	0.213 (0.461)
6	Soilrite	14.217 (3.767)	18.383 (4.283)	13.367 (3.633)	6.413 (2.531)	0.180 (0.412)
CD		0.239	0.382	0.742	0.568	0.169
SEmt		0.078	0.124	0.241	0.184	0.005

* Values in paranthesis represents $\sqrt{x}$ transformed ones

** Mud pots were used as containers

*** Shoots were not treated with any auxin

cocofibe media (50%) followed by vermiculite (20.8%) and the least in soilrite (5.95%). Early rooting was observed in sand (10.4 days). The number of roots was maximum in potting mixture though statistically on par with cocofibe (5.3 to 5.5). Maximum length and thickness of roots were (14.62 cm, 0.21 cm respectively) obtained in cocofibe closely followed by potting mixture and soilrite though all the three were statistically on par with respect to the characters studied.

4.5 Hardening of plantlets

The plantlets obtained were retained in the mist chamber for 60 days after which they were transferred to bigger mud pots (Plate 10) and kept in net houses (50% light) with frequent irrigation and were treated with nutrient solution at fortnightly intervals. It was observed that, plants grown in pro-trays when transferred to bigger mud pots were smaller than others and with lesser number of leaves. However, after 80-100 days, there was no much difference between the plants morphologically. Vining and flowering of plants were observed.

4.6 Indirect morphogenesis

4.6.1 Induction of callus

The data on the experiments on induction of callus in *H. annulare* is presented in Tables 35a to 44.

4.6.1.1 Effect of growth regulators

The data on the effect of the use of different auxins such as NAA and 2,4-D at 4 levels of 0.5, 1.0, 2.5 and 5.0 mg l^{-1} on initiation of callus is presented in Tables 35a, 35b, 36a, 36b, 38 and Fig.7. Callus index was maximum when NAA

Plate 10. Plants transferred to bigger pots

Plate 10

Table 35a. Effect of different concentrations of 2,4-D on callus induction from axenic explants of *H. annulare* cultured under light conditions

Treatment No.	Treatment		% culture initiating callus	Days to initiate callus	Callus index		Morphology of callus
	Content-ration of 2,4-D (mg l^{-1})	Explant used*			7th day	20th day	
1	2	3	4	5	6	7	8
1	0.5	S	82.1	6.9	0.107	2.999	Watery, translucent changing to yellow
2	0.5	P	62.35	7.2	0.031	1.247	Watery, translucent
3	0.5	YL	67.5	10.6	0.000	0.068	Watery, translucent
4	0.5	L+P	90.0	11.7	0.000	1.800	Cream coloured, watery, translucent with white friable portions
5	0.5	L	60.0	12.1	0.000	0.900	Watery, translucent and white friable
6	1.0	S	86.0	6.85	0.129	2.15	Watery, translucent changing to yellow
7	1.0	P	85.0	7.3	0.034	0.85	Watery, translucent
8	1.0	YL	49.0	12.1	0.000	0.044	Watery, translucent
9	1.0	L+P	89.7	11.7	0.000	0.036	Watery, translucent
10	1.0	L	75.7	8.2	0.008	0.076	Watery, translucent and with pinkish colouration
11	2.5	S	89.7	7.1	0.089	2.691	Watery, translucent and with yellow colouration
12	2.5	P	77.6	11.65	0.000	26.384	Highly watery, translucent and yellow
13	2.5	YL	91.25	12.05	0.000	1.095	Watery, translucent, some nodular callus

Contd.

Table 35a. Continued

1	2	3	4	5	6	7	8
14	2.5	L+P	84.9	11.8	0.000	1.104	Watery, translucent
15	2.5	L	80.5	10.95	0.000	0.644	Watery, translucent and with white friable portions
16	5.0	S	80.2	6.81	0.016	1.444	Watery, translucent later yellow
17	5.0	P	75.1	11.56	0.000	0.075	Watery, translucent
18	5.0	YL	79.3	12.01	0.000	0.793	Watery, yellow
19	5.0	L+P	82.1	11.8	0.000	0.821	Watery, yellow
20	5.0	L	78.5	12.31	0.000	0.118	Watery, translucent

* Explants include S - stem segment, P - petiole segments, YL - whole young leaves, L+P - leaf segments with petiole attached and L - leaf segments

Table 35b. Effect of different concentrations of 2,4-D on callus induction from axenic explants of *H. annulare* cultured under dark conditions

Treatment No.	Treatment		% cultures initiating callus	Days to initiate callus	Callus index		Morphology of callus
	Concentration of 2,4-D (mg l^{-1})	Explant used*			7th day	20th day	
1	2	3	4	5	6	7	8
1	0.5	S	79.25	6.6	0.099	1.585	Watery, translucent, changing to yellow
2	0.5	P	85.50	6.91	0.026	0.086	Watery, yellow
3	0.5	YL	78.80	11.1	0.000	0.394	Watery, translucent and white friable
4	0.5	L+P	82.5	11.65	0.000	0.660	Watery, translucent
5	0.5	L	80.1	12.1	0.000	0.801	Watery, translucent
6	1.0	S	75.3	6.32	0.113	2.636	Watery, translucent and yellow
7	1.0	P	80.5	7.5	0.024	1.006	Watery, translucent
8	1.0	YL	81.2	12.3	0.000	0.649	Watery, translucent
9	1.0	L+P	85.7	11.66	0.000	1.028	Watery, translucent
10	1.0	L	83.5	12.05	0.000	0.443	Watery, translucent
11	2.5	S	85.9	7.2	0.086	1.289	Watery, translucent and yellow
12	2.5	P	81.2	12.1	0.057	0.812	Watery, translucent and yellow
13	2.5	YL	70.5	12.2	0.000	0.353	Watery, translucent and yellow
14	2.5	L+P	80.5	11.95	0.000	0.807	Watery, yellow
15	2.5	L	69.2	10.25	0.000	0.692	Watery, translucent

Contd.

Table 35b. Continued

1	2	3	4	5	6	7	8
16	5.0	S	85.85	6.75	0.000	1.374	Watery, translucent and yellow
17	5.0	P	62.5	10.95	0.000	0.781	Watery, translucent
18	5.0	YL	80.25	11.98	0.000	0.080	Watery, translucent
19	5.0	L+P	81.3	11.75	0.000	1.524	Watery, translucent
20	5.0	L	59.65	12.52	0.000	0.597	Watery, translucent

*Explants include S - stem segments, P - petiole segments, YL - whole young leaves, L+P - leaf segment with petiole attached, L - leaf segments

Table 36a. Effect of different concentrations of NAA on callus induction from axenic explants of *H. annulare* cultured under light conditions

Treatment No.	Treatment		% culture initiating callus	Days to initiate callus	Callus index			Morphology of callus
	Concentration of NAA (mg l^{-1})	Explant used*			7th day	20th day	30th day	
1	0.5	S	80.4	6.35	0.402	40.2		Watery translucent changing to creamy powdery callus in 8-10 days. In 60% of cultures 3-5 hairy or smooth roots observed
2	0.5	P	79.6	7.00	0.016	23.88		
3	0.5	YL	81.2	6.50	0.008	1.624		
4	0.5	L+P	80.2	10.10	0.000	1.604		
5	0.5	L	67.6	10.00	0.000	1.352		,,
6	1.0	S	89.8	6.1	0.269	23.797		,,
7	1.0	P	87.6	6.8	0.009	1.314		,,
8	1.0	YL	86.5	8.2	0.000	0.865		,,
9	1.0	L+P	90.2	10.3	0.000	0.902		,,
10	1.0	L	88.75	10.1	0.000	1.331		,,
11	2.5	S	84.5	5.9	0.761	42.25	169.00	,,
12	2.5	P	85.6	6.3	0.086	29.96	158.36	,,
13	2.5	YL	83.25	6.75	0.008	4.163	8.325	,,
14	2.5	L+P	81.6	6.3	0.008	36.72	146.88	,,
15	2.5	L	80.75	6.45	0.008	40.375	121.125	,,
16	5.0	S	81.25	5.8	0.406	60.938	142.88	,,
17	5.0	P	80.3	6.7	0.402	40.15	108.41	,,
18	5.0	YL	79.65	6.9	0.008	7.169	59.738	,,
19	5.0	L+P	81.25	7.0	0.244	48.75	121.875	,,
20	5.0	L	82.35	7.0	0.165	41.175	144.113	,,

* Explants include S - stem segments, P - petiole segments, YL - whole young leaves, L+P - leaf segment with petiole attached, L - leaf segment

Table 36b. Effect of different concentrations of NAA on callus induction from axenic explants of *H. annulare* cultured under dark conditions

Treatment No.	Treatment		% cultures initiating callus	Days to initiate callus	Callus index		Morphology of callus
	Concentration of NAA (mg l^{-1})	Explant used*			7th day	20th day	
1	0.5	S	81.2	7.8	0.244	34.916	50 per cent of cultures produced (3-5) hairy or smooth roots, watery callus change to cream powdery callus in 5-6 days
2	0.5	P	79.6	7.1	0.016	31.84	
3	0.5	YL	65.8	7.0	0.019	2.632	
4	0.5	L+P	78.2	9.5	0.000	2.346	,,
5	0.5	L	66.3	10.35	0.000	0.995	,,
6	1.0	S	87.8	11.1	0.000	1.905	,,
7	1.0	P	88.1	10.5	0.000	0.881	,,
8	1.0	YL	84.5	10.85	0.000	0.676	,,
9	1.0	L+P	86.2	11.2	0.000	0.689	,,
10	1.0	L	87.1	10.1	0.000	0.348	,,
11	2.5	S	80.5	7.6	0.121	4.025	,,
12	2.5	P	72.3	7.1	0.072	5.061	,,
13	2.5	YL	71.5	10.9	0.000	1.43	,,
14	2.5	L+P	60.4	11.5	0.000	0.604	,,
15	2.5	L	81.5	10.8	0.000	2.038	,,
16	5.0	S	82.6	7.1	0.372	12.39	,,
17	5.0	P	83.7	7.2	0.251	7.533	,,
18	5.0	YL	81.2	6.9	0.041	1.624	,,
19	5.0	L+P	60.7	7.3	0.006	0.789	,,
20	5.0	L	61.7	10.2	0.000	1.234	,,

*Explants include S - stem segments, P - petiole segments, YL - whole young leaves, L+P - leaf segment with petiole attached, L - leaf segments

Table 37. Effect of different cytokinins and auxins on callus growth and morphology in *H. annulare*

Treatment No.	Treatment		Callus index		Percentage of cultures established	Morphology
	Media Growth regulator	Concentration (mg l^{-1})	20th day	30th day		
1	MS+BA	2.5	29.72	40.668	40.0	Cream with green tinge
2	MS+BA	5.0	27.777	50.004	41.67	Very green, compact
3	MS+BA	10.0	24.0	88.32	32.0	Green, compact
4	MS+BA	20.0	21.75	89.915	43.5	Green, compact with white specks
5	MS+KIN	0.25	1.475	18.430	14.75	Cream
6	MS+KIN	0.5	0.424	7.42	10.60	Creamish green
7	MS+KIN	2.5	3.045	31.538	21.75	Creamy
8	MS+KIN	5.0	1.5	20.1	30.0	Creamy
9	MS+2iP	0.25	17.36	27.125	21.70	Greenish calli changing to cream
10	MS+2iP	0.5	13.013	27.48	20.82	Cream
11	MS+2iP	1.0	25.529	53.064	26.4	Cream
12	MS+2iP	2.5	15.868	35.24	24.3	Cream, fresh calli
13	MS+BA+IAA	0.5+1.0	20.556	45.292	26.8	Greenish calli changing to cream
14	MS+BA+IAA	0.5+2.0	15.385	28.96	18.1	Cream
15	MS+BA+IAA	1.0+1.0	9.639	37.842	35.7	Creamy turning to yellow
16	MS+BA+NAA	0.25+0.5	16.20	27	21.6	Cream
17	MS+BA+NAA	0.5+1.0	21.75	27.188	14.5	Cream
18	MS+BA+NAA	0.5+2.0	7.395	14.918	12.75	Fresh cream and white friable
19	½MS+KIN	1.0	0.290	0.387	9.67	Cream
20	MS+TDZ	0.05	66.885	121.03	63.7	Green compact with white friable specks

Table 38. Effect of auxin concentration on induction and growth of callus of *H. annulare*

Treatment No.	Treatment		Percentage of cultures initiating callus	Callus Index*
	Auxin	Concentration (mg l^{-1})		
1	NAA	0.5	76.010	14.139
2	NAA	1.0	87.655	3.271
3	NAA	2.5	78.191	16.663
4	NAA	5.0	77.470	22.175
5	2,4-D	0.5	76.811	0.984
6	2,4-D	1.0	79.161	0.892
7	2,4-D	2.5	81.125	3.587
8	2,4-D	5.0	76.475	0.761

* Callus index observed 20 days after inoculation

Table 39. Effect of different growth regulators on callus induction of explants of *H. annulare* taken from glasshouse and cultured under light

Treatment No.	Treatment: Growth regulator	Treatment: Concentration (mg l^{-1})	Treatment: Explent*	% of culture initiating callus	Days for callus initiation	Callus index: 7th day	Callus index: 20th day	Callus index: 30th day	Morphology
1	2	3	4	5	6	7	8	9	10
1	2,4-D	0.5	S	57.0	6.7	1.14	19.95	28.5	Pinkish coloured
2	2,4-D	0.5	P	60.3	6.9	0.006	24.12	30.15	Compact and white friable
3	2,4-D	0.5	YL	49.7	7.1	0.049	2.485	4.97	Watery, translucent and white friable
4	2,4-D	0.5	L+P	62.3	6.6	21.961	77.875	124.60	Compact cream
5	2,4-D	0.5	L	61.75	5.5	2.161	40.138	64.838	Watery, translucent
6	2,4-D	1.0	S	52.50	5.25	0.683	47.276	78.75	Cream, compact and white friable
7	2,4-D	1.0	P	51.63	4.67	7.67	39.600	84.054	Translucent and watery
8	2,4-D	1.0	YL	48.1	9.8	0.000	1.924	48.1	Translucent watery and creamy
9	2,4-D	1.0	L+P	49.7	7.2	2.485	42.245	74.55	"
10	2,4-D	1.0	L	60.2	7.4	0.015	9.632	31.605	"
11	NAA	1.0	S	47.3	6.7	29.799	40.678	93.654	Cream powdery with green patches 3-4 roots in 50% cultures
12	NAA	1.0	P	51.45	6.3	25.725	38.588	90.038	
13	NAA	1.0	YL	49.75	7.0	21.393	44.775	74.625	"
14	NAA	1.0	L+P	55.23	7.1	44.184	82.845	124.268	"
15	NAA	1.0	L	59.8	7.2	5.98	44.85	104.65	"
16	2iP	0.5	S	49.7	7.4	0.089	30.814	59.64	Green compact changed to cream by 20 days
17	2iP	0.5	P	60.2	6.9	0.120	30.1	36.12	
18	2iP	0.5	YL	67.8	10.25	0.000	1.017	33.9	"

Contd.

Table 39. Continued

1	2	3	4	5	6	7	8	9	10
19	2iP	0.5	L+P	70.2	8.1	1.053	52.65	70.2	Green compact changed to cream
20	2iP	0.5	L	61.3	11.5	0.000	19.616	42.91	by 20 days
21	2iP	2.0	S	48.75	6.9	0.488	15.6	40.95	Translucent, watery and white friable
22	2iP	2.0	P	61.3	6.8	1.226	18.39	45.975	"
23	2iP	2.0	YL	59.7	7.1	0.299	14.93	37.611	70% of cultures greenish
24	2iP	2.0	L+P	60.8	9.8	0.000	7.904	25.354	Green nodular calli
25	2iP	2.0	L	57.6	10.15	0.000	0.576	14.4	"
26	BA	2.0	S	60.7	7.2	3.035	30.35	60.7	Cream nodular calli
27	BA	2.0	P	61.3	9.1	0.092	7.66	30.65	"
28	BA	2.0	YL	62.7	11.3	0.000	0.063	37.62	"
29	BA	2.0	L+P	49.75	10.8	0.000	0.498	22.89	"
30	BA	2.0	L	70.1	10.2	0.000	1.753	52.58	"
31	BA	4.0	S	66.7	6.9	3.335	33.35	66.7	Green compact calli
32	BA	4.0	P	62.3	6.8	1.246	1.869	31.15	"
33	BA	4.0	YL	61.46	11.7	0.000	30.073	30.73	Green, compact with white friable patches
34	BA	4.0	L+P	52.3	5.95	2.092	54.706	62.3	Cream nodular, some green patches
35	BA	4.0	L	65.1	10.25	bleached by 7th day			
36	BA	10.0	S	71.3	6.7	2.139	53.475	89.125	Green compact and with white friable
37	BA	10.0	P	70.4	6.88	0.704	70.4	105.6	specks
38	BA	10.0	YL	61.2	10.5	0.000	0.000	3.06	"
39	BA	10.0	L+P	53.76	11.27	0.000	2.688	26.88	"
40	BA	10.0	L	60.78	9.98	bleached by 7th day			

* Explant include S - stem segments; P - Petiole segments; YL - Whole Young leaves
L+P - Leaf segment with petiole attached; L - Leaf segment

Table 40. Effect of different explants on induction and growth of callus of *H. annulare*

Treatment No.	Treatment Explants	Percentage of cultures initiating callus	Callus Index*
1	Stem segments S	83.272	14.743
2	Petiole segments P	79.159	10.741
3	Young leaves YL	76.963	1.479
4	Leaf segments with petiole attached L+P	80.966	6.262
5	Leaf segment L	75.211	5.819

Table 41. Effect of cytokinin concentration on induction and growth of callus of *H. annulare***

Treatment No.	Treatment		Percentage of cultures initiating callus	Callus index*
	Cytokinin	Concentration (mg l^{-1})		
1	2iP	0.5	61.840	26.839
2	2iP	2.0	57.630	11.479
3	BA	2.0	60.910	8.065
4	BA	4.0	61.772	18.599
5	BA	10.0	63.488	25.313

* Callus index observed 20 days after inoculation
** Explants were taken from glasshouse

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Table 42. Effect of auxin concentrations on induction and growth of callus from axenic and glasshouse explants of *H. annulare*

Treatment	Treatment			Percentage of cultures initiating callus	Callus Index*
	Explant source	Auxin	Concentration (mg l^{-1})		
1	Axenic	NAA	1.0	88.570	5.642
2	Axenic	2,4-D	0.5	72.390	1.263
3	Axenic	2,4-D	1.0	77.080	0.631
4	Glasshouse	NAA	1.0	52.706	50.347
5	Glasshouse	2,4-D	0.5	58.210	32.914
6	Glasshouse	2,4-D	1.0	52.430	28.136

*Callus index observed 20 days after inoculation

Table 43. Effect of light on induction and growth of callus from axenic explants of *H. annulare*

Treatment No.	Treatment			Percentage of cultures initating callus	Callus Index*
	Auxin	Concentration (mg l^{-1})	Culture condition L/D**		
1	NAA	0.5	L	77.800	13.732
2	NAA	0.5	D	74.220	14.546
3	NAA	1.0	L	88.569	5.642
4	NAA	1.0	D	86.740	0.899
5	NAA	2.5	L	83.140	30.694
6	NAA	2.5	D	73.240	2.632
7	NAA	5.0	L	80.960	39.636
8	NAA	5.0	D	73.980	4.714
9	2,4-D	0.5	L	72.390	1.263
10	2,4-D	0.5	D	81.230	0.705
11	2,4-D	1.0	L	77.080	0.631
12	2,4-D	1.0	D	81.240	1.153
13	2,4-D	2.5	L	84.790	6.384
14	2,4-D	2.5	D	77.460	0.791
15	2,4-D	5.0	L	79.040	0.650
16	2,4-D	5.0	D	73.910	0.871

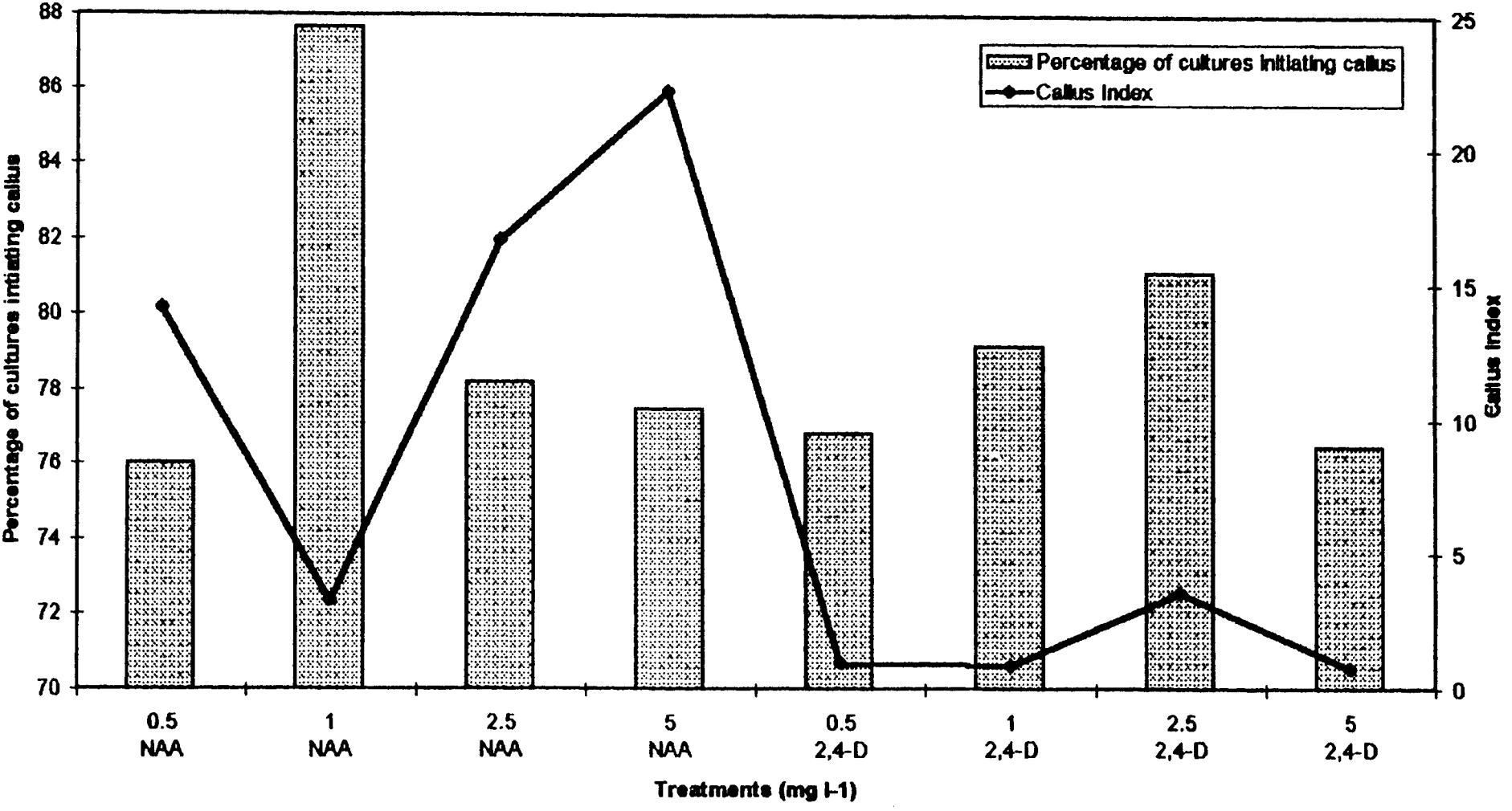
* Callus index observed 20 days after inoculation
** L indicates light and D indicates dark

Table 44. Effect of orientation of leaf explants on induction and growth of callus in *H. annulare*

Treatment No.	Treatment		Percentage of cultures initiating callus	Callux index*
	Explant**	Orientation**		
1	L + P	Abaxial	80.96	1.174
2	YL	Abaxial	76.96	0.454
3	L + P	Adaxial	61.35	0.798
4	YL	Adaxial	49.6	0.169

* Callus index observed 20th day after inoculation
** L+P indicates leaf segment with petiole attached to it and YL indicates whole young leaf
***Abaxial/Adaxial surface of the explant touched the medium

Fig.7. Effect of auxin concentration on induction and growth of *H.annulare*



was used especially at the highest concentration of 5.0 mg l^{-1} (22.2). 2,4-D in general, produced lesser quantity of callus. Among the different concentrations of 2,4-D tried, 2.5 mg l^{-1} produced higher amount of callus (3.6). At other concentrations, the callus formed ranged from 0.76 to 0.98, minimum when 2,4-D was used at the highest concentration of 5.0 mg l^{-1}.

The morphology of the callus produced also varied (Tables 35a, 35b, 36a, 36b and Plates 11, 12). When NAA was used, the callus initially induced was soft, watery and transparent and it soon changed to creamy, powdery callus which was not very responsive to the later attempts to regenerate. Callus growth rate was higher in medium supplemented with NAA, and was being produced upto 30 days. Calli produced with NAA could be easily subcultured while that produced in 2,4-D supplemented medium were very soft, watery and as such was difficult to subculture. Also, root regeneration was observed in callus initiated in medium supplemented with NAA. The morphology of roots produced varied. They were hairy or smooth. Those roots produced when cultures were exposed to light were light green in colour while the cultures exposed to dark conditions white or creamy white in colour. Forty to fifty per cent of cultures produced roots in media supplemented with NAA. Calli produced in media supplemented with 2,4-D remained watery, soft and transparent. A few cultures produced greenish or pinkish tinged calli. Some also had white friable or white nodular portions. Such cultures were found to be better responsive to embryogenesis on further subculturing to appropriate media.

The data on the effect of different growth regulators on induction of callus from explants directly taken from the stock plants kept in glasshouse, are

Plate 11. Translucent callus with nodular portions in MS medium + 2,4-D

Plate 12. Hairy roots on callus in MS medium + NAA

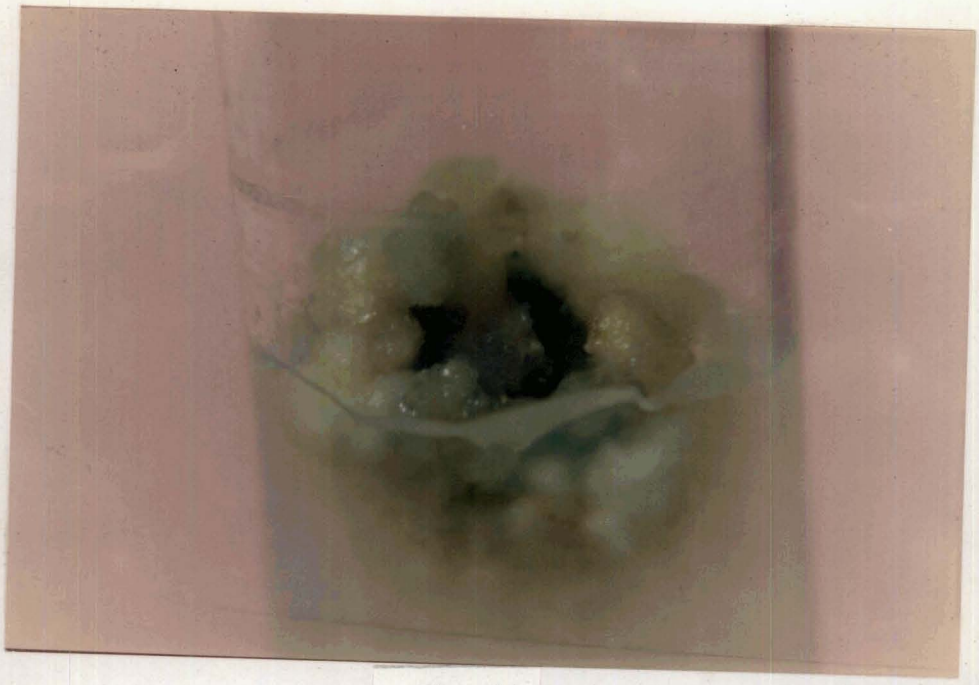


Plate 11

Plate 12

presented in Tables 39 and 41. Callus index was maximum when 2iP at a concentration of 0.5 mg l^{-1} was incorporated in medium (26.8) closely followed by BA at very high concentration of 10.0 mg l^{-1} (25.3). Number of cultures initiating callus were almost equal in all the treatments tried (Table 41).

The morphology of the callus initiated in media containing cytokinin was typical. They were green and compact and with white powdery portions (Table 37).

When TDZ was incorporated to MS medium at the rate of 0.1 mg l^{-1}, it produced a callus index of 112.5 in 20 days when leaf segments from axenic plants were used as explants. Callus remained fresh, green and compact even upto 60 days acquiring a callus index of 225 (3 g).

4.6.1.2 Standardisation of explants

The data on the response of different types of explants to forming callus are presented in Table 40. Among the different explants screened, maximum callusing was observed with stem bits (callus index (14.7) followed by petiole bits (10.7). Least responsive was whole young leaf (1.5).

4.6.1.3 Effect of source of explants

In general, explants taken from plants grown in the glasshouse produced better quantity of callus than explants taken from axenic cultures irrespective of the growth regulator used. For example, when NAA at 1 mg l^{-1} was supplemented in MS medium, the callus index of *in vitro* explant was only 5.6 while that of glasshouse explant was 10 times higher ie. 50.3. So was the case when 2,4-D too was supplemented in MS medium. However, the percentage of cultures initiating

callus was much higher (79.3%) when *in vitro* explants were used. The percentage establishment of glasshouse explants was comparatively lesser ie. 54.4% (Table 42).

4.6.1.4 Effect of light

In most cases, maximum callus index was observed when cultures were exposed to light than when exposed to dark (Table 43). When NAA 2.5 mg l^{-1} concentration was incorporated to MS medium and when callus was initiated in such medium both under light and dark conditions, callus index was much higher in light (30.7) than in dark (2.6). On an average the callus index under light conditions was (12.3) as against dark (3.3).

4.6.1.5 Effect of orientation of foliar explants

The results of the effect of the orientation of the leaf explants on the media are presented in Table 44. Irrespective of the explant used, maximum number of callus initiating cultures and maximum quantity of callus (as expressed by callus index) was produced when abaxial surface of the explant was placed touching the media.

4.6.2 Proliferation of callus

4.6.2.1 Proliferation of callus in solid media

Callus that was induced in MS medium supplemented with either 2,4-D or NAA could be further proliferated either in same medium or in a medium having higher/lower concentration of the same growth regulator. Each subculturing was done at 20 days interval inoculating approximately 0.001 g of callus to the fresh medium.

The data on the effect of different treatments tried for proliferation studies are presented in Table 45a. In general, NAA produced better callus than 2,4-D when supplemented in MS medium. Callus index in NAA supplemented media was on an average 102.4 as against 63.4 in 2,4-D supplemented media during first subculture.

The morphology of callus produced varied among treatments. Cultures induced in medium supplemented with 2,4-D produced watery, soft callus while that with NAA produced profuse cream callus (Plate 13a). Rhizogenesis was also induced in most cultures produced in MS medium supplemented with NAA. Callus induced in medium containing TDZ were further subcultured to lower concentration (0.05 mg l^{-1}) of the same growth regulator (Plate 13b) producing an average callus index of 84 within 20 days and it continued to produce fresh green callus upto 45 days (callus index 160).

Proliferated callus obtained either directly from induction medium or after further proliferation in medium with same, lower or higher concentration of growth regulator could be subjected to morphogenesis trials.

4.6.2.2 Proliferation of callus as suspension culture

The results of the effect of callus proliferation in liquid medium through production and maintenance of suspension culture are presented in Table 45b and Plate 13c.

The proliferation rate was ascertained with respect to the increase in packed cell volume expressed as percentage.

Table 45a. Effect of different auxins on callus proliferation in *H. annulare* (solid media)

Treatment No.	Treatment					
	Induction medium MS		First * subculture MS	Second * subculture MS	Callus index in first subculture	Callus index in second subculture
	Growth regulator	Cocnentration ($mg\ l^{-1}$)	concentration ($mg\ l^{-1}$)	Concentration ($mg\ l^{-1}$)		
1	2,4-D	0.5	0.5		70.335	
2	2,4-D	0.5	1.0		72.379	
3	2,4-D	0.5	1.0	1.0	108.0	109.5
4	2,4-D	1.0	1.0		67.600	
5	2,4-D	2.5	2.5		33.170	
6	2,4-D	2.5	5.0		42.700	
7	2,4-D	5.0	5.0		16.298	
8	2,4-D	5.0	5.0	1.0	96.500	87.5
9	NAA	0.5	0.5		101.700	
10	NAA	0.5	1.0		102.600	
11	NAA	0.5	1.0	1.0	97.800	100.25
12	NAA	1.0	1.0		109.700	
13	NAA	2.5	2.5		115.200	
14	NAA	2.5	5.0		90.350	
15	NAA	5.0	5.0		89.750	
16	NAA	5.0	5.0	1.0	111.800	110.25

* Subculturing was done at 20 days intervals

Plate 13a. Proliferation of callus in MS medium + NAA

Plate 13b. Proliferation of callus in MS medium + TDZ

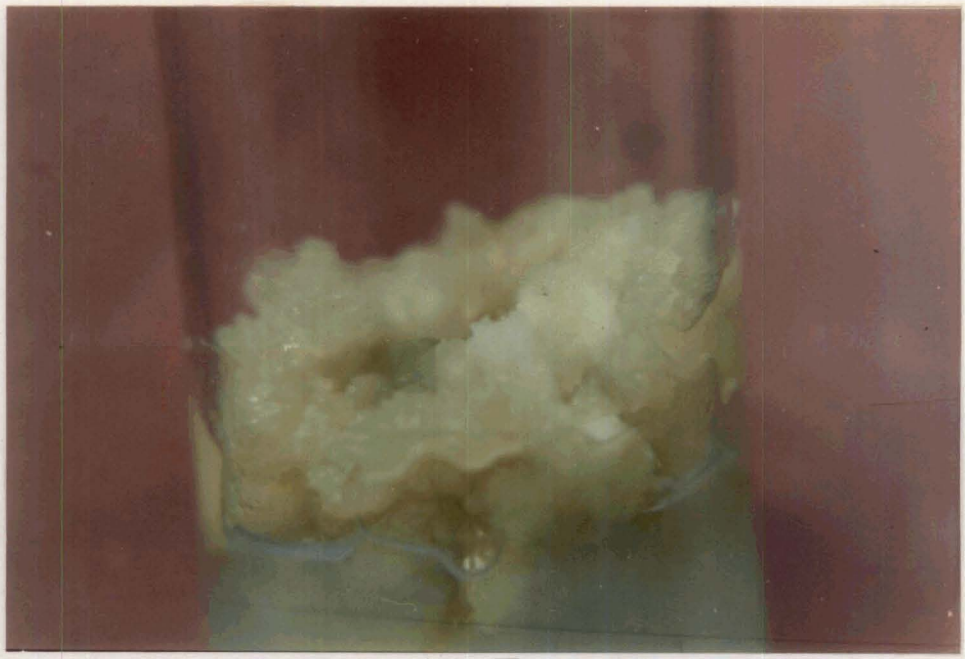


Plate 13a

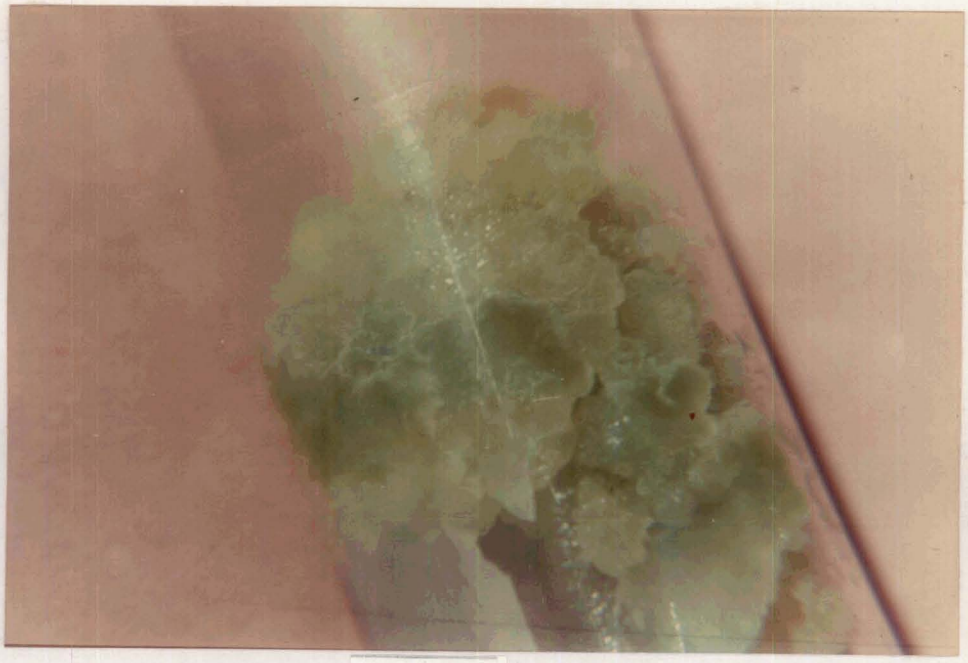


Plate 13b

Table 45b. Effect of different auxins on callus proliferation in *H. annulare* (liquid media)

Treatment No.	Treatment: Induction medium MS (solid) + Growth regulator	Concentration ($mg\ l^{-1}$)	Treatment: Medium for initial suspension MS (solid) + Growth regulator	Concentration ($mg\ l^{-1}$)	PCV* of initial suspension	Medium to which suspension was subcultured MS (liquid) +		Final PCV	Increase in PCV expressed as percentage
1	TDZ	0.1	2,4-D	0.5	0.5	2,4-D	0.5	1.0	50
2	TDZ	0.1	2,4-D	5.0	0.4	(a) MS basal (b) 2,4-D	5.0	1.0	60
3	TDZ	0.1	2,4-D+ BA	0.5+ 0.5	0.25	2,4-D+ BA	0.5+ 0.5	2.5	225
4	BA+IAA	2.5+1.0	2,4-D+ BA	0.5+ 5.0	0.5	2,4-D+ BA	0.5+ 5.0	1.5	100
5	2,4-D	0.5	2,4-D	0.5	0.35	(a) MS basal (b) 2,4-D (c) 2,4-D+ BA	5.0 0.5+ 5.0	26.3	2590
6	2,4-D**	0.5	2,4-D+BA	0.5+5.0	0.125	2,4-D+BA	0.5+5.0	2.0	187.5
7	2,4-D**	1.0	2,4-D+BA	5.0+5.0	0.1	2,4-D+BA	5.0+5.0	0.4	27.5
8	2,4-D**	2.5	2,4-D+BA	5.0+0.5	0.25	2,4-D+BA	5.0+0.5	0.5	25
9	2,4-D**	5.0	2,4-D+BA	5.0+5.0	0.20	2,4-D+BA	5.0+5.0	0.4	17.5
10	NAA**	1.0	2,4-D+BA	0.5+5.0	0.20	2,4-D+BA	0.5+5.0	1.0	80
11	NAA**	1.0	2,4-D+BA	5.0+0.5	0.37	2,4-D+BA	5.0+0.5	1.0	63

* PCV indicates packed cell volume

** Cultures were initiated in the dark

Plate 13c. Proliferation of callus in liquid MS (suspension culture)

Plate 14a. Callus initiating embryoids (solid MS medium + 2,4-D)

Plate 13c

Plate 14a

Maximum increase in packed cell volume was observed when callus was induced in MS medium supplemented with 0.5 mg l^{-1} 2,4-D and further subcultured on to liquid MS supplemented with the same concentration of 2,4-D followed by subculturing into three different media, basal MS medium, MS medium supplemented with a combination of 2,4-D (0.5 mg l^{-1}) and BA (5.0 mg l^{-1}). The final PCV was 26.25. Thus the increase in PCV expressed as percentage was as high as 2590 per cent.

Least effective was that of callus induced in 2,4-D 5.0 mg l^{-1} and further maintained in MS medium (liquid) supplemented with BA and 2,4-D each at 5.0 mg l^{-1} (17.5%).

4.6.3 Regeneration

4.6.3.1 Organogenesis

Among the different treatments tried for induction of organoids from the callus formed, no treatment was responsive in the formation of organoids. Treatments with TDZ and BA however resulted in the callus mass acquiring greenish coloration and a compact nature. However, there was no further development and formation of organoids.

4.6.3.2 Embryogenesis

The results of the attempts to induce regeneration through embryogenesis are summarised in Tables 46, 47, 48 and 49.

Table 46. Effect of different auxins on induction of embryoids in *H. annulare*

Treatment No.	Media in which explants were raised originally MS solid + Growth regulator	Concentration (mg l^{-1})	Explant*	Callus induction media Growth regulator	Concentration (mg l^{-1})	Subculture No.	Subculture media Growth regulator	Concentration (mg l^{-1})	Embryogenesis Stage of embryo	No. of embryos produced	Days for embryo production
1	KIN	1.0	L+P	2,4-D	1.00***	1	2,4-D	1.0	Globular	2	23
2	KIN	1.0	P	2,4-D	2.5	1	2,4-D	2.5	Globular Reverted to callus by 38th day	4	22
3	KIN	1.0	YL	2,4-D	2.5	1	2,4-D	2.5	Globular Contaminated	4	19
4	TDZ	0.1	L+P	2,4-D	2.5	1	2,4-D	2.5	White nodular structures		
						2	2,4-D	1.0	Globular	2	22
						3	MS basal (liquid)		Globular white smooth embryoid floating on the surface	18	19
5	TDZ	0.1	L+P	2,4-D	1.0	1	2,4-D	1.0	Globular	2	20
6	TDZ	0.1	L+P	2,4-D	1.0	1	2,4-D	1.0	Globular	3	18
						2	2,4-D	1.0	Reverted to callus		15
7	TDZ	0.1	L+P**	2,4-D	5.0	1	2,4-D	5.0	Globular	5	26
8	TDZ	0.1	L	2,4-D	2.5	1	2,4-D	2.5	Globular	7	22
9	TDZ	0.1	L	2,4-D	2.5	1	2,4-D	2.5	Globular, heart	8, 2	20
						2	2,4-D	1.0	Heart	4	12
						3	MS basal (solid)		Elongated	2	9
10	TDZ	0.05	L+P**	2,4-D	5.0	1	2,4-D	5.0	Globular Reverted to callus	4	12 20

* Explants include L+P - Leaf segment with petiole attached, P - petiole segment; YL - whole young leaf; L - leaf segment

** Adaxial surface of the explant touched the medium

*** Cultures kept in dark conditions for induction

Table 47. Effect of explant source on induction of embryoids in *H. annulare*

Treatment No.	Treatment*		Percentage of embryogenesis
	Growth regulator	Concentration (mg l^{-1})	
1	BA	1.0	0
2	KIN	1.0	20.15
3	2iP	1.0	0
4	TDZ	0.1	46.667
5	TDZ	0.05	6.67

*Treatments denote the media combinations in which explants were originally raised

Table 48. Effect of different explants on subsequent embryoid formation in *H. annulare*

Treatment No.	Treatment Explant		Percentage of embryogenesis
1	Stem segment	S	0.000
2	Petiole segment	P	6.667
3	Leaf segment with petiole attached	L + P	40.05
4	Leaf segment	L	13.33
5	Young leaf	YL	6.667

Table 49. Effect of orientation of explants on subsequent embryoid formation in *H. annulare*

Treatment No.	Treatment		Percentage of embryogenesis
	Explant*	Orientation**	
1	L + P	Abaxial	26.667
2	L + P	Adaxial	13.330
3	L	Abaxial	13.330
4	L	Adaxial	0.000

* L+P indicates leaf segment with petiole attached and L indicates leaf segment
** Abaxial/Adaxial surface of the explant touching the medium

4.6.3.2.1 Formation of embryoids

The explants taken from axenic plants produced in MS medium supplemented with either TDZ or KIN alone initiated globular structure when cultured on media with 2,4-D and later removed to fresh media with either the same or lower concentration of 2,4-D. Maximum percentage of embryo formation was obtained when explants were cultured in MS medium supplemented with TDZ at 0.1 mg l^{-1} concentration (46.7%) followed by KIN 1.0 mg l^{-1} (20.2%). When concentration of TDZ was only 0.05 mg l^{-1}, percentage of embryoid formation was also decreased (6.7%). Callus formed initially in media supplemented with BA and 2iP did not form embryoids subsequently even when transferred to media with 2,4-D.

When leaf bit with a portion of petiole attached (taken form culture produced in MS media supplemented with TDZ 0.1 mg l^{-1}) were used for callus initiation in MS medium supplemented with 2.5 mg l^{-1} of 2,4-D soft watery callus was formed. This when subcultured to the same medium, produced white nodular structures on the calli. Such calli on further subculture to a lower concentration (1.0 mg l^{-1}) of 2,4-D produced two globular creamy white smooth embryoids within 22 days. On subculture to liquid basal MS medium and maintaining as suspension culture, larger number of (18) globular white embryoids floating on the surface of the culture were produced within 19 days of subculture.

The next best treatment was T_9. When leafbits produced in MS media supplemented with TDZ 0.1 mg l^{-1} was induced to produce callus in 2.5 mg l^{-1} 2,4-D supplemented MS media and subjected to the same series of subcultures (as that of treatment 4) excepting the third subculture which instead of MS basal liquid

medium, a solid basal MS medium was used. However, the number of embryoids produced was lesser. But four heart shaped embryoids along with six globular embryoids were produced, by second subculture and of these two got elongated in third subculture.

4.6.3.2.2 Effect of explants

The explant used for induction of callus had profound effect on the subsequent formation of embryoids (Table 48). When leaf bits with portions of the petiole attached were used for callus initiation and when the callus formed were induced to form embryoids, the highest percentage of embryoids were formed from such sources (40.05%) and when leaf bits alone were used for callus initiation, the subsequent embryoid formation from such callus was reduced (13.33%). Callus induced from petiole bits and whole young leaves were on par with respect to the percentage of embryoids formed (7.6%). The callus induced from stem bits, was not conducive to embryoid formation at all.

The orientation of the explant when put on the media for callusing also had an effect on the subsequent formation of embryoids (Table 49). The callus formed when leaf bits were oriented with the abaxial surface in contact with the medium produced a mean percentage of around 20 per cent of embryoid while from the callus produced when the adaxial surface was in contact with the medium only around 6.7 per cent embryoid formation was observed. Callus formed from leaf bits with petiole attached and with the abaxial surface in contact with the medium produced higher percentage of embryoid formation (26.7%).

The results showed that when globular embryoids (Plates 14a and 14b) formed in 2,4-D medium was retained in the same medium or subcultured to the medium containing same concentration of 2,4-D there were chances of embryoids to get reverted to callus phase (Table 46). This was the case observed in treatments 2 and 10. However, if subcultured to medium containing lower concentration of the same auxin produced heart shaped embryoids which later on elongated (Plates 14c and 14d).

The embryoids formed however did not develop further.

4.7 Direct morphogenesis

4.7.1 Direct organogenesis

When whole young leaf was cultured in MS medium supplemented with 0.05 mg l^{-1} TDZ it produced a shoot bud directly in 32 days (Plate 15).

4.8 Encapsulation studies

4.8.1 Production of synthetic seeds

An assessment of the various concentrations of sodium alginate (2-3% w/v) and calcium chloride (50-100 mM) for the formation of beads is presented in Table 50a. A 2.5 per cent solution of sodium alginate upon complexation with 75 mM of $CaCl_2.2H_2O$ solution gave optimal, firm and round beads within an ion exchange duration of 30 min (Plate 16a). The diameter of the beads ranged between 0.6 and 0.8 cm. Lower concentration of sodium alginate (2%) resulted in the formation of very fragile beads, whereas concentration higher than 2.5 per cent proved to be too viscous for the free flow of the dispersed explant. Lower levels of

Plate 14b. Embryoid at globular stage (x 40)

Plate 14c. Embryoid at heart shaped stage (x 40)

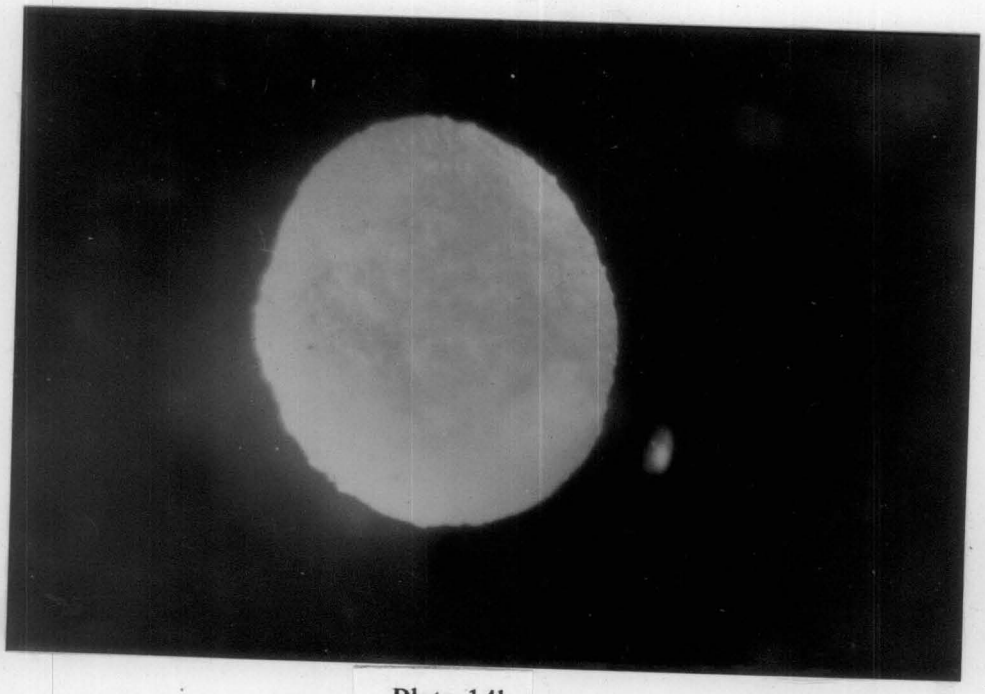


Plate 14b

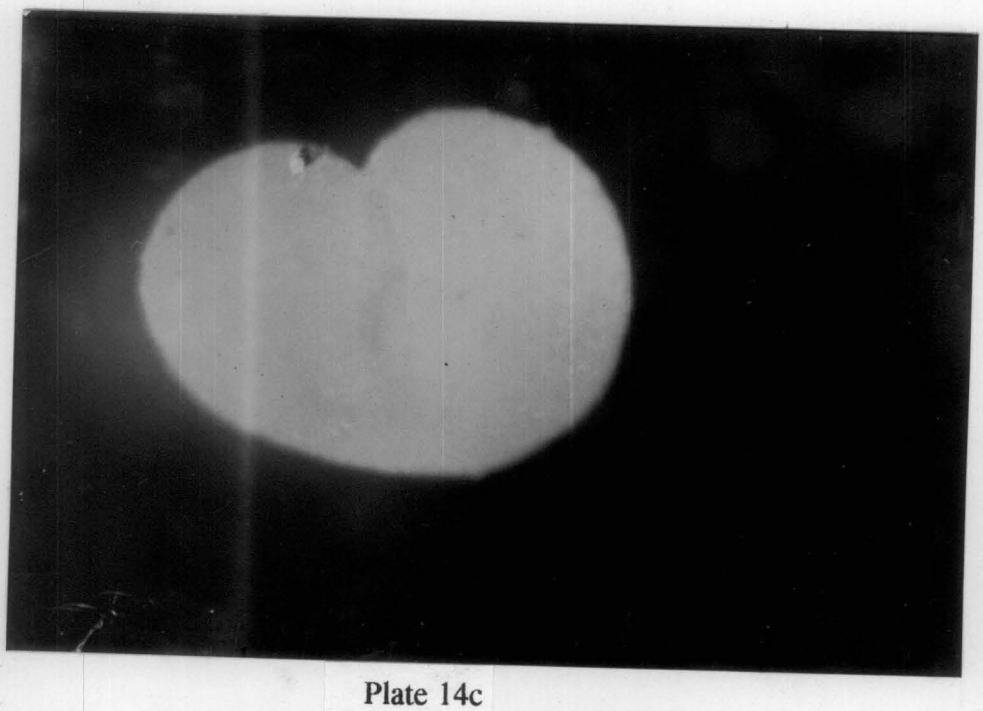


Plate 14c

Plate 14d. Elongation of embryoids in solid media (x 25)

Plate 15. Direct organogenesis from young leaf explant in MS medium + TDZ

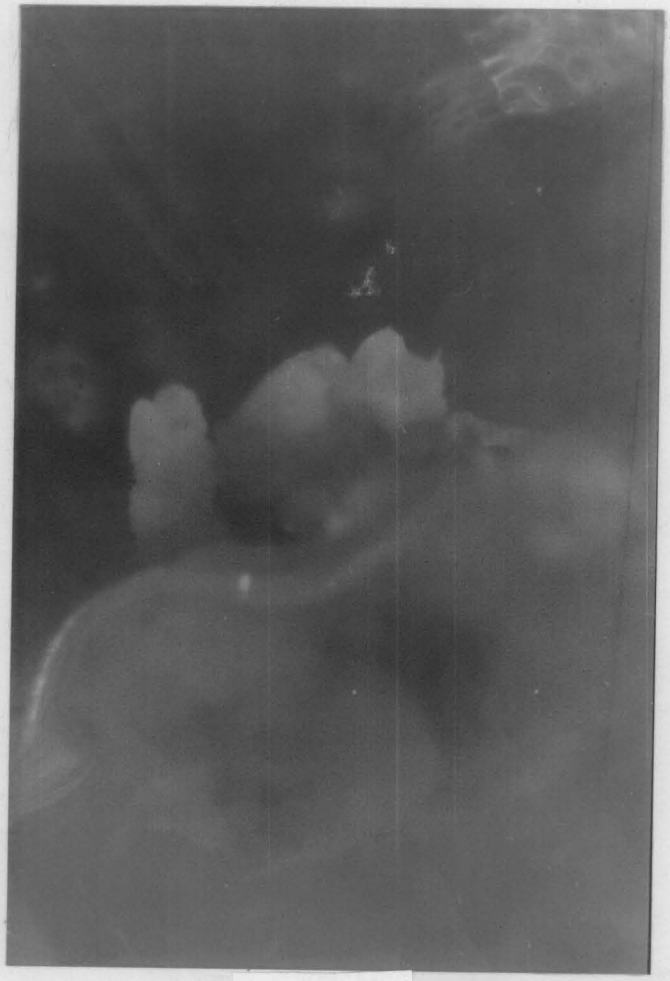


Plate 14d

Plate 15

Table 50a. Effect of different concentration of sodium alginate and calcium chloride on bead formation in *H. annulare*

$CaCl_2.2H_2O$ (mM)	Sodium alginate (% w/v)		
	2.0	2.5	3.0
50	++	+++	+
75	+++	++++	+++
100	+++	++++	+++

Observations were taken after 30 min complexation duration

+ refers to bead quality in terms of shape, size and firmness

Table 50b. Effect of different substrata and storage temperature on retention of viability of encapsulated beads of *H. annulare*

Treatment No.	Treatment		Days to which viability is retained
	Storage stubstrate	Temperature (°C)	
1	Cotton wool + liquid MS	30	15
2	,,	26	22
3	,,	4	40
4	Solid MS basal	30	12
5	,,	26	19
6	,,	4	38

Plate 16a. Encapsulated alginate beads of *H. annulare*

Plate 16b. Shoot initiation in alginate beads of *H. annulare*

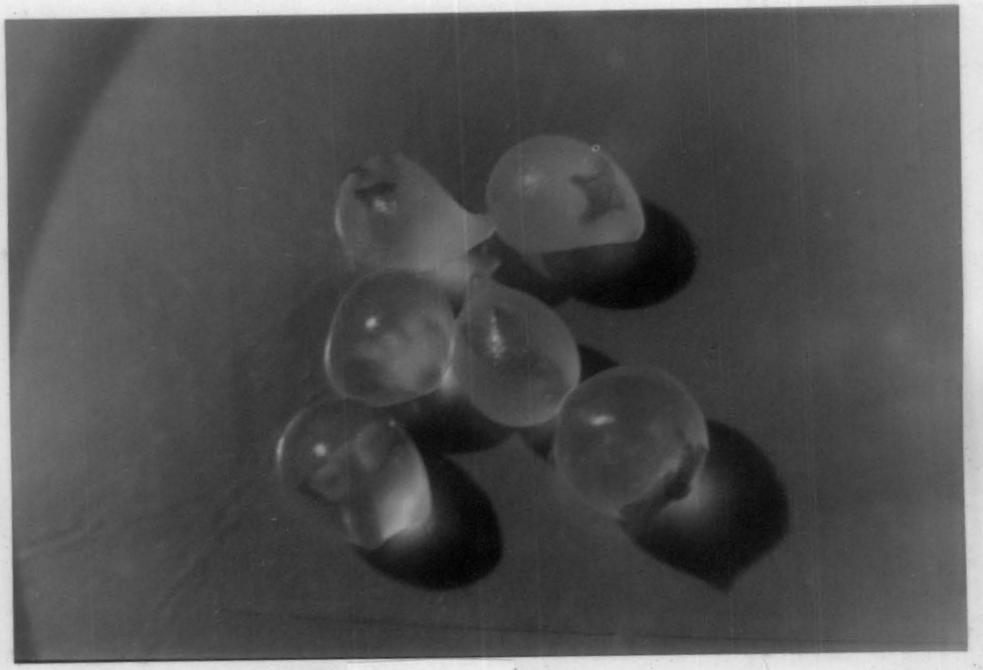


Plate 16a

Plate 16b

$CaCl_2.2H_2O$ on the other hand, not only adversely affected the bead quality, but also prolonged the complexation time.

4.8.2 Storage and plantlet conversion of synthetic seeds

The encapsulated beads stored at room temperature (30°C) retained their regeneration capacity for 15 days when put on cotton wool moistened with MS basal salts, while those kept in solid MS basal medium lost viability in 12 days. When the beads were stored at 26°C, regeneration capacity was extended to 22 days in cotton soaked with liquid MS media and 19 days for the beads stored in solid MS basal salts (Table 50b). When they were stored at 4°C the regeneration capacity was further extended upto 40 days in cotton wool soaked in liquid MS media and upto 38 days when stored on solid MS basal salts. The encapsulated beads when put on basal MS media with 0.5 mg l^{-1} BA regenerated into well formed shoots within 8 days (Plate 16b).

4.9 Biochemical studies

4.9.1 Poly acrylamide gel electrophoresis

The peroxidase isozyme patterns are represented as bands in Plate 17. The pattern of the bands was more or less similar in the tissues compared, namely, leaves and roots taken from *in vitro* raised plants and mother plants. Three comparatively broad bands were obtained in the case of tissues taken from the leaves and two broad bands were obtained in the case of tissues taken from the roots.

Plate 17. Poly acrylamide gel electrophoresis of peroxidase isozyme

1. *In vitro* leaf
2. *In vitro* root
3. *In vitro* root
4. *In vitro* root
5. *In vivo* root
6. *In vivo* leaf
7. *In vivo* root
8. *In vivo* root

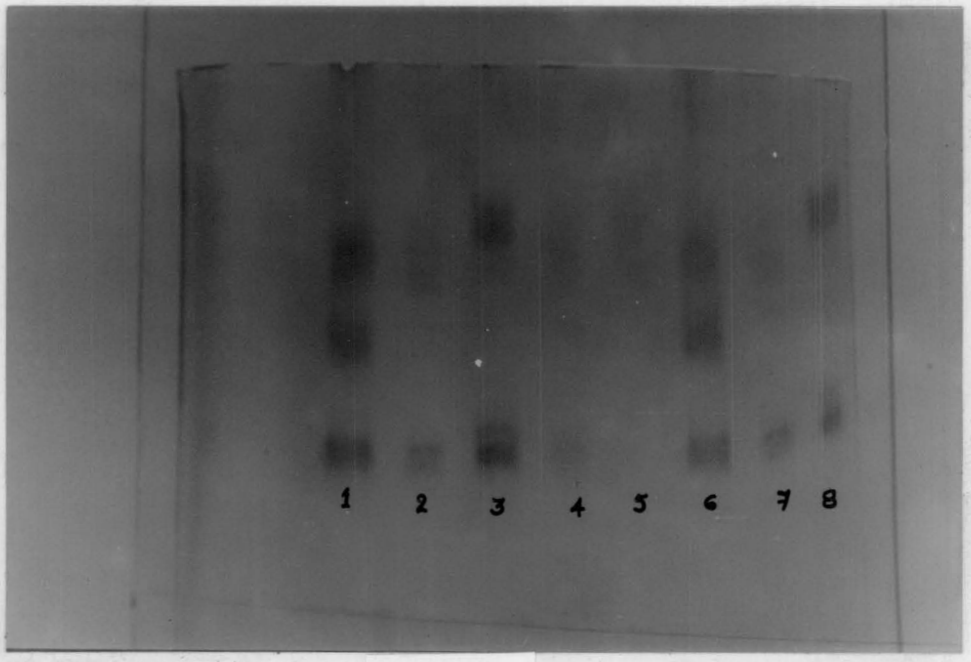


Plate 17

Discussion

DISCUSSION

The present study on standardisation of *in vitro* techniques for the rapid multiplication of *Holostemma annulare* K. Schum was carried out at the Plant Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara from 1993 to 1995.

Holostemma annulare K. Schum is a medicinal plant belonging to the family Asclepiadaceae which is widely used for cure of different ailments of the eye and as a general tonic. The roots of this plant are reported to possess cooling, tonic and lactative properties. Plant as such is highly valued and the entire demand is met through collection from wild sources. The plant is not domesticated and can be considered as an endangered species. *Holostemma annulare* is propagated through vine and root cuttings to a limited extent and seed set is very sparse.

The use of tissue culture techniques for clonal propagation has become the most widely used application of tissue culture technology in horticulture in recent years (Thorpe, 1990). Clonal plants could be produced at a very rapid rate compared to conventional methods by adopting micropropagation techniques (Chaturvedi and Sharma, 1988). Micropropagation can be achieved by enhancing axillary bud breaking, production of adventitious buds directly or indirectly through a callus phase or by somatic embryogenesis directly or indirectly (Murashige, 1974).

The present studies were carried out to standardise rapid multiplication methods using *in vitro* techniques in *Holostemma annulare* K. Schum.

The results obtained in the studies are discussed in this chapter.

5.1 Standardisation of surface sterilisation of explants

The explants collected from the field harbour a variety of microorganisms which have to be removed before inoculation on to the culture medium. Though general sterilisation procedures have been outlined by various workers (Dodds and Roberts, 1982; George and Sherrington, 1984), specific sterilization procedures have to be evolved based on the tissues being handled. Hence, in the present study, the sterilization procedure with respect to the concentration and duration of exposure was standardised. Among the different treatments tried, the best sterilization treatment varied with respect to the source of explants. For two to three month old explants taken from the glasshouse, sterilization treatment with 0.1 per cent mercuric chloride for 5 min or 10 min was found better than others with reference to the least percentage of contamination and the maximum percentage of explants showing growth. Treatment with chlorine water for 7 min was also effective in completely preventing contamination but around 40 per cent of the explants were seen bleached with this treatment. For ten to fourteen month old explants taken from the glasshouse, the effectiveness of the sterilization treatments differed. The best treatment for these explants was found to be with 0.1 per cent Bavistin for 30 min followed by a dip in 1.0 per cent Cetrimide for 10 min and then in 0.1 per cent mercuric chloride for 15 min which gave the highest percentage of contamination free cultures. Addition of 0.1 per cent streptomycin to the medium considerably reduced the extent of bacterial contamination. However, the percentage of establishment of cultures with this treatment was seen reduced.

In respect of the explants taken from plants grown in the field, combination treatments with different sterilants had to be given for effective results.

Treatment with 0.1 per cent mercuric chloride for 5 or 10 min was not effective. For these explants, the most effective sterilization treatment was to treat them first with 0.1 per cent Bavistin for 30 min followed by one per cent Cetrimide for 10 min, 0.1 per cent Norfloxacin for 30 min, an alcohol wipe followed by a dip in 0.1 per cent mercuric chloride for 13 min with respect to the higher percentage of establishment and cultures showing growth. Of the different sterilization treatments tried for root explants, none were found effective in producing contamination free cultures.

Mercuric chloride has been effectively used as a surface sterilant for various medicinal plants. Sudha and Seeni (1994) reported that a treatment with 1.0 per cent Labolene for 6-8 min followed by washing with tap water and treatment with 0.1 per cent mercuric chloride for 15 min produced contamination free cultures. Sharma and Chandel (1992) found that a teepol wash for 15 min followed by treatment with 0.1 per cent mercuric chloride for 7 min was an effective sterilization procedure for *Tylophora* sp. The results obtained in the present study agree with these reports in respect of the effectiveness of mercuric chloride as a surface sterilant. Chlorine water has been successfully used as a surface sterilant in crops like *Aristolochia indica* where treatment for 15 min was effective. However, in the present study, use of chlorine water resulted in a large number of cultures getting bleached.

Combinations of ethyl alcohol and mercuric chloride have been used with success as sterilant treatments in *Duboisia myoporoides* (Kukreja *et al.*, 1986), *Coscinium fenustratum* (Nair *et al.*, 1992), *Vetiveria zizanioides* (Keshavachandran and Abdul Khader, 1993) etc. Likewise, explants have been treated with antibiotics or fungicides prior to putting in a surface sterilant solution for more effective control

of contamination as was reported in *Commiphora wightii* (Barve and Mehta, 1993) and *Ananas comosus* (Prabha, 1993). Incorporation into media or treatment of explants with antibiotics in the present study resulted in lower percentages of establishment of the explants.

The percentage of contamination observed for the explants collected in the different months showed variation. The explants collected in the drier months of the year from January to April showed comparatively lower contamination while those collected in the wetter rainy months showed higher contamination rates. The rates of contamination observed with explants taken in the months from May to October could be reduced by using a combination sterilization treatment with 0.1 per cent Bavistin for 30 min followed by Norfloxacin 0.1 per cent for 30 min, an alcohol wipe followed by 0.1 per cent mercuric chloride for 13 min. Differences in the rates of contamination in different months have been reported in other crops also. Barve and Mehta (1993) found in the case of *Commiphora wightii* that the contamination rate was higher for the cultures initiated during July-August and September-October. Prabha (1993) reported that for *Ananas comosus* the explants collected in the wetter rainy months showed higher contamination rates. Likewise, the contamination rate was found to be lowest in the present study when collected in the months of January and February.

5.2 Culture establishment

5.2.1 Standardisation of media and growth regulators

In most of the reports on medicinal plants, the media used was Murashige and Skoog (1962) medium. In the present study also for culture of nodal segments and shoot tip explants MS medium was better than WPM with respect to

the percentage of establishment and growth. MS medium has been successfully reported to be the most suitable in *Aristolochia indica, Tylophora indica, Aegle marmelos, Gomphrena officinalis, Valeriana wallichii, Nardostachys jatamansi* and *Piper longum* tissue cultures. Physical nature of media also has been found to be a very important criterion for morphogenesis. In the present study, early bud release was observed in stationery liquid media irrespective of the explants used. However, growth was better in solid media.

In the present study, maximum percentage of establishment of nodal segment explants was observed in MS medium supplemented with KIN 1 mg l^{-1}. However, the growth of cultures was better in MS medium supplemented with BA. Precocity in bud release was observed when BA was incorporated in the medium. BA when incorporated at lower concentration of 1 mg l^{-1} resulted in the production of more number of longer shoots, buds and nodes. In *Aegle marmelos*, the shoots produced in BA medium were elongated but were thin compared to those induced in KIN medium as reported by Hossain *et al.* (1994). In the present study when KIN alone was incorporated into the medium, the shoots produced were short and robust while with BA at lower concentration (upto 2.5 mg l^{-1}) elongated shoots which were light green coloured and with long internodes were produced. In *Eucalyptus grandis*, when nodal explants were cultured in medium containing BA alone, multiple shoots were produced as reported by Lakshmi Sita and Shoba Rani (1985). In *Pelargonium graveolens* where nodal explants were reported to form more multiple shoots with a combination of BAP and IAA incorporated in the medium (Satyakala *et al.*, 1995).

In the present study, BA when incorporated at higher concentrations of 5.0 and 10.0 mg l^{-1}, large number of multiple shoot buds were produced but no

further elongation of shoots was observed and the shoots were thin, light green coloured, stunted and with weak nodes. These had to be subcultured as a clump to MS media containing low concentration of 0.5 mg l^{-1} BA or to basal MS media for elongation and production of strong nodes.

Hu and Wang (1983) reported that elongation of micropropagated shoots is often inhibited by higher cytokinin levels in the media. The present study conforms with this observation. In *Woodfordia fruticosa*, Krishnan and Seeni (1994) have reported that the number of shoots regenerated per explant increased concomittantly with the concentration of BAP.

Irrespective of the growth regulator used, callus formation was observed at the base of the explant. It was profuse when BA was incorporated in the MS media and sparse when KIN was used.

Extremely low concentrations of TDZ have been reported to stimulate axillary shoot proliferation in many woody species including *Juglans nigra* (Driver and Kumyuki, 1984). In the present study also TDZ at low concentration of 0.1 or 0.2 mg l^{-1} produced moderate number of (7-8) nodes on an average.

The use of additives did not have any significant improvement in the production of longer shoots, buds or nodes in *Holostemma annulare*. However, the callus production was reduced significantly when activated charcoal was incorporated in MS media followed by silver nitrate and then ascorbic acid. In the present study it was observed that the treatments to suppress callus production also resulted in suppression of shoot growth. Among the treatments tried, MS medium without any additives gave the best percentage of establishment followed by MS

medium incorporated with 2 mg l^{-1} of adenine sulphate. Rate of growth of the shoots was better in medium containing 15 per cent coconut water. Skoog and Tsui (1948) have reported that adenine sulphate when added to the medium, often can enhance growth and shoot formation. Multiple shoot induction in *Dioscorea floribunda* was reported by Sinha and Chaturvedi (1979) in a medium supplemented with BAP, adenine sulphate and NAA. Addition of coconut water at 15 per cent level was found to be beneficial to shoot proliferation in *Dendrobium* orchid (Lakshmidevi, 1992). Dornelas and Viera (1994) have reported that coconut water had an important role on the morphogenic capacity of *Passiflora* root explants. These are reports which indicate the promoting effects of additives on establishment and growth of cultures as seen in the present study. The ability of certain additives like activated charcoal, silver nitrate and ascorbic acid to inhibit callus growth have been observed in the present study as also in the report by Fridborg and Eriksson (1975), who have reported that charcoal inhibits growth of soyabean.

The effect of growth regulators on the establishment and growth of shoot tip explants was similar to that on nodal segment explants. The growth of shoots was best when lower concentrations of BA (0.25-2.5 mg l^{-1}) was used. At lower concentrations, the shoot length was more while with higher BA concentrations, shoots were shorter but with more shoot buds. KIN and 2iP produced relatively shorter shoots. At half the salt concentration, bud break was earlier than at full salt concentration of MS.

In the present study, treatments involving adenine sulphate was superior to those without any other additives unlike nodal segment explants. However, silver nitrate was found to be inhibitory to the growth of shoot tip cultures.

Comparing the performance of nodal segments and shoot tips, it was found that nodal segments were better in respect of early release of buds, more number of longer shoots, nodes and buds. However, shoot tip explants produced lesser amount of callus. The number of explants that could be multiplied from a mother plant was comparatively less with shoot tip explants compared to nodal segments.

5.2.2 Culture conditions

In general, tropical plant species require a higher range of temperature during culture. Durahvila *et al.* (1992) reported that higher incubation temperature (between 21°C and 30°C) was required for bud culture and callus induction in sweet orange. In the present study higher temperature (30 ± 1°C) proved better than lower temperature (26 ± 1°C) for the growth of cultures.

In *Passiflora edulis*, Dornelas and Vieira (1994) reported that except for cotyledenary explants, all others needed exposure to light which alone favoured shoot regeneration. Similar was the case with *Holostemma* where exposure to light was found necessary for early release of buds and for healthy growth of the shoots. Exposure to dark resulted in etiolation of shoots.

5.3 Proliferation

5.3.1 Standardisation of growth regulators

In the present study, it was clearly seen that as the concentration of BA in the media was measured, the number of shoots, buds and nodes produced was also increased. BA at 20 mg l^{-1} resulted in cent per cent of the shoots being very short swollen, stunted and light green in colour. BA at lower concentrations (0.25-

2.5 mg l^{-1}) produced longer shoots. BA at 1 mg l^{-1} produced around 4 shoots, 7 buds and shoots of 16 cm length with 10-11 nodes in 3 weeks but callusing was profuse. KIN at higher concentrations produced very less number of shoot buds. Among the different concentrations of KIN tried, 2 mg l^{-1} was better. KIN and 2iP produced morphologically similar shoots which were shorter and more robust than those produced in medium containing BA. When IAA was incorporated in MS medium containing KIN, the shoots produced were longer and with more number of nodes and with more number of shoot buds than when KIN alone was used. When instead of IAA, NAA was used, callusing was very high.

Multiple shoots in large numbers could be obtained from excised hypocotyls of young *Eucalyptus terreticornis* seedlings in media containing BA alone as reported by Subbaiah and Minocha (1990). When a comparison was made between various cytokinins, it was found that KIN was not so effective on bud induction in *Taxus* spp. (Chee, 1995). In *Aegle marmelos*, Hossain *et al.* (1994) reported that adventitious shoots could be developed in a medium supplemented with BA or with a combination of auxins of which IAA was the best. Kukreja *et al.* (1986) reported in *Duboisia myoporoides* that KIN and BAP at lower levels favoured only callusing when used in combination with IAA, IBA or NAA. But at higher levels of KIN and BAP in combination with lower levels of IAA, extensive shoot bud regeneration was reported. The synergestic action of KIN and IAA at equal concentrations promoted vigorous growth of shoot buds in *Clitorea ternatea* as reported by Lakshmanan and Dhanalakshmi (1990). This was in conformity with the results of the present study. A combination of KIN and NAA could produce the highest number of shoot buds in *Valeriana wallichii* through indirect organogenesis as reported by Mathur and Ahuja (1991).

In the present study, when shoots were proliferated in MS medium containing TDZ, long shoots with approximately 7 nodes were produced. Shoots of *Miscanthus xogiformis* when transferred from medium containing BA to TDZ formed significantly more axillary shoots than shoots grown continuously on either medium or transferred from TDZ to BA (Nielsen *et al.*, 1995).

Full strength MS medium with or without agar proved better than half strength MS with respect to more number of nodes and lesser internodal length in *Holostemma*. Solid MS medium alone produced shoot buds but increased length of shoot, more number of nodes and lesser callus were observed in liquid MS medium.

Among the additives used, coconut water was proved to be better than others with respect to longer shoots and more number of nodes. No shoot bud production was observed. The shoots were dark purplish green in colour.

5.3.2 Effect of growth regulators and additives on serial subculturing

BA proved to be the best growth regulator for obtaining maximum proliferation in all subcultures tried. In all excepting the first subculture, BA at higher concentration was good for more number of nodes per culture. For the first subculture, a combination of BA and IAA at 0.25 and 0.5 mg l^{-1} respectively, gave the best response. For the second, third and fifth subculture, BA at 10 mg l^{-1} was the best but for the fourth one BA at 20 mg l^{-1} was the best. Thus in the present study, cultures of single nodal segment forms the best method of proliferation. There are similar reports in other crops also. George and Sherrington (1984) have reported that single node culture is of possible value for propagating species that produce elongated shoots in culture. Report on certain viny medicinal plants proliferated by

this method are *Dioscorea floribunda* (Chaturvedi, 1975); *Passiflora* spp. (Drew, 1991); *Tylophora indica* (Sharma and Chandel, 1992) and *Aristolochia indica* (Kavitha and Raju, 1995). George *et al.* (1993) reported in *Gardenia jasminodes* that multiple shoot porliferation was maximum after two subcultures in high BA medium. To maintain high multiplication rate, subculture to the same medium was sufficient. However, the concentration had to be reduced in further subcultures to produce healthy shoots. This conforms with the present study. In *Woodfordia fruticosa*, Krishnan and Seeni (1994) have reported that reculture of the nodal explants added a higher propagation rate to shoot tip culture and together formed the basis of the rapid micropropagation protocol in the crop.

In the present study if the best treatment in each subculture was given in sequence, approximately 2 crores 37 lakh nodes could potentially be obtained over a period of 225 days. Among the different treatments tried in each subculture, an assessment of the maximum number of nodes that could be obtained at the end of each subculture was made and it was found that a maximum of 148, 1600, 27766, 485899 and 3356744 nodes respectively could be produced at the end of first, second, third, fourth and fifth subcultures.

As obtained in the present study, higher multiplication rates have been obtained in other medicinal crops as well. In *Glycirrhiza glabra*, Shah and Dalal (1980) indicated that from a single axillary bud, more than 2,00,000 plants can be obtained in the flasks in six months through enhanced release of axillary buds. Assuming that 50 per cent of the plants survive, it is possible to have an yield of 100,000 plants within eight months, starting from a single bud. Similarly in *Dioscorea floribunda* from a single node stem segment, about 25,60,000 true to type

plants could be obtained (Chaturvedi *et al.*, 1982). In *Pelargonium graveolens*, by enhanced axillary shoot multiplication there is enormous potential for mass multiplication, with a high rate of upto 50 to 60 shoots per subculture and about 95 per cent rooting has been reported (Satyakala *et al.*, 1995). Thus it could be inferred that rapid propagation could be obtained through *in vitro* culture of single nodal segments in those medicinal plants producing elongated shoots in culture.

Among the additives tried, silver nitrate proved to be inhibitory to shoot growth if incorporated in solid MS medium while the inhibitory action of silver nitrate was not pronounced in stationary liquid medium. When added to liquid medium along with BA, the effect of cytokinin was more pronounced. None of the additives in any subculture proved superior for proliferation. Moderate number of nodes were produced when media were supplemented either with coconut water or adenine sulphate. But they could not produce as much nodes as could be obtained in a medium containing BA.

5.4 Rooting

5.4.1 *In vitro* rooting

In the present study, maximum rooting could be obtained with full strength MS basal medium (90%) while minimum rooting was observed in MS medium supplemented with activated charcoal (30%). Early rooting and more number of longer roots could be obtained in MS basal medium. Rooting was faster in solid media than stationary liquid media. The number and length of roots were more in solid media. Basal part of the shoot gave better rooting than the top portion.

Morphology of the roots produced differed among the treatments tried. Roots induced in MS medium supplemented with NAA were thick, short, creamish in colour and were produced in clusters while those in medium supplemented with IBA were thin, white in colour and with rootlets. Roots produced in MS basal medium were thin, long, unbranched, coiled and light green in colour.

Various workers have obtained *in vitro* rooting in medicinal plants. In *Clitorea ternatea*, efficient rooting could be obtained in media supplemented with 0.5 mg l^{-1} of IBA. In *Glycirrhiza glabra*, Shah and Dalal (1980) reported that NAA if incorporated in MS medium produced short thick roots. In the present study also, NAA produced morphologically similar roots. Maximum root length was obtained in MS basal medium with half the salt concentration, but the roots so produced were very slender as reported in *Gardenia jasminodes* by George *et al.* (1993). In the present study also the longest roots was obtained in MS basal medium without any auxin but with full salt strength and the roots so produced were slender and coiled. Prolonged exposure of *Commiphora wightii* shoots to auxin levels which promoted root initiation was found inhibitory for further root growth. Hence transfer of cultures to auxin free medium was necessary as reported by Barve and Mehta (1993). Ravishankar and Jagadish (1989) have reported that transfer of shoots to auxin free medium was necessary for rooting in Indian rose wood.

5.4.2 *Ex vitro* rooting

Deberg and Maene (1981) observed that the most labour intensive part of micropropagation is *in vitro* rooting while as per Rajeevan and Pandey (1986), the major cost of producing *in vitro* plants lies in the rooting and hardening stages. Separation of individual shoots from multiple shoot cultures and rooting them *in*

vitro are both time and labour consuming. The shoots produced *in vitro* could be rooted under *ex vitro* conditions by treating the shoots as microcuttings without using aseptic conditions. Rooting under *ex vitro* conditions would also facilitate the combining of the rooting stage with the acclimatization (George and Sherrington, 1984). In the present study, *ex vitro* rooting was found better than *in vitro* rooting. *Ex vitro* rooting was attempted with different media, growth regulators and containers. Maximum rooting was obtained with IBA 1000 mg l^{-1}. This treatment resulted in maximum number, length and thickness of roots (at 60 days after rooting) as well as earlier rooting. Minimum rooting was obtained with the proprietory dust formulation viz., Grow root powder. Even without any growth regulator treatment, there was rooting. A preliminary trial was made with three different media viz. sand, potting mixture and vermiculite and it was found that maximum percentage of rooting and early rooting were obtained in sand while maximum number, length and thickness were obtained in potting mixture.

Considering the combined effect of the growth regulators and the media used, the best performance with respect to more rooting percentage, early rooting and more number of roots was obtained when shoots were treated with 1000 mg l^{-1} IBA for 60 s followed by planting in sand. However, length and thickness of the roots were slightly lesser in this case.

5.4.2.1 Potting mixes and containers

The type of potting mixes used for transplanting *in vitro* raised shoots is an important factor which determines the efficiency of rooting and growth of shoots kept for *ex vitro* rooting. In the present study, cocofibe produced maximum percentage of rooting, number, length and thickness of the roots though sand produced

roots earlier than cocofibe. The type of containers may also affect the rooting and growth of shoots kept *ex vitro*. In the present study, the best container was plastic pot which produced maximum rooting, number and length of the root. Thickness was maximum when kept in mud pots. However, earliest rooting was obtained when protrays were used. The number, length and thickness of roots were however, the minimum when protrays were used. Considering the effect of media and containers together it was found that the treatments differed with the parameters studied. Maximum rooting was obtained for shoots kept in mud pots and filled with cocofibe. However, maximum number of roots were obtained in shoots kept in plastic pots filled with sand. Length and thickness were maximum for shoot kept in plastic pots filled with cocofibe and vermicompost, respectively. Early rooting was observed for protrays filled with vermicompost.

In *Holostemma annulare, ex vitro* rooting is the best rooting method. Thus *ex vitro* rooting under nonaseptic conditions help to reduce the time and labour involved in *in vitro* rooting and in the subsequent hardening stages thereby substantially reducing the cost of production of *in vitro* plantlets. It also facilitates the combining of the rooting stage with that of acclimatization which is an essential part of the micropropagation procedure (George and Sherrington, 1984).

Ex vitro rooting has been successfully used as an efficient rooting method only in a few medicinal plants. Kavitha and Raju (1995) have reported in *Aristolochia indica* that the shoots produced *in vitro* could be planted in autoclaved wet soilrite mixed with IBA 0.1 mg l^{-1} and covered with polythene sheet to provide high humdiity and rooted *ex vitro*. Prabha (1993) found that the commercially available rooting powder, Rooton induced the fastest rooting and the maximum

length of the root in *ex vitro* rooting in *Ananas comosus*. In the present study, shoots pretreated with growth regulators were kept in the mist chamber to provide congenial conditions for rooting.

Various workers have used different potting mixes to successfully induce roots *ex vitro*. George and Sherrington (1984) have described that the *in vitro* produced shoots could be inserted into rooting media such as peat, perlite, vermiculite or a mixture of these compounds and kept in a highly humid environment for rooting. A potting mix of peat and purmice was found successful in *Pinus radiata* (Aitkenchristie and Thorpe, 1984) and soilrite in *Aristolochia indica* (Kavitha and Raju, 1995). Prabha (1993) has reported in *Ananas comosus* that the potting mixes such as cocopeat, soilrite, biofibe and vermiculite were found better for inducing vigorous growth of the *in vitro* grown plantlets. The better results obtained with potting mixes such as cocofibe and sand in the present study may be due to the improved rhizosphere provided by these media having better ventilation and drainage which would have created a favourable condition for better root growth and the consequent vigorous growth of the plantlets.

Various workers have used different types of containers to transfer shoots or plantlets raised *in vitro*. Plastic pots have been used to transfer plantlets of different species (Yadav *et al.*, 1990). Ramesh *et al.* (1993) also used plastic pots for successful transfer of jack plantlets and reported mudpots as the least effective. In *Ananas comosus*, Prabha (1993) found that the plantlets could be successfully transferred into protrays for rapid growth of more number of plantlets in the initial stages and then transplanted into plastic pots or mudpots for vigorous growth. From the present study it can be stated that though early rooting was observed when pro-

trays were used, the percentage of rooting obtained was poor and hence planting in plastic pot was the best for *Holostemma annulare*.

Considering the potting mixes and containers together, from the present study, it could be seen that shoots produced *in vitro* after giving a quick dip (1 min) in IBA 1000 mg l^{-1} should be planted in plastic pots filled with sand in the initial stages for early rooting and then transplanted into plastic pots or mud pots filled with cocofibe for vigorous growth of root and shoot portions. Plantlets could be transferred to bigger mud pots 60 days after rooting and kept in net houses. These were treated with nutrient starter solution frequently for boosting up the vigour.

5.5 Callus induction, proliferation and regeneration

5.5.1 Induction

In the present study, callus induction was obtained with different auxins and cytokinins from different explants but the intensity, morphology and regenerative ability of calli varied. Maximum callus index could be obtained when NAA was used as the auxin while 2,4-D produced very less callus but were more responsive to embryogenesis trials. Among the cytokinins, callus index was higher when 2iP was used. But TDZ produced the highest callus under relatively lower concentrations. NAA produced watery callus initially which soon changed to powdery and cream coloured callus. 2,4-D produced soft, translucent watery callus with nodular portions which was difficult to subculture. TDZ and BA produced green, compact, hard callus with white powdery portions on the top. Calli produced in the medium containing TDZ remained fresh for a longer period than that induced in any other growth regulator.

Among the different explants screened, maximum callus was observed with stem bits. But leaf segments produced more regenerative callus. In general, explants taken from plants grown in the glasshouse produced better quantity of callus than explants taken from axenic cultures. But the percentage establishment of glasshouse explants was comparatively lesser. In the case of foliar explants, maximum callusing was obtained when abaxial surface of the explant was placed touching the media.

Maximum callus index was obtained when cultures were exposed to light than when exposed to dark.

5.5.2 Proliferation

Callus proliferation was more in solid MS medium supplemented with NAA but further regeneration was not successful in that case. The callus could be held for longer duration without subculturing in MS medium supplemented with TDZ. When callus proliferation was attempted using liquid MS medium as suspension culture, maximum increase in packed cell volume was observed when callus induced in solid MS medium supplemented with 2,4-D was proliferated in liquid MS medium containing 2,4-D and further prolfierated in MS medium supplemented with or without 2,4-D or a combination of 2,4-D and BA.

A number of workers have reported callus induction and proliferation in media supplemented with auxins in many crops such as *Solanum nigrum* (Mandal and Gadgil, 1979), *Rauvolfia serpentina* (Ilahi and Akram, 1987), *Plumbago rosea* (Satheeshkumar and Bhavanandan, 1989) and *Catharanthus roseus* (Mumtaz *et al.*, 1990).

Skoog and Miller (1957) have reported that the levels of plant growth regulating substances in the culture medium, particularly high auxin and low cytokinins, often lead to callus formation. Mathur (1993) has reported that auxin alone or a high auxin:cytokinin ratio were responsible for continued inorganized callus proliferation or callus growth accompanied by rhizogenesis while high levels of cytokinin alone produced dark green nodular structure on callus surface with meristematic zones in them which failed to develop further, when transferred to the same fresh medium. The results obtained in the present study also conform to this.

5.5.3 Regeneration

5.5.3.1 Indirect organogenesis

Treatments with TDZ and BA resulted in callus acquiring green coloration but no further development was obtained. There was no development of organoids in any of the treatment tried.

5.5.3.2 Indirect somatic embryogenesis

In the present study, embryoids were initiated mostly from axenic leafbit explants (with or without petiole) only from explants produced in MS medium supplemented with TDZ or KIN. These explants when kept in MS medium supplemented with 2,4-D, watery, soft calli with nodular portions were produced which when subcultured in the medium containing lower concentrations of 2,4-D, produced a few globular smooth embryoids. Further production was triggered when these were again subcultured to solid or liquid MS basal medium but the increase in embryoid production from the latter medium was much higher and globular embryoids were seen floating on the surface. These embryoids had to be transferred

to solid media for further development. Some of the globular embryoids initiated in solid MS media on a second subculture were transferred to heart shaped embryoids of which some got elongated. Orientation of foliar explants was important and embryoids were intiated only in those oriented with the abaxial surface touching the media.

Thus from the present study it was shown that embryogenic calli were intiated in the medium containing higher levels of 2,4-D but differentiation to embryoids took place either at lower concentrations of 2,4-D or in the basal media. The report by Ammirato (1983) conforms with the present study. He reported that in *Hyoscyamus niger*, callus growth was induced in an auxin enriched medium and somatic embryogenesis took place only upon transfer of callus to a medium free of the auxin. In *Cayratia japonica*, Zhou *et al.* (1994) have reported spontaneous somatic embryogenesis when embryogenic callus (formed in MS medium supplemented with 2,4-D alone or in combination with TDZ) was subcultured on to the basal medium.

Somatic embryogenesis was attempted in a number of medicinal plants, and the positive result include the reports of Chang and Hsing (1980) in *Panax ginseng*, Mhatre *et al.* (1984) in *Tylophora indica*, Reinbothe *et al.* (1990) in *Digitalis lanata*, Giri *et al.* (1993) in *Aconitum* spp. and Jasrai *et al.* (1993) in *Plantago ovata*. Anu (1993) obtained embryoids of torpedo stage in *Gymnema sylvestre* but further development was not obtained. Embryoids either turned brown and dried up or turned to a callus phase. In the present study, the elongated embryoids did not develop further when subcultured on to the regeneration media. Some of the embryoids which were retained in higher concentrations of 2,4-D got reverted to callus phase on in the case of *Gymnema* cultures.

5.6 Direct organogenesis

In the present study when the axenic whole young leaf explant was cultured in MS medium supplemented with 0.05 mg l^{-1} TDZ, one of the cultures produced a shoot bud directly from the leaf in 28 days. In many woody species including *Malus domestica*, it has been reported that a range of TDZ concentrations from 0.1 to 20 μm has been used to stimulate shoot organogenesis from leaves (Fasolo *et al.*, 1988). Further development of the shoot bud was not however obtained.

5.7 Encapsulation studies

The healthy shoots produced *in vitro* could be cut into small, single nodal segments and encapsulated into beads using sodium alginate at 2.5 per cent and calcium chloride at 75 mM with an ion exchange duration of 30 min. The beads formed were firm and round. The beads when put on regeneration media started differentiating into shoots within 8 days. The encapsulated beads could be successfully stored at room temperature on cotton wool moistened with MS basal salts without loosing their regeneration capacity for 15 days while at 4 $^{\circ}$C it could be stored for upto 40 days. Various workers have used the technique of encapsulation in medicinal and aromatic plants such as *Hyoscyamus muticus, Dioscorea floribunda* (Ahuja *et al.*, 1989), *Vetiveria zizanioides* (Keshavachandran *et al.*, 1993) and *Valeriana* (Mathur *et al.*, 1989a). Prabha (1993) has produced encapsulated beads of *Ananas comosus* that could be stored at room temperature for 30 days without loosing the regeneration capacity. Encapsulated beads of vetiver could be stored at 4 $^{\circ}$C for 45 days without loss of viability (Keshavachandran *et al.*, 1993).

Mathur *et al.* (1989a) have reported in *Valeriana wallichi* that encapsulation of apical and axial shoot buds has resulted in scaling up the micro-propagation technique while at the same time economising the medium, space and time requirements. As per their report, encapsulated explants utilize only about 200 ml of MS medium for the preparation of nearly 350 beads each of which contains an explant, thereby producing 350 plantlets under *in vitro* conditions. When non-encapsulated explants were used, nearly 4.5 l of MS medium were required for producing the same number of plants. The culture space requirement can also be reduced to 1/6 in an Erlenmeyer flask of 250 ml capacity, which can accomodate only 5 non-encapsulated explants but can hold 30 beads. He could store the beads for over 6 months without affecting viability either at cold temperature (4-6 $^{\circ}$C) or in mineral oil. In addition, plants from encapsulated explants could be easily retrieved by picking with a forceps or just shaking the beads out. Also, the technique offers easy transportation of large number of plants in low bulk.

5.8 Biochemical studies

Isozymes provide a means of marking the sequential developmental process in plant tissue culture and therefore represent the differential gene expression of differentiating cells (Srivastava and Steinhauer, 1981). The enzyme pattern of an organism changes during development and differentiation. Changes of isozyme patterns are manifested as the appearance and disappearance of individual isozymes. Such changes in isozymes suggest that genes involved in the synthesis of these enzymes are differentially activated in development (Chawla, 1988).

In the present study, the peroxidase isozyme pattern of the leaves and roots from the *in vitro* plantlets and *in vivo* plantlets were similar having the same number of bands. This is suggestive of the clonal identity of the *in vitro* raised plantlets through shoot bud culture with the mother plants.

Summary

SUMMARY

Studies were conducted on standardisation of *in vitro* techniques for the rapid multiplication of *Holostemma annulare* K. Schum. at the Plant Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara. The salient findings of the study are summarised in this chapter.

1. Among the different surface sterilization treatments tried, the best treatment varied with respect to the source of explant.

2. For two to three month old explants taken from the glasshouse, the best sterilization treatment was found to be with 0.1 per cent mercuric chloride for 5 min or 10 min with reference to least percentage of contamination and the maximum percentage of explants showing growth. Treatments with chlorine water resulted in bleaching of cultures.

3. For ten to fourteen month old explants taken from the glasshouse, the best treatment was found to be with 0.1 per cent Bavistin for 30 min followed by a dip in 1.0 per cent Cetrimide for 10 min and then in 0.1 per cent mercuric chloride for 15 min.

4. Addition of streptomycin in the medium reduced the extent of bacterial contamination but the establishment percentage of cultures was reduced.

5. In respect of the explants taken from plants grown in the field, combination treatments with different sterilants had to be given for effective results. The most effective was a combination involving Bavistin (0.1%) for 30 min,

Cetrimide (1.0%) for 10 min, Norfloxacin (0.1%) for 30 min, ethyl alcohol (70%) wipe, mercuric chloride (0.1%) for 13 min respectively in sequence.

6. Of the different sterilization treatments tried for root explants, none were found effective in producing contamination free cultures.

7. The explants collected in the months of January to April gave lower contamination and maximum survival percentages. Contamination rate was lowest in the months of January and February.

8. Early release of buds was observed in stationary liquid media but further growth of the shoot was better in solid media when either nodal segment or shoot tip was used as the explant.

9. The early release of buds and further growth of nodal segment and shoot tip explants was better in MS media supplemented with BA as the cytokinin. At lower concentrations of BA, longer shoots with moderate number of nodes and buds were produced but at higher concentrations, the length of the shoot was drastically reduced while the number of shoots and buds were more, resulting in more number of nodes which were weak.

10. Shoots produced in medium supplemented with KIN were short, robust and darker in colour. Number of multiple shoots and buds produced were lesser compared to that in medium supplemented with BA.

11. Irrespective of the growth regulator or explant, callus formation was observed at the base profusely when BA was incorporated in the media and sparsely when KIN was used.

12. Extremely low concentrations of TDZ (0.05-0.2 mg l^{-1}) could stimulate axillary bud proliferation.

13. Among the different additives tried for increasing the rate of production of shoots and nodes, none was found to be effective. Additives such as silver nitrate and activated charcoal could drastically reduce callus production in culture but the shoot growth was also reduced concomittantly with these additives.

14. Nodal segments were better in respect of early release of buds, more number of longer shoots, nodes and buds compared to shoot tips.

15. The number of explants that could be multiplied from a mother plant was comparatively less with shoot tip explants than nodal segments.

16. Higher temperature (30 $\pm$ 1 $^{\circ}$C) proved better than lower temperature (26 $\pm$ 1 $^{\circ}$C) for the growth of cultures.

17. Exposure to light alone favoured healthy growth of shoots.

18. Proliferation rate was higher at higher concentrations of BA but the shoots produced were very swollen, weak and could not be subcultured as single nodal segment but as a clump in media supplemented with lower concentrations of BA for getting healthy shoots. Shoots could also be proliferated in MS media supplemented with extremely low concentration (0.05 mg l^{-1}) of TDZ.

19. Full strength MS medium proved better than half strength MS with respect to the production of more number of nodes and lesser internodal length.

20. Comparing the media consistency, it was found that shoot buds were produced only in solid MS medium. However, in liquid MS, the internodal length was longer and with lesser callus.

21. BA proved to be the best growth regulator for obtaining maximum proliferation in all the five subcultures tried. Excepting the first subculture, in all others tried, BA at higher concentrations proved better for proliferation. For the first subculture, a combination of BA and IAA at low concentrations was sufficient.

22. If the best treatment in each subculture was given in sequence, approximately 2 crores 37 lakh nodes could potentially be obtained over a period of 225 days.

23. Maximum percentage of rooting, early rooting and more number of longer roots could be obtained in solid MS basal media than in any other media tried for *in vitro* rooting.

24. Roots induced in MS medium supplemented with NAA were thick, short, creamish in colour and produced in clusters, while those produced with IBA were thin, white and branched. Roots produced in basal MS medium were thin long, unbranched, coiled and light green in colour.

25. Among the auxins tried as pretreatment of shoots kept for *ex vitro* rooting, IBA 1000 mg l^{-1} quick dip for 60 seconds was the best for early rooting, maximum number, length and thickness of the roots induced.

26. Among the three media used for preliminary trial, sand proved to be the best.

27. Among the different potting mixes tried, cocofibe produced better percentage of rooting and more number, length and thickness of the root while sand produced roots earlier than cocofibe.

28. Among the different containers tried, plastic pot was better than others with respect to the percentage of rooting while early rooting was observed for those in protrays.

29. Considering the potting mixes and containers together, from the present study, it can be said that shoots produced *in vitro* after giving quick dip (60 s) in IBA 1000 mg l^{-1} should be planted in plastic pots filled with sand in the initial stages for early rooting and then transplanted into plastic or mudpots filled with cocofibe for vigorous growth of root and shoot portions. Plants hardened were transferred successfully.

30. 2,4-D proved better than NAA for obtaining more regenerative callus.

31. Among the cytokinins, TDZ produced highest callus index at relatively lower concentrations. TDZ and BA produced hard, green coloured, compact callus which however did not regenerate.

32. Leaf segments (with or without petiole attached) produced more regenerative callus. The orientation of leaf explant was important, the best response being with their abaxial surface touching the medium.

33. Callus index was maximum when cultures were exposed to light than when cultured in the dark.

34. Callus proliferation could be obtained in liquid or solid media.

35. Leaf bit explants (produced in MS medium supplemented with TDZ or KIN) when cultured in MS medium supplemented with 2,4-D initially at higher concentrations and subsequently at lower concentrations produced globular embryoids. Embryoid production were triggered on further subculture to liquid MS basal medium.

36. Direct organogenesis was obtained from one of the cultures, when whole young leaf was cultured in 0.05 mg l^{-1} of TDZ.

37. Single nodal segments could be encapsulated into beads using 2.5 per cent sodium alginate and 75 mM calcium chloride.

38. Encapsulated beads could be successfully stored at room temperature in cotton wool moistured with liquid basal MS for 15 days which could be prolonged (40 days) if stored at 4 °C.

39. The peroxidase isozyme pattern of the leaves and roots from the *in vitro* plantlets and *in vivo* plantlets were similar having the same number of bands indicative of the clonal identity of the *in vitro* raised plantlets through shoot bud culture with the mother plants.

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* Originals not seen

Appendices

APPENDIX-I

Meteorological parameters of the experimental site at the College of Horticulture, Vellanikkara, for the period from January, 1994 to December 1995

Year	Months											
	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
Mean air temperature (°C)												
1994	27.8	28.9	30.0	29.7	29.2	25.9	25.5	26.4	27.5	27.5	27.6	27.2
1995	27.7	29.4	30.7	30.8	28.7	27.4	26.6	27.1	26.8	28.2	26.9	26.9
Mean relative humidity (%)												
1994	58	59	59	74	75	90	91	85	78	80	68	58
1995	59	60	60	71	78	86	89	86	82	78	80	57
Rainfall (mm)												
1994	19.4	1.7	21.0	165.2	124.2	955.1	1002.1	509.2	240.5	358.2	125.3	0.0
1995	0	0.5	2.8	118.7	370.5	500.4	884.7	448.7	282.5	110.4	88.4	0.0
Number of rainy days												
1994	1	0	1	10	7	27	29	20	8	20	5	0
1995	0	0	0	5	13	19	26	22	13	8	5	0
Evaporation (mm)												
1994	222.2	169.2	209.2	144.7	137.0	84.2	86.1	91.4	113.9	97.1	137.9	169.6
1995	178.5	172.2	190.2	164.3	129.3	103.7	88.5	96.4	97.7	113.8	89.1	195.9
Mean sunshine (hours)												
1994	9.1	8.7	9.3	8.0	8.0	2.1	1.4	3.0	7.3	6.7	8.1	10.6
1995	9.6	10.0	9.3	9.1	6.5	3.7	2.1	3.7	6.1	8.3	6.5	10.3

APPENDIX-II

Abstract of analysis of variance for the effect of different treatments

Sl. No.	Character	Treatment DF	Treatment MSS	Error DF	Error MSS	Level of significance
1	2	3	4	5	6	7
I. Surface sterilization						
1. Effect of surface sterilants on the establishment of explants of *H. annulare* (2-3 month old from glasshouse)						
a) Contammination (%)		19	2367.83	40	9.49	0.01
b) Bacterial contamination (%)		19	1401.37	40	2.54	0.01
c) Fungal contamination (%)		19	1371.93	40	3.31	0.01
d) Establishment (%)		19	2123.79	40	19.04	0.01
e) Cultures bleached (%)		19	1479.87	40	6.18	0.01
f) Cultures showing growth (%)		19	2146.93	40	16.87	0.01
2. Effect of surface sterilants on the establishment of explants of *H. annulare* (10-14 month old from glasshouse)						
a) Contamination (%)		4	788.98	10	7.91	0.01
b) Bacterial contamination (%)		4	506.44	10	2.69	0.01
c) Fungal contamination (%)		4	3401.63	10	12.67	0.01
d) Establishment (%)		4	788.98	10	2.32	0.01
e) Cultures showing growth (%)		4	803.42	10	6.23	0.01
3. Effect of surface sterilants on the establishment of explants of *H. annulare* (explants from field)						
a) Contamination (%)		11	1605.81	24	2.05	0.01
b) Bacterial contamination (%)		11	772.96	24	15.85	0.01
c) Fungal contamination (%)		11	1017.17	24	11.4	0.01
d) Establishment (%)		11	1142.35	24	17.22	0.01
e) Cultures showing growth (%)		11	738.20	24	20.49	0.01
4. Effect of season of collection and age on establishment of *H. annulare* explants		5	317.75	12	25.82	0.01
5. Effect of combination of surface sterilant treatments in different seasons on explants of *H. annulare*		11	3094.63	24	13.66	0.01

Appendix-II. Continued

1	2	3	4	5	6	7
II.A. Establishment of nodal segment explants						
1.	Effect of different growth regulators on establishment of nodal segments of *H. annulare*	31	490.47	64	37.45	0.01
2.	Effect of different growth regulators on growth of nodal segments of *H. annulare*					
a)	Days to bud release	31	9.96	64	0.53	0.01
b)	Number of shoots	31	0.48	64	0.18	0.01
c)	Number of shoot buds	31	7.97	64	0.72	0.01
d)	Length of the longest shoot (cm)	31	26.97	64	3.11	0.01
e)	Average length of a shoot (cm)	31	11.28	64	2.29	0.01
f)	Number of nodes	31	8.62	64	0.99	0.01
g)	Weight of callus (g)	31	1.43	64	0.10	0.01
3.	Effect of media combination on establishment of nodal segments of *H. annulare*	3	121.69	8	30.19	NS
4.	Effect of media combination on growth of nodal segments of *H. annulare*					
a)	Days to bud release	3	9.42	8	0.33	0.01
b)	Number of shoots	3	1.34	8	0.05	0.01
c)	Number of shoot buds	3	14.52	8	0.15	0.01
d)	Length of the longest shoot (cm)	3	69.47	8	2.04	0.01
e)	Average length of a shoot (cm)	3	4.99	8	2.09	NS
f)	Number of nodes	3	1.46	8	0.10	0.01
g)	Weight of callus (g)	3	1.15	8	0.01	0.01
5.	Effect of different additives on establishment of nodal segments of *H. annulare*	7	398.61	16	18.73	0.01
6.	Effect of different additives on growth of nodal segments of *H. annulare*					
a)	Days to bud release	7	12.25	16	0.89	0.01
b)	Number of shoots	7	0.08	16	0.06	NS
c)	Length of the longest shoot (cm)	7	32.10	16	1.09	0.01
d)	Average length of a shoot (cm)	7	17.08	16	1.54	0.01
e)	Number of nodes	7	5.88	16	1.85	0.05
f)	Callus	7	0.83	16	0.02	0.01

Appendix-II. Continued

1	2	3	4	5	6	7
II.B. Establishment of shoot tip explants						
1. Effect of different growth regulators on establishment of shoot tip explants of *H. annulare*		31	761.35	64	20.26	0.01
2. Effect of different growth regulators on growth of shoot tip explants of *H. annulare*						
a) Days to bud release		31	69.89	64	3.17	0.01
b) Number of shoots		31	0.38	64	0.15	0.01
c) Number of shoot bud		31	11.98	64	0.75	0.01
d) Length of the longest shoot (cm)		31	35.04	64	6.72	0.01
e) Average length of shoot (cm)		31	11.84	64	3.08	0.01
f) Number of nodes		31	7.56	64	3.46	0.01
g) Weight of callus (g)		31	3.11	64	0.59	0.01
3. Effect of different media on establishment of shoot tip explants of *H. annulare*		3	372.75	8	19.09	0.01
4. Effect of different media on growth of shoot tip explants of *H. annulare*						
a) Days to bud release		3	96.12	8	6.91	0.01
b) Number of shoots		3	0.69	8	0.12	0.05
c) Number of shoot bud		3	8.33	8	0.58	0.01
d) Length of the longest shoot (cm)		3	17.26	8	4.36	NS
e) Average length of shoot (cm)		3	8.68	8	0.23	0.01
f) Number of nodes		3	13.86	8	3.96	NS
g) Weight of callus (g)		3	2.53	8	0.05	0.01
5. Effect of different additives on establishment of shoot tip explants of *H. annulare*		7	360.81	16	13.05	0.01
6. Effect of different additives on growth of shoot tip explants of *H. annulare*						
a) Days to bud release		7	56.20	16	4.69	0.01
b) Number of shoots		7	0.01	16	0.004	NS
c) Length of the longest shoot (cm)		7	13.65	16	0.75	0.01
d) Average length of a shoot (cm)		7	5.17	16	0.68	0.01
e) Number of nodes		7	3.96	16	0.75	0.01
f) Weight of callus (g)		7	1.70	16	0.03	0.01

Contd.

Appendix-II. Continued

1	2	3	4	5	6	7
III. Proliferation						
1. Effect of different growth regulators on proliferation of nodal segments of *H. annulare*						
a) Number of shoots		34	26.46	70	0.07	0.01
b) Number of shoot buds		34	37.32	70	0.22	0.01
c) Length of the longest shoot (cm)		34	27.45	70	0.72	0.01
d) Number of nodes		34	34.48	70	0.47	0.01
e) Weight of callus (g)		34	2.59	70	0.09	0.01
2. Effect of varying media concentration on proliferation of nodal segments of *H. annulare*						
a) Number of shoots		9	2.530	20	0.021	0.01
b) Number of shoot buds		9	14.717	20	0.054	0.01
c) Length of the longest shoot (cm)		9	35.378	20	1.597	0.01
d) Number of nodes		9	10.822	20	0.528	0.01
e) Weight of callus (g)		9	1.911	20	0.005	0.01
3. Effect of physical nature of media on proliferation of nodal segments of *H. annulare*						
a) Number of shoots		3	0.636	8	0.100	0.05
b) Number shoot buds		3	2.616	8	0.019	0.01
c) Length of the longest shoot (cm)		3	0.367	8	0.117	NS
d) Number of nodes		3	1.087	8	0.026	0.01
e) Weight of callus (g)		3	4.265	8	0.159	0.01
4. Effect of different additives on proliferation of nodal segments of *H. annulare*						
a) Number of shoots		9	0.359	20	0.031	0.01
b) Number of shoot buds		9	2.131	20	0.074	0.01
c) Length of the longest shoot (cm)		9	14.863	20	0.383	0.01
d) Number of nodes		9	4.137	20	0.435	0.01
e) Weight of callus (g)		9	1.941	20	0.045	0.01

Contd.

Appendix-II. Continued

1	2	3	4	5	6	7
5.	Effect of growth regulators on serial subculturing (first subculture) for proliferation of nodal segments of *H. annulare*)					
a)	Number of shoots	19	39.994	40	1.645	0.01
b)	Number of shoot buds	19	47.109	40	1.445	0.01
c)	Length of the longest shoot (cm)	19	45.678	40	2.344	0.01
d)	Number of nodes	19	99.532	40	3.723	0.01
e)	Weight of callus (g)	19	2.718	40	0.140	0.01
6.	Effect of growth regulators on serial subculturing (second subculture) for proliferation of nodal segments of *H. annulare*					
a)	Number of shoots	19	29.861	40	0.644	0.01
b)	Number of shoot buds	19	43.106	40	0.061	0.01
c)	Length of the longest shoot (cm)	19	52.61	40	1.232	0.01
d)	Number of nodes	19	68.534	40	1.415	0.01
e)	Weight of the callus (g)	19	3.622	40	0.213	0.01
7.	Effect of additives on serial subculturing (second subculture) for proliferation of nodal segments of *H. annulare*					
a)	Number of shoots	5	0.755	12	0.214	0.05
b)	Number of shoot buds	5	2.900	12	0.043	0.01
c)	Length of the longest shoot (cm)	5	18.318	12	0.588	0.01
d)	Number of nodes	5	26.632	12	0.337	0.01
e)	Weight of the callus (g)	5	1.770	12	0.015	0.01
8.	Effect of growth regulators on serial subculturing (third subculture) for proliferation of nodal segments of *H. annulare*					
a)	Number of shoots	22	39.608	46	0.466	0.01
b)	Number of shoot buds	22	120.290	46	1.013	0.01
c)	Length of the longest shoot (cm)	22	60.901	46	1.87	0.01
d)	Number of nodes	22	26.034	46	4.867	0.01
e)	Weight of the callus (g)	22	3.414	46	0.662	0.01

Contd.

Appendix-II. Continued

1	2	3	4	5	6	7
9. Effect of additives on serial subculturing (third subculture) for proliferation of nodal segments of *H. annulare*						
a) Number of shoots		22	1.246	46	0.183	0.01
b) Length of the longest shoot (cm)		22	25.465	46	2.793	0.01
c) Number of nodes		22	8.699	46	2.438	0.01
d) Weight of the callus 9g)		22	1.347	46	0.014	0.01
10. Effect of growth regulators on serial subculturing (fourth subculture) for proliferation of nodal segments of *H. annulare*						
a) Number of shoots		11	52.375	24	2.113	0.01
b) Number of shoot buds		11	89.881	24	1.929	0.01
c) Length of the longest shoot (cm)		11	39.830	24	0.418	0.01
d) Number of nodes		11	111.148	24	0.899	0.01
e) Weight of callus (g)		11	7.547	24	0.199	0.01
11. Effect of additives on serial subculturing (fourth subculture) for proliferation of nodal segments of *H. annulare*						
a) Number of shoots		7	0.187	16	0.168	NS
b) Length of the longest shoot (cm)		7	16.247	16	2.243	0.01
c) Number of nodes		7	11.998	16	2.094	0.01
d) Weight of callus 9g)		7	0.687	16	0.002	0.01
12. Effect of growth regulators on serial subculturing (fifth subculture) for proliferation of nodal segments of *H. annulare*						
a) Number of shoots		10	14.369	22	0.121	0.01
b) Number of shoot buds		10	20.573	22	0.005	0.01
c) Length of the longest shoot (cm)		10	37.213	22	1.671	0.01
d) Number of nodes		10	17.803	22	1.058	0.01
e) Weight of callus (g)		10	3.401	22	0.066	0.01

Contd.

Appendix-II. Continued

1	2	3	4	5	6	7
13.	Effect of additives on serial subculturing (fifth subculture) for proliferation of nodal segments of *H. annulare*					
a)	Number of shoots	5	1.333	12	0.026	0.01
b)	Length of the longest shoot (cm)	5	21.716	12	1.122	0.01
c)	Number of nodes	5	5.079	12	0.283	0.01
d)	Weight of the callus (g)	5	0.570	12	0.139	0.05
14.	Effect of serial subculturing (first subculture) on number of nodes produced from *H. annulare* in different media	32	3305.502	66	89.916	0.01
15.	Effect of serial subculturing (second subculture) on number of nodes produced from *H. annulare* in different media	38	626468.35	78	10767.66	0.01
16.	Effect of serial subculturing (third subculture) on number of nodes produced from *H. annulare* in different media	61	77125967	124	91870.46	0.01
17.	Effect of serial subculturing (fourth subculture) on number of nodes produced from *H. annulare* in different media	25	3.44×10^{9}	52	5.2×10^{10}	0.01
18.	Effect of serial subculturing (fifth subculture) on number of nodes produced from *H. annulare* in different media	26	1.166×10^{12}	54	4.75×10^{9}	0.01
IV.	*In vitro* rooting					
1.	Effect of different auxins on *in vitro* root growth in *H. annulare*					
a)	Days to root	8	59.109	18	22.201	0.05
b)	Number of roots	8	16.432	18	12.930	NS
c)	Length of the longest root (cm)	8	14.049	18	4.418	0.05
d)	Average length of a root 9cm)	8	11.339	18	4.114	0.05
e)	Weight of callus (g)	8	0.700	18	0.393	NS

Contd

Appendix-II. Continued

1	2	3	4	5	6	7
2.	Effect of different additives on *in vitro* root growth in *H. annulare*					
a)	Days to root	2	44.528	6	201.056	NS
b)	Number of roots	2	75.444	6	3.722	0.01
c)	Length of the longest root (cm)	2	59.086	6	1.488	0.01
d)	Average length of a root 9cm)	2	42.968	6	0.704	0.01
e)	Weight of the callus (g)	2	0	6	0	NS
3.	Effect of physical nature of media on *in vitro* root growth in *H. annulare*					
a)	Days to root	1	121.41	4	5.591	0.01
b)	Number of roots	1	77.042	4	1.388	0.01
c)	Length of the longest root (cm)	1	7.833	4	6.167	NS
d)	Average length of a root (cm)	1	0.589	4	2.940	NS
e)	Weight of the callus (g)	1	0	4	0	NS
V.	*Ex vitro* rooting					
1.	Effect of different auxins on *ex vitro* rooting in *H. annulare*	5	0.09	36	0.01	0.01
2.	Effect of different auxins on *ex vitro* root growth in *H. annulare* (30 days after rooting)					
a)	Days to root	5	0.23	36	0.02	0.01
b)	Number of roots	5	1.43	36	0.17	0.01
c)	Length of the longest root (cm)	5	1.12	36	0.19	0.01
d)	Average length of a root (cm)	5	1.44	36	0.18	0.01
e)	Thickness of the thickest root (cm)	5	0.02	36	0.01	0.01
3.	Effect of different auxins on *ex vitro* root growth in *H. annulare* (60 days after rooting)					
a)	Days to root	5	0.23	36	0.02	0.01
b)	Number of roots	5	1.57	36	0.40	0.01
c)	Length of the longest root (cm)	5	0.69	36	0.38	NS
d)	Average length of a root (cm)	5	0.10	36	0.15	NS
e)	Thickness of the thickest root (cm)	5	0.14	36	0.02	0.01
4.	Effect of different media on *ex vitro* rooting in *H. annulare*	2	0.56	36	0.12	0.01

Appendix-II. Continued

1	2	3	4	5	6	7
5.	Effect of different media on *ex vitro* root growth in *H. annulare* (30 days after rooting					
a)	Days to root	2	1.03	36	0.02	0.01
b)	Number of roots	2	19.96	36	0.17	0.01
c)	Length of the longest root (cm)	2	1.67	36	0.19	0.01
d)	Average length of a root (cm)	2	1.03	36	0.18	0.01
e)	Thickness of the thickest root (cm)	2	1.5	36	0.01	0.01
6.	Effect of different media on *ex vitro* root growth in *H. annulare* (60 days after rooting)					
a)	Days to root	2	1.03	36	0.02	0.01
b)	Number of roots	2	17.08	36	0.40	0.01
c)	Length of the longest root	2	1.79	36	0.38	0.05
d)	Average length of a root	2	2.35	36	0.15	0.01
e)	Thickness of the thickest root	2	9.97	36	0.05	0.01
7.	Effect of different auxins and media on *ex vitro* rooting in *H. annulare*	10	0.12	36	0.01	0.01
8.	Effect of different auxins and media on *ex vitro* root growth in *H. annulare*					
a)	Days to root	10	0.05	36	0.02	0.05
b)	Number of roots	10	3.33	36	0.17	0.01
c)	Length of the longest root (cm)	10	1.82	36	0.19	0.01
d)	Average length of a root (cm)	10	2.80	36	0.18	0.01
e)	Thickness of the thickest root (cm)	10	0.03	36	0.01	0.01
9.	Effect of different auxins and media on *ex vitro* root growth (60 days after rooting)					
a)	Days to root	10	0.05	36	0.02	0.05
b)	Number of roots	10	3.96	36	0.40	0.01
c)	Length of the longest root	10	0.54	36	0.38	NS
d)	Average length of root	10	0.82	36	0.15	0.01
e)	Thickness of the thickest root	10	0.33	36	0.05	0.01
10.	Effect of different potting mixes on *ex vitro* rooting in *H. annulare*	5	0.73	48	0.01	0.01

Contd

Appendi-II. Continued

1	2	3	4	5	6	7
11.	Effect of different potting mixes on *ex vitro* root growth in *H. annulare* (30 days after rooting)					
a)	Days root	5	0.32	48	0.05	0.01
b)	Number of roots	5	0.42	48	0.05	0.01
c)	Length of the longest root	5	3.17	48	0.08	0.01
d)	Average length of a root	5	2.63	48	0.11	0.01
e)	Thickness of the thickest root	5	0.06	48	0.01	0.01
12.	Effect of different containers on *ex vitro* rooting in *H. annulare*	3	0.47	48	0.01	0.01
13.	Effect of different containers on *ex vitro* root growth (30 days after rooting) in *H. annulare*					
a)	Days to root	3	1.30	48	0.05	0.01
b)	Number of roots	3	33.97	48	0.05	0.01
c)	Length of the longest root	3	1.23	48	0.08	0.01
d)	Average length of a root	3	3.95	48	0.11	0.01
e)	Thickness of the thickest root	3	0.03	48	0.01	0.05
14.	Effect of different potting mixes and containers on *ex vitro* rooting in *H. annulare*	15	0.26	48	0.01	0.01
15.	Effect of different potting mixes and containers on *ex vitro* root growth in *H. annulare* (30 days after rooting)					
a)	Days to root	15	0.07	48	0.05	NS
b)	Number of roots	15	0.21	48	0.05	0.01
c)	Length of the longest root	15	0.42	48	0.08	0.01
d)	Average length of a root	15	0.32	48	0.11	0.01
e)	Thickness of the thickest root	15	0.01	48	0.01	NS
16.	Effect of different potting mixes on *ex vitro* rooting in *H. annulare* (30 days after rooting) (shoots not treated with any auxin)	5	0.100	12	0.009	0.01

Contd.

Appendix-II. Continued

1	2	3	4	5	6	7
17.	Effect of different potting mixes on *ex vitro* root growth in *H. annulare* (30 days after rooting) (shoots not treated with any auxin)					
a)	Days to root	5	0.130	12	0.018	0.01
b)	Number of roots	5	0.619	12	0.046	0.01
c)	Length of the longest root	5	1.447	12	0.174	0.01
d)	Average length of a root	5	1.304	12	0.102	0.01
e)	Thickness of the thickest root	5	0.052	12	0.009	0.01

NS - Non significant

APPENDIX-III

Media combination tried for proliferation of nodal segments of *H. annulare*

Abbreviation	Composition of media		
	Basal media	Growth regulator/ additive	Concentration (mg l^{-1})
1	2	3	4
M_2	½ MS basal		
M_3	MS basal		
M_{92}	MS basal (liquid)		
M_{47}	MS basal	BA	0.25
M_6	MS basal	BA	0.5
M_4	MS basal	BA	1.0
M_{54}	MS basal	BA	2.0
M_7	MS basal	BA	2.5
M_{55}	MS basal	BA	4.0
M_8	MS basal	BA	5.0
M_{56}	MS basal	BA	10.0
M_{65}	MS basal	BA	20.0
M_{95}	½ MS basal	BA	1.0
M_{85}	MS (liquid)	KIN	1.0
M_{48}	MS basal	KIN	0.25
M_9	MS basal	KIN	0.5
M_{10}	MS basal	KIN	1.0
M_{60}	MS basal	KIN	2.0
M_{11}	MS basal	KIN	2.5
M_{97}	MS basal	KIN	4.0
M_{89}	MS basal	KIN	10.0
M_{12}	MS basal	KIN	5.0
M_{90}	½ MS basal	KIN	1.0

Appendix-III. Continued

1	2	3	4
M_{49}	MS basal	2iP	0.25
M_{13}	MS basal	2iP	0.5
M_{14}	MS basal	2iP	1.0
M_{57}	MS basal	2iP	2.0
M_{15}	MS basal	2iP	2.5
M_{59}	MS basal	2iP	4.0
M_{16}	MS basal	2iP	5.0
M_{58}	MS basal	2iP	10.0
M_{25}	MS basal	NAA	0.5
M_{70}	MS basal	NAA	1.0
M_{27}	MS basal	NAA	2.5
M_{28}	MS basal	NAA	5.0
M_{67}	MS basal	NAA	0.25
M_{74}	½MS basal	Charcoal	1000 (.1%)
M_{71}	MS basal	Charcoal	1000 (.1%)
M_{75}	MS basal	Silver nitrate	5
M_{76}	MS basal	Ascorbic acid	100
M_{77}	MS basal	Coconut water	150000 (15%)
M_{81}	½ MS basal	Adenine sulphate	2
M_{83}	½ MS (liquid)	Adenine sulphate	2
M_{84}	½ MS basal	Silver nitrate + BA	5 + 1.0
M_{78}	MS basal	Silver nitrate + BA	5 + 1.0
M_{79}	MS (liquid)	Silver nitrate + BA	5 + 1.0
M_{86}	MS basal	Silver nitrate + KIN	5 + 1.0
M_{91}	MS (liquid)	Adenine sulphate	2
M_{98}	MS basal	Adenine sulphate	2

Contd

Appendix-III. Continued

1	2	3	4
M_{29}	MS basal	BA + NAA	0.5 + 0.5
M_{30}	MS basal	BA + NAA	0.5 + 1.0
M_{31}	MS basal	BA + NAA	1.0 + 0.5
M_{32}	MS basal	BA + NAA	1.0 + 1.0
M_{33}	MS basal	BA + NAA	2.5 + 0.5
M_{34}	MS basal	BA + NAA	2.5 + 1.0
M_{35}	MS basal	KIN + NAA	0.5 + 0.5
M_{36}	MS basal	KIN + NAA	0.5 + 1.0
M_{37}	MS basal	KIN + NAA	1.0 + 0.5
M_{38}	MS basal	KIN + NAA	1.0 + 1.0
M_{39}	MS basal	KIN + NAA	2.5 + 0.5
M_{40}	MS basal	KIN + NAA	2.5 + 1.0
M_{50}	MS basal	BA + NAA	0.25 + 0.5
M_{51}	MS basal	BA + NAA	0.25 + 1.0
M_{52}	MS basal	BA + IAA	0.25 + 0.5
M_{53}	MS basal	BA + IAA	0.25 + 1.0
M_{62}	MS basal	BA + NAA	0.5 + 2.0
M_{63}	MS basal	BA + IAA	0.5 + 1.0
M_{64}	MS basal	BA + IAA	0.5 + 2.0
M_{80}	WPM		
M_{82}	WPM	BA	1.0
M_{87}	MS basal	BA + IAA	1.0 + 1.0
M_{88}	MS basal	BA + Streptomycin	0.5 + 0.1%
M_{93}	MS basal	BA + IAA	2.5 + 1.0
M_{94}	MS basal	KIN + IAA	2.5 + 1.0
M_{96}	MS basal	KIN + IAA	1.0 + 1.0

STANDARDISATION OF *IN VITRO* TECHNIQUES FOR RAPID MULTIPLICATION OF *Holostemma annulare* K. Schum

By

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ABSTRACT OF A THESIS

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ABSTRACT

Studies were conducted on standardisation of *in vitro* techniques for the rapid multiplication of *Holostemma annulare* K. Schum. at the Plant Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikakra during 1993-1995.

Surface sterilization was standardised for explants from different sources. For two to three month old explants from the glasshouse, treatment with 0.1 per cent mercuric chloride for 5 min or 10 min was found to be better. A combination of sterilants was necessary for mature explants taken either from the glasshouse or field. Explants collected in the months of January and February gave the lowest contamination rate.

Early release of buds and further growth of nodal segments and shoot tip explants was better in MS media supplemented with BA. Cultures in medium containing KIN were short, robust, darker and with lesser number of buds and shoots than those in medium containing BA. Extremely low concentrations of TDZ could stimulate axillary bud proliferation. Additives like silver nitrate and activated charcoal could drastically reduce callus production in culture, but the shoot growth was also reduced with these additives. Nodal segments were better in respect of early release of buds, more number of longer shoots, nodes and buds than shoot tips. Higher temperature proved better than lower temperature for the growth of cultures. Also exposure to light was favourable for healthy growth of shoots.

Proliferation rate was higher at higher concentrations of BA but the shoots were very swollen, weak and had to be subcultured as a clump into media containing lower concentrations of BA for healthy growth of shoots. Shoots could be proliferated at extremely low concentrations of TDZ. MS basal with full concentration of salts was better for better growth of shoots. When the best treatment in each subculture was given in sequence approximately 2 crores 37 lakh nodes could potentially be obtained over a period of 225 days.

Maximum rooting, early rooting and more number of longer roots could be obtained in solid MS basal media when shoots were kept for *in vitro* rooting. *Ex vitro* rooting of shoots was successful when they were treated with IBA 1000 mg l^{-1} as quick dip followed by planting in plastic pots filled with sand in the initial stages for early rooting and then transplanted to plastic or mud pots filled with cocofibe for vigorous growth of root and shoot portions.

TDZ produced the highest callus index at relatively lower concentrations. The callus produced was hard, green in colour and compact. 2,4-D proved better than NAA for obtaining more regenerative callus among the auxins tried. Leaf segments (with or without petiole attached) oriented with the abaxial surface touching the solid medium supplemented with 2,4-D and exposed to light alone produced embryoids after one or two subcultures into MS medium with lower concentrations of 2,4-D. The embryoid production could be triggered if the calli were subcultured to liquid MS basal medium and when further transferred to solid media alone produced elongation of such embryoids. But the original explants had to be raised in MS medium supplemented with either TDZ or KIN as cytokinin for the embryoids to form subsequently.

Encapsulated beads were successfully formed with nodal segments using 2.5 per cent sodium alginate and 75 mM calcium chloride with a complexation time of 30 min and the beads could be stored successfully for 15 days at room temperature and upto 40 days at 4 °C.

The peroxidase isozyme pattern of the leaves and roots from *in vitro* plantlets and *in vivo* plantlets were similar having the same number of bands.