STANDARDISATION OF *IN VITRO* TECHNIQUES FOR RAPID MULTIPLICATION OF *Trichopus zeylanicus* Gaertn.

By

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THESIS

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DECLARATION

I here by declare that the thesis entitled "**Standardisation of *in vitro* techniques for rapid multiplication of *Trichopus zeylanicus* Gaertn.**" 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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We, the undersigned members of the Advisory Committee of **Miss.Seema,B.J.**, a candidate for the degree of **Master of Science in Horticulture**, with major in **Plantation Crops and Spices**, agree that the thesis entitled **"Standardisation of *in vitro* techniques for rapid multiplication of *Trichopus zeylanicus* Gaertn."** may be submitted by Miss.Seema,B.J., in partial fulfilment of the requirement for the degree.

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ABBREVIATIONS

BA	- benzyl adenine
BAP	- benzyl amino purine
°C	- degree celsius
CH	- casein hydrolysate
cm	- centimeter
CW	- coconut water
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}	- milligram(s) per litre
ml l^{-1}	- millilitre(s) per litre
min	- minute(s)
mM	- milli molar
MS	- Murashige and Skoog's (1962) medium
1/2 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
s	-	second(s)
U-v	-	ultra violet
v/v	-	volume in volume
WP	-	wettable powder
WPM	-	woody plant medium (Lloyd and McCown, 1980)
w/v	-	weight in volume
μM	-	micromolar

INTRODUCTION

Trichopus zeylanicus Gaertn. is a rare, herbaceous, perennial and rhizomatous wild plant belonging to family Trichopodaceae. The plant is of limited geographical distribution and is found on damp forest floors at elevations upto 1100 m in the Travancore and Tinnevelly hills of the Western Ghats in South India, Sree Lanka and Malay Peninsula (Wills, 1973; Krishnan *et al.*, 1995).

The forest dwelling local Kani tribe call this plant Arogyappacha meaning inducer of health. Earlier workers named the plant as Kerala ginseng due to its medicinal properties being similar to that of ginseng.

The plant is endowed with unique medicinal properties. The fresh kernels of tender fruits are eaten by tribals to get instant energy, stamina and vitality (Pushpangadan *et al.*, 1988). It is also claimed by the tribals that a regular intake of the kernels and certain other preparations made of the leaf, stem and rhizomes of *T. zeylanicus* would enable one to enjoy youthful health, vitality, resistance from diseases and longevity. The seeds are particularly rich in saponins and is reported to be a safe antistress, antifatigue, apetite promoting and restorative herbal tonic. Adaptogenic activity or antistress properties of seeds of *Trichopus* has been experimentally proved by Sharma *et al.* (1989) in rats and mice.

Due to the ethno-medicinal importance and potential, there is need to conserve and propagate this herb on a large scale. The rapid pace of deforestation activities in the Western Ghats region in recent years has depleted the wild stands and hampered the natural regeneration potential of *Trichopus zeylanicus*. Poor seed

set, slow maturation of seeds lasting 4-5 months, loss of seeds due to rodents and inundation also limits natural regeneration (Krishna *et al.*, 1995).

Plant tissue culture is useful for conservation and rapid propagation of rare and endangered medicinal plants (Arora and Bhojwani, 1989; Sudha and Seeni, 1994). Standardisation of suitable *in vitro* techniques for rapid multiplication of this plant for the production of large amount of planting material is hence important and was thus undertaken in *Trichopus zeylanicus* with the following objectives.

1. To standardise the method of *in vitro* seed germination.
2. To standardise the method of rapid multiplication through enhanced release of axillary buds, organogenesis and somatic embryogenesis.
3. To standardise the rooting and hardening of plantlets derived *in vitro*.

REVIEW OF LITERATURE

Trichopus zeylanicus Gaertn. is a rare, herbaceous, perennial and rhizomatous wild plant growing in the Agasthyar hill forests of Kerala. The forest dwelling local Kani tribe call the plant 'Arogyappacha' meaning inducer of health/elixir of life and use it as a health tonic. It was also called as the Kerala ginseng by previous workers (Pushpangadan *et al.*, 1988). Kernels of tender fruits are eaten by the tribal people to get instant energy, stamina and vitality. It is also claimed by the tribals that a regular intake of the kernels and certain other preparations made of the leaf, stem and rhizomes of *T. zeylanicus* would enable one to enjoy youthful health, vitality, resistance from diseases and longevity. The seeds are particularly rich in saponins.

T. zeylanicus is an endemic of limited geographical distribution. The rapid pace of deforestation activities in the Western ghat region in recent years has depleted the wild stands and hampered the natural regeneration potential of *T. zeylanicus*. Due to its ethno-medicinal importance and potential, there is need to conserve and propagate this herb on a large scale (Krishnan *et al.*, 1995). Plant tissue culture is useful for conservation and rapid propagation of rare and endangered medicinal plants (Arora and Bojwani, 1989; Sudha and Seeni, 1994). The only report available on rapid propagation of *Trichopus* is by Krishnan *et al.* in 1995 who attempted rapid propagation through shoot tip culture and this enabled an estimated harvest of 7848 buds from a single shoot tip in 28 months. Literature available on the use of tissue culture technique for propagation of other medicinal plants is furnished in this chapter.

1.1 *In vitro* culture

The concept of totepotency inherited in the cell theory of Schleiden (1838) and Schwann (1839), is the foundation for plant tissue culture.

Haberlandt (1902) for the first time isolated, fully differentiated cells in a nutrient medium containing glucose, peptone and Knop's salt solution and developed the concept of *in vitro* culture. Pioneering investigators in the field of plant tissue culture include White (1943), Gautheret (1939), Miller *et al.* (1956), Reinert (1959), Steward *et al.* (1958), Bergmann (1960), Vasil and Hildebrandt (1965) etc.

Success achieved in tissue culture would be regarded as incomplete without understanding the role of plant growth regulators. Skoog and Miller (1957) proposed the concept of hormonal control of organ formation. They showed that root and bud formation were conditioned by a balance between auxin and cytokinin and that organ formation could be regulated by changing the relative concentration of these substances in the media.

Completely defined nutrient medium was first developed by Murashige and Skoog (1962).

Several aspects of plant tissue culture are being applied in agriculture. The best commercial application of tissue culture technique has been in the production of true to type plants at a very rapid rate compared to conventional method (Levy, 1981). Plants produced by tissue culture methods are reported to grow faster and mature earlier than seed propagated plants (Vasil and Vasil, 1980).

According to Murashige (1974), there are three possible routes available for *in vitro* propagule multiplication namely,

i) enhanced release of axillary buds

ii) production of adventitious shoots through organogenesis

iii) somatic embryogenesis.

Morel (1960) reported the application of shoot apex culture for rapid clonal multiplication of plants for the first time.

Shoot tip culture ensures genetic uniformity while somatic embryogenesis 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).

1.2 Factors influencing success of *in vitro* propagation

1.2.1 Explant source

Genotype as well as the degree of tissue differentiation can influence the ability of plant tissues to undergo morphogenesis. Takayama and Misawa (1979) examined the ability of different explants of *Lilium aurantum* and *L. specisoum* to produce bulbs *in vitro*. Fifty per cent of the peduncle explants, 75 per cent of 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 conditions while the explants from bulbs showed 100 per cent success.

For *in vitro* propagation of *Hypoxis rooperi* in which normal vegetative propagation is not feasible and seeds of which show dormancy, wounded young

flower parts (perianth, stamens and styles) were used as explants to induce callus mediated organogenesis (Page and Staden, 1986).

Mathur and Ahuja (1991) reported that in *Valeriana wallichi*, the petiole explants after removing leaf lamina did not produce any callus, but 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.

For propagation of *Withania somnifera* by organogenesis, various explants like germinating seeds, hypocotyl, mature seeds etc. were used. Adventitious multiple shoots were induced in germinating seeds and intact seedlings (Kulkarni *et al.*, 1995).

In *Chlorophytum borivilianum*, where natural regeneration through tubers and seeds is very poor, use of young shoot bases as explants proved to be the ideal explant for *in vitro* clonal multiplication. A four fold rate of multiplication has been achieved during every subculture (Purohit and Dave, 1995). Propagation of *Trichopus zeylanicus* was achieved by culturing shoot tips (0.3-0.5 cm) of 2 month old seedlings by Krishnan *et al.* (1995).

Adventitious bud formation was obtained from stem internodal explants of *Ephedra fragilis* as reported by O'Dowd and Richardson (1993).

Ovules collected 90-120 days after pollination was the best explant for organogenetic differentiation in *Aegle marmalos* as reported by Hossain *et al.* (1994).

Morphogenetic cultures from single pollen grains of *Hyosciamus niger* cultured in liquid medium was reported by Raghavan and Nagami (1983).

Donato and Perucco (1986) found that when half seeds of *Asparagus officinalis* with or without embryo at different stage of maturity were cultured *in vitro*, callus formation was greatest from immature (gelatinous), endosperm in the presence of embryo.

Explants taken from newly originated organe are 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 Takayama and Misawa (1982), most segments derived from young leaves of Begonia produced buds and roots, where as those derived from mature leaves usually dried. Callus derived from seedling and inflorescence explants of many Cymbopogon species had a higher morphogenic capacity than that arising from seeds, culm, root or rhizomes (Jagdish Chandra and Sreenath, 1987).

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 tissue several physiological changes occur which may influence the *in vitro* behaviour of the explants (David, 1982).

Hossain *et al.* (1994) reported that in *Aegle marmelos* shoot differentiation efficiency varied remarkably when cotyledons from seedlings of different ages

were cultured. Explants from 10 day old seedlings showed maximum regeneration of 90 per cent.

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

Bhat *et al.* (1995) investigated the morphogenetic potential of root, leaf node and internode explants of *Piper longum, Piper nigrum* and *Piper betle* and found that the highest number of shoot buds was produced on root explants followed by node, internode and leaf explants. *Piper longum* showed the highest regeneration response followed by *Piper betle* and *Piper nigrum*.

A comparative study of plant regeneration efficiency of two *Digitalis* species was done by Onisei *et al.*, 1992. Culture response by the two species was similar but *D. purpurea* explants exhibited a stronger organogenetic capacity and survival of regenerated plants exceeded 95 per cent.

Tissues taken from field grown plants are not equally amenable to tissue culture conditions throughout the year. Also there is difference in the *in vitro* response of explants taken from field and those from plants grown under controlled conditions. Maliarcikova (1981) found that for taking explants in strawberry, August was more suitable than July or September. 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 explant collected during September-October as reported by Barve and Mehta (1993).

Anu (1993) reported that months from January to April was the best period for culture establishment of *Gymnema sylvestre*. Similar influence of season on establishment of explants is reported in Cardamon (Reghunath, 1989), Banana (Bhaskar, 1991) and Holostemma (John, 1996).

1.1.2 Surface sterilization

The explants collected from the field harbour numerous microorganisms which when inoculated onto the nutrient medium contaminate the entire *in vitro* system. Hence surface sterilization is resorted prior to inoculation of explants. The aim of surface sterilization is to remove all the microorganisms present on the explant, with a minimum damage to the plant or part to be cultured. 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).

Sodium hypochlorite (0.5 to 2 per cent w/v), calcium hypochlorite (5 to 10 per cent w/v) and mercuric chloride (0.05 to 0.1 w/v) are the most commonly used surface sterilants. Since the sterilants are toxic to plant cells, it is necessary to wash the tissue twice or thrice with sterile distilled water to ensure removal of the chemical (Hu and Wang, 1983). Sommer and Caldas (1981) reported that for softer tissues, dilution to a lower strength may be needed. Alcohol alone or with other surface sterilants has also been used for disinfection (Bonga, 1982).

Fungicides and antibiotics are also used either as surface sterilants or as media additives. However, most of the systemic fungicides and some of the antibiotics inhibit growth of the plant cultures. Several workers have reported the

use of various fungicides in cultures for reducing fungal contamination (Brown *et al.*, 1982; Sheilds *et al.*, 1984) Dodds and Roberts (1985) suggested to avoid the use of antibiotics for sterilization since they or their degradation products may be metabolized by plant tissue with unpredictable results.

Krishnan and Seeni (1994) reported that, for surface sterilization of shoot cuttings of *Woodfordia fruticosa*, washing the excised shoot tips first in 1 per cent Labolene detergent for 5-6 minutes and in running tap water for 10 minutes followed by surface sterilization by passage through 0.1 per cent mercuric chloride for 5 minutes and washing 6-8 times in sterile distilled water was effective.

In *Adhatoda beddomei*, Sudha and Seeni (1994) reported that a treatment with 1 per cent Labolene for 6-8 minutes followed by tap water washing and treatment with 0.1 per cent $HgCl_2$ for 15 minutes could produce contamination free cultures.

In *Piper longum*, Bhat *et al.* (1992) reported that washing in water for 5-10 minutes followed by treatment with 0.1 per cent $HgCl_2$ for 20 minutes was an effective sterilization method.

Teepol detergent wash for 15 minutes at slow speed on magnetic stirrer and washing in running tap water for 2 h followed by surface disinfestation with 0.1 per cent $HgCl_2$ for 10 minutes and rinsing several times with sterile distilled water was effective for surface sterilization of *Gentiana kurroo* as reported by Sharma *et al.* (1993).

Combination of ethyl alcohol and $HgCl_2$ was also effective as reported in *Duboisia myoporoides* (Kukreja *et al.*, 1986), *Coscinum fenestratum* (Nair *et al.*, 1992) and *Vetiveria zizanioides* (Keshavachandran and Abdul Khader, 1993).

Mathur (1993) reported that in *Nardastachys jatamansi*, 2 per. cent sodium hypochlorite treatment for 7 minutes was effective.

Surface sterilization by immersing in 70 per cent ethyl alcohol for 30 seconds followed by a rinse in sterile distilled water, 10 minutes in a 10 per cent solution of commercial sodium hypochlorite and 2 rinses of sterile water was effective for *Trillum sp.* (Pence and Sootup., 1993)

Kavitha and Raju (1995) have reported in *Aristolochia indica* that chlorine water with a few drops of Triton-x-100 treatment for 15 minutes was very effective.

1.2.3 Basal media

Selection of culture medium depends upon the plant species and purpose of cell, tissue or organ culture resorted to. A wide variety of media have been reported to be used. The earliest and widely used basal media were Whites (1943) and Heller's (1953). Since 1960, most researchers have been using MS (Murashige and Skoog, 1962) medium, B5 (Gamborg *et al.*, 1968) or SH (Schenk and Hildebrandt, 1972) medium. MS medium is characterised by high concentration of mineral salts, so some workers found it beneficial to reduce its strength by half (Skirvin, 1980, Griffis *et al.*, 1981). After 1986, 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.

Murashige and Skoog (1962) medium is the most commonly used media for *in vitro* propagation of many endangered medicinal plants like *Dioscorea sp., Smilax oldhami, Panax ginseng, Chlorophytum borivilianum, Curculigo orcheodes, Rauwolfia caffra* etc. Rapid propagation of *Solanum xanthocarpum* was achieved by Rao and Narayanaswamy (1968) in White's medium.

Nitsch medium along with 15 per cent coconut water (Sopory and Maheswari, 1976) was found to be effective in *Datura innoxia*. Spontaneous somatic embryogenesis and plant regeneration of *Peucedanum palustre* was achieved in B_5 medium (Vuorela *et al.*, 1993).

Ilahi and Akram (1987) reported that buds of *Rauwolfia serpentina* developed new leaves when cultured on Abou-Mandour's (AM) and MS media.

Sudha and Seeni (1994) obtained axillary bud release in *Adhatoda beddomei* when inoculated on SH (Schenk and Hildebrandt, 1972) medium. Krishnan *et al.* (1995) obtained plantlet regeneration of *Trichopus zeylanicus* in Woody plant medium.

1.2.4 Growth regulators

The growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium and the growth substance produced endogenously by cultured cells. Selection and addition of growth regulators at the optimum level is one of the crucial factors for a successful plant tissue culture (Krikorian, 1982). Commonly used growth regulators in tissue culture include auxins, cytokinins, gibberellins, ethylene and abscissins. BAP has been the most effective cytokinin for meristem, shoot tip and bud cultures followed by KIN

(Murashige, 1974). Cytokinin has been utilised to overcome the apical dominance of shoot to enhance the branching of lateral buds from leal axils (Murashige, 1974). Lo *et al.* (1980) reported that a high content of cytokinin was deleterous to the initiation and elongation of roots of both monocotyledons and dicotyledons. For axillary bud proliferation, exogenous auxin was not always needed. Although exogenous auxins do not promote axillary shoot proliferation, culture growth has been improved by their presence (Wang and Hu, 1980).

In general, monocotyledonous species do not show a pronounced response to cytokinins and require high concentration of potent auxins such as 2,4-D to achieve changes in the development of cultured tissues (Harms, 1982).

One of the possible role of auxin at the elongation stage is to nullify the suppressive effect of high cytokinin concentration, thereby restoring normal shoot growth (Lundergan and Janick, 1980).

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 break, but also induce callus formation.

Method for *in vitro* cloning of *Valeriana wallichi* DC was described by Viola and Fritz (1991). Best proliferation results were obtained on MS medium containing BA (5 mg l^{-1}), while KIN, 2iP, zeatin and PBA were less effective.

Best growth regulator combination for induction of nodular tissue in *Smilax oldhami* culture was 0.4 mg NAA and 0.3 mg BA l^{-1} in liquid MS medium and NAA and BA each at 0.5-2.0 mg l^{-1} in solid MS medium as reported by Kuroda and Kawamura (1994). Maximum shoot cluster induction was achieved when media

was supplemented with 0.5 mg l^{-1} BA alone and NAA had no effect on shoot cluster formation.

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).

Although cytokinin alone was effective in inducing adventitious buds, significant difference was observed on the effectiveness of various cytokinins. The number of shoots formed from embryo explants and their subsequent development was affected by the concentration and duration of exposure to BA (Lu *et al.*, 1991).

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

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).

Murashige (1974) observed that a variety of auxins including IAA, NAA, IBA and 2,4-D could be used either alone or in combination for growth of cultured cells, but among these 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.

Callus from root of *Panax ginseng* was induced and maintained on MS medium containing 2,4-D at 1 ppm and KIN 0.1 ppm which on transfer to medium without 2,4-D developed many nodules,shoot differentiation and plantlet formation was promoted in medium containing KIN at 1 ppm (Furoya *et al.*, 1986).

Bhat *et al.* (1992) have 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.

Effect of a range of growth regulator on callus production in various species of *Ephedra* was investigated by O'Dowd *et al.* (1993). All species produced callus on modified MS medium supplemented with 0.25 μm KIN and 5 μm 2,4-D or NAA. Neither IAA nor IBA induced significant callus formation, but the latter maintained growth of established callus cultures in several species.

Germinated seedlings of *Artemisia pallens* gave 3 types of callus on MS medium supplemented with different plant growth hormones. Medium containing BA and 2,4-D stimulated unorganised callus. BA and IAA medium gave semi-organised callus interspersed with shoot buds and medium with BA, NAA and IAA developed complete shoot cultures (Benjamin *et al.*, 1990).

Effect of different concentration of NAA in combination with BA on the induction of callus from leaf explants of *Rauwolfia caffra* on MS medium was investigated by Upadhyay *et al.* (1992). Best response for callus induction and growth was obtained on medium containing 2 mg NAA + 2 mg BA l^{-1}.

A proper balance between the kind and concentration 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 is required (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 embryoids (Kavathekar *et al.*, 1978; Krikorian and Kaun, 1981). Studies on *Nardostachys jatamansi* by Mathur (1993) has proved this. Auxin alone or a high auxin cytokinin ratio was responsible for continued unorganised callus proliferation accompanied by rhizogenesis while high levels of cytokinin alone produced dark green nodular structures on the callus surface with meristematic zones in them but failed to develop further when transferred to the same medium but a combination of auxin and cytokinin (NAA-KIN) proved effective in differentiation of somatic embryos, thus conforming to the findings of earlier workers.

Combination and levels of exogenous hormones were found to control the mode of embryogenesis in *Freesia refracta* (Wang *et al.*, 1989). On medium with IAA and BA some of the epidermal cells began to exhibit the features of embryogenic cells. These cells produced embryoids and developed into plantlets through embryogenesis. In medium containing IAA, BA and NAA, pale yellow transluscent nodular callus appeared which on transfer to medium with IAA and BA formed embryoids which further developed into plantlets.

Ammirato (1983) has reported that the callus growth was induced in an auxin enriched medium and somatic embryogenesis could occur only upon transfer

of callus to a medium free of auxin. The presence of auxin in the induction medium seems essential for the tissue to develop embryo in the embryo development medium.

1.2.5 Carbon source

Sucrose is the most commonly used carbon energy source for plant tissue culture. Most of the workers have used 20-30 g l^{-1} sucrose in the medium. Glucose and fructose may be substituted in some cases, but most other sugars are reported to be less effective (George and Sherrington, 1984). Takayama and Misawa (1979) reported that organogenesis in Liliaceae was inhibited at high sucrose levels and sucrose at 90 g l^{-1} increased root dry weight.

Favourable effect of maltose on embryo formation of *Diglalis lanata* was reported by Reinbothe *et al.* (1990). In asparagus, shoot growth was promoted at high sucrose levels (2, 3, 5 and 7% sucrose) and high sucrose levels upto 9 per cent produced the best rooting of 95 per cent in the shortest time (Desjardine and Tiessen, 1985).

1.2.6 Other organic compounds

Conger (1981) reported the role of complex organic compounds for successful growth of tissues and organs. These include casein hydrolysate, coconut water, yeast, malt extract and fruit and vegetable juice. Role of these complex compounds are usually unpredictible and repeatability is also very poor, therefore, it has been recommended to avoid their use as far as possible (Gamborg and Shyluk, 1981).

Coconut water was reported to be promoting growth and differentitation in *Datura* embryos (Van Overbeek *et al.*, 1941). The discovery by 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. Illahi and Akram (1987) reported that addition of 100 ml l^{-1} coconut milk along with biotin, NAA, BAP and sodium di-ethyl-dithio carbonate to the medium enhanced root formation in *Rauwolfia serpentina* bud cultures. In the same crop, shoot regeneration from roots was induced in media with 10 per cent coconut milk and 5 mg l^{-1} biotin (Akram *et al.*, 1993). Measurement of freshmass of axenic seedlings of *Drosera spathulata* cultured on MS medium with 5 per cent coconut milk, 5 per cent grape-wine exudate and 5 per cent carrot extract revealed that the freshmass was highest on media supplemented with carrot extract (Blehova *et al.*, 1992).

In cases where nutritional requirements have not been established, mixtures of amino acids such as casein hydrolysate may be added between 0.5 and 0.1 per cent (Huang and Murashige, 1977). In *Eucalyptus camaludensis*, addition of casein hydrolysate was found to be superior to other growth regulators (Kumar, 1993).

Adenine sulfate when added to the medium, often can enhance growth and shoot formation (Skoog and Tsui, 1948). Jain and Chaturvedi (1985) reported that rhizome tissue explants of *Costus speciosus* callused within 30 days of incubation in a modified Schenk and Hildebrandt medium supplemented with 10 mg adenine sulfate, 0.25 mg BA, 0.5 mg IAA and 100 mg malt extract per litre.

In *Asparagus cooperi*, shoot regeneration from callus required BA, L-arginine, adenine and a low level of NAA in the media (Ghosh and Sen, 1992).

Embryoid formation from callus of *Panax quinquefolius* was highest when cultured on media containing 2,4-D, 500 mg l^{-1} lactoalbumin hydrolysate and 3 per cent sucrose (Yan and Lu, 1987).

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

Addition of activated charcoal to plant tissue culture media may have either beneficial or harmful effects. Activated charcoal frequently promotes the *in vitro* growth and organogenesis of plant tissues by the absorption of compounds from the culture medium and/or from the container atmosphere (Druart and Wulf, 1993). Stimulation of growth, rooting, organogenesis and embryogenesis in a wide variety of species and tissues including onion (Fridborg and Eriksson, 1975), carrot (Fridborg *et al.*, 1978), *Artemesia montana* (Conchou *et al.*, 1992) etc. have been reported.

Activated charcoal has been found to bind hormones and other metabolites (Weatherhead *et al.*, 1978). Activated charcoal added to liquid medium at concentrations ranging from 0.1 per cent to 5 per cent reduced IAA and IBA concentration by more than 97 per cent (Scott and Ellen, 1990).

Addition of activated charcoal to the medium for shoot multiplication of *Artemesia montana* promoted shoot development but inhibited shoot initiation (Conchou *et al.*, 1992).

Sucrose hydrolysis which normally reaches 10 per cent during auto-claving increases to 95 per cent in the presence of 1 per cent activated charcoal. This gives rise to the acidification of the solution due to a specific reaction of the formed fructose. Changing the available carbon source from the initial sucrose to a mixture of fructose, glucose and sucrose, causes a osmolarity increase, a drop in the agar gelling and the formation of furfural derivatives which are the indirect consequences of sucrose hydrolysis affecting tissue culture media (Druart and Wulf, 1993).

1.2.7 Culture conditions

The physical form of the medium, whether liquid or semisolid, medium pH, other environmental factors like light, temperature, relative humidity and season of culture etc play an important role in *in vitro* growth and differentiation.

Light requirement for differentiation involve a combination of several components, namely intensity, quality and duration (Murashige, 1974). According to Murashige (1977), the optimum day light period required is 16 hours for a wide range of plants. Yeoman (1986) reported that the environmental temperature of the species in the original habitat should be taken into consideration during *in vitro* culture also. However most of the *in vitro* cultures are grown successfully at temperatures around 25 + 2°C.

For induction of multiple shoots in *Smilax*, a temperature of 25°C with a 16 h photoperiod was effective (Kuroda and Kawamura, 1994). Maintenance of

cultures in a 16 h light/8 h dark cycle was reported by Mumtaz *et al.* (1990) in *Catharanthus roseus*.

Calli of *Drosera spathulata* cultured in dark and light were found to behave differently (Blehova *et al.*, 1992). In calli cultured in the dark, roots differentiated before shoots. The reverse occured in calli cultured under long day conditions.

When *Digitalis obscura* hypocotyl sections were cultured under both light and dark conditions for embryoid production, Arrilaga *et al.* (1986) found that light accelerated embryogenesis and also increased the number of embryoids formed.

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 is an important factor in hardening and planting out of *in vitro* raised cultures.

1.3 Rooting of *in vitro* shoots

In vitro produced shoots can be rooted either through *in vitro* methods itself or through *ex vitro* methods. There are three phases involved in rhizogenesis, namely induction, initiation and elongation.

Generally auxins favour rhizogenesis. Among the different auxins, NAA has been the most effective one for root induction (Ancora *et al.*, 1981). The concentration of hormone required is often critical to provide sufficient stimulus to initiate roots while preventing the excessive formation of callus (Yeoman, 1986). Root elongation has been found to be very sensitive to auxin concentration. High

concentration of auxin inhibited root elongation (Thimman, 1977). The use of NAA produced short thick roots in liquorice (Shah and Dalal, 1980). In *Duboisa myoporoides*, NAA was necessary for rooting of shoots (Kukreja *et al.*, 1986) while IAA or IBA could not substitute NAA.

Rhizogenesis in *Plumbago rosea* (Satheesh Kumar and Bhavanandan, 1989) and in *Chlorophytum borivilianum* (Purohit and Dave, 1995) was achieved by adding IBA to the culture medium.

Sometimes a combination of growth regulators may give better response (Gupta *et al.*, 1980; Mathew and Hariharan, 1990). Slabbert *et al.* (1990) found that highest percentage of rooting of Asparagus shoots was obtained by using MS medium supplemented with 0.1 mg NAA, 0.1 mg KIN and 1.25 mg ancymidol. Mumtaz *et al.* (1990) reported the use of GA_3 and BAP in rooting medium of *Catharanthus roseus*.

Usually a medium with low salt concentration promotes rooting (Hu and Wang, 1983). Abundant rooting was observed when the salt concentration in the medium was reduced to half, one third or one fourth of the standard strength (Lane, 1979; Skirvin and Chu, 1979). For rooting stage, sugar content of the medium is also lowered in most of the cases (Roy *et al.*, 1990). Watanabe *et al.* (1991) reported that white root formation in asparagus cultures was reduced with increasing sugar levels. Lowering of N content in the culture media promoted root formation in *Digitalis purpurea* (Rucker, 1982).

Activated charcoal has a profound influence on rooting of shoots *in vitro*. Activated charcoal may absorb toxic substances in the medium thereby improving

root regeneration and development (Jaiswal and Amin, 1987). It may also absorb residual cytokinin from the shoot and it also shades *in vitro* roots from light, which in high intensity may inhibit root growth (Hu and Wang, 1983). Root formation of *Artemesia annua* shoots produced *in vitro* was positively affected by addition of 2 g activated charcoal per litre of the rooting medium (Mathe and Lasloffy, 1992).

Concentration of agar used for rooting varies from 0 (liquid media) to 0.9 per cent. Liquid medium facilitates free diffusion of toxic plant wastes and when used with filter paper bridge system provides excellent aeration for root development (Hu and Wang, 1983).

1.4 Hardening and planting out

Acclimatization is important in the case of micropropagated plants because *in vitro* plant matured is not adopted for *ex vitro* conditions. 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 newly transferred plantlets. Three methods of controlling relative humidity are the polyethene tent, misting and fogging. In polyethene tent, as the aerial weaning environment is closed, it is possible to take advantage of the Co_2 enrichment during hardening (Lakso *et al.*, 1986).

Standardisation of rhizosphere environment is also necessary for getting better growth of plants (Zimmerman and Fordham, 1985). Transfer of *in vitro* raised plantlets of *Nepenthes khasiani* to a potting mixture containing sand, top soil and cattle manure (1:1:1) in 5 x 5 cm earthern nursery pots together with providing a suitable environment led to the survival of 90-95 per cent of the plants. The potted plants were kept in a 9 cm diameter polypropylene dish with a thin film of water, received diffused sunlight from the sides and were under sprinkled irrigation (Latha and Seeni, 1994).

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

Nutrition of the micropropagules during rooting and hardening has been shown to be species dependent and Scott (1987) has shown that inclusion of fertilizers during hardening can be detrimental for Kalmia, improve plant quality of Rhododendron and is essential for quality Magnolia. 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 plants with the application of half strength MS nutrients as well as NPK fertilizer solution (10:5:10 g l^{-1}) at weekly intervals. However, better vigour of the plantlets was observed by application of the latter solution.

Success of acclimatization depends upon not only the post transfer condition but also the pre-transfer culture conditions (Zhu, 1986).

Hardening treatment for effective planting out of *in vitro* plantlets of vetiver was standardised by Keshavachandran (1993). Immersing the roots of the plantlets in sterile water for 12 h followed by transfer into pots and keeping this in 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 longer leaves, the largest diameter of the bush and the highest number of tillers/ plant.

In *Pelargonium graveolens*, Satyakala *et al.* (1995) reported that the rooted plants should be transferred to ½ 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 at 25°C. After 25 days, the hardened plants were gradually transferred to pots.

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).

Effect of containers on survival was studied by Prabha (1993) on tissue cultured pineapple plantlets. Plantlets grown in plastic pots showed maximum vigour followed by those in mud pots and polybags.

MATERIALS AND METHODS

The present study on standardisation of *in vitro* propagation technique in *Trichopus zeylanicus* Gaertn. was carried out in the Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara, Thrissur, during 1995 to 1997.

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 Glassware

Borosilicate glassware of Corning/Borosil brand were used for the study. The glassware were cleaned initially by soaking in potassium dichromate solution for 12 h followed by thoroughly 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 glassware were then dried in hot air oven at 100°C for 24 h. They were then stored in cupboards away from contaminants and dust until use.

3.1.2 Culture media

3.1.2.1 Composition of media

Murashige and Skoog (MS) medium (1962), Schenk and Hildebrandt (SH) medium (1972), B_5 medium (Gamborg *et al.* 1968) and Woody Plant medium (WPM) (Llyod and McCown,1980) were used for the study. The nutrient media included inorganic salts, vitamins, growth regulators, organic supplements and a carbon source. The composition of different basal media tried are given in Appendix I.

3.1.2.2 Preparation of media

The stock solutions for macro and micro nutrients, vitamins, iron-EDTA and growth regulators were prepared with sterile distilled water.

To prepare 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 5.5-5.8 as required using 0.1 N NaOH and 0.1 N HCl. The volume was made up to one litre. Agar (0.75%) was weighed out, added to the medium and melted by keeping the solution in a water bath maintained at 90-95°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°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 use.

3.1.3 Growth regulators

Auxins (2,4-D, NAA, IBA, IAA) and cytokinins (BA, 2iP, KIN) and a new steroid growth substance, brassinolide were incorporated in the media at various stages of cultures for direct and indirect morphogenesis, bud and shoot formation from floral buds, rooting etc.

GA_3 was added to the media for seed germination and shoot elongation. Addition of GA_3 to the media was done by filter sterilization. For this the micro-filter was first cleaned and washed thoroughly with distilled water. It was then sterilized in an autoclave and dried in the hot air oven. Inside the laminar air flow cabinet, the required quantity of GA_3 was pipetted and added to the sterilised and melted medium after filtering through microfilter.

3.1.4 Organic supplements

Adenine sulfate, coconut water, casein hydrolysate and yeast extract were tried for their effect on shoot bud multiplication and shoot elongation.

3.1.5 Carbon source

Sucrose was used as the main source of carbon for the study and was added to the medium at the rate of 3 per cent for all media except for B_5 in which 2 per cent sucrose was used.

3.1.6 Transfer area and aseptic manipulations

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

Plate 1. Stock plants of *Trichopus zeylanicus*

1

under the hood of a clean laminar air flow cabinet. The working table of the laminar airflow cabinet was first surface sterilized with absolute alcohol and then by putting on the UV light for 30 min. The petri dishes as well as the instruments used for inoculation were first steam sterilised in an autoclave at 15 PSI at 121°C for 30 min and then flame sterilised before each inoculation. The hands were also scrubbed with alcohol before each inoculation.

3.1.7 Culture room

The cultures were incubated at 27°C in an air conditioned culture room with a 16 h photoperiod of around 2000 lux supplied by cool, day light fluorescent tubes. Cultures were also incubated under dark to study the effect of light and dark conditions on culture establishment and growth, callus development, callus regeneration etc.

3.1.8 Source of explant

The explants were taken from stock plants maintained in the glass house. The stock plants were obtained from Regional Research Institute of the Central Council for Research on Ayurveda and Sidha at Poojapura and from Tropical Botanical Garden and Research Institute, Palode. The plants brought were planted in pots containing potting mixture (1:1:1 sand, soil and farmyard manure) and kept in the glass house giving daily irrigation and frequent fertilisation (Plate 1). Plants were frequently sprayed with systemic fungicide Bavistin 50 WP (Carbendazim) 0.3 per cent and contact fungicide Fytolan (copper oxychloride) at 0.1 per cent concentration. Whenever insect attack (mainly mealy bugs and scales) was noticed, the plants were sprayed with Dimecron (0.05%). Rodents were also found to

damage the plants by cutting the entire plant at soil surface. Poison baiting with Roban was adopted to control the rats.

3.2 Methods

3.2.1 Preparation of explant

Mature dark green shoots, semimature purplish green shoots, tender purple shoots with unopened but, developed leaves, as well as very small basal shoots with the bract were taken for the study. Shoots were first washed thoroughly with distilled water to remove soil from the base of the shoots. Then they were immersed in Teepol solution for five minutes. After washing off the Teepol solution with distilled water, the explants were subjected to surface sterilization treatments. Explants used include leaf segments with midrib, petiole segments, root segments and floral buds.

3.2.2 Standardisation of surface sterilization

The explants were subjected to various surface sterilants at different concentrations for different durations as given below:

1. Mercuric chloride 0.05 per cent for 2, 5 and 10 min
2. Mercuric chloride 0.1 per cent for 2, 5 and 10 min
3. Chlorine water for 2, 5 and 10 min

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

3.2.2.1 Effect of season on explant survival

The explants were collected round the year during different seasons and

cultured after surface sterilization using 0.1 per cent mercuric chloride. The difference in contamination as affected by season was studied. Observations on percentage contamination, survival and establishment were recorded.

3.3 Routes of micro propagation attempted

For *in vitro* multiplication of *Trichopus*, different routes of micro-propagation such as enhanced release of axillary buds and direct and indirect organogenesis/embryogenesis were tried. *In vitro* seed germination was also attempted.

3.3.1 *In vitro* seed germination

Mature, hard and black coloured capsules were collected from glass house grown stock plants. The capsules were washed in running tap water to remove adhering soil and then immersed in Teepol solution for 5 minutes. They were again washed with distilled water and subjected to surface sterilisation treatments. The sterilized capsules were washed in four to five changes of sterile, distilled water and dried on sterile filter paper. Seeds were extracted from the capsules after drying and inoculated.

To enhance germination of seeds, soaking of capsules and seeds in sterile water, inoculation in different media and incubation under light and dark were tried. Seeds were transferred to fresh media at monthly intervals.

Observations on percentage germination of seeds and average time taken for germination were recorded.

Media tried for seed germination are

1. ½ MS

2. ½ MS + GA 2 mg l^{-1}

3.3.2 Axillary bud release

3.3.2.1 Culture establishment

The following growth regulator combinations were tried in five different basal media for enhanced release of axillary buds:

i) Media

MS, WPM, SH, B_5 and ½ MS

ii) Growth regulators

1. BA 0.5, 1.0, 2.5 and 5.0 mg l^{-1}
2. KIN 0.5, 1.0, 2.5 and 5.0 mg l^{-1}
3. 2iP 0.5, 1.0, 2.5 and 5.0 mg l^{-1}
4. MS + NAA 0.5 mg l^{-1} + BA 0.5, 1.0, 2.5 and 5.0 mg l^{-1}
5. MS + IAA 0.5 mg l^{-1} + BA 0.5, 1.0, 2.5 and 5.0 mg l^{-1}
6. MS + NAA 0.5 mg l^{-1} + 2iP 0.5, 1.0, 2.5 and 5.0 mg l^{-1}
7. MS + IAA 0.5 mg l^{-1} + 2iP 0.5, 1.0, 2.5 and 5.0 mg l^{-1}
8. MS, WPM, SH, B_5 and ½ MS without growth regulators

The cultures were inoculated both under light and dark. The buds that survived and started to develop were subcultured into new media.

Observations were recorded with respect to culture establishment, number of days for bud burst, number of buds or shoots (if any).

3.3.2.2 Proliferation and elongation

3.3.2.2.1 Effect of media and BA

For multiple bud/shoot production, the buds after establishment were transferred to the following media combinations:

1. MS + BA 0.5, 1.0, 2.5 and 5.0 mg l^{-1}
2. WPM + BA 0.5, 1.0, 2.5 and 5.0 mg l^{-1}
3. SH + BA 0.5, 1.0, 2.5 and 5.0 mg l^{-1}
4. SH + GA 2.0 and 5.0 mg l^{-1}

The buds were subcultured to the same media at one month interval for further proliferation as the growth and multiplication of buds was slow.

The number of shoots and buds developed and the length of shoots were recorded.

3.3.2.2.2 Effect of additives

For proliferation and elongation of buds and shoots, the following additives were incorporated in SH medium along with BA at four concentrations 0.5, 1.0, 2.5 and 5.0 mg l^{-1}.

1. Adenine sulfate 50 and 100 mg l^{-1}
2. Casein hydrolysate 100 and 500 mg l^{-1}
3. Yeast extract 100 and 500 mg l^{-1}
4. Coconut water 10 per cent and 20 per cent

The number of shoots and buds developed and the length of shoots were recorded.

3.3.3 Indirect morphogenesis

3.3.3.1 Induction of callus

3.3.3.1.1 Standardisation of explant and surface sterilization

Explants used such as leaf bits with midrib, leaf base and petioles were used for callus induction. The explants were washed in water to which few drops of Teepol was added and then washed with distilled water. Various surface sterilization treatments were tried as in the case of buds. After surface sterilization the leaves and petioles were dried on sterile filter paper and cut into pieces of 0.5-0.75 cm^2 and 0.5-1.0 cm respectively.

3.3.3.1.2 Standardisation of media and growth regulators

The following media and growth regulators were tried for callus induction.

i) Media

MS, SH, WPM and ½ MS

ii) Growth regulators

1. 2,4-D 0.5, 1.0, 2.5 and 5.0 mg l^{-1}
2. NAA 0.5, 1.0, 2.5 and 5.0 mg l^{-1}
3. 2,4-D + BA (0.5+0.5, 1.0+1.0, 2.5+2.5 and 5.0+5.0 mg l^{-1})
4. NAA + BA (0.5+0.5, 1.0+1.0, 2.5+2.5 and 5.0+5.0 mg l^{-1}
5. 2,4-D + BA + KIN (0.5+0.5+0.5, 2.5+2.5+2.5, 5.0+5.0+5.0 mg l^{-1})

Cultures were incubated both under light and dark conditions. Observations like percentage of cultures initiating callus, number of days taken for callus initiation, callus index 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 and G is the growth score. Scoring was 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 four months in culture tubes (Transfer of callus to new media was done at 20-25 days interval).

3.3.3.2 Callus proliferation and regeneration (embryogenesis/organogenesis)

Subculturing of developing callus was done at 20-25 days interval for proliferation of callus.

3.3.3.2.1 Organogenesis

Uniform bits of calli were subcultured into media containing different growth regulators to initiate organoids. Following were the growth regulators tried. All the treatments were tried in MS medium.

1. BA 0.5, 2.5 and 5.0 mg l^{-1}
2. KIN 0.5, 2.5 and 5.0 mg l^{-1}
3. 2iP 0.5, 2.5 and 5.0 mg l^{-1}
4. BA 0.5 mg l^{-1} + NAA 0.1 mg l^{-1}
5. BA 0.5 mg l^{-1} + IAA 0.1 mg l^{-1}
6. BA 0.5 mg l^{-1} + IBA 0.1 mg l^{-1}
7. BA 0.5 mg l^{-1} + Brassinolide 0.001, 0.0005 and 0.0001 mg l^{-1}
8. MS without growth regulators

Observations on percentage organogenesis and days for bud development from callus were recorded. Number of buds, shoots and shoot length after two months were also recorded. Subculturing to the same media was done at an interval of 25 days. Shoots with well developed leaves were rooted *in vitro*.

3.3.3.2.2 Embryogenesis

Calli bits were subcultured into the following media for embryogenesis studies:

1. MS + 2,4-D 0.5 and 1 mg l^{-1}
2. MS + BA 2.5 mg l^{-1}
3. MS + BA 2.5 mg l^{-1} + coconut water 10 per cent

Observations on the number of embryoids produced and stage of embryo were studied. Developing embryos were transferred to basal MS medium for germination.

3.3.3.3 Induction of rooting

The regenerated shoots with fully developed leaves were put in different rooting treatments either singly or as a clump.

The percentage of root initiation, days to root initiation, number and length of roots were recorded for the different cultures subjected to different treatments. Medium tried was ½ MS. The treatments were as follows:

1. NAA + IBA (0.5+0.5, 1.0+1.0, 1.0+0.5, 0.5+1.0 mg l^{-1})
2. IBA 1.0 mg l^{-1}

3. IBA + NAA + brassinolide (0.5+0.5+0.0005, 0.5+0.5+0.001 mg l^{-1}) .

4. Brassinolide (0.0005, 0.001 mg l^{-1}) transferred to IBA 1 mg l^{-1} after 1 week

5. ½ MS

3.3.3.4 Hardening

Rooted plantlets were either taken of the media, washed with sterile water to remove media adhering to the plant parts, dipped in Fytolan 0.1 per cent for 5 minutes and then transferred to sterile mud pots or transferred from rooting media to basal medium (½ MS) with 1.5 per cent sucrose, kept under increased light intensity for two weeks and then transferred to mud pots after again dipping in Fytolan as done earlier. The potted plants were kept in the hardening unit where the plants were sprayed with water at 20 min interval. MS solution (1/10th strength) was applied to the surviving plants at biweekly interval.

3.4 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 technique. Logit, $\sqrt{x}$ and $\sqrt{x+½}$ transformation were made wherever necessary before analysis.

RESULTS

The results of the studies on standardisation of *in vitro* propagation techniques in *Trichopus zeylanicus* Gaertn. conducted during 1995-97 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 Standardisation of surface sterilization of shoot buds

The results of the trial for surface sterilization of shoot buds are presented in Table 1a. No contamination was observed in cultures receiving treatments T_7 (chlorine water 2 min), T_8 (chlorine water 5 min) and T_9 (chlorine water 10 min), but establishment and growth of cultures in these treatments were very poor (24% in cultures receiving T_7 and 0% with T_8 and T_9). As a result of these treatments, most of the cultures dried up. Establishment was maximum (87.5%) in cultures receiving T_5 treatment (mercuric chloride 0.1% 5 min). Cultures contaminated (12.5%) and dried (0%) were also less in this treatment and T_5 was on par with T_6 (mercuric chloride 0.1% 10 min). Explants when cultured (in appropriate media), without any sterilization treatment resulted in cent per cent contamination.

As the duration of exposure to mercuric chloride increased, the percentage of cultures that dried up also increased (10% in T_6 treatment).

Table 1a. Effect of surface sterilants on the establishment of buds of *Trichopus zeylanicus*

Treatment No.	Treatments			Observations**				
	Sterilant	Concentration (%)	Duration (mts)	Fungal contamination (%)	Bacterial contamination (%)	Contamination (%)	Cultures bleached/ dried (%)	Establishment (%)
1	Mercuric chloride	0.05	2	33.33 (-0.693)*	33.33 (-0.693)	66.66 (0.693)	0 (-3.135)	33.33 (-0.693)
2	Mercuric chloride	0.05	5	18.33 (-1.498)	36.37 (-0.549)	54.99 (0.203)	0 (-3.036)	45.00 (-0.203)
3	Mercuric chloride	0.05	10	0 (-3.296)	28.58 (-0.916)	28.58 (-0.747)	7.145 (-2.544)	64.29 (0.602)
4	Mercuric chloride	0.1	2	11.11 (-2.08)	22.22 (-1.253)	38.33 (-0.693)	0 (-3.571)	66.66 (0.693)
5	Mercuric chloride	0.1	5	12.5 (-1.940)	0 (-3.43)	12.5 (-1686)	0 (-3.432)	87.5 (1.946)
6	Mercuric chloride	0.1	10	0 (-2.944)	10.0 (-2.165)	10.0 (-1.648)	10.0 (-2.165)	80.0 (1.386)
7	Chlorine water	-	2	0 (-3.135)	0 (-3.135)	0 (-2.397)	74.99 (1.151)	25.0 (-1.152)
8	Chlorine water	-	5	0 (-3.375)	0 (-3.375)	0 (-2.647)	100.00 (3.366)	0 (-3.375)
9	Chlorine water	-	10	0 (-3.135)	0 (-3.135)	0 (-23.97)	100.00 (3.135)	0 (-3.135)
10	Control (No sterilant)	-		66.66	33.33	100	0	0
	CD			0.126	0.789	0.6	1.167	0.599
	Sem±			0.04	0.25	0.19	0.37	0.19

* Values in paranthesis represent logit transformed ones

** Observations recorded three weeks after inoculation

4.1.2 Surface sterilization of leaf and petiole bits

Results of the work on surface sterilization of leaves and petioles are summarised in Table 1b. Of the various surface sterilization treatments tried, contamination was minimum (0%) in the case of cultures receiving T_5 (mercuric chloride 0.1% 5 min), T_6 (mercuric chloride 0.1% 10 min) and T_7 (chlorine water 2 min) and these were on par with T_8 (chlorine water 5 min) and T_9 (chlorine water 10 min). Percentage of cultures bleached and dried was high (62.5 to 80.36%) in cultures treated with chlorine water for different durations.

Establishment was maximum (86.61%) for cultures receiving T_5 (mercuric chloride 0.1% 5 min) as in the case of shoot buds.

All the treatments tried for buds, leaves and petiole bits were tried for very tender basal shoots and roots taken from potted plants in glass house. None of such shoots and roots survived. They were either contaminated or dried up.

4.1.3 Surface sterilization of seeds and capsules

Data on the effect of surface sterilization of capsules are presented in Table 1c. Among the different treatments tried, T_5 (mercuric chloride 0.1% 5 min) and T_6 (mercuric chloride 0.1% 10 min) gave maximum establishment (100%) of seeds. Contamination was nil for these two treatments. Inoculation without sterilization resulted in complete contamination. As the concentration of sterilant and duration of treatment increased there was decrease in the percentage of contamination.

Table 1b. Effect of surface sterilants on the establishment of leaf and petiole segments of *I. zeylanicus*

Treat-ment No.	Treatments			Observations**				
	Sterilant	Concent-ration (%)	Duration (mts)	Fungal contami-nation (%)	Bacterial contami-nation (%)	Contami-nation (%)	Cultures bleached/ dried (%)	Establ-ishment (%)
1	Mercuric chloride	0.05	2	12.5 (-1.946)*	18.75 (-1.522)	31.25 (-0.805)	6.25 (-2.689)	62.5 (0.549)
2	Mercuric chloride	0.05	5	13.4 (-1.869)	6.25 (-2.689)	19.65 (-1.445)	13.4 (-1.869)	66.96 (-0.714)
3	Mercirue chloride	0.05	10	0 (-3.364)	19.65 (-1.445)	19.65 (-1.445)	0 (-3.364)	80.36 (1.445)
4	Mercirue chloride	0.1	2	21.43 (-1.354)	0 (-3.296)	21.43 (-1.354)	0 (-3.296)	78.57 (1.354)
5	Mercuric chloride	0.1	5	0 (-3.364)	0 (-3.364)	0 (-3.364)	13.4 (-1.869)	86.61 (1.869)
6	Mercuric chloride	0.1	10	0 (3.432)	0 (-3.432)	0 (-3.432)	25.0 (-1.099)	75.0 (1.099)
7	Chlorine water	-	2	0 (-3.432)	0 (-3.432)	0 (-3.432)	62.5 (0.511)	37.5 (-0.511)
8	Chlorine water	-	5	6.25 (-2.689)	0 (-3.364)	6.25 (-2.621)	80.36 (1.445)	13.4 (-1.869)
9	Chlorine water	-	10	6.25 (-2.689)	0 (-3.432)	6.25 (-2.689)	62.5 (0.511)	31.25 (-0.805)
10	Control (No sterilant)			53.57 (0.144)	46.43 (-0.144)	100 (3.366)	0 (-3.364)	0 (-3.364)
	CD			1.167	0.946	1.23	0.82	0.883
	Sem±			0.37	0.3	0.39	0.26	0.28

* Values in paranthesis represent logit transformed ones

** Observations recorded three weeks after inoculation

Table 1c. Effect of surface sterilants on the establishment of seeds of *T. zeylanicus*

Treat-ment No.	Treatments			Observations**			
	Sterlant	Concent-ration (%)	Duration (mts)	Fungal contami-nation (%)	Bacterial contami-nation (%)	Contami-nation (%)	Establish-ment (%)
1	Mercuric chloride	0.05	2	45.00 (-0.203)*	32.50 (-0.752)	77.50 (1.242)	22.50 (-1.242)
2	Mercuric chloride	0.05	5	10.00 (-2.047)	55.00 (0.203)	65.00 (0.693)	35.00 (-0.693)
3	Mercuric chloride	0.05	10	7.14 (-2.565)	10.75 (-2.178)	17.86 (-1.545)	82.14 (1.545)
4	Mercuric chloride	0.1	2	0 (-2.555)	25.00 (-1.099)	25.00 (-1.099)	75.00 (1.099)
5	Mercuric chloride	0.1	5	0 (-3.137)	0 (-3.137)	0 (-3.137)	100.00 (3.135)
6	Mercuric chloride	0.1	10	0 (-2.708)	0 (-2.708)	0 (-2.708)	100.00 (2.708)
7	Control (No sterilant)			65.00 (0.626)	35.00 (-0.626)	100.00 (3.664)	0 (-3.664)
	CD			0.84	0.99	1.14	0.84
	SEm±			0.28	0.33	0.38	0.28

* Values in paranthesis represent logit transformed ones

** Observations recorded three weeks after inoculation

Percentage establishment of cultures was maximum (87.5% and 86.6% for shoot buds and leaf and petiole bits, respectively) when treated with mercuric chloride 0.1 per cent for 5 min. Percentage of cultures dried increased with increasing duration of mercuric chloride treatment. Treatment of shoot buds, leaf and petiole bits with chlorine water resulted in drying up of cultures (62.5%-100%). Mercuric chloride 0.1 per cent for 5 min and 10 min resulted in maximum percentage (100%) of survival of seeds.

4.1.4 Seasonal variation in contamination of shoot buds

The data on the seasonal influence on culture establishment of buds are presented in Table 2a. Sterilization treatment with 0.1 per cent mercuric chloride for 5 min was repeated at monthly interval. Survival of shoot buds was highest during January to April with maximum in April (95%) followed by January (90%). Survival was lower during the months from June to September with minimum during September (35%) (Fig.1).

4.1.5 Seasonal variation in contamination of leaves and petioles

Data on seasonal variation in contamination of leaves and petioles are given in Table 2b. As in the case of shoot buds, leaves and petioles were treated with mercuric chloride 0.1 per cent for 5 min and inoculated in suitable media. Maximum survival was found during February (90%) followed by January (88.88%). During the dry months, contamination was less compared to the rainy season.

Survival of explants was maximum during January to April in the case of shoot buds, leaves and petioles. Highest survival of shoot buds (95%) was observed

Table 2a. Effect of season on establishment of buds of *T. zeylanicus*

Treatment No.	Month	Contamination (%)	Fungal contamination (%)	Bacterial contamination (%)	Cultures dried (%)	Establishment (%)
1	January	5.00 (-2.45)	5.00 (-2.93)	0 (-3.66)	5.00 (-2.93)	90.00 (2.20)
2	February	10.00 (-1.95)	10.00 (-2.20)	0 (-3.66)	5.00 (-2.93)	85.00 (1.79)
3	March	15.00 (-1.67)	10.00 (-2.20)	5.00 (-2.93)	0 (-3.66)	85.00 (1.79)
4	April	5.00 (-2.45)	5.00 (-2.93)	0 (-3.66)	0 (-3.66)	95.00 (2.93)
5	May	10.00 (-1.95)	10.00 (-2.20)	0 (-3.66)	10.00 (-2.20)	80.00 (1.39)
6	June	30.00 (-0.92)	15.00 (-1.79)	15.00 (-1.79)	10.00 (-2.20)	60.00 (0.41)
7	July	80.00 (-0.90)	15.00 (-1.79)	15.00 (-1.79)	10.00 (-2.20)	60.00 (0.41)
8	August	45.00 (-0.20)	25.00 (-1.12)	20.00 (-1.39)	10.00 (-2.20)	45.00 (-0.20)
9	September	65.00 (0.63)	40.00 (-0.41)	25.00 (-1.12)	0 (-3.66)	35.00 (-0.63)
10	October	15.00 (-1.67)	5.00 (-2.93)	10.00 (-2.20)	5.00 (-2.93)	80.00 (1.52)
11	November	35.00 (-0.63)	10.00 (-2.20)	25.00 (-1.12)	0 (-3.66)	65.00 (0.63)
12	December	30.00 (-0.90)	15.00 (-1.80)	15.00 (-1.79)	0 (-3.66)	70.00 (0.90)
	CD	1.11	1.33	0.96	1.14	1.26
	Sem±	0.36	0.43	0.31	0.37	0.41

* Values in parenthesis represent logit transformed ones

Fig.1. Effect of season on establishment of buds of *Trichopus zeylanicus*

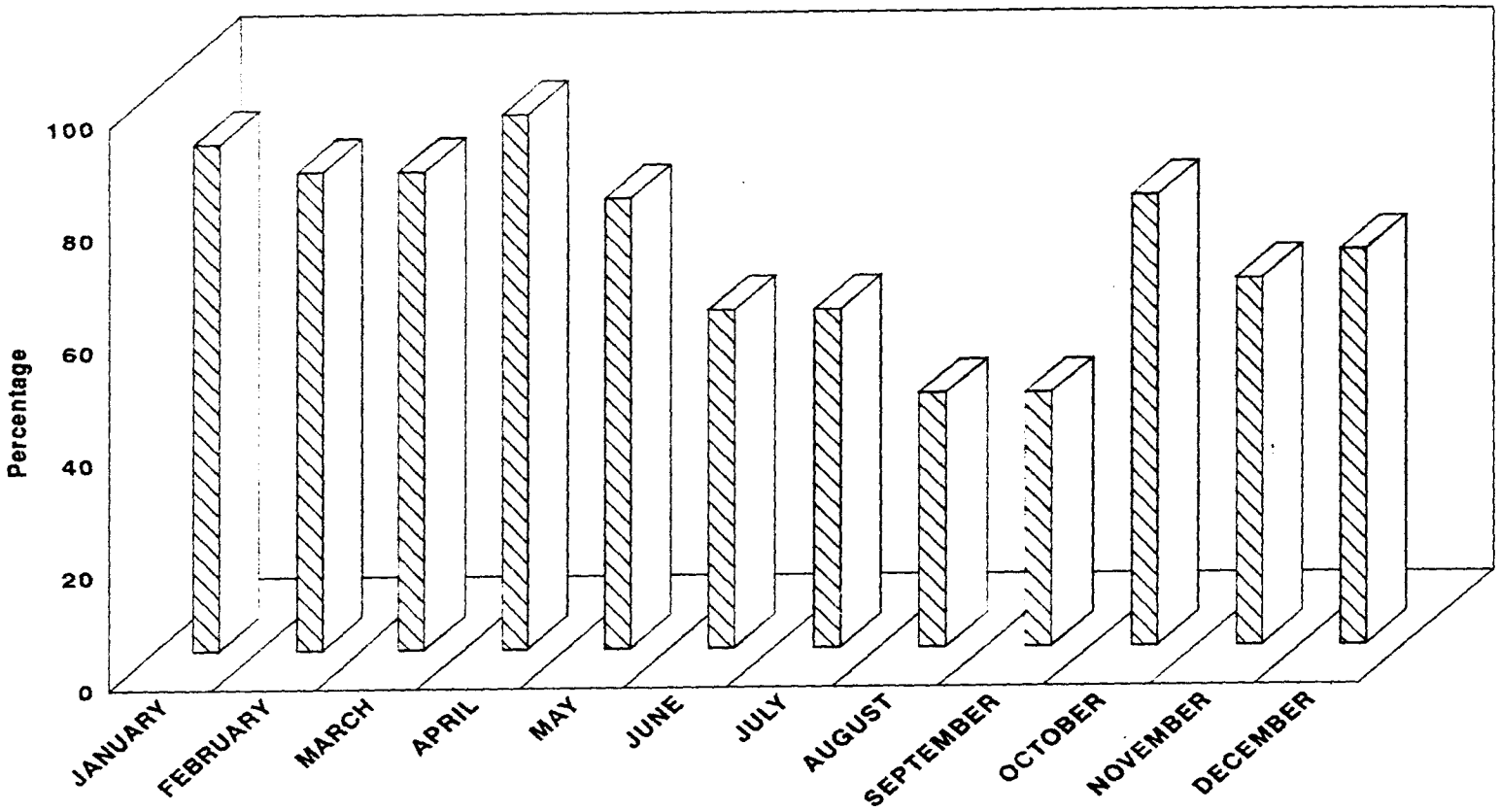

Table 2b. Effect of season on establishment of leaf and petiole bits of *T. zeylanicus*

Treatment No.	Month	Contamination (%)	Fungal contamination (%)	Bacterial contamination (%)	Cultures dried (%)	Establishment (%)
1	January	11.11 (-2.33)*	5.55 (-2.82)	5.55 (-3.56)	0 (-3.56)	88.88 (2.08)
2	February	10.00 (-1.95)	0 (-3.66)	10.00 (-2.20)	0 (-3.66)	90.00 (2.20)
3	March	8.33 (-1.95)	0 (-2.45)	8.33 (-2.46)	7.15 (-2.46)	84.52 (1.70)
4	April	14.30 (-1.52)	7.15 (-2.54)	7.15 (-2.54)	0 (-3.30)	85.71 (1.80)
5	May	22.26 (-1.15)	22.26 (-1.25)	0 (-3.97)	7.42 (-2.53)	70.33 (0.86)
6	June	29.17 (-0.90)	17.36 (-1.60)	11.81 (-2.01)	11.81 (-2.01)	59.03 (0.37)
7	July	38.18 (-0.48)	24.09 (-1.18)	14.09 (-1.85)	19.09 (-1.45)	42.73 (-0.29)
8	August	29.16 (-0.90)	22.21 (-1.25)	5.55 (-2.82)	16.67 (-1.67)	55.55 (0.24)
9	September	55.55 (0.22)	38.89 (-0.46)	16.67 (-1.67)	0 (-3.56)	44.44 (-0.22)
10	October	30.00 (-0.85)	10.00 (-2.20)	20.00 (-1.39)	0 (-3.66)	70.00 (0.85)
11	November	24.09 (-1.18)	14.55 (-1.84)	9.55 (-2.25)	0 (-3.71)	75.91 (1.18)
12	December	23.22 (-1.22)	7.15 (-2.54)	14.29 (-1.79)	0 (-3.30)	77.00 (1.35)
	CD	0.86	1.33	1.29	0.71	0.65
	SEm±	0.28	0.43	0.42	0.23	0.21

* Values in paranthesis represent logit transformed values

during April and that of leaf and petiole bits (90% survival) during February. During the rainy season from June to September, contamination percentage was found to increase.

4.2 *In vitro* seed germination

Results of *in vitro* seed germination trial are presented in Table 3a and 3b. Percentage germination of seeds was maximum (87.5) in T_1 (1/2 MS) and T_2 (1/2 MS + GA 2.0 mg). Soaking of seeds in water was not found to increase germination of seeds and it increased the contamination of seeds. Only 62.5 per cent of the surviving seeds germinated. Incubation under dark also showed lower germination per cent (62.5%). However, soaking of seeds decreased the days for germination of seeds and minimum number of days for germination (96.8 days) was observed in T_4 (soaking seeds for one hour in water and incubation in 1/2 MS) (Plates 2a, 2b and 2c).

4.3 Axillary bud release

4.3.1 Establishment of shoot buds

4.3.1.1 Effect of explant maturity on establishment and growth of shoot buds

The data on the effect of explant and its maturity on culture establishment and growth are presented in Table 4. Parts of tender, pink purple coloured shoots showed maximum establishment percentage (Plate 3). As the maturity of shoots increased, establishment of cultures decreased and most of the cultures dried within 2-3 weeks after culturing whereas tender explants showed signs of growth.

Table 3a. Effect of GA, incubation under dark and seed soaking on percentage germination in *T. zeylanicus*

Treatment No.	Treatments	Concentration (mg l^{-1})	% germination
1	½ MS + GA	2	87.5
2	½ MS		87.5
3	½ MS + soaking seeds in water for 30 mts		62.5
4	½ MS + soaking seeds in water for 1 hr		62.5
5	½ MS, incubation under dark		62.5

Table 3b. Effect of GA, incubation under dark and seed soaking on days for germination in *T. zeylanicus*

Treatment No.	Treatments	Concentration (mg l^{-1})	Days for germination
1	½ MS + GA	2	175.00 (13.22)*
2	½ MS		146.00 (12.06)
3	½ MS + 30 mts soaking seeds in water		131.40 (11.46)
4	½ MS + soaking seeds for 1 hr in water		96.80 (9.72)
5	½ MS, incubation under dark		119.40 (10.85)
	CD		1.47
	SEm±		0.5

* Values in paranthesis represent logit transformed values

Plate 2a. Mature seeds of *T. zeylanicus*

Plate 2b. Stages of *in vitro* seed germination

Plate 2c. *In vitro* seedling of *T. zeylanicus*

2a

2c

2b

Table 4. Effect of explant maturity on establishment and growth of cultures

Treat-ment No.	Explant	Maturity stage	Establish-ment (%)	Cultures showing callus initiation/ bud burst (%)	Contami-nation (%)	Dried (%)
1	Leaf bits	Tender	70.00 (0.896)	60.00 (0.405)	7.50 (-2.571)	32.50 (-0.896)
2	Leaf base	Tender	96.59 (3.345)	40.18 (-0.399)	19.65 (-1.445)	40.18 (-0.399)
3	Petiole	Tender	95.34 (3.018)	58.33 (0.346)	25.00 (-1.152)	16.66 (-1.610)
4	Bud	Tender	70.00 (0.896)	70.00 (0.896)	15.00 (-2.022)	15.00 (-2.022)
5	Leaf	Semi mature	0 (-3.157)	0 (-3.137)	33.33 (-0.693)	66.66 (0.693)
6	Leaf base	Semi mature	21.43 (-1.354)	0 (-3.296)	21.43 (-1.354)	78.57 (1.354)
7	Petiole	Semi mature	0 (-3.296)	0 (-3.296)	0 (-3.296)	100.00 (3.296)
8	Bud	Semi mature	12.50 (-2.165)	12.50 (2.165)	27.50 (-1.386)	60.00 (0.405)
9	Leaf	Mature	0 (-3.434)	0 (-3.434)	0 (-3.434)	100.00 (3.434)
10	Leaf base	Mature	0 (-3.135)	0 (-3.135)	16.66 (-1.610)	83.33 (1.609)
11	Petiole	Mature	0 (-2.944)	0 (-2.944)	0 (-2.944)	100.00 (2.944)
12	Bud	Mature	0 (-3.296)	0 (-3.296)	21.43 (-1.354)	78.57 (1.354)
	CD		0.80	0.77	1.17	1.08
	Sem±		0.29	0.25	0.38	0.35

Plate 3. Explants used for *in vitro* multiplication of *T. zeylanicus*


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Tender shoots/flower buds were most responsive compared to semi-mature and mature buds. Seventy per cent of tender buds showed establishment and bud development whereas mature buds did not establish at all.

Shoot buds from tender, purple coloured shoots with unopened leaves were observed to give maximum establishment and growth (70%) whereas buds taken from semimature and mature shoots gave only much low percentage establishment (12.5 and 0%).

4.3.1.2 Effect of light

Shoot buds were cultured under both light and dark. Establishment percentage was lower under dark compared to that under light (Table 5a and 5b). Buds cultured in MS + BA 0.5 mg l^{-1} to 5.0 mg l^{-1} under light resulted in 50 to 83.33 per cent establishment whereas under dark the percentage establishment was only 10 to 45.45 per cent (Plates 4a, 4b). For buds cultured in MS + 2iP and MS + KIN and incubated under dark, the establishment per cent was only 0 to 8.3 and 0 to 6.66 per cent, respectively.

As the establishment per cent was observed to be low under dark, for further experiments, shoot buds were incubated under light.

4.3.1.3 Effect of media and growth regulators

The data on percentage establishment of buds in different basal media supplemented with cytokinins and auxins are presented in Table 5b. Better establishment of buds was observed in all the media when supplemented with BA (50-100%) compared to KIN (0-71.43%) and 2iP (0-66.66%). Establishment was

Table 5a. Effect of the cytokinins on establishment of buds of *T. zeylanicus* under dark

Treatment No.	Media and growth regulator	Concentration (mg l^{-1})	Establishment (%)
1	MS + BA	0.5	45.45
2	MS + BA	1.0	16.66
3	MS + BA	2.5	10.00
4	MS + BA	5.0	10.00
5	MS + KIN	0.5	0.00
6	MS + KIN	1.0	8.30
7	MS + KIN	2.5	0.00
8	MS + KIN	5.0	0.00
9	MS + 2iP	0.5	0.00
10	MS + 2iP	1.0	0.00
11	MS + 2iP	2.5	0.00
12	MS + 2iP	5.0	6.66

Table 5b. Effect of growth regulators on establishment of buds of *T. zeylanicus*

Treatment No	Media	Growth regulators	Concentration (mg l^{-1})	Establishment (%)
1	2	3	4	5
1	MS	BA	0.5	83.33
2	MS	BA	1.0	83.33
3	MS	BA	2.5	64.71
4	MS	BA	5.0	50.00
5	WPM	BA	0.5	87.50
6	WPM	BA	1.0	87.50
7	WPM	BA	2.5	66.66
8	WPM	BA	5.0	66.66
9	SH	BA	0.5	100.00
10	SH	BA	1.0	90.90
11	SH	BA	2.5	81.81
12	SH	BA	5.0	83.33
13	B5	BA	0.5	72.22
14	B5	BA	1.0	66.66
15	B5	BA	2.5	66.66
16	B5	BA	5.0	61.54
17	½ MS	BA	0.5	62.50
18	½ MS	BA	0.5	62.50
19	½ MS	BA	1.0	60.00
19	½ MS	BA	2.5	75.00
20	½ MS	BA	5.0	50.00
21	MS	KIN	0.5	0
22	MS	KIN	1.0	50.00
23	MS	KIN	2.5	60.00
24	MS	KIN	5.0	71.43

Contd.

Table 5b Continued

1	2	3	4	5
25	WPM	KIN	0.5	0
26	WPM	KIN	1.0	16.66
27	WPM	KIN	2.5	20.00
28	WPM	KIN	5.0	50.00
29	SH	KIN	0.5	14.29
30	SH	KIN	1.0	18.29
31	SH	KIN	2.5	20.00
32	SH	KIN	5.0	41.67
33	½ MS	KIN	0.5	0
34	½ MS	KIN	1.0	0
35	½ MS	KIN	2.5	11.11
36	½ MS	KIN	5.00	15.38
37	B5	KIN	0.5	0
38	B5	KIN	1.0	0
39	B5	KIN	2.5	10.00
40	B5	KIN	5.0	8.33
41	MS	2iP	0.5	40.00
42	MS	2iP	1.0	40.00
43	MS	2iP	2.5	50.00
44	MS	2iP	5.0	30.00
45	WPM	2iP	0.5	33.33
46	WPM	2iP	1.0	36.36
47	WPM	2iP	2.5	33.33
48	WPM	2iP	5.0	66.66
49	SH	2iP	0.5	50.00
50	SH	2iP	1.0	40.00
51	SH	2iP	2.5	20.00
52	SH	2iP	5.0	16.66

Contd.

Table 5b. Continued

1	2	3	4	5
53	½ MS	2iP	0.5	0
54	½ MS	2iP	1.0	0
55	½ MS	2iP	2.5	16.66
56	½ MS	2iP	5.0	8.33
57	MS	BA NAA	0.5 0.5	33.33
58	MS	BA NAA	1.0 0.5	50.00
59	MS	BA NAA	2.5 0.5	80.00
60	MS	BA NAA	5.0 0.5	28.57
61	MS	BA IAA	0.5 0.5	50.00
62	MS	BA IAA	1.0 0.5	33.33
63	MS	BA IAA	2.5 0.5	20.00
64	MS	BA IAA	5.0 0.5	11.11
65	MS	2iP NAA	0.5 0.5	33.33
66	MS	2iP NAA	1.0 0.5	41.67
67	MS	2iP NAA	2.5 0.5	20.00
68	MS	2iP NAA	5.0 0.5	15.38

Contd.

Table 5b. Continued

1	2	3	4	5
69	MS	2iP IAA	0.5 0.5	0
70	MS	2iP IAA	1.0 0.5	0
71	MS	2iP IAA	2.5 0.5	16.66
72	MS	2iP IAA	5.0 0.5	14.29
73	MS	-	-	0
74	WPM	-	-	0
75	SH	-	-	0
76	½ MS	-	-	0
77	B5	-	-	0

Plate 4a. Establishment of buds in MS + BA 0.5 mg l^{-1}

Plate 4b. Stage of bud development from node

4a

4b

high at lower concentration of BA (0.5-1.0 mg l^{-1}). Maximum percentage of establishment (100%) was observed in T_9 (SH + BA 0.5 mg l^{-1}) followed by T_{10} (SH + BA 1.0 mg l^{-1}) (90.9). Also, shoots cultured in SH medium along with BA were found to remain greener and healthier for longer duration.

4.3.1.3.1 Effect of BA

The data on the effect of BA on establishment of buds in five different basal media are presented in Table 5b and 5c.

The number of days for the first bud to burst was minimum (11.8) in MS medium followed by SH medium (14.7). In B_5 and 1/2 MS, the number of days for bud burst was more (23 and 28 days respectively).

Number of days for bud burst was minimum when BA was used at lower concentrations (0.5 - 2.5 mg l^{-1}). Minimum number of days for bud burst (6.33) was observed in T_1 (MS medium, supplemented with BA 0.5 mg l^{-1}) followed by T_2 (11.7) (MS + BA 1.0 mg l^{-1}) which was on par with T_3 (MS + BA 2.5 mg l^{-1}), T_4 (MS + BA 5.0 mg l^{-1}), T_5 (WPM + BA 0.5 mg l^{-1}, T_9 (SH + BA 0.5 mg l^{-1}), T_{10} (SH + BA 1.0 mg l^{-1}) and T_{11} (SH + BA 5.0 mg l^{-1}). Maximum number of buds (4.0) was observed in T_9 (SH + BA 0.5 mg l^{-1}) followed by T_6 (WPM + BA 1.0 mg l^{-1}) (2.67). This was on par with T_5 (WPM + BA 0.5 mg l^{-1}) (2.5) (Fig.2).

Buds formed in SH medium supplemented with BA 0.5 mg l^{-1} was found to be stout, short and creamy white where as buds cultured in WPM and MS medium were slender and elongated.

Table 5c. Effect of BA on establishment of shoot buds of *T. zeylanicus*

Treatment No.	Treatment			Days to bud release	No. of buds
	Media	Growth regulator	Concent-ration ($mg\ l^{-1}$)		
1	2	3	4	5	6
1	MS	BA	0.5	6.33 (2.46)	1.33 (1.14)
2	MS	BA	1.0	11.17 (3.21)	1.00 (1.00)
3	MS	BA	2.5	14.50 (3.79)	1.67 (1.24)
4	MS	BA	5.0	15.50 (3.84)	1.33 (1.12)
5	WPM	BA	0.5	13.33 (3.61)	2.50 (1.54)
6	WPM	BA	1.0	22.50 (4.72)	2.67 (1.57)
7	WPM	BA	2.5	18.00 (4.22)	1.17 (1.07)
8	WPM	BA	5.0	22.67 (4.73)	1.17 (1.07)
9	SH	BA	0.5	12.50 (3.51)	4.00 (1.91)
10	SH	BA	1.0	14.33 (3.71)	2.00 (1.37)
11	SH	BA	2.5	11.17 (3.27)	1.83 (1.31)
12	SH	BA	5.0	20.83 (4.54)	1.83 (1.35)

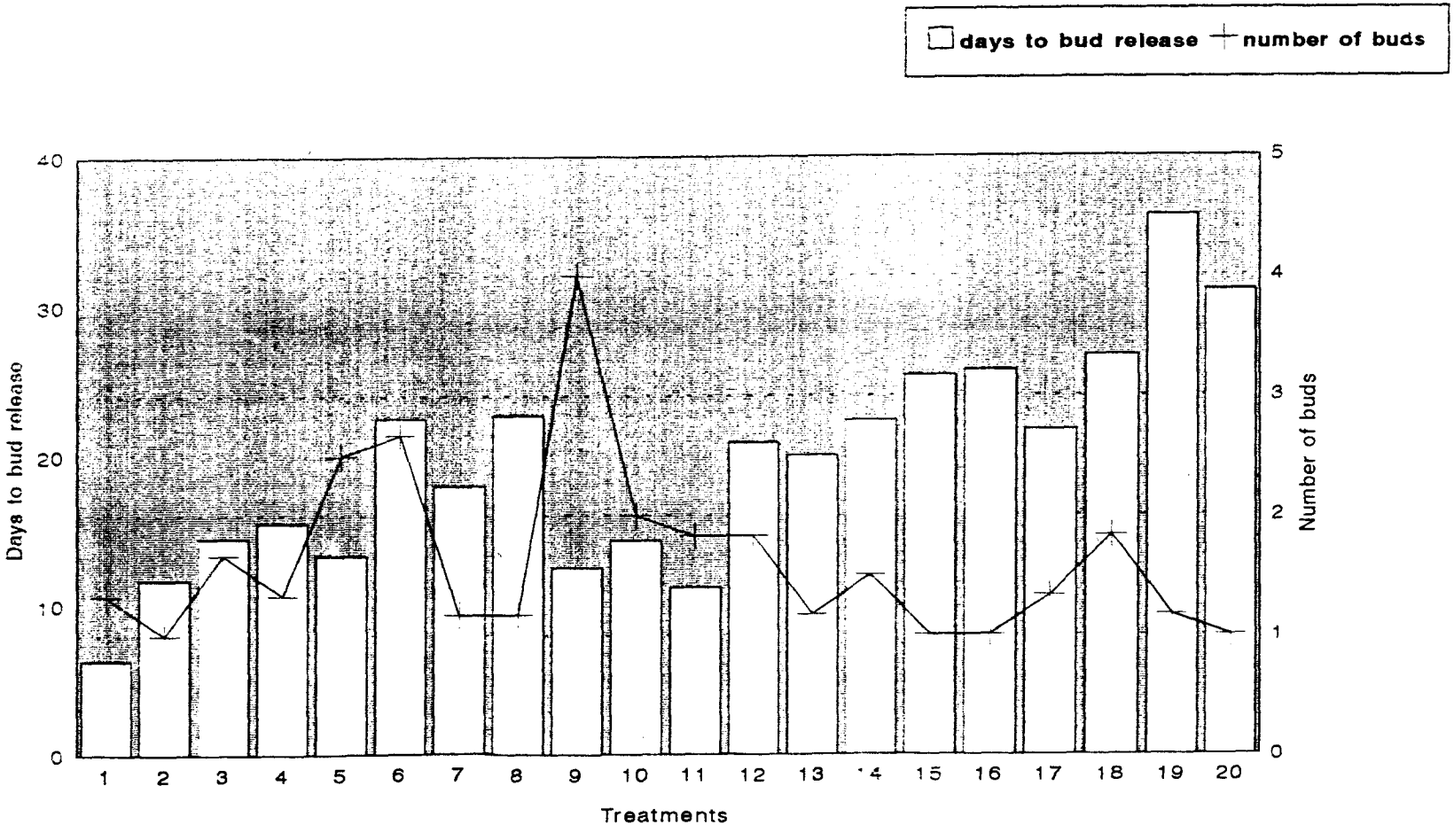
Contd.

Table 5c. Continued

1	2	3	4	5	6
13	B5	BA	0.5	20.00 (4.47)*	1.17 (1.07)
14	B5	BA	1.0	22.33 (4.68)	1.50 (1.21)
15	B5	BA	2.5	25.33 (5.02)	1.00 (1.00)
16	B5	BA	5.0	25.67 (5.05)	1.00 (1.00)
17	½ MS	BA	0.5	21.67 (4.64)	1.33 (1.14)
18	½ MS	BA	1.0	26.67 (5.14)	1.83 (1.31)
19	½ MS	BA	2.5	36.00 (5.99)	1.17 (1.07)
20	½ MS	BA	5.0	31.00 (5.54)	1.00 (1.00)
	CD			0.702	0.340
	SEm±			0.253	0.121

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

Fig.2. Effect of BA on establishment of shoot buds of *T. zeylanicus*



4.3.1.3.2 Effect of KIN

The data on the effect of KIN on establishment of shoot buds are summarised in Table 5b and 5d. At low concentrations of KIN (0.5 mg l^{-1}), culture establishment was nil in all the three media tried. Minimum number of days for bud burst (10) was observed in T_5 (WPM + 2.5 mg l^{-1} KIN) which was on par with T_4 (WPM + 1.0 mg l^{-1} KIN). Number of buds per tube was less compared to BA and maximum number of buds (2) was observed in T_3 (MS + KIN 5.0 mg l^{-1}) and this was on par with T_8 (SH + KIN 2.5 mg l^{-1}).

4.3.1.3.3 Effect of 2iP

The data on the effect of 2iP on bud establishment are presented in Table 5b and 5e. Number of days for establishment was minimum at the lowest concentrations tried (0.5 - 2.5 mg l^{-1} 2iP) in all the three media, with minimum number of days (8) being observed in T_1 (MS + 2iP 0.5 mg l^{-1}). Maximum number of buds (2) developed in T_3 (MS + 2iP 2.5) which was on par with T_4 (MS + 2iP 5.0). The buds formed in these treatments were slender and elongated. However, percentage establishment was low comparable to that of media containing BA. Better establishment and bud formation was observed in MS medium compared to WPM and SH medium.

4.3.1.3.4 Effect of combination of cytokinins along with auxins

The data on establishment of buds in media containing cytokinins along with auxins are presented in Table 5b and 5f. Percentage establishment of cultures in media supplemented with BA at varying concentrations along with low concentration of auxins (NAA and IAA 0.5 mg l^{-1}) was lower than that in media containing BA

Table 5d. Effect of KIN on establishment of buds of *T. zeylanicus*

Treatment No.	Media	Concentration of KIN (mg l^{-1})	Days to bud release	No. of buds
1	MS	1.0	18.67 (4.26)	1.00 (1.00)
2	MS	2.5	20.00 (4.44)	1.00 (1.00)
3	MS	5.0	18.00 (4.23)	2.00 (1.36)
4	WPM	1.0	12.00 (3.46)	1.00 (1.00)
5	WPM	2.5	10.00 (3.15)	1.00 (1.00)
6	WPM	5.0	12.33 (3.49)	1.33 (1.12)
7	SH	1.0	16.80 (4.08)	1.20 (1.08)
8	SH	2.5	25.00 (5.00)	1.50 (1.21)
9	SH	5.0	22.50 (4.73)	1.00 (1.00)

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

** KIN at 0.5 mg l^{-1} concentration was tried but did not show response

*** As the treatment had unequal number of replication CD is not shown

Table 5e. Effect of 2iP on establishment of buds of *T. zeylanicus*

Treatment No.	Media	Concentration of 2iP (mg l^{-1})	Days to bud release	No. of buds
1	MS	0.5	8.00 (2.81)	1.00 (1.00)
2	MS	1.0	21.33 (4.53)	1.33 (1.14)
3	MS	2.5	27.67 (5.19)	2.00 (1.38)
4	MS	5.0	31.67 (5.62)	1.67 (1.28)
5	WPM	0.5	16.33 (4.02)	1.00 (1.00)
6	WPM	1.0	12.00 (3.41)	1.00 (1.00)
7	WPM	2.5	15.00 (3.85)	1.17 (1.07)
8	WPM	5.0	26.00 (5.10)	1.00 (1.00)
9	SH	0.5	17.83 (4.21)	1.00 (1.00)
10	SH	1.0	27.00 (5.19)	1.00 (1.00)
11	SH	2.5	25.00 (4.95)	1.00 (1.00)
12	SH	5.0	27.00 (5.20)	1.00 (1.00)

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

** CD not shown because treatments had unequal replications

Table 5f. Effect of combination of cytokinins with auxinson establishment of buds of *T. zeylanicus*

Treatment No.	Media	Growth regulator	Concentration (mg l^{-1})	Days for bud burst	No. of buds
1	MS	BA + NAA	0.5 + 0.5	44.50 (6.63)	1.00 (1.00)
2	MS	BA + NAA	1.0 + 0.5	23.60 (4.81)	1.20 (1.08)
3	MS	BA + NAA	2.5 + 0.5	16.00 (3.98)	1.17 (1.07)
4	MS	BA + NAA	5.0 + 0.5	33.50 (5.48)	1.50 (1.21)
5	MS	BA + IAA	0.5 + 0.5	19.20 (4.26)	1.20 (1.08)
6	MS	BA + IAA	1.0 + 0.5	9.00 (3.00)	1.00 (1.00)
7	MS	BA + IAA	2.5 + 0.5	15.00 (3.87)	2.50 (1.57)
8	MS	BA + IAA	5.0 + 0.5	21.00 (4.58)	1.50 (1.21)
9	MS	2iP + NAA	0.5 + 0.5	22.00 (4.69)	1.25 (1.10)
10	MS	2iP + NAA	1.0 + 0.5	25.40 (5.04)	1.00 (1.00)
11	MS	2iP + NAA	2.5 + 0.5	28.00 (5.29)	1.50 (1.21)
12	MS	2iP + NAA	5.0 + 0.5	27.00 (5.19)	1.00 (1.00)
13	MS	2iP + IAA	2.5 + 0.5	68.00 (8.25)	2.00 (1.40)
14	MS	2iP + IAA	5.0 + 0.5	91.00 (9.53)	2.50 (1.57)

alone. However, combinations of BA with NAA in MS medium resulted in better establishment (28-80%) compared to BA with IAA (11-50%), 2iP along with IAA and NAA (0-41%), KIN and 2iP in the same medium.

Days for establishment and first bud to burst was minimum for T_6 (1.0 BA + 0.5 IAA). Considering the number of buds and days for establishment, T_7 (BA 2.5 + IAA 0.5) was found to be the best treatment (15 days for establishment with an average of 2.5 buds). 2iP at 2.5 to 5.0 mg l^{-1} concentration when added to the media along with IAA resulted in more number of buds (2 and 2.5 respectively for 2.5 and 5.0 mg l^{-1} 2iP) but required maximum number of days for bud burst (68 and 91 days respectively).

Nodal explants cultured in different basal media showed no signs of establishment and bud burst. They remained healthy for one to two weeks and later dried up.

In a few of the cultures, first few buds developed into flowers, but later on, whitish, green coloured shoot buds developed from the base of flower buds. Culturing of nodes with buds protruding out led invariably to the development of flower buds. Inoculation of nodes without buds protruding out resulted mostly in the development of cream or white coloured buds which developed into shoots with a few buds developing into flowers (Plates 5a, 5b).

Thus it can be seen that establishment of buds under light was higher upto 100 per cent whereas under dark it was lower (less than 50%). Establishment of buds was maximum (100%) in SH medium supplemented with BA 0.5 compared to KIN, 2iP and combination of BA with auxins, establishment of buds was better in

Plate 5a. Flower buds developing from node

Plate 5b. Shoot buds and shoot development from the base of flower buds

5a

5b

media supplemented with BA (50-100% establishment). Number of days for bud burst was minimum (6.33) in MS medium supplemented with 0.5 mg l^{-1} BA. The number of buds were maximum (4) in SH medium supplemented with 0.5 mg l^{-1} BA. KIN at 0.5 mg l^{-1} did not show response with respect to the establishment of buds.

4.3.2 Proliferation and elongation of buds and shoots

4.3.2.1 Standardisation of media and growth regulators

The results of the experiment on the effect of different concentrations of BA in three different media on the proliferation of shoot buds are presented in Table 6a and 6b. The number of buds developed, increased with increasing the concentrations of BA in the medium. Maximum number of buds (12.5) were observed in WPM with BA 5.0 mg l^{-1}. This was on par with SH medium containing BA at the same level (11.33). At lower concentrations of BA, eventhough the number of buds were lower, the number of shoots developed were found to be higher (3 and 4.67 in SH medium supplemented with BA 0.5 and 1.0 mg l^{-1} respectively). The length of shoots was also observed to be more (1.58-1.17 cm) at lower concentrations of BA (0.5 to 1.0 mg l^{-1}). The highest number of shoots (4.67) was observed in SH medium containing 1.0 mg l^{-1} BA with an average shoot length of 1.25 cm. Maximum shoot length (1.58 cm) was observed in SH medium containing 0.5 mg l^{-1} BA. Treatments T_1 (SH + 0.5 mg l^{-1} BA), T_2 (SH + 1.0 mg l^{-1} BA), T_5 (WPM + 0.5 mg l^{-1} BA), T_6 (WPM + 1.0 mg l^{-1} BA), T_9 (MS + 0.5 mg l^{-1} BA) and T_{10} (MS + BA 2.5) were comparable with respect to the number of shoots and length of shoots (Fig.3).

Table 6a. Effect of media and BA on proliferation of buds of *T. zeylanicus*

Treatment No.	Media	Growth regulator	Concentration (mg l^{-1})	No. of buds/tube
1	SH	BA	0.5	3.00 (1.858)
2	SH	BA	1.0	2.83 (1.816)
3	SH	BA	2.5	4.00 (2.105)
4	SH	BA	5.0	11.33 (3.419)
5	WPM	BA	0.5	4.33 (2.134)
6	WPM	BA	1.0	4.17 (2.086)
7	WPM	BA	2.5	4.33 (2.178)
8	WPM	BA	5.0	12.50 (3.591)
9	MS	BA	0.5	2.00 (1.559)
10	MS	BA	1.0	2.50 (1.625)
11	MS	BA	2.5	5.67 (2.430)
12	MS	BA	5.0	8.17 (2.908)
CD				0.51
SEm±				0.18

* Values in paranthesis represent $\sqrt{x} + \frac{1}{2}$ transformed values

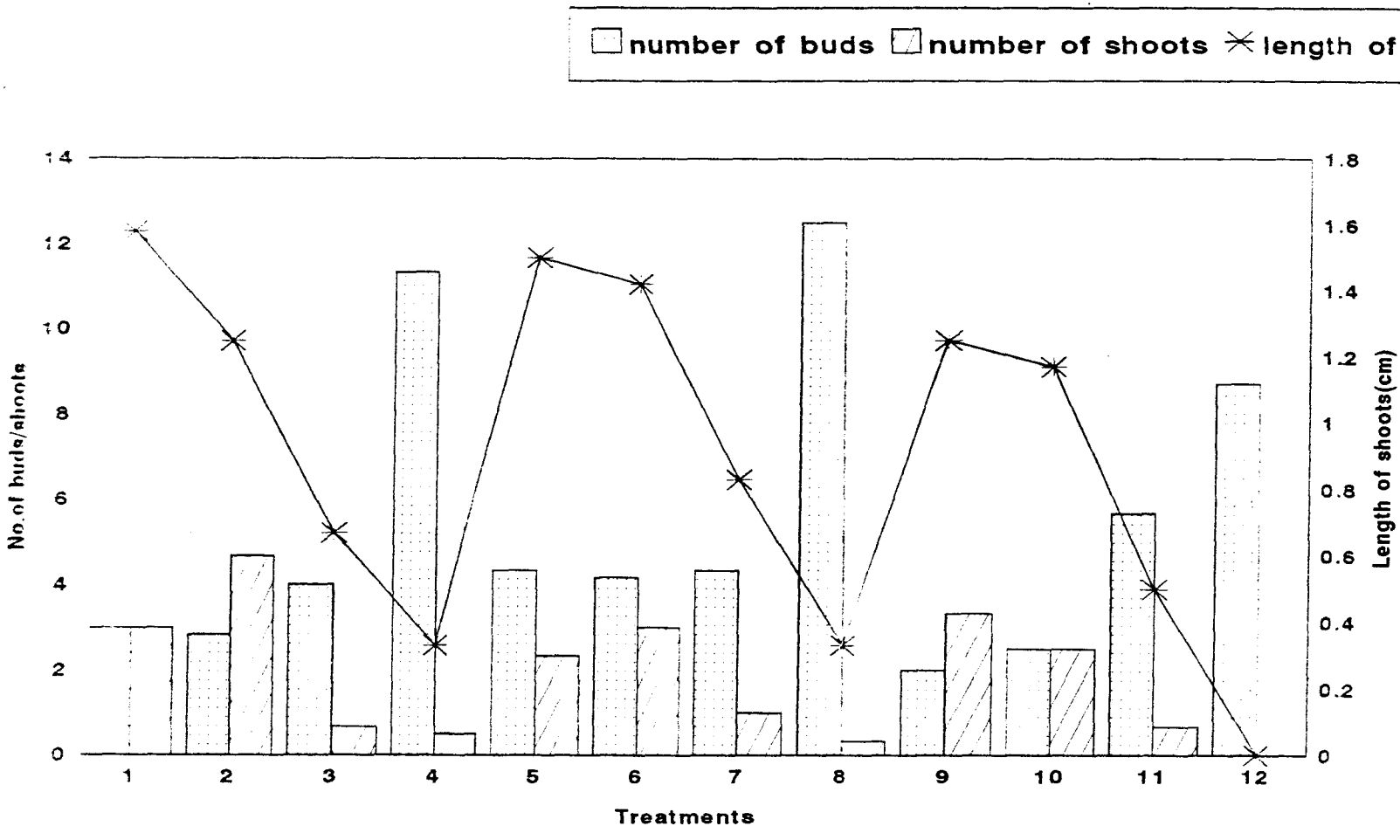
Table 6b. Effect of media and BA on development and elongation of shoots of *T. zeylanicus*

Treatment No.	Media	Growth regulator	Concentration (mg l^{-1})	No. of shoots/ tube	Average length of shoots (cm)
1	SH	BA	0.5	3.00 (1.794)	1.58 (1.438)
2	SH	BA	1.0	4.67 (2.170)	1.25 (1.319)
3	SH	BA	2.5	0.67 (1.025)	0.67 (1.029)
4	SH	BA	5.0	0.50 (0.939)	0.33 (0.880)
5	WPM	BA	0.5	2.33 (1.642)	1.50 (1.407)
6	WPM	BA	1.0	3.00 (1.775)	1.42 (1.379)
7	WPM	BA	2.5	1.00 (1.171)	0.83 (1.115)
8	WPM	BA	5.0	0.33 (0.880)	0.33 (0.880)
9	MS	BA	0.5	3.33 (1.937)	1.25 (1.319)
10	MS	BA	1.0	2.50 (1.654)	1.17 (1.288)
11	MS	BA	2.5	0.67 (1.025)	0.50 (0.966)
CD				0.57	0.255
SEm±				0.20	0.09

* Values in paranthesis represent $\sqrt{x} + ½$ transformed ones

** In MS medium BA 5 mg l^{-1} was also tried but no shoot development was observed

Fig.3. Effect of media and BA on prolieration of buds of *T. zeylanicus*



4.3.2.2 Effect of additives

The data on the effect of additives on proliferation of buds and shoot development are summarised in Table 6c and 6d. Out of the four additives used at two concentrations each along with BA, adenine sulfate was found to give maximum results, that too at lower concentrations of BA (0.5-2.5 mg l^{-1}) (Plate 6). Maximum number of buds (36.33) were observed in T_2 (SH + BA 1.0 mg l^{-1} + adenine sulfate 50 mg l^{-1}) followed by T_1 (SH + BA 0.5 mg l^{-1} + 100 mg l^{-1}) (21.5). This was comparable with T_3 (BA 2.5 mg l^{-1} + adenine sulfate 50 mg l^{-1}) (20.67), T_4 (BA 5.0 + adenine sulfate 50 mg l^{-1}) (16.17) and T_7 (BA 2.5 + adenine sulfate 100 mg l^{-1}) (16.33).

The highest number of shoots (13.5) was observed in T_5 (BA 0.5 mg l^{-1} + adenine sulfate 100 mg l^{-1}). Treatments T_1 to T_4 (BA 0.5-5.0 mg l^{-1} + adenine sulfate 50 mg l^{-1}) and T_7 (BA 2.5 + adenine sulfate 100 mg l^{-1}) were on par with T_5 with respect to the number of shoots. However, length of shoots was maximum (2.7) in T_{18} (BA 2.5 + coconut water 20%) which was on par with T_5 (BA 0.5 + adenine sulfate 100 mg l^{-1}) (2.38) and T_1 (BA 0.5 + adenine sulfate 50 mg l^{-1}) (2.08) (Fig.4).

Minimum number of buds (3.67) was observed in cultures receiving T_{27} (BA 2.5 + coconut water 10%), T_{13} (BA 0.5 + yeast extract 500 mg l^{-1}), T_{19} (BA 0.5 + CH 2.5), T_{24} (BA 5.0 mg l^{-1} + CW 20%) and T_{28} (BA 1.0 + CW 10%), were comparable with respect to the number of buds formed.

The number of buds induced, shoots arising and the length of the shoots were found to be more when adenine sulfate was used along with BA than when BA

Table 6c Effect of BA and different additives on proliferation of buds of *T. zeylanicus*

Treatment No.	Treatment					No. of shoot buds
	Media	Growth regulator	Concentration (mg l^{-1})	Additive	Concentration (mg l^{-1})	
1	2	3	4	5	6	7
1	SH	BA	0.5	Adenine sulfate	50	21.50 (4.48)
2	SH	BA	1.0	Adenine sulfate	50	36.33 (5.98)
3	SH	BA	2.5	Adenine sulfate	50	20.67 (4.53)
4	SH	BA	5.0	Adenine sulfate	50	16.17 (3.93)
5	SH	BA	0.5	Adenine sulfate	100	6.67 (2.65)
6	SH	BA	1.0	Adenine sulfate	100	15.00 (3.82)
7	SH	BA	2.5	Adenine sulfate	100	16.33 (4.02)
8	SH	BA	5.0	Adenine sulfate	100	7.83 (2.80)
9	SH	BA	0.5	Yeast extract	100	5.17 (2.33)
10	SH	BA	1.0	Yeast extract	100	8.00 (2.80)
11	SH	BA	2.5	Yeast extract	100	4.67 (2.24)

Contd

Table 6c. Continued

1	2	3	4	5	6	7
12	SH	BA	5.0	Yeast extract	100	4.83 (2.27)
13	SH	BA	0.5	Yeast extract	500	4.33 (2.16)
14	SH	BA	1.0	Yeast extract	500	4.50 (2.20)
15	SH	BA	2.5	Yeast extract	500	4.67 (2.22)
16	SH	BA	5.0	Yeast extract	500	2.00 (1.56)
17	SH	BA	0.5	Casein hydrolysate	100	5.83 (2.48)
18	SH	BA	1.0	Casein hydrolysate	100	16.33 (3.98)
19	SH	BA	2.5	Casein hydrolysate	100	4.50 (2.21)
20	SH	BA	5.0	Casein hydrolysate	100	4.66 (2.11)
21	SH	BA	0.5	Casein hydrolysate	500	10.17 (3.04)
22	SH	BA	1.0	Casein hydrolysate	500	12.50 (3.67)
23	SH	BA	2.5	Casein hydrolysate	500	6.67 (2.63)
24	SH	BA	5.0	Casein hydrolysate	500	4.17 (1.99)

Contd.

Table 6c Continued

1	2	3	4	5	6	7
25	SH	BA	0.5	Coconut water	10%	6.17 (2.56)
26	SH	BA	1.0	Coconut water	10%	6.83 (2.69)
27	SH	BA	2.5	Coconut water	10%	3.67 (2.02)
28	SH	BA	5.0	Coconut water	10%	4.33 (2.15)
29	SH	BA	0.5	Coconut water	20%	9.50 (2.07)
30	SH	BA	1.0	Coconut water	20%	7.33 (2.69)
31	SH	BA	2.5	Coconut water	20%	10.33 (3.18)
32	SH	BA	5.0	Coconut water	20%	10.67 (3.29)
CD						0.91
SEm±						0.33

* Values in paranthesis represent $\sqrt{x} + ½$ transformed values

Table 6d. Effect of BA and different additives on shoot development on elongation of *T. zeylanicus*

Treat-ment No.	Treatment					No. of shoots	Length of shoots (cm)
	Media	Growth regulator	Concent-ration (mg l^{-1})	Additive	Concent-ration (mg l^{-1})		
1	2	3	4	5	6	7	8
1	SH	BA	0.5	Adenine sulfate	50	11.50 (3.29)	2.08 (1.60)
2	SH	BA	1.0	Adenine sulfate	50	11.17 (3.50	1.50 (1.41)
3	SH	BA	2.5	Adenine sulfate	50	3.50 (1.86)	1.27 (1.32)
4	SH	BA	5.0	Adenine sulfate	50	0.67 (1.03)	0.53 (0.98)
5	SH	BA	0.5	Adenine sulfate	100	13.50 (3.65)	2.38 (1.69)
6	SH	BA	1.0	Adenine sulfate	100	12.83 (3.57)	1.33 (1.34)
7	SH	BA	2.5	Adenine sulfate	100	10.00 (3.15)	1.37 (1.35)
8	SH	BA	5.0	Adenine sulfate	100	1.83 (1.47)	1.00 (1.23)
9	SH	BA	0.5	Yeast extract	100	3.17 (1.81)	1.05 (1.24)
10	SH	BA	1.0	Yeast extract	100	4.00 (1.95)	1.03 (1.24)
11	SH	BA	2.5	Yeast extract	100	1.50 (1.39)	1.00 (1.23)
12	SH	BA	5.0	Yeast extract	100	2.50 (1.69)	1.00 (1.23)

Contd.

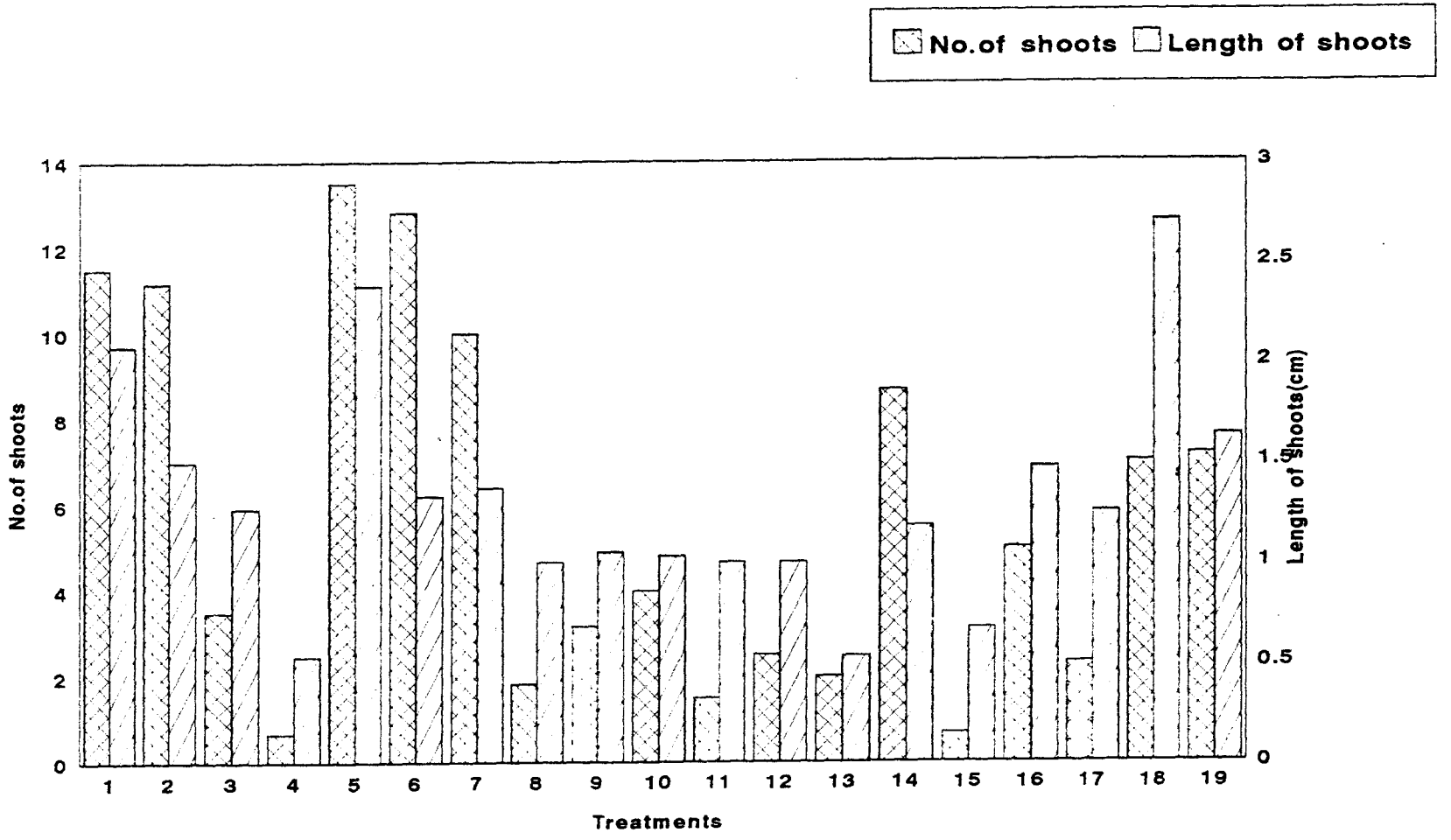
Table 6d. Continued

1	2	3	4	5	6	7	8
12	SH	BA	0.5	Caseine hydrolysate	100	2.00 (1.37)	0.53 (0.98)
13	SH	BA	1.0	Caseine hydrolysate	100	8.67 (2.85)	1.18 (1.27)
14	SH	BA	0.5	Coconut water	10%	0.67 (1.03)	0.67 (1.03)
15	SH	BA	0.5	Coconut water	20%	5.00 (2.28)	1.47 (1.39)
17	SH	BA	1.0	Coconut water	20%	2.33 (1.57)	1.25 (1.31)
18	SH	BA	2.5	Coconut water	20%	7.00 (2.64)	2.70 (1.77)
19	SH	BA	5.0	Coconut water	20%	7.17 (2.70)	1.63 (1.48)
CD						0.87	0.22
SEm±						0.31	0.08

* Values in paranthesis represent $\sqrt{x+½}$ transformed ones

Plate 6. Proliferation of buds and shoot development in SH + BA 0.5 mg l^{-1} + adenine sulfate 100 mg l^{-1}

Fig.4. Effect of BA and different additives on shoot development and elongation of *T.zeylanicus*



was used alone. Number of buds induced, shoots arising and length of the shoots were 21.5, 11.5, 2.08 cm and 3, 3, 1.58 cm for SH + BA 0.5 + adenine sulfate 50 mg l^{-1} and SH + BA 0.5, respectively.

Yeast extract and casein hydrolysate at higher concentrations (500 mg l^{-1}) were not found to promote shoot development. Callus development at the base of the bud clump was also noticed in cultures developing in media containing casein hydrolysate.

Established buds were transferred to media containing GA at two levels 2.0 mg l^{-1} and 5.0 mg l^{-1}. But proliferation and elongation of buds were not observed with these treatments. Developing buds were also transferred to MS and SH media without any growth regulators but the buds remained as such and did not show any further proliferation and later on dried up.

From the above experiments it can be seen that SH and WPM showed comparable results in terms of the number of buds developed when BA 0.5-5.0 mg l^{-1} was added to the media (2.83-11.33 and 4.17-12.5 buds in SH and WPM respectively). However, the number of shoots was more in SH medium (4.67) compared to WPM (3.00 at BA 1.0 mg l^{-1}).

Addition of organic supplements along with BA, especially adenine sulfate resulted in better proliferation compared to BA alone. Maximum number of buds (36.33) was observed in SH medium supplemented with BA 1.0 mg l^{-1} and adenine sulfate 50 mg l^{-1}. Number of shoots (13.5) and length of shoots (2.38) was maximum when adenine sulfate 100 mg l^{-1} was used along with BA 0.5 mg l^{-1}. At higher concentration of yeast extract (500 mg l^{-1}) and casein hydrolysate (500 mg l^{-1}) shoot development was nil.

4.4 Indirect morphogenesis

4.4.1 Induction of callus

The data on the experiments on induction of callus in *Trichopus zeylanicus* are presented in Table 7a, 7b and 7c.

4.4.1.1 Effect of explant and explant maturity on callus induction

Leaf bits with mid rib, leaf base and petiole bits were used as explants for callus induction. Callus initiation was observed in 40-60 per cent of tender explants whereas with the semimature and mature explants, callus initiation was nil. Of the different explants tried, callus formation was maximum in the case of leaf bits with mid rib portion (60%) which was on par with that of petioles (58.33%). Only 40 per cent of leaf bases showed callus initiation (Plates 7a, 7b).

4.4.1.2 Effect of media and growth regulators

Percentage of cultures showing callus induction was very low (0-33.33%) when 2,4-D and NAA alone were added to the media irrespective of the media used. At 2,4-D 5.0 mg l^{-1} 33.33 per cent of the cultures were found to initiate callus in WPM. Callus formed was white or cream in colour and hard in media supplemented with 2,4-D but the callus growth was very poor and dried on subsequent subculturing. Response to 2,4-D, eventhough low, was observed in MS and WPM. 0-25 per cent cultures initiated callus in MS + 2,4-D and 16.66-33.33 per cent in WPM supplemented with 2,4-D. In 1/2 MS and SH media containing 2,4-D, purple leaf bits turned green and showed some enlargement, without any callus initiation.

Table 7a. Effect of auxins and cytokinins on callus induction of *T. zeylanicus*

Treatment No.	Media	Growth regulator	Concent-ration (mg l^{-1})	% culture initiating callus	Nature of callus
1	2	3	4	5	5
1	MS	2,4-D	0.5	0.00	
2	MS	2,4-D	1.0	14.29	White, powdery
3	MS	2,4-D	2.5	8.33	White, powdery
4	MS	2,4-D	5.0	25.00	White, hard, granular
5	MS	NAA	0.5	12.50	Creamy white, cream roots developed
6	MS	NAA	1.0	8.33	Creamy white, cream roots developed
7	MS	NAA	2.5	0.00	
8	MS	NAA	5.0	0.00	
9	WPM	2,4-D	0.5	16.66	Cream hard callus, granular
10	WPM	2,4-D	1.0	20.00	Cream hard callus, granular
11	WPM	2,4-D	2.5	33.33	Whitish cream callus
12	WPM	2,4-D	5.0	33.33	Whitish cream callus
13	WPM	NAA	0.5	12.50	Whitish cream callus; slender roots developed
14	WPM	NAA	1.0	20.00	Whitish cream callus; slender roots developed
15	WPM	NAA	2.5	20.00	Whitish cream callus; slender roots developed
16	WPM	NAA	5.0	25.00	Whitish cream callus; slender roots developed

Contd.

Table 7a. Continued

1	2	3	4	5	6
17	MS	BA+2,4-D	0.5+0.5	30.77	Creamy yellow, granular, hard callus
18	MS	BA+2,4-D	1.0+1.0	63.16	Creamy yellow, granular, hard callus
19	MS	BA+2,4-D	2.5+2.5	72.22	Creamy yellow and white, granular
20	MS	BA+2,4-D	5.0+5.0	54.17	Yellow, granular, hard
21	MS	BA+NAA	0.5+0.5	75.00	Creamy and white hard nodular callus
22	MS	BA+NAA	1.0+1.0	58.57	Creamy and white hard nodular callus
23	MS	BA+NAA	0.5+0.5	35.72	Cream nodular callus
24	MS	BA+NAA	5.0+5.0	33.33	Creamy yellow callus
25	MS	BA+KIN + 2,4-D	0.5+0.5 +0.5	41.66	Cream, hard callus
26	MS	BA+KIN + 2,4-D	5.0+5.0 +5.0	55.00	Cream, hard callus
27	WPM	BA+2,4-D	0.5+0.5	15.38	Yellowish cream granular callus
28	WPM	BA+2,4-D	1.0+1.0	80.00	Yellowish cream granular callus
29	WPM	BA+2,4-D	2.5+2.5	60.00	Yellowish cream granular callus
30	WPM	BA+2,4-D	5.0+5.0	25.00	Yellow, granular hard callus
31	WPM	BA+NAA	0.5+0.5	57.14	Creamy white hard nodular callus

Contd.

Table 7a. Continued

1	2	3	4	5	6
32	WPM	BA+NAA	1.0+1.0	75.00	Yellowish cream granular callus
33	WPM	BA+NAA	2.5+2.5	66.66	Creamy yellow granular hard callus
34	WPM	BA+NAA	5.0+5.0	30.80	Creamy yellow granular hard callus
35	WPM	BA+KIN + 2,4-D	0.5+0.5 +0.5	50.00	Cream granular hard callus
36	WPM	BA+KIN + 2,4-D	5.0+5.0 +5.0	66.66	Cream granular hard callus
37	SH	BA+2,4-D	0.5+0.5	30.77	Cream hard callus
38	SH	BA+2,4-D	1.0+1.0	53.85	Cream hard callus
39	SH	BA+2,4-D	2.5+2.5	44.44	Cream granular hard callus
40	SH	BA+2,4-D	5.0+5.0	30.00	Cream granular hard callus
41	SH	BA+NAA	0.5+0.5	40.00	Yellow nodular hard callus
42	SH	BA+NAA	1.0+1.0	50.00	Yellow nodular hard callus
43	SH	BA+NAA	2.5+2.5	57.14	Yellow granular hard callus
44	SH	BA+NAA	5.0+5.0	66.66	Yellow granular hard callus
45	SH	BA+KIN + 2,4-D	0.5+0.5 +0.5	30.00	Cream granular hard callus
46	SH	BA+KIN + 2,4-D	5.0+5.0 +5.0	44.44	Cream granular hard callus
47	½ MS	2,4-D+BA	0.5+0.5	40.00	Creamy white granular hard callus
48	½ MS	2,4-D+BA	1.0+1.0	40.00	Creamy white granular hard callus

Contd.

Table 7a. Continued

1	2	3	4	5	6
49	½ MS	2,4-D+BA	2.5+2.5	57.14	Cream granular hard callus
50	½ MS	2,4-D+BA	5.0+5.0	50.00	Yellowish cream granular hard callus
51	½ MS	BA+NAA	0.5+0.5	75.00	White and cream granular and nodular callus
52	½ MS	BA+NAA	1.0+1.0	67.14	White and cream granular and nodular callus
53	½ MS	BA+NAA	2.5+2.5	67.14	White and cream granular and nodular callus
54	½ MS	BA+NAA	5.0+5.0	50.00	Creamy yellow, hard granular callus
55	½ MS	BA+KIN 2,4-D	0.5+0.5 +0.5	44.44	Creamy yellow, hard granular callus
56	½ MS	BA+KIN 2,4-D	5.0+5.0 +5.0	54.54	Creamy yellow, hard granular callus

* 2,4-D and NAA were also tried in ½ MS and SH but there was no response

** MS, WPM, SH and ½ MS without any growth regulator did not show response

Table 7b. Effect of combination of auxins and cytokinins on callus induction and growth of *T. zeylanicus*

Treatment No.	Media	Growth regulator	Concentration (mg l^{-1})	Days for callus induction	Callus index*
1	2	3	4	5	6
1	MS	BA+2,4-D	0.5+0.5	36.00 (5.998)	37.75 (6.14)
2	MS	BA+2,4-D	1.0+1.0	41.00 (6.49)	99.71 (9.01)
3	MS	BA+2,4-D	2.5+2.5	57.75 (7.57)	126.35 (11.01)
4	MS	BA+2,4-D	5.0+5.0	49.00 (6.98)	243.75 (15.50)
5	MS	BA+NAA	0.5+0.5	51.25 (7.14)	150.00 (11.90)
6	MS	BA+NAA	1.0+1.0	52.25 (2.23)	110.00 (10.45)
7	MS	BA+NAA	2.5+2.5	44.50 (6.66)	147.74 (12.05)
8	MS	BA+NAA	5.0+5.0	41.00 (6.39)	150.00 (12.16)
9	MS	BA+KIN +2,4-D	0.5+0.5 +0.5	52.00 (7.09)	83.16 (9.07)
10	MS	BA+KIN +2,4-D	5.0+5.0 +5.0	73.50 (8.50)	55.50 (7.41)
11	WPM	BA+2,4-D	0.5+0.5	72.50 (8.50)	57.14 (7.56)
12	WPM	BA+2,4-D	1.0+1.0	52.00 (7.09)	160.00 (12.65)

Contd.

Table 7b. Continued

1	2	3	4	5	6
13	WPM	BA+2,4-D	2.5+2.5	51.50 (7.14)	166.66 (12.91)
14	WPM	BA+2,4-D	5.0+5.0	55.00 (7.37)	47.92 (6.91)
15	WPM	BA+NAA	0.5+0.5	51.25 (7.14)	199.99 (14.11)
16	WPM	BA+NAA	1.0+1.0	43.00 (6.54)	225.00 (15.00)
17	WPM	BA+NAA	2.5+2.5	54.75 (7.40)	100.00 (9.86)
18	WPM	BA+NAA	5.0+5.0	56.75 (7.52)	114.06 (10.64)
19	WPM	BA+KIN +2,4-D	0.5+0.5 +0.5	75.50 (8.66)	135.00 (11.57)
20	WPM	BA+KIN +2,4-D	5.0+5.0 +5.0	53.25 (7.29)	155.36 (12.31)
21	SH	BA+2,4-D	0.5+0.5	72.00 (8.48)	64.68 (7.92)
22	SH	BA+2,4-D	1.0+1.0	60.75 (7.78)	96.28 (9.76)
23	SH	BA+2,4-D	2.5+2.5	54.75 (7.40)	33.33 (6.70)
24	SH	BA+2,4-D	5.0+5.0	56.75 (7.52)	46.33 (6.80)
25	SH	BA+NAA	0.5+0.5	69.75 (8.35)	82.50 (8.86)

Contd.

Table 7b. Continued

1	2	3	4	5	6
26	SH	BA+NAA	1.0+1.0	49.00 (7.00)	120.00 (10.88)
27	SH	BA+NAA	2.5+2.5	51.50 (7.17)	547.43 (6.86)
28	SH	BA+NAA	5.0+5.0	67.50 (8.21)	60.00 (7.73)
29	SH	BA+KIN +2,4-D	0.5+0.5 +0.5	69.00 (8.30)	100.00 (9.66)
30	SH	BA+KIN +2,4-D	5.0+5.0 +5.0	71.25 (8.40)	91.67 (9.44)
31	½ MS	BA+2,4-D	0.5+0.5	48.50 (6.96)	66.52 (8.14)
32	½ MS	BA+2,4-D	1.0+1.0	51.00 (7.14)	46.00 (6.77)
33	½ MS	BA+2,4-D	2.5+2.5	50.75 (7.12)	53.66 (7.31)
34	½ MS	BA+2,4-D	5.0+5.0	54.00 (7.35)	72.00 (8.34)
35	½ MS	BA+NAA	0.5+0.5	43.50 (6.57)	137.44 (11.47)
36	½ MS	BA+NAA	1.0+1.0	46.00 (6.78)	100.00 (9.73)
37	½ MS	BA+NAA	2.5+2.5	47.50 (6.89)	85.71 (8.94)
38	½ MS	BA+NAA	5.0+5.0	59.74 (7.73)	71.43 (8.34)

Contd.

Table 7b. Continued

1	2	3	4	5	6
39	½ MS	BA+KIN +2,4-D	0.5+0.5 +0.5	53.50 (7.30)	50.00 (6.97)
40	½ MS	BA+KIN +2,4-D	5.0+5.0 +5.0	54.25 (7.33)	47.92 (6.91)
CD				0.82	2.22
SEm±				0.30	0.81

* Callus index was calculated 4 months after initial culture of explant

** Values in paranthesis represent √x transformed ones

Table 7c. Effect of different concentrations of auxins and cytokinins on callus induction of *T. zeylanicus* under dark

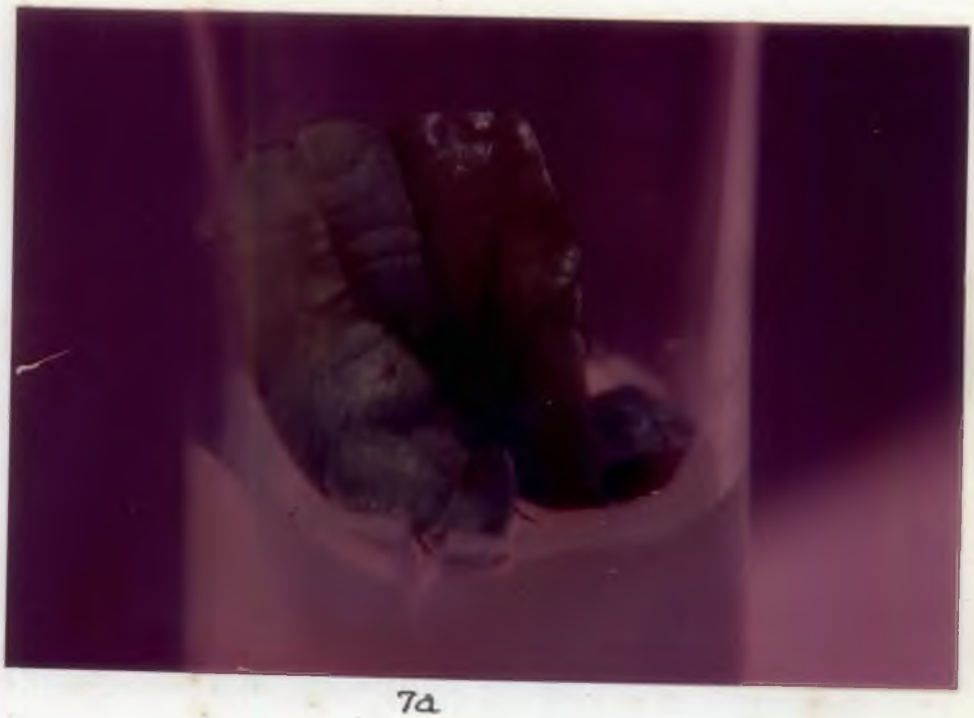
Treatment No.	Treatment: Media + growth regulator	Treatment: Concentration (mg l^{-1})	Days to initiate callus	Callus index*	Morphology of callus
1	MS+BA+ 2,4-D	0.5+0.5	40.00 (6.323)	75.00 (83.536)	White and pale cream
2	MS+BA+ 2,4-D	1.0+1.0	43.50 (6.594)	113.63 (10.606)	Granular hard callus
3	MS+BA+ 2,4-D	2.5+2.5	58.00 (7.609)	150.00 (2.185)	Granular hard callus
4	MS+BA+ 2,4-D	5.0+5.0	52.00 (7.206)	200.00 (14.071)	Granular hard callus
5	MS+BA+ NAA	0.5+0.5	48.50 (6.964)	125.00 (11.124)	Pale, cream, nodular hard callus
6	MS+BA+ NAA	1.0+1.0	50.00 (7.068)	166.66 (12.844)	Pale, cream, nodular hard callus
7	MS+BA+ NAA	2.5+2.5	48.50 (6.960)	100.00 (10.000)	Pale, cream, nodular hard callus
8	MS+BA+ NAA	5.0+5.0	49.50 (7.035)	83.36 (9.082)	Pale, cream, nodular hard callus
9	MS+BA+ KIN + 2,4-D	0.5+0.5 +0.5	50.50 (7.104)	83.36 (9.082)	Pale, cream, nodular hard callus
10	MS+BA+ KIN+ 2,4-D	2.5+2.5 +2.5	52.00 (7.211)	55.00 (8.536)	Pale, cream, nodular hard callus
11	MS+BA+ KIN+ 2,4-D	5.0+5.0 +5.0	73.00 (8.528)	50.00 (7.071)	Pale, cream, nodular hard callus
CD			0.75	3.45	
SEm±			0.24	1.11	

* Callus index was calculated four months after initial culture

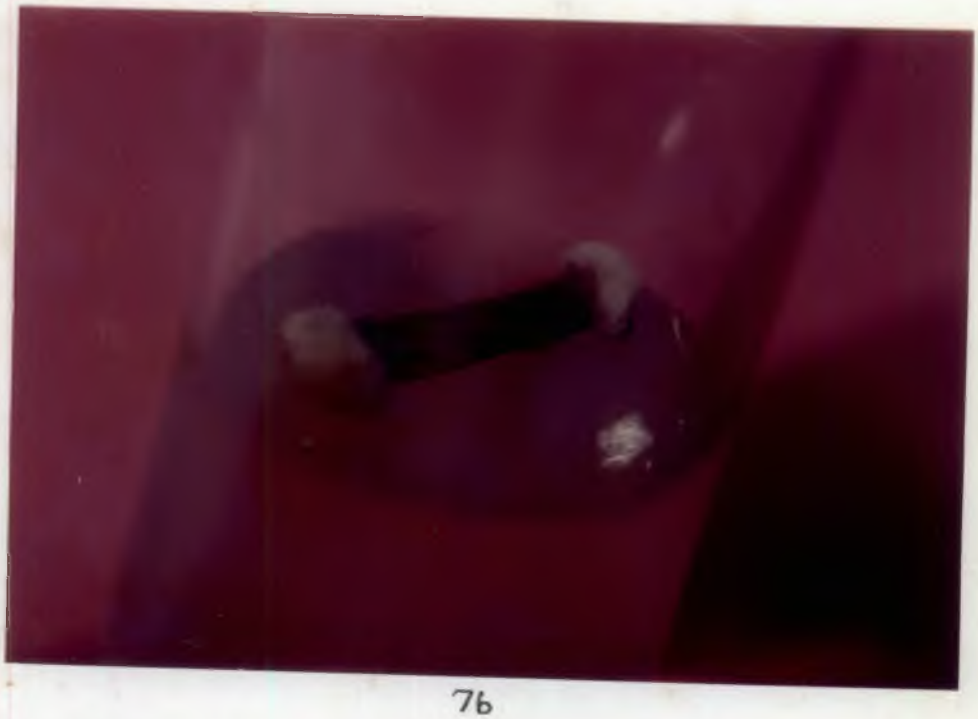
** Values in paranthesis represent $\sqrt{x}$ transformed ones

Plate 7a. Enlargement and greening of tender leaf segments in media

Plate 7b. Callus initiation at cut edges of petiole



7a



7b

Similar response was observed in media supplemented with NAA also. When NAA was supplemented in different media, creamy white to creamish coloured hard and granular callus developed. Percentage of cultures initiating callus and callus growth was low (0-12.5% in MS + NAA and 12.5-25% in WPM + NAA). However, cultures in which callus initiation occurred, thin, slender, cream coloured roots started developing within a month after callus initiation (Plate 8). Callus initiation was maximum (25%) in WPM at 5.0 mg l^{-1} NAA.

Callus initiation was higher in cultures receiving combinations of auxins with cytokinins irrespective of the media used. Maximum callus initiation (85%) was observed in T_{28} (WPM + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} 2,4-D) followed by T_{32} (WPM + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} NAA), T_{51} (1/2 MS + 0.5 mg l^{-1} BA + 0.5 mg l^{-1} NAA) and T_{21} (MS + 0.5 mg l^{-1} BA + 0.5 mg l^{-1} BA) (75%). At low to medium concentrations (0.5-2.5 mg l^{-1}) of BA, along with 2,4-D and NAA, maximum callus initiation (15.38-80%) was observed in all the media tried. Addition of KIN along with BA and 2,4-D was found to show similar or better response with respect to percentage of cultures initiating callus. In MS medium, percentage of cultures initiating callus was 30.7 and 54.17 when BA 0.5 + 2,4-D 0.5 mg l^{-1} and BA 5.0 + 2,4-D 5.0 mg l^{-1} where added, whereas for BA 0.5 + 2,4-D 0.5 + KIN 0.5 mg l^{-1} and BA 5.0 + 2,4-D 5.0 + KIN 5.0 mg l^{-1}, it was 41.66 and 55 per cent respectively.

Callus initiation was very slow and took a minimum of 36 days in T_1 (MS + BA 0.5 + 2,4-D 0.5). Treatment T_1 was on par with T_2 (MS + BA 1.0 + 2,4-D), T_7 (MS + BA 2.5 + NAA 2.5), T_8 (MS + BA 5.0 + NAA 5.0), T_{15}

Plate 8. Root initiation from callus in media supplemented with NAA

(WPM + BA 0.5 + NAA 0.5), T_{31} (1/2 MS + BA 0.5 + 2,4-D 0.5) and T_{32} (1/2 MS + BA 1.0 + 2,4-D 1.0). Maximum number of days for initiation of callus (75.5 days) was observed in T_{19} (WPM + BA 0.5 + KIN 0.5 + 2,4-D 0.5).

Since callus growth was slow, subculturing to same media was done at an interval of 25-30 days. Nature of callus was different in cultures receiving different growth regulators (granular, hard in medium supplemented with BA, 2,4-D and nodular in media with BA and NAA) and at different concentrations (nodular callus at low 0.5-1.0 mg l^{-1} of BA and NAA and granular at 2.5-5.0 mg l^{-1} BA + 2.5-5.0 mg l^{-1} NAA) irrespective of the media (Plates 9a, 9b). When BA and NAA at lower concentrations were added to different media, the callus formed was nodular and in subsequent subcultures to the same media, buds started developing.

Direct organogenesis was observed in 1/2 MS medium supplemented with BA 0.5 mg l^{-1} + NAA 0.5 mg l^{-1} on the cut edges of leaf explants in 50-60 per cent of cultures along with callus formation on the other side of the same leaf bit (Plate 10).

Callus index was maximum (243.75) in T_4 (MS + BA 5.0 + 2,4-D 5.0). This was on par with T_{16} (WPM + BA 0.5 + NAA 0.5) and T_5 (WPM + BA 0.5 + NAA 0.5). Treatments T_{12} (WPM + BA 1.0 + 2,4-D 1.0), T_{13} (WPM + 2.5 BA + 2.5 2,4-D), T_{19} (WPM + BA 0.5 + KIN 0.5 + 2,4-D 0.5), T_{20} (WPM + KIN 5.0 + BA 5.0 + 2,4-D 5.0), T_3 (MS + BA 2.5 + 2,4-D 2.5), T_5 (MS + 0.5 BA + 0.5 NAA), T_7 (MS + BA 2.5 + NAA 2.5), T_8 (MS + BA 5.0 + NAA 5.0) and T_{35} (1/2 MS + BA 0.5 + NAA 0.5) had comparable and moderate callus growth. Minimum callus index (37.75) was observed in T_1 (MS + BA 0.5 + 2,4-D 0.5).

Plate 9a. Proliferation of callus in media supplemented with 2,4-D and BA

Plate 9b. Proliferation of callus in media supplemented with NAA and BA

9a

9b

Plate 10. Direct organogenesis from leaf segments in ½ MS + NAA 0.5 mg l^{-1} + BA 0.5 mg l^{-1}

4.4.1.3 Effect of light

Cultures incubated under light and dark showed only slight variations in callus initiation and development. In MS + BA + 2,4-D number of days for callus initiation was more under dark and took minimum 40 days for callus initiation whereas it was 36 days under light. Callus index was higher (243) under light compared to that under dark (200) at the highest concentration (5.0 mg l^{-1}) of BA and 2,4-D used, whereas at lower concentrations of BA + 2,4-D (0.5-2.5) callus index was higher under dark (75-150).

Minimum callus index (50-83.36) was observed when KIN was added along with BA and 2,4-D. T_{11} (MS + BA 5.0 + KIN 5.0 + 2,4-D 5.0) took maximum number of days (73) for initiation of callus under dark. Callus developed under dark had lighter colour compared to that induced under light.

Proliferation of callus in liquid medium was also tried but due to the hard nature of callus, suspension cultures were not formed.

Thus from this experiment it can be seen that initiation of callus was low (0-33.33%) when 2,4-D and NAA alone were added to the media. In combination of NAA and 2,4-D with BA and KIN, induction of callus was higher. Percentage of cultures showing callus initiation was maximum (80%) in WPM supplemented with BA 1.0 mg l^{-1} and 2,4-D 1.0 mg l^{-1}. Days for initiation of callus was minimum (36) in MS medium supplemented with 0.5 mg l^{-1} BA and 0.5 mg l^{-1} 2,4-D but with low callus index (37.75). Callus index was maximum (243.75) in MS medium supplemented with 5.0 mg l^{-1} BA and 2,4-D.

4.4.2 Regeneration

4.4.2.1 Induction of organogenesis

The results of the attempts to induce regeneration through organogenesis are summerised in Table 8a and 8b. Among the different treatments tried, maximum regeneration (100%) was observed in MS medium supplemented with BA 0.5 mg l^{-1} followed by KIN 2.5 mg l^{-1} (85%). The number of buds (10.25) and shoots (16.5) formed was also observed to be high in BA 0.5 mg l^{-1}. Days for initiation of buds (46.5) was also comparable with that of KIN 2.5 mg l^{-1} (42.00). However the length of shoots was observed to be less (1.5 cm) and the shoots formed were slender and unhealthy whereas at 2.5 mg l^{-1} concentration of KIN, the shoots formed were healthy and longer (3.25 cm). Number of buds (8.75), shoots (10.25) and days for bud initiation (42.00) in T_5 (MS + KIN 2.5) were also comparable with T_1 (MS + BA 0.5) (Plates 11a, 11b, 11c and 11d). At higher concentrations (5 mg l^{-1}) of BA eventhough buds developed (4.25), they did not develop into shoots.

Among the other cytokinins tried, 2iP was not effective in inducing indirect organogenesis in *Trichopus zeylanicus*. Eventhough greening of callus in 42-50 per cent of cultures and development of a few buds (3.5, 1.75 and 5 in 0.5, 2.5 and 5.0 mg l^{-1} 2iP respectively) were observed, shoot development was nil in all these treatments.

Combinations of BA with NAA showed moderate response with respect to percentage of regeneration (70%), number of buds (7.5), number of shoots (3.5) and shoot length (2.53) (Plate 11a). Days taken for regeneration was observed to be more (60.5) with IBA in combination with BA as also the least number of shoots (0).

Table 8a. Effect of growth regulators on regeneration of shoots from callus of *T. zeylanicus*

Treatment No.	Treatment		
	Media and growth regulator	Concentration (mg l^{-1})	Regeneration (%)
1	MS + BA	0.5	100.00
2	MS + BA	2.5	70.00
3	MS + BA	5.0	50.00
4	MS + KIN	0.5	50.00
5	MS + KIN	2.5	85.00
6	MS + KIN	5.0	80.00
7	MS + 2iP	0.5	42.86
8	MS + 2iP	2.5	50.00
9	MS + 2iP	5.0	42.86
10	MS + BA + NAA	0.5 + 0.1	70.00
11	MS + BA + IAA	0.5 + 0.1	63.63
12	MS + BA + IBA	0.5 + 0.1	45.45
13	MS + BA + Brassanolide	0.5 + 0.001	75.00
14	MS + BA + Brassanolide	0.5 + 0.0005	75.00
15	MS + BA + Brassanolide	0.5 + 0.0001	70.00
16	MS	-	60.00

Table 8b. Effect of growth regulators on regeneration of shoots from callus of *T. zeylanicus*

Treatment No.	Treatment: Media + growth regulator	Treatment: Concentration (mg l^{-1})	Days for bud initiation	No. of buds	No. of shoots	Length of shoots (cm)
1	2	3	4	5	6	7
1	MS+BA	0.5	46.50 (6.85)	10.25 (3.18)	16.50 (3.81)	1.50 (1.39)
2	MS+BA	2.5	56.25 (7.46)	9.75 (3.02)	2.50 (1.68)	1.38 (1.37)
3	MS+BA	5.0	61.75 (7.89)	4.25 (2.11)	0.00 (0.71)	0.00 (0.71)
4	MS+KIN	0.5	57.50 (7.60)	4.75 (2.20)	1.75 (1.48)	2.13 (1.61)
5	MS+KIN	2.5	42.00 (6.49)	8.75 (2.98)	10.25 (3.20)	3.25 (1.89)
6	MS+KIN	5.0	60.00 (7.77)	10.75 (3.33)	1.50 (1.40)	1.00 (1.23)
7	MS+2iP	0.5	39.75 (6.34)	3.50 (1.98)	0.00 (0.71)	0.00 (0.71)
8	MS+2iP	2.5	55.55 (7.48)	1.75 (1.48)	0.00 (0.71)	0.00 (0.71)
9	MS+2iP	5.0	65.25 (8.10)	1.75 (1.48)	0.00 (0.71)	0.00 (0.71)
10	MS+BA+NAA	0.5+0.1	32.00 (5.69)	7.50 (2.82)	3.50 (1.86)	2.53 (1.65)
11	MS+BA+IAA	0.5+0.1	50.50 (7.13)	2.25 (1.65)	1.00 (1.14)	0.875 (1.10)

Contd

Table 8b. Continued

1	2	3	4	5	6	7
12	MS+BA+IBA	0.5+0.1	60.50 (7.75)	4.50 (2.22)	0.00 (0.71)	0.00 (0.71)
13	MS+BA+ Brassinolide	0.5+0.001	30.00 (5.52)	3.75 (1.94)	1.50 (1.36)	0.50 (0.97)
14	MS+BA+ Brassinolide	0.5+0.0005	28.00 (5.32)	5.50 (2.43)	2.50 (1.53)	1.13 (1.23)
15	MS+BA+ Brassinolide	0.5+0.00001	27.25 (5.25)	6.50 (2.57)	6.50 (2.41)	2.13 (1.55)
16	MS	-	27.75 (5.31)	6.50 (2.57)	3.00 (1.61)	0.63 (1.01)
	CD		0.82	0.88	1.08	0.45
	SEm±		0.29	0.31	0.38	0.16

*Values in paranthesis represent $\sqrt{x+½}$ transformed ones

Plate 11a. Shoot bud differentiation from callus in MS + KIN 2.5 mg l^{-1}

Plate 11b. Shoot and root development from callus in MS + KIN 2.5 mg l^{-1}

11a

11b

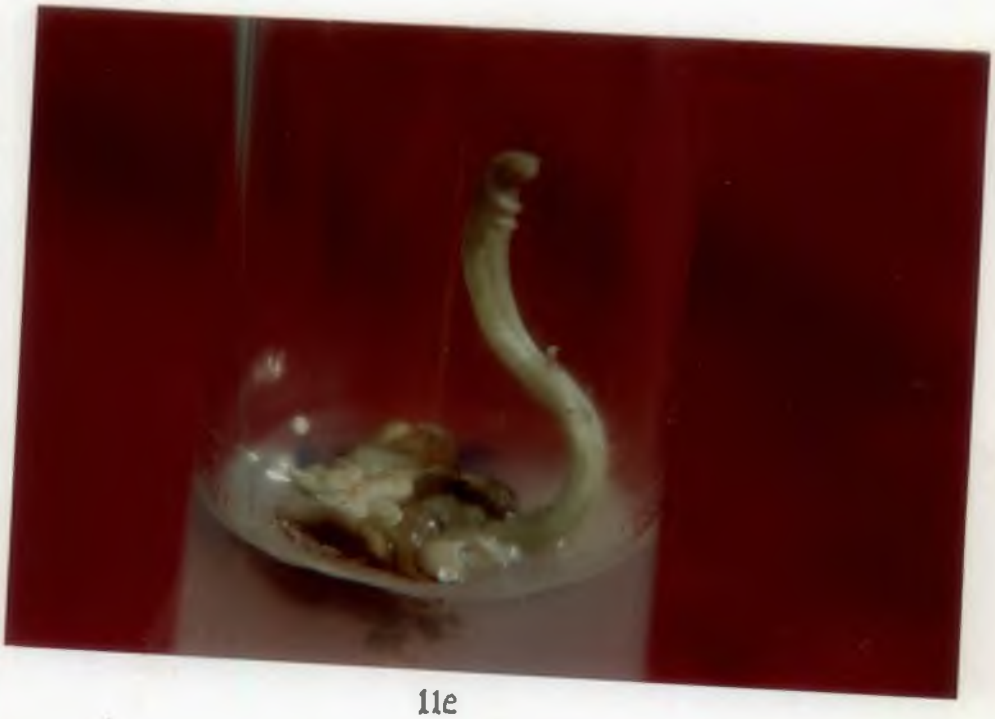
Plate 11c. Shoot development from callus in MS + KIN 2.5 mg l^{-1}

Plate 11d. Shoot development from callus in MS + BA 0.5 mg l^{-1}

11c

11d

Plate 11e. Shoot development from callus in MS + BA 0.5 mg l^{-1} + NAA 0.1 mg l^{-1}



11e

Addition of brassinolide along with BA was found to be effective in reducing the number of days for bud initiation (27-30 days). However, the number of buds and shoots were lower compared to BA alone. Number of buds (6.5), shoots (6.5) and length of shoots (2.13 cm) were higher when brassinolide was used at the lowest concentration (0.0001 mg l^{-1}).

Regeneration of shoots from callus was thus observed to be maximum (100%) in MS medium supplemented with BA 0.5 mg l^{-1} followed by KIN 2.5 mg l^{-1} (85%). However, healthy and longer (3.25 cm)shoots were formed in MS medium supplemented with KIN. In media supplemented with BA along with brassinolide, days for bud development from callus was less with minimum number of days (27.25) in MS + BA 0.5 + brassinolide 0.0001 mg l^{-1}.

4.4.2.2 Induction of embryogenesis

The results of the attempts to induce regeneration through embryogenesis are summerised in Table 9. Callus obtained in MS medium supplemented with BA 5.0 and 2,4-D 5.0 was used for further study. In T_1 (MS + 2,4-D 0.5 mg l^{-1}) and T_2 (MS + 2,4-D 1.0 mg l^{-1}) no response was observed. Slight green and white colour development was observed on yellowish cream coloured callus but no embryoid formation occurred. In T_3 and T_4, embryoid development was observed. Minimum days for embryoid formation (58 days) was observed in T_4 (MS + BA 2.5 + coconut water 10%) with numerous globular embryoids developing. In T_3 also a similar response was observed. In T_3 (MS + BA 2.5), the number of embryoids developed were less (10) whereas in T_4, numerous embryoids were found

Table 9. Effect of growth regulators on induction of embryoids in *T. zeylanicus*

Treatment No.	Media in which explants were raised originally		Subculture media		No. of embryoids produced	Days for embryo production
	Growth regulator	Concentration (mg l^{-1})	Growth regulator	Concentration (mg l^{-1})		
1	MS + 2,4-D	5.0	MS + 2,4-D	0.5	Nil	
2	MS + 2,4-D	5.0	MS + 2,4-D	0.5		
3	MS + 2,4-D	5.0	MS + BA	2.5	10 (globular)	73
4	MS + 2,4-D	5.0	MS + BA + coconut water	2.5 + 10%	Numerous	58

developing. The globular stage embryoids were transferred to basal MS medium in which they germinated (Plates 12a, 12b and 12c).

4.4.3.1 Induction of rooting

Shoots obtained by both axillary bud release and indirect organogenesis were cultured in different media for obtaining rooting of shoots (Plates 13a, 13b and 13c).

Data relating to the experiments on induction of rooting are presented in Table 10a, 10b and 10c. Of the different concentrations of growth regulators tried, T_9 (1/2 MS + brassinolide 0.001, transferred to 1.0 IBA 1 week later) proved far superior (85.7%) with respect to rooting of shoots. This was followed by T_8 (1/2 MS + brassinolide 0.0005, transferred to IBA 1 week later) with an average of 67.14 per cent and T_5 (1/2 MS + IBA 1.0 mg l^{-1}) with 60 per cent rooting. Minimum rooting (12.5%) was observed in T_2 and T_3 (1/2 MS + NAA 1.0 + IBA 1.0, 1/2 MS + NAA 1.0 + IBA 0.5) (Fig.5).

Minimum number of days for root initiation (45.67) and maximum length of roots (0.92 cm) were observed in T_9 (1/2 MS + brassinolide 0.001 mg l^{-1} transferred to IBA 1.0 mg l^{-1}). This was on par with T_8 (1/2 MS + brassinolide 0.0005 to IBA 1.0 mg l^{-1}). However, the number of roots was less compared to T_5 (IBA 1.0 mg l^{-1}) in which the number of roots was 10. Days for root initiation in T_5 (49.00) was on par with T_8 (47.67) and T_9 (45.67).

Root initiation was earlier and better in liquid medium compared to solid medium. More number (3.5) of longer (1.5 cm) roots were formed in minimum number of days (17.0) when liquid medium was used.

Plate 12a. Embryoid development and germination

Plate 12b. Embryoids detached from callus and dispersed in medium

Plate 12c. Germinating embryo showing root and shoot initiation

12a

12 b

12c

Plate 13a. Shoot cluster kept for rooting

Plate 13b. *In vitro* root formation from single shoot

Plate 13c. *In vitro* root initiation in shoot cluster

13a

13b

13c

Table 10a. Effect of growth regulators on *in vitor* root growth of *T. zeylanicus*

Treatment No	Media and growth regulators	Concentration (mg l^{-1})	% rooting
1	½ MS + NAA + IBA	0.5 + 0.5	37.50
2	½ MS + NAA + IBA	1.0 + 1.0	12.50
3	½ MS + NAA + IBA	1.0 + 0.5	12.50
4	½ MS + NAA + IBA	0.5 + 1.0	20.00
5	½ MS + IBA	1.0	60.00
6	½ MS + NAA + IBA + Brassinolide	0.5 + 0.5 + 0.0005	20.00
7	½ MS + NAA + IBA + Brassinolide	0.5 + 0.5 + 0.001	16.67
8	½ MS + Brassinolide (transferred to IBA 1 week later)	0.0005 + 1.0	67.14
9	½ MS + Brassinolide (transferred to IBA 1 week later)	0.001 + 1.0	85.70
10	½ MS	-	0.00

Table 10b. Effect of growth regulators on *in vitro* root growth in *T. zeylanicus*

Treatment No.	Media and growth regulator	Concentration (mg l^{-1})	No. of roots	Length of roots(cm)	Days to root
1	½ MS + NAA + IBA	0.5 + 0.5	4.33 (1.99)	0.67 (0.85)	114.00 (10.62)
2	½ MS + NAA + IBA	1.0 + 1.0	2.00 (1.41)	0.63 (0.79)	94.00 (9.70)
3	½ MS + NAA + IBA	1.0 + 0.5	3.00 (1.62)	0.67 (0.81)	95.50 (9.77)
4	½ MS + NAA + IBA	0.5 + 1.0	3.00 (1.62)	0.63 (0.79)	88.00 (9.38)
5	½ MS + IBA	1.0	10.00 (3.13)	0.75 (0.86)	49.00 (7.00)
6	½ MS + IBA + NAA + Brassinotide	0.5 + 0.5 + 0.0005	1.50 (1.21)	0.75 (0.86)	68.33 (8.26)
7	½ MS + IBA + NAA + Brassinotide	0.5 + 0.5 + 0.001	1.00 (1.00)	0.50 (0.71)	53.00 (7.27)
8	½ MS + Brassinolide (transferred to IBA 1 week later)	0.0005 1.0	3.00 (1.72)	0.83 (0.91)	47.67 (6.90)
9	½ MS + Brassinolide (transferred to IBA 1 week later)	0.001 1.0	2.67 (1.63)	0.92 (0.96)	45.67 (6.76)

* Values in paranthesis represent $\sqrt{x+½}$ transformed ones

** The treatments had unequal replication. So CD and Sem not shown above.

Table 10c. Effect of nature of media on *in vitro* root growth of *T. zeylanicus*

Treatment No.	Media and growth regulators	Concentration mg l^{-1}	No. of roots	Length of roots cm	Days to root
1	½ MS liquid + brassinoloide (Transferred to IBA 1 week later)	0.001 1.0	3.50	1.50	17.00
2	½ MS solid + brassinoloide (Transferred to IBA 1 week later)	0.001 1.0	2.67	0.92	45.67

Shoots were also transferred to basal ½ MS medium without any growth regulators. But no positive response with respect to rooting was observed. However, shoot clumps with an average shoot length of 0.5-1.5 cm were found to put forth new healthy normal shoots of 2-3 cm or more in length in this medium.

4.4.4 Hardening of plantlets

Rooted plantlets were transferred to pots containing sterilized sand and were transferred to hardening units. Only 10 per cent of the plants survived when plantlets were transferred from rooting media directly to pots. When the rooted plantlets were transferred first to 1/2 MS media containing reduced sucrose (1.5%), kept under increased light intensity for two weeks and thereafter transferred to pots and kept in the hardening unit, the survival was found to be better (33.33%) (Plates 14a, 14b and 14c).

Table 11. Effect of pretreatments on survival of *in vitro* raised plantlets of *T. zeylanicus*

Treatment No.	Treatment	Survival
1	Rooted plants directly to pots in hardening and	10
2	Rooted plants to media with reduced sucrose and higher light intensity followed by transfer to hardening unit	33.33

Fig.5. Effect of growth regulators on in vitro root growth of *T.zeylanicus*

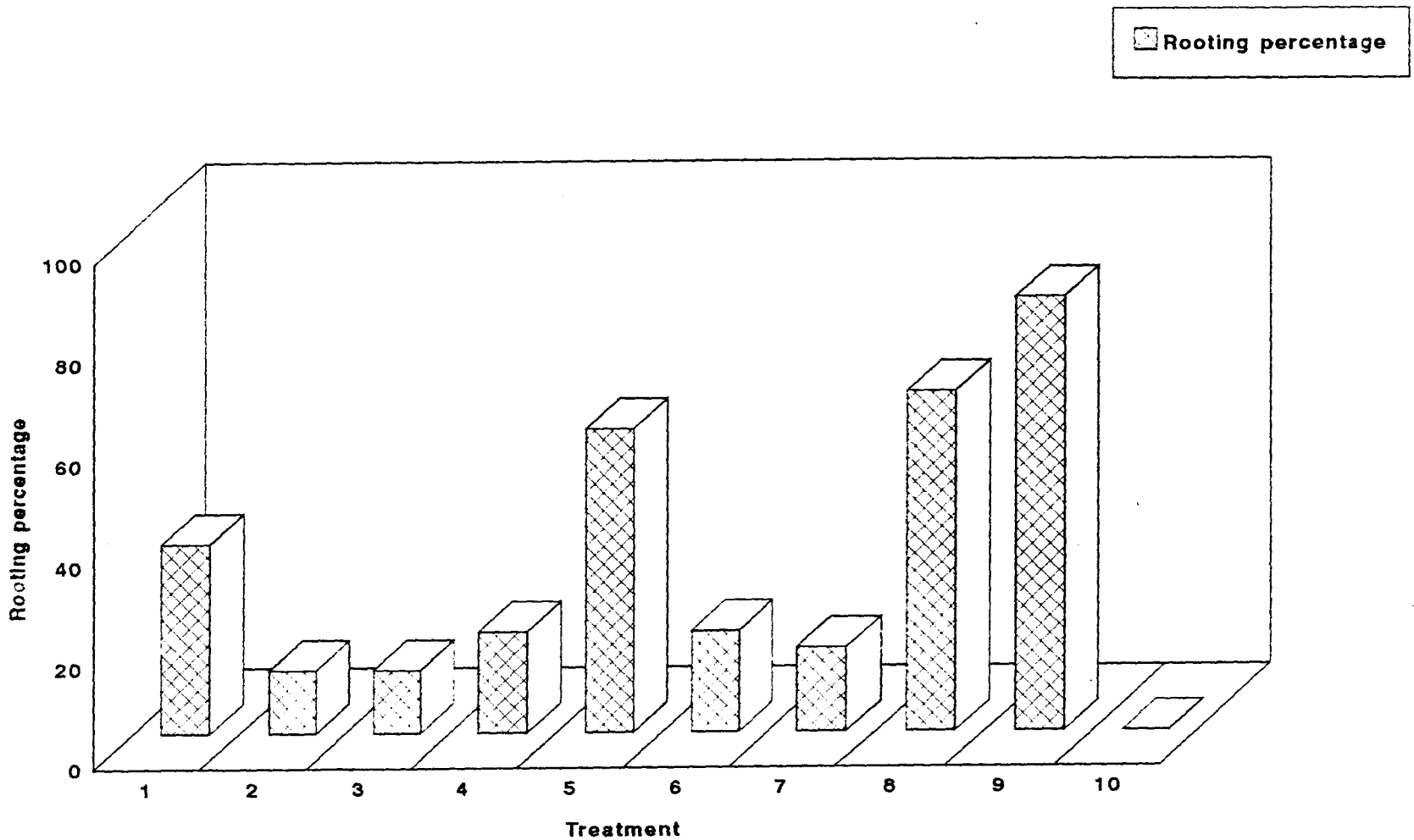


Plate 14a. Pre-treatments of plantlets in ½ MS (liquid) with reduced sucrose

Plate 14b. Rooted plantlets ready for planting out

Plate 14c. Hardened plantlet

14 a

14c

14 b

DISCUSSION

The present study on standardisation of *in vitro* techniques for rapid multiplication of *Trichopus zeylanicus* Gaertn was carried out at the Plant Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara from 1995 to 1997.

Trichopus zeylanicus Gaertn is a medicinal plant belonging to the family Trichopodaceae, the fruits of which is reported to be a safe antistress, antifatigue, apetite promoting and restorative health tonic. The availability of planting material is a major problem in the domestication of this crop. Standardisation of suitable *in vitro* technique for rapid multiplication of this plant would help in the production of large number of plants.

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 *Trichopus zeylanicus* Gaertn. 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 micro organisms which have to be removed before inoculation on to the culture medium. Though general standardisation procedures have been outlined by various workers (Dodds and Roberts, 1982; George and Sherrington, 1984), specific sterilization procedures have be to 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 various surface sterilization treatments tried, mercuric chloride 0.1 per cent for 5 minutes was found to be the best for both shoot buds and leaves and petioles giving 87.5 per cent and 86.6 per cent establishment respectively. In the case of seeds, mercuric chloride 0.1 per cent for 5 minutes and 10 minutes were equally effective. Chlorine water treatment for durations of two to ten minutes eventhough reduced the contamination percentage, most of the leaves and petiole explants were found to be bleached. Establishment of shoot buds was also very low when chlorine water treatment was given.

Mercuric chloride has been effectively used as a surface sterilant for various medicinal plants. Krishnan and Seeni (1994) reported that washing the excised shoot tips first in one per cent Labolene detergent for 5-6 minutes and in running tap water for 10 minutes followed by surface sterilization by passage through 0.1 per cent mercuric chloride for 5 minutes and again washing 6-8 times in sterile distilled water was effective for surface sterilization of *Woodfordia fruticosa*. In *Adhatoda beddomei* Sudha and Seeni (1994) reported that treatment with one per cent Labolene for 6-8 minutes followed by tap water washing and treatment with 0.1 per cent mercuric chloride for 15 minutes could produce contamination free cultures.

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 minutes was effective (Kavitha and Raju, 1995). In the present study, use of chlorine water resulted in a large number of cultures getting bleached. Bleaching of cultures by treatment with chlorine water has been reported in *Holostemma* (John, 1996).

The percentage contamination of the explants collected during different months showed variation. Survival of shoot buds was highest during January to April with maximum survival in April. For leaves and petiole, maximum survival was observed during February. Survival of explants was low during June to September. Explants collected during the drier months showed comparatively lower contamination. Vegetative buds of *Commiphora wightii* collected during April-June gave a good response whereas sprouting was reduced for explants collected during September-October as reported by Barve and Mehta (1993). Anu (1993) reported that the months from January to April was the best period for culture establishment of *Gymnema sylvestre*. Similar influence of season on establishment of explants is reported in cardamom (Reghunath, 1989) and *Holostemma* (John, 1996).

5.2 *In vitro* seed germination

In nature, seed set in *Trichopus* is poor and maturation of seeds is slow, lasting four to five months. Due to the sweet nature of seeds, they are often damaged by rodents (Krishnan *et al.*, 1995). Hence, *in vitro* germination of seeds was tried in *Trichopus*. In the present study, maximum percentage germination was observed in ½ MS and ½ MS + GA under light, but the number of days taken for germination was lower when incubated under the dark. Soaking of seeds in water or

in 100 ppm GA_3 was attempted in *Panax ginseing* to enhance seed germination as reported by Sruamsiri *et al.* (1995). Krishnan *et al.* (1995) reported germination of seeds of *Trichopus* under dark to get axenic seedlings. In the case of *Capsella* sp. light was found to have no effect on germination of seeds (Yang and Kin, 1993).

5.3 Culture establishment

5.3.1 Effect of explant and explant maturity

Shoot buds taken from young, tender, purple coloured shoots were found to be the most responsive with respect to establishment and growth whereas buds from mature shoots did not establish at all. During the maturation process of tissues, several physiological changes occur which may influence the *in vitro* behaviour of explants (David, 1982). Juvenile tissues have a higher degree of morphogenic competence than older tissues. 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).

5.3.2 Effect of media and growth regulators

Maximum percentage of establishment of buds was observed in SH medium supplemented with BA. Shoots cultured in SH medium along with BA was found to remain greener and healthier for longer duration. Number of buds formed was also higher in SH medium containing BA at lowest concentration tried (0.5 mg l^{-1}). Number of days for bud burst was minimum and maximum number of buds was obtained at 0.5 to 1.0 mg l^{-1} BA. Also, better establishment of buds was observed when the media was supplemented with BA compared to 2iP and KIN. SH medium have been reported to be used in plants like adhatoda, costus etc.

Clonal propagation of *Adhatoda beddomei* was achieved through callus free axillary meristem proliferation from stem node explants of field grown plants cultured in SH medium supplemented with BA 3.0 mg l^{-1}, 2iP 0.5 mg l^{-1} and IAA 1.0 mg l^{-1} (Sudha and Seeni, 1994). In *Costus speciosus*, multiple shoots were obtained on SH medium supplemented with KIN 0.1 mg l^{-1} and IBA 0.1 mg l^{-1} (Pal and Roy, 1991). Best proliferation results in *Valeriana wallichi* Dc was obtained on MS medium containing 5.0 mg l^{-1} BA while KIN, 2iP and zeatin were less effective (Viola and Fritz, 1991). BAP was found to be superior to other growth regulators for shoot differentiation from root segments of *Aegle marmelos* and incorporation of auxins in the combination of BA and KIN stimulate shoot number and their growth (Bhati *et al.*, 1992). Krishnan *et al.* (1995) reported that in *Trichopus zeylanicus* maximum number of shoot buds was obtained with 2.0 mg l^{-1} BAP. The caulogenic response declined as the concentration of BAP increased and at 10 mg l^{-1} shoot bud formation was completely inhibited. The results in the present study agree with the above said reports in respect of the effect of BA. BA in combination with auxins has been reported to have better results compared to BA alone in medicinal plants like *Piper longum* (Bhat *et al.*, 1992), *Kielmeyera coriacea* (Arella and Puito, 1993), Costus (Pal and Roy, 1991) etc. But in *Trichopus*, addition of auxins along with BA was not found to have a better effect.

5.3.3 Effect of light

In *Trichopus* establishment of cultures was poor under dark and exposure to light was found necessary for good growth of buds. Similar results were obtained in *Passiflora edulis* by Dornellas and Vieira (1994). In *Passiflora* all the explants except cotyledonary explants needed exposure to light for shoot regeneration.

5.4 Proliferation

5.4.1 Standardisation of media and growth regulators

In the present study, it was seen that as the concentration of BA in the media increased, the number of buds also increased. SH and WPM showed similar response with respect to the number of buds developed at highest concentration (5.0 mg l^{-1}) of BA tried. However, the number of shoots and shoot length was maximum in SH medium supplemented with 0.5 to 1.0 mg l^{-1} BA. Multiple shoots in large numbers could be obtained from excised hypocotyls of young *Eucalyptus* seedlings in media containing BA alone as reported by Subbaiah and Minocha (1990). Shoot multiplication of *Gentiana kurro* was achieved on MS medium containing 8-9 μm BA and 1.1 μm NAA (Sharma *et al.*, 1993). Most suitable media for shoot formation in *Symphytum* species was MS with 0.3 mg l^{-1} BA (Harris *et al.*, 1989).

5.4.2 Effect of organic supplements

Addition of organic compounds as supplements to BA was found to be more effective for enhanced production of multiple buds and shoots. Of the different additives tried, adenine sulfate (50 to 100 mg l^{-1}) used with lower concentrations (0.5 to 1.0 mg l^{-1}) of BA gave much better results compared to other additives as well as BA alone. In media supplemented with yeast extract and casein hydrolysate at higher concentrations (500 mg l^{-1}), no shoot formation was observed. Addition of GA also did not give any positive results. Favourable effect of adenine sulfate has been reported by earlier workers. Skoog and Tsui (1948) have reported that adenine sulfate when added to the medium, often 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 sulfate and

NAA. In *Aspargus cooperi*, shoot regeneration from callus required BA, L-arginine, adenine and a low level of NAA in the medium (Ghosh and Sen, 1992). Maximum number of multiple shoots in *Asparagus cooperi* was obtained from shoot tip explants in medium containing 2.0 mg BA, 80 mg adenine sulfate and 0.2 mg NAA (Ghosh and Sen, 1994). Beneficial effect of additives such as coconut water and casein hydrolysate have been reported in many medicinal plants. But in the present study, of the different additives tried, adenine sulphate was found to be the most beneficial.

5.5 Callus initiation and proliferation

In the present study, callus induction was obtained with different auxins and cytokinins. When 2,4-D and NAA alone were added to the media, callus initiation was observed to be low. Callus initiation and proliferation was higher in cultures receiving combinations of auxins (NAA and 2,4-D) with cytokinins (BA) irrespective of the media used. Combinations of cytokinins with auxins on callus initiation and proliferation have been reported in many medicinal plants. Best response for callus induction and growth in *Rauwolfia caffra* was obtained in media containing 2.0 mg NAA and 2.0 mg BA l^{-1} (Upadhyay *et al.*, 1992). Callus cultures of *Kaempferia galanga* was initiated on MS medium supplemented with 2,4-D and BA as reported by Vincent *et al.* (1992). In *Aristolochia bracteolata*, callusing was observed on culturing young leaves and nodes on MS medium containing combinations of KIN and NAA. Similar results of callusing in combinations of auxins and cytokinins have been reported in *Eucommia ulmoides* (Wang *et al.*, 1994), *Hemidesmus indicus* (Sarsan *et al.*, 1994), *Costus sperocisus* (Jain and Chaturvedi, 1985), *Panax ginseng* (Furaya *et al.*, 1986) etc.

Tender leaf and petiole pieces were found to respond better compared to mature and semimature ones in terms of callus initiation and development. Explants taken from newly originated organs are most likely to be capable of organogensis.

Callus derived from seedlings and inflorescence explants of various *Cymbopogon species* had a higher morphogenetic capacity than that arising from seeds, culms, roots or rhizomes (Jagaddishchandra and Sreenath, 1987). In the experiments of Takayama and Misawa (1982), most segments derived from young leaves of *Begonia* produced buds and roots whereas those derived from mature leaves normally dried. Nature of callus was different in combination of BA with NAA and BA with 2,4-D. In media cotaining BA and 2,4-D and 2,4-D alone, granular hard callus was obtained whereas in media containing BA and NAA at lower concentrations (0.5 and 1.0 mg l^{-1}) nodular callus was obtained. Differences in the morphology of callus depending on the concentration and kind of growth regulators added have been reported by many workers. In *Eucommia ulmoides*, two types of calli, one which was white and non-embryogenic, and the other yellow-green coloured and embryogenic were obtained in media supplemented with high concentrations of BA along with NAA and low concentrations of BA along with NAA respectively (Wang *et al.*, 1994). 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.

5.6 Callus regeneration

5.6.1 Organogenesis

In the present study, maximum regeneration of shoots from callus was obtained when callus was transferred to media containing BA but healthier, longer shoots were formed in media containing KIN. In media supplemented with

brassinolide along with BA, days for regeneration was less. Regeneration was also obtained in MS medium without growth regulator, but the percentage regeneration was lower. The results of the present study is in conformity with results obtained in other medicinal plants in which subculturing of callus to media containing cytokinins or combination of cytokinins with auxins resulted in shoot regeneration. In *Panax ginseng*, shoot differentiation and plantlet formation from callus were promoted on media containing KIN (Donato and Perucco, 1986). Regeneration of plants from leaf callus of *Plumbago rosea* was obtained in medium containing BA alone (Satheesh Kumar and Bhavanandan, 1989). Shoot development from callus regenerated roots of *Nardostachys jatamansi* was achieved in medium containing 2.0-6.0 mg l^{-1} KIN (Mathur, 1993).

5.6.2 Embryogenesis

Among the different routes of micropropagation, somatic embryogenesis is the most rapid mode of regeneration. In the present study, development of embryoids was observed in media containing BA and BA + coconut water. In media containing low concentration of 2,4-D, embryogenesis was not observed. Developing embryoids on transfer to basal (MS) medium germinated. Chuang and Chang (1987) have reported the occurrence of spontaneous embryoid formation in medium containing 0.1-0.5 mg l^{-1} 2,4-D. But in the present study, 2,4-D at low concentration had no positive effect on embryoid development. Callus transferred from the medium containing IAA, BA and NAA to that containing 2.0 mg IAA and 3.0 mg BA embryoids were formed in *Freesia refracta* which further developed into plantlets (Wang *et al.*, 1989). In *Aconitum heterophyllum*, somatic embryos appeared after transfer of callus obtained in 2,4-D or NAA to medium containing BAP 1.0 mg l^{-1} and NAA 0.1 mg l^{-1} (Giri *et al.*, 1993). In the present study,

embryoid formation occurred in MS media containing BA alone, but number of embryoids formed was more in media containing coconut water in addition to BA. Germination of embryoids in hormone free basal medium as in the present study have been reported in a number of medicinal plants like *Peucedanum palustre* (Vuorela *et al.*, 1993), *Oplopanax elatus* (Kim *et al.*, 1991), *Rauwolfia caffra* (Upadhyay *et al.*, 1992) etc.

5.7 Induction of rooting

In the present study, maximum rooting and minimum days for rotoing could be obtained when shoots were cultured in ½ MS + brassinolide for one week and later transfered to IBA 1.0 mg l^{-1}. The number of roots were maximum in IBA 1.0 mg l^{-1}. Minimum rooting was seen in combinations of IBA with NAA. In *Madhuca longifolia* excised shoots were rooted on ½ MS with IBA 1.0 mg l^{-1} (Rout and Das, 1993). Gelled MS medium containing 10 mg l^{-1} IBA was optimum for rooting of shoots in *Gomphrena officinalis* (Mercier *et al.*, 1992). Rooting was generally accelerated in stationary liquid medium containing 0.2 mg l^{-1} IBA or IAA in *Adhatoda beddomii* (Sudha and Seeni, 1994).

In maize, brassinolide induced significant stimulation of root growth, but auxin was reported to inhibit this action (Cerena *et al.*, 1983, Cerena *et al.*, 1984, Romani *et al.*, 1981). Growth in azuki bean epicotyls were affected by both brassinolide and auxin (Cerena *et al.*, 1983). Yopp *et al.* (1981) pointed out that strong synergism existed between auxin and brassinolide and this synergism occurs only when tissues are first treated first with brassinolide and then with auxin (IAA). The results of the present study on rooting confirms these reports.

5.8 Hardening

Hardening is important in the case of *in vitro* plants as they are not adapted to *ex vitro* conditions. Light temperature and relative humidity have to be controlled during acclimatization for better survival of plants. In the present study, better survival of plants was obtained when rooted plantlets were transferred to ½ MS solution for two weeks and thereafter transferred to the hardening unit where the plants were frequently sprayed with water. In *Pelargonium graveolens*, Satyakala *et al.* (1995) reported that transfer of rooted plants to ½ MS solution for one week and then planted in autoclaved soilrite and kept in a growth chamber at 80 per cent relative humidity at 25°C gave better success.

Summary

SUMMARY

Studies were conducted on standardisation of *in vitro* techniques for rapid multiplication of *Trichopus zeylanicus* Gaertn.at 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 found was with 0.1 per cent mercuric chloride for 5 minutes in the case of shoot buds as well as leaf and petiole bits.

2. Chlorine water treatment resulted in bleaching of a high percentage of cultures.

3. For seeds, treatment with mercuric chloride 0.1 per cent for 5 minutes or 10 minutes was found to be equally effective.

4. Of the different sterilization treatments tried for very small basal shoots and root explants, none were found effective for producing contamination free cultures.

5. Explants collected during the months of January to April gave lower contamination and maximum survival.

6. Percentage germination of seeds was higher in ½ MS and ½ MS + GA incubated under light, where as soaking in water and incubation under dark gave lower percentage of germination.

7. Days for seed germination was lowest when the seeds were soaked in water for one hour before inoculation.

8. Buds from young, tender, purple coloured shoots showed maximum establishment and growth compared to buds from mature shoots for which the establishment percentage was nil.

9. Establishment percentage of buds was maximum in all the media tried when supplemented with BA as the cytokinin. Establishment was lower when, KIN, 2iP or combination of BA and 2iP with auxins were added to the medium.

10. Maximum number of buds was obtained when BA was used at lower concentrations. The highest number of buds was obtained in SH medium supplemented with BA and minimum number of days for bud burst was observed in MS medium.

11. The number of days for bud burst were more and number of buds were low in media supplemented with KIN, 2iP and BA and 2iP in combination with auxins.

12. Establishment of buds was favoured by light and under dark establishment of buds was low.

13. Proliferation of buds was higher at higher concentration of BA (5 mg l^{-1}) in SH and WPM.

14. The number of shoots developed and length of shoots were found to be higher in SH medium supplemented with BA 0.5 and 1 mg l^{-1}.

15. Among the different additives tried for increasing the proliferation of buds and development of shoots, adenine sulfate proved to be the best at lower concentrations of BA.

16. Yeast extract and casein hydrolysate at high concentration did not promote shoot growth.

17. Tender leaf and petiole explants were found to respond better than mature explants.

18. Callus initiation with the addition of NAA and 2,4-D into the media was noticed only in MS and WPM.

19. Rhizogenesis from callus was observed in media containing NAA.

20. Percentage of cultures initiating callus and higher callus index was observed in combinations of NAA and 2,4-D with BA.

21. Callus index was maximum in MS medium supplemented with BA and 2,4-D. Minimum days for callus initiation was observed in the same medium with lower concentrations of growth regulators.

22. Nature of callus differed in media containing 2,4-D and BA and NAA and BA.

23. Cultures incubated under dark and light did not show much variation with respect to callus index and days to initiate callus.

24. Direct organogenesis was observed in ½ MS supplemented with BA and NAA.

25. Organogenesis from callus was found to be maximum in media containing BA.

26. Healthy and longer shoots were obtained in media containing KIN.

27. Number of days for shoot regeneration was less in media containing brassinolide along with BA and in basal MS medium.

28. Somatic embryogenesis was observed in media containing BA and BA + coconut water, and embryoid germination was obtained in MS basal medium.

29. Maximum rooting of shoots was obtained by culturing shoots in media containing brassinolide for one week and there after transfer to ½ MS containing IBA. Number of roots were more in media containing IBA. Also, rooting was found to be earlier in liquid medium.

30. Better survival of *in vitro* raised plantlets was obtained when rooted plantlets were kept in ½ MS with reduced sucrose and increasedlight intensity in the culture room for two weeks followed by transfer to the hardening unit.

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* Originals not seen

APPENDIX-I

Composition of various basal media tried for *in vitro* culture of *Trichopus zeylanicus*

Ingredients mg l^{-1}	MS mg/l	B_5	SH	WPM
KNO_3	1900	2500	2500	-
NH_4NO_3	1650	-	-	400
$CaCl_2.2H_2O$	440	150	200	22
$MgSO_4.7H_2O$	370	250	400	1850
KH_2PO_4	170	-	-	340
$(NH_4)_2SO_4$	-	134	-	-
$NaH_2PO_4.2H_2O$	-	150	300	-
$Ca(NO_3)_2.4H_2O$	-	-	-	556
$MnSO_4.4H_2O$	22.30	10	10.0	22.3
$ZnSO_4.7H_20$	8.60	2.0	1.0	8.60
H_3BO_3	6.20	3.0	5.0	6.20
KI	0.83	0.75	1.0	-
$CuSO_4.5H_2O$	0.025	0.025	0.2	0.25
$Na_2MO_4.2H_2O$	0.25	0.025	0.1	0.25
$CaCl_2.6H_2O$	0.025	0.25	0.1	-
$FeSO_4.6H_2O$	27.80	-	15.0	27.80
Na_2 EDTA.$2H_2O$	37.30	0.28	10	37.30
Myo Inositol	100	100	1000	100
Thiamine-HCl	0.1	10.0	5.0	1.0
Nicotinic acid	0.5	1.00	5.0	0.5
Pyridoxine-HCl	0.5	1.00	5.0	0.5
Glycine	2.0	-	-	2.0
Surcrose	30,000	20,000	30,000	30,000

APPENDIX-II

Abstract of analysis of variance for the effect of different treatments

Sl. No.	Character	Treatment DF	Treatment MS	Error DF	Error MS	Level of significance
1	2	3	4	5	6	7
1	Surface sterilization					
1	Effect of surface sterilants on establishment of shoot buds					
a)	Fungal contamination (%)	9	805.38	10	0.57	0.05
b)	Bacterial contamination (%)	9	3595.06	10	13.48	0.05
c)	Total contamination (%)	9	1897.88	10	16.29	0.05
d)	Cultures bleached (%)	9	3831.14	10	30.93	0.05
e)	Establishment (%)	9	2077.06	10	29.11	0.05
2	Effect of surface sterilants on establishment of leaf and petiole bits					
a)	Fungal contamination (%)	9	478.25	10	21.72	0.05
b)	Bacterial contamination (%)	9	390.61	10	20.51	0.05
c)	Total contamination (%)	9	1582.90	10	37.89	0.05
d)	Cultures bleached (%)	9	1727.64	10	10.47	0.05
e)	Establishment (%)	9	1815.64	10	59.31	0.05
3	Effect of surface sterilants on sterilization of seeds					
a)	Fungal contamination (%)	6	1151.84	7	28.09	0.05
b)	Bacterial contamination (%)	6	694.98	7	53.93	0.05
c)	Total contamination (%)	6	2931.00	7	89.63	0.05
d)	Establishment (%)	6	2816.78	7	69.71	0.05
4	Seasonal variation in contamination of shoot buds					
a)	Fungal contamination (%)	11	189.84	12	23.69	0.05
b)	Bacterial contamination (%)	11	150.26	12	23.18	0.05
c)	Total contamination (%)	11	574.62	12	71.88	0.05
d)	Culture dried (%)	11	22.79	12	7.03	0.05
e)	Establishment (%)	11	946.85	12	89.84	0.05

Contd.

Appendix-II

1	2	3	4	5	6	7
5	Seasonal variation in contamination of leaf and petiole bits					
a)	Fungal contamination (%)	11	219.49	12	82.46	0.05
b)	Bacterial contamination (%)	11	55.98	12	22.87	0.05
c)	Total contamination (%)	11	314.28	12	27.76	0.05
d)	Cultures dried (%)	11	70.68	12	9.64	0.05
e)	Establishment (%)	11	553.88	12	38.56	0.05
6	Effect of light, soaking in water and GA on seed germination					
a)	Days for seed germination	4	4285.46	20	560.86	0.05
7	Effect of explant and explant maturity on growth of cultures					
a)	Establishment (%)	11	3115.05	12	34.54	0.05
b)	Cultures showing callus initiation/bud development (%)	11	1605.88	12	22.14	0.05
c)	Contamination (%)	11	178.99	12	51.06	0.05
d)	Cultures dried (%)	11	1867.452	12	51.52	0.05
8	Effect of BA on establishment of buds					
a)	Days for establishment	12	1297.68	100	108.14	0.05
b)	Number of buds	12	2.03	100	0.89	0.05
9	Effect of KIN on establishment of buds					
a)	Days for establishment	8	67.79	28	19.36	0.05
b)	Number of buds	8	0.58	28	0.45	0.05
10	Effect of 2ip on establishment of buds					
a)	Days for establishment	11	246.31	45	25.99	0.05
b)	Number of buds	11	0.55	45	0.15	0.05
8	Effect of combination of cytokinins with auxins on establishment					
a)	Days for establishment	13	1211.98	35	79.82	0.05
b)	Number of buds	13	0.55	35	0.18	0.05

Contd.

Appendix-II. Continued

1	2	3	4	5	6	7
11	Effect of BA on proliferation of buds					
a)	Number of buds	11	71.68	60	5.31	0.05
b)	Number of shoots	10	12.5	55	3.22	0.05
c)	Length of shoots (cm)	10	1.32	55	0.23	0.05
12	Effect of additives on proliferation of and elongation of shoots					
a)	Number of buds	31	301.63	160	34.39	0.05
b)	Number of shoots	18	113.17	95	16.60	0.05
c)	Length of shoots (cm)	18	1.98	95	0.28	0.05
13	Effect of growth regulators on callus induction					
a)	Days for callus initiation	27	281.31	120	80.42	0.05
b)	Callus index	27	7841.25	120	1197.18	0.05
14	Effect of growth regulators on callus initiation under dark					
a)	Days for callus initiation	10	145.28	11	28.77	0.05
b)	Callus index	10	4141.63	11	1192.3	0.05
15	Effect of growth regulators on callus regeneration					
a)	Days for bud formation	15	769.96	48	70.37	0.05
b)	Number of buds	15	35.37	48	12.24	0.05
c)	Number of shoots	15	80.66	48	18.01	0.05
d)	Length of shoots	15	4.18	48	0.74	0.05
16	Effect of growth regulators on induction of roots					
a)	Number of roots	8	18.72	15	4.26	0.05
b)	Length of roots	8	0.039	15	0.032	0.05
c)	Days for root initiation	8	1797.10	15	119.23	0.05

APPENDIX-III

Meteorological parameters of the experimental site at the College of Horticulture, Vellanikkara, for the period from January, 1996 to December 1996

Month	Mean maximum temperature (°C)	Mean minimum temperature (°C)	Mean relative humidity (%)	Rainfall (mm)	Number of rainy days	Mean sunshine (h)
January	33.1	22.4	53	0	0	9.4
February	34.7	23.4	53	0	0	9.9
March	36.4	24.3	60	0	0	9.3
April	34.6	25.0	73	152.0	7	8.3
May	32.8	25.2	77	95.6	4	7.7
June	30.5	23.8	85	400.3	16	4.7
July	28.8	23.1	90	588.7	25	2.7
August	29.1	23.6	87	310.0	20	3.7
September	29.2	23.7	84	391.6	17	4.3
October	30.1	22.9	82	219.8	12	6.0
November	31.5	23.6	72	23.1	2	7.1
December	30.5	21.8	68	60.8	2	6.8

STANDARDISATION OF *IN VITRO* TECHNIQUES FOR RAPID MULTIPLICATION OF *Trichopus zeylanicus* Gaertn.

By

SEEMA, B.J.

ABSTRACT OF THESIS

Submitted in partial fullfilment of the requirement for the degree of

MASTER OF SCIENCE IN HORTICULTURE

Faculty of Agriculture
Kerala Agricultural University

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Vellanikkara, Thrissur

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ABSTRACT

Studies were conducted on standardisation of *in vitro* techniques for rapid multiplication of *Trichopus zeylanicus* Gaertn at the Plant Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara during 1995-1997.

Surface sterilization was standardised for different explants. Treatment with 0.1 per cent mercuric choloride for 5 min was found to be the best for all the explants. Explants collected during January to April showed lower contamination and maximum survival.

Soaking seeds in water for one hour was found to reduce the number of days for germination but lower germination percentage.

Young, purple shoots were observed to show maximum establishment and growth. Establishment percentage and maximum number of buds was observed to be highest in SH media supplemented with BA compared to 2iP and KIN. Also exposure to light was favourable for better establishment of buds.

Proliferation rate was higher at higher concentration of BA but shoot development was better at lower concentration of BA. Addition of adenine sulfate increased the proliferation rate of buds and development of shoots but supplements like yeast extract and casein hydrolysate were not effective in promoting shoot growth.

Tender leaf and petiole explants were found to respond better, than mature explants and percentage of callus initiation and callus index was higher in combinations of NAA and 2,4-D with BA. Direct organogenesis was observed in ½ MS supplemented with BA and NAA. Regeneration of healthy and longer shoots were obtained in MS medium supplemented with KIN.

Somatic embryogenesis was observed in media containing BA and BA + coconut water, and embryoid germination was obtained in MS medium.

Maximum rooting was obtained by culturing shoots in media containing brassinolide for one week and thereafter transfer to IBA. Earlier rooting was obtained in liquid medium.

Keeping in ½ MS with reduced sucrose and increased light intensity in the culture room for two weeks before transfer to hardening unit resulted in better survival of plantlets.