STANDARDISATION OF TISSUE/MERISTEM CULTURE TECHNIQUES IN IMPORTANT HORTICULTURAL CROPS

By

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THESIS

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DECLARATION

I hereby declare that this thesis entitled "Standardisation of tissue/meristem culture techniques in important horticultural crops" 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, associateship, fellowship or other similar title of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled "Standardisation of tissue/meristem culture techniques in important horticultural crops" is a record of research work done independently by Mr. K. RAJMOHAN under my guidance and supervision, and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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INTRODUCTION

Vegetative propagation ensures genetic uniformity among the progeny and is preferred to seed propagation for the multiplication of heterozygous genotypes having superior traits. Nevertheless, a number of the important horticultural crops of Kerala, in spite of being cross pollinated, are propagated through seeds. Methods of vegetative propagation like air layering, rooting of cuttings, budding and grafting are possible in several species; but are generally cumbersome and do not have commercial feasibility, except in the case of crops like cashew, jack, mango and pepper.

There has been an increasing interest in recent years in the application of tissue culture techniques as an alternative means of asexual propagation of economically important plants. The exploitation of the concept of totipotency (suggested by Haberlandt, 1902) has progressed from a future possibility to a rapidly expanding reality as is evident from the number of species now being successfully propagated through tissue culture.

Even in cases where conventional methods of vegetative propagation have reached commercial acceptability, tissue culture techniques have been shown to have definite advantages as they ensure an extremely rapid rate of

multiplication which is not season-dependent and require only a limited quantity of plant tissue as the initial explant. Tissue culture techniques also can aid in the production of disease-free plants and in the cryopreservation of germplasm. Tissue culture mediated genetic modifications serve as complementary to the conventional plant breeding methods to a great extent.

Most of the species that are currently being propagated through tissue culture are herbaceous horticultural crops. The commercial adoption of micropropagation, especially in woody perennials, depends to a large extent on solving the problems related to culture establishment, polyphenol oxidation, influence of the physiological age of the explant, systemic presence of pathogens, and planting out. Long term evaluation of the field performance of the tissue cultured plants is also required.

Attempts for standardising tissue culture procedures have not been made for most of the important horticultural crops of Kerala. The present investigations, therefore, aimed at standardising tissue culture techniques in some of these crops, namely, jack, mussaenda, breadfruit, pepper and nutmeg. Detailed investigations were made for standardising

the techniques for jack (*Artocarpus heterophyllus* Lam.) and mussaenda (*Mussaenda erythrophylla* Schum. & Thonn.). In the case of breadfruit (*Artocarpus altilis* L.), pepper (*Piper nigrum* L.) and nutmeg (*Myristica fragrans* Houtt.), preliminary studies were made to understand the *in vitro* behaviour of the explants.

REVIEW OF LITERATURE

The concept of totipotency, which is inherent in the cell theory of Schleiden (1838) and Schwann (1839), is the basis for plant tissue culture. In 1902, Haberlandt suggested the use of embryo sac fluids for inducing divisions in vegetative cells and the culture of isolated plant cells, and prophesied that 'artificial embryos' could be grown from isolated mature plant cells. Since then, plant tissue culture has evolved as a powerful research tool in fundamental and applied aspects of Agriculture, Horticulture, Forestry and drug manufacture.

Widespread successes with plant tissue culture were reported after the discovery of auxins and cytokinins and after the revelation by Skoog and Miller (1957) that regeneration of shoots and roots in cultured cells could be manipulated simply by varying the proportions of these growth substances in the nutrient media (Murashige, 1982). The pioneering experiments of White (1934, 1939), Gautheret (1938), Nobecourt (1939), Reinert (1958), Steward et al. (1958) and Morel (1960) are often cited as the landmarks in the developmental phase of plant tissue culture.

In the early experiments on plant tissue culture, the basic nutrient media were often supplemented with

complex mixtures of natural origin (coconut milk, yeast extract, fruit juice etc.) in order to obtain optimal growth and to induce organogenesis (Steward et al., 1958; Hildebrandt, 1963; Vasil and Hildebrandt, 1966; Butenko, 1968; Street, 1969). Most of these media were modifications of the formulations used by White (1932, 1939), Gautheret (1939) and Hildebrandt et al. (1946). The first major completely defined nutrient medium was developed by Murashige and Skoog (1962). Most of the nutrient media now being used for tissue culture are chemically defined and can be easily adapted to various uses and needs by slight modifications (Vasil and Vasil, 1980).

Several aspects of tissue culture are currently being applied to Agriculture. By far, the best commercial application of tissue culture techniques has been in the production of clonal plants at a very rapid rate compared to the conventional methods. Such plants are reported to grow faster and to 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: (a) enhanced release of axillary buds, (b) production of adventitious shoots through organogenesis and (c) somatic embryogenesis. Callus mediated somatic

organogenesis is not recommended for clonal propagation; but may be ideal for recovery of useful variant lines. In shoot tip culture, genetic uniformity is favoured. Somatic embryogenesis is limited to a few species but results in the most rapid mode of plant regeneration (Evans et al., 1981).

Currently, in vitro clonal propagation procedures have been standardised for a number of important plant species. Excellent reviews on the subject have been made by Murashige (1974; 1978), Vasil and Vasil (1980), Hu and Wang (1983), Styer and Chin (1983) and Sharp et al. (1984). The orchid industry now relies almost exclusively on tissue culture to propagate orchids that are difficult to breed. The greatest success using this technique has been achieved in herbaceous horticultural species (Hu and Wang, 1983). Compared to herbaceous plants, the micropropagation of woody species lags behind. Woody or tree species of angiosperms and gymnosperms have proved to be rather difficult to culture and regenerate. Success in obtaining viable plantlets from mature trees has been more recent (Bonga, 1982).

All species in which organogenesis and plant formation can be achieved in vitro may not be suitable for large scale clonal propagation (Vasil and Vasil, 1980).

For some species the process is too expensive, the rate of multiplication slow and the mortality of plants at the time of their transfer to soil high. Genetic variation may sometimes take place in the clonal population of plants as a result of aneuploidy and polyploidy introduced during cell proliferation *in vitro* (Murashige, 1974).

Not much attempts have been made on the *in vitro* propagation of jack, mussaenda, breadfruit, pepper and nutmeg. With a few exceptions, the tissue culture procedures for trees and woody plants are similar to those for other plants. Hence, the present review deals with other crops to cover various aspects of *in vitro* propagation, factors influencing success of *in vitro* propagation, *in vitro* rooting, cytology and morphology of regenerated plantlets and the planting out of the plantlets.

I. Routes of *in vitro* propagation

A. Enhanced release of axillary buds

Morel (1960) was the pioneer in applying shoot tip culture as a tool for clonal multiplication. In addition, he could standardise a procedure to render the orchid, *Cymbidium* free of virus. Since then, *in vitro* clonal multiplication gained momentum. The greatest success using this technique has been achieved in herbaceous

horticultural species. This success has been partially due to the weak apical dominance and strong root regenerating capacity of many herbaceous plants (Hu and Wang, 1983).

Wickson and Thimann (1958) discovered that cytokinins could release the lateral buds from apical dominance. In the presence of a cytokinin, the dormant buds of a vegetative apex are stimulated to grow and to elongate, as are those that form on the new axis. This finding was effectively utilised for large scale clonal propagation in strawberry by Boxus (1974). This work was a significant contribution for the development of the most widely used tissue culture procedure for clonal propagation of plants (Hussey, 1980).

There are only preliminary reports on the application of shoot tip culture for the micropropagation of jack (Artocarpus heterophyllus Lam.). Rao et al. (1981 b) and Doreswamy (1983) were successful in inducing multiple shoots from the shoot tips of mature jack trees when cultured on MS medium supplemented with a cytokinin and an auxin (BA 30.0 ppm + IAA 0.5 to 5.0 ppm or 2 iP 30.0 ppm + NAA 1.0 ppm). Only chance rooting of shoots occurred in their attempts to induce in vitro rooting, using medium containing IBA and NAA (0.1, 0.5, 1.0, 5.0 and 10.0 ppm).

Yie and Liaw (1977) established papaya seedling shoot tips *in vitro* and obtained proliferative growth on MS medium containing 0.3 μM IAA and either 23 μM kinetin or 2.2-4.4 μM BA. Litz and Conover (1977, 1978) developed a procedure for establishment and culture of excised shoot tips from field grown, mature papaya. Shoot tips were cultured on establishment medium consisting of MS basal medium with 87.6 mM sucrose, 47 μM kinetin and 10.8 μM NAA. Enlarged explants were subcultured after two to three months on shoot proliferation medium (MS medium with 2.2 μM BA and 0.54 μM NAA). Establishment time and rate of proliferation were both dependent on the age of the stock plants, the time of the year, the sex type and the presence of bacterial/viral contaminants (Litz and Conover, 1981). The staminate plants responded more rapidly than the pistillate plants. Explants from stock plants during active period of growth responded the best. The rate of proliferation was observed to be seven to eight fold between the subcultures. The ability of the cultures to maintain proliferation was lost after eight to thirteen subcultures. Pandey and Rajeevan (1983) obtained prolifically growing shoot cultures when the shoot apices of papaya seedlings of the varieties Ranchi Selection 1-45D and

Coorg Honey Dew, cultured on MS medium supplied with NAA 5 /uM and kinetin 50 /uM (establishment medium) were transferred after a period of three months to the same basic medium with NAA 0.5 /uM and BAP 2.5 /uM (proliferation medium). The establishment period could be reduced to two weeks when the kinetin level was doubled in the establishment medium.

Ma and Shii (1972) reported the in vitro formation of adventitious buds in banana shoot apex following decapitation. Berg and Bustamante (1974) could recover only a single plant per excised shoot apex while attempting the micropropagation of banana. The applicability of the excised shoot tip culture technique to a number of banana clones was assessed by de Guzman et al. (1976). Krikorian and Cronauer (1984) could induce multiple shoots by releasing dormant buds at the leaf bases. Subculturing could be carried out from the proliferating mass of shoots. Protocorm-like bodies were formed at the newly formed shoot bases which, in turn, produced multiple shoots. Jarret et al. (1985) initiated shoot tip cultures of two clones of banana (Saba and Pelipita) on a modified MS medium supplemented with 3 mg/l BA and 1 mg/l IAA. Propagation cultures were initiated by splitting shoot tips along their longitudinal axis and reculturing the individual pieces on basal medium supplemented with 5 mg/l BA.

Enhanced release of axillary buds from the shoot apices of Carrizo citrange was induced by Kitto and Young (1981) using a medium consisting of Knop's macro nutrients and organic growth factors and MS micro nutrients, supplemented with 5.0 mg/l BA. Barlass and Skene (1982) observed that the optimum concentration of BA was 10 mg/l to induce multiple shoot formation from the explants of various Citrus spp, and hybrids.

Initial attempts on micropropagation of pineapple (Ananas comosus) were made by Lakshmi Sita et al. (1974), Teo (1974), Pannetier and Lanaud (1976) and Mathews et al. (1976). Mathews and Rangan (1979) found that the basal medium of MS supplemented with 1.8 mg/l NAA, 2.0 mg/l IBA and 2.1 mg/l kinetin was suitable both for establishment and proliferation of shoots. About 75.7% of the cultures recorded a proliferation rate of 4.5 shoots. Drew (1981) could induce 30 to 50 multiple shoots within a period of one month when the explant was cultured on MS medium containing 2.5 mg/l BA and 1.9 mg/l NAA. Shoots from the proliferating cultures, cut longitudinally to four segments, could also be regenerated.

A novel method for rapid multiplication of grapes (Vitis vinifera) involving the induction of adventive buds from fragmented apical meristem explants was described by Barlass and Skene (1978). In a period of two weeks, four

fold proliferation of shoots occurred at the basal end of the leaf like structures originating from the apical fragments. The chemical and physical parameters for shoot production have been revised and optimized for several cultivars and species (Krul and Mowbray, 1984). Nodal explants of grapes were also used for in vitro multiple shoot production (Mullins et al., 1979).

Proliferation of apple shoots in vitro, followed by rooting of these shoots, was accomplished by Abbott and Whiteley (1976). Meristem tips were excised from seedling- and adult-phase trees of Cox's Orange Pippin and cultured on MS medium with simplified organic constituents, no auxin, and 0.5 - 4.6 /uM kinetin. Most of the subsequent work with apples used BA as the cytokinin (Zimmerman, 1984). Jones (1976) reported the striking effect of phloridzin and phloroglucinol on proliferation of M.7 and M.26 rootstock shoots. Shoot proliferation occurred on a modified MS medium containing BA and IAA; addition of phloridzin or phloroglucinol increased the number, length and weight of shoots, whereas elimination of IAA stopped proliferation. An in vitro system having the potential of producing 60000 shoots in eight months from a single shoot apex of M.26 apple rootstock was proposed by Jones et al. (1977). Subsequent research extended the technique to numerous other rootstock and scion

cultivars (James and Thurbon, 1981; Werner and Boe, 1980; Norton and Boe, 1982; Zimmerman, 1984). Snir and Erez (1980) found no response to phloroglucinol in their cultures. The most effective cytokinin in stimulating shoot proliferation in apple cultivars was found to be BA, the least effective being 2 iP. Kinetin was found to be intermediate in its effectiveness. However, the effect of these on shoot elongation was in the reverse order (Lundergan and Janick, 1980). The shoots produced at the most effective concentrations of BA (13.2-22.2 μM) for proliferation were found to be stunted. Normal growth could be obtained by transferring the cultures to medium containing 4.4 μM BA and 4.9 μM IBA.

Anderson (1980) perfected tissue culture propagation procedure for rhododendron. He observed that the explants did not respond to their maximum potential when grown on MS medium. They were apparently affected by general salt toxicity as shown by foliage chlorosis and browning of the stems. Anderson found it necessary to modify the MS formula by reducing the ammonium nitrate and potassium nitrate to approximately 1/4 strength and by adding twice the strength of ferrous sulphate and Na_2 EDTA. These changes dramatically improved the propagule multiplication and culture health.

The first report on rapid clonal multiplication of pomegranate (Punica granatum) from mature trees by tissue

culture was made by Gupta *et al*. (1981). In another study by Mascarenhas *et al*. (1981) on two varieties (Ganesh and Muscat) of mature pomegranate trees, multiple shoot formation was obtained on MS medium containing 500 mg/l casein hydrolysate, 0.5 mg/l kinetin and 1.0 mg/l BA. However, elongation of shoots did not take place on this medium. Transfer to a medium with lower casein hydrolysate (100 mg/l) with same concentrations of cytokinins brought about elongation. Under these conditions, the leaves turned bright green and the shoots elongated.

The pioneer work on the application of *in vitro* techniques on coffee (*Coffea* spp.) was published by Staritsky (1970). However, the first report on enhanced release of axillary buds was made by Custer *et al*. only in 1980. When nodal explants of aseptically grown 3-month old *Coffea arabica* plants were cultured on MS medium supplemented with 44 /uM BA and 0.6 /uM IAA, shoot development occurred on the average of 2.2 per node after two to five weeks. Similar techniques have been used by Dublin (1980) with *Arabusta* plants. Shoot development was observed in medium supplemented with malt extract (400 mg/l) and BA (4.4 /uM). Kartha *et al*. (1981) induced multiple shoots in isolated apical meristems from seedlings of *Coffea arabica* cv. Caturra Rojo and Catui, cultured on MS medium containing 5.0-10.0 /uM BA or Zeatin and 1.0 /uM NAA.

Multiple shoot formation was reported by Madgauda *et al.* (1983) from *in vitro* grown cardamom (*Elettaria cardamomum*) shoot tips. Young sprouted buds were excised and cultured on MS medium supplemented with 0.5 mg/l BA, 0.5 mg/l kinetin, 2.0 mg/l IAA, 5.0% coconut water, 0.1 mg/l calcium pantothenate and 0.1 mg/l biotin. The buds grew to a length of over 40 mm in eight weeks. Shoot tips from elongated buds or rooted plantlets, when excised and transferred to the same medium, gave rise to multiple shoots. Kumar *et al.* (1985) reported the direct formation of shoots from the immature panicles (measuring 0.5 to 5.0 cm after initiation) of cardamom, on MS medium supplemented with 0.5 mg/l NAA, 0.5 mg/l kinetin, 1.0 mg/l BA, 0.1 mg/l calcium pantothenate, 0.1 mg/l folic acid and 10% coconut water. The shoots, when subcultured on MS medium containing 2.0 mg/l NAA and 0.05 mg/l kinetin, and incubated in dark for five days, formed roots.

Meristem culture techniques, as first applied to cassava (*Manihot esculenta*) cultivars, employed the MS medium containing vitamins as in the B5 medium (Gamborg *et al.*, 1968), supplemented with NAA, BA and GA at 1.0, 0.5 and 0.1 μM, respectively (Kartha *et al.*, 1974). Later, it was observed that BA was the cytokinin best suited for plant regeneration in the presence of NAA and GA, and that GA had a stimulating effect on shoot growth from meristem cultures (Nair *et al.*, 1979). The use of upto 0.5 μM BA promoted shoot initiation

and growth. Further increase of BA concentration retarded shoot growth, inhibited rooting and stimulated callus formation. Addition of NAA to this medium further enhanced callus growth and rooting in some varieties (Roca, 1984). Meristem tips cultured in MS medium with 0.05 /uM NAA and increasing concentrations of BA gradually developed into rosette cultures, with shortened shoots and many nodes (CIAT, 1979). Further growth of the axillary buds at each node occurred when the concentration of BA was reduced to 0.25 /uM in the presence of 0.1 /uM GA and 0.1 /uM NAA in rotated liquid MS medium. This gave rise to multiple shoot cultures. It was found that sucrose interacted with BA in meristem culture of cassava (CIAT, 1980). At low BA concentrations, shoot elongation was practically doubled when sucrose was increased from 0.03 - 0.06 /uM.

Chaturvedi et al. (1978) presented a preliminary report on the propagation of bougainvillea (Bougainvillea glabra) by tissue culture. Sharma et al. (1981) could induce an average of ten shoots from the shoot apex of B. glabra 'Magnifica' cultured on a basal medium supplemented with 0.5 mg/l BA and 1.5 mg/l IAA.

Tissue culture of Eucalyptus spp. has been described by Aneja and Atal (1969), de Fossard (1978), Lakshmisita (1979),

Lakshmisita and Vaidyanathan (1979) and Hartney and Barker (1980). However, in all these studies, organogenesis was obtained only from juvenile seedling tissues or embryos and not from tissues of mature trees. Gupta et al. (1981) described for the first time the propagation of mature trees by tissue culture. Multiple shoots were obtained from terminal buds of 20 year-old trees of Eucalyptus citriodora on MS medium supplemented with calcium pantothenate (0.1 mg/l) BA (0.3 mg/l) and kinetin (0.2 mg/l). Incubation at 15°C with continuous illumination followed by growth in agitated liquid cultures was essential for inducing shoot development in the primary terminal buds. These treatments were not necessary in later subcultures or with explants from seedlings. In the same experiment they observed that at the higher cytokinin levels tried the buds turned brown within a week. Gupta et al. (1984) formulated a practical estimate of producing over 50,000 plants from a single explant in an year through the enhanced release of axillary buds.

Micropropagation of teak (Tectona grandis) was attempted by Gupta et al. (1980). They could induce multiple shoot formation from excised seedling explants as well as from excised terminal buds of 100-year old trees on MS medium containing 0.1 mg/l BA and 0.1 mg/l kinetin. Buds were cultured on a primary MS medium (without growth substances but

with 2% sucrose) for three to four weeks, after which they were transferred to the liquid MS proliferating medium with continuous agitation and illumination (1000 lux). Shoots obtained on this medium were excised and induced to root on a low salt medium containing three auxins. Nodal explants from these rooted shoots were subcultured on fresh medium with 0.5 mg/l kinetin and 1.0 mg/l BA where they proliferated to form multiple shoots.

Vegetative propagation of various palm species through tissue culture methods was attempted only during the last decade. Techniques have been standardised for oil palm (Jones, 1974; Corley et al., 1976; Rabechault and Martin, 1976), date palm (Tisserat, 1979; 1981) and coconut (Blake and Eeuwens, 1981; Raju et al. 1984). The technique developed by Jones (1974) is being used on a pilot scale for production of oil palm planting material in Malaysia (Choo et al., 1981). All the published work on palms showed the initiation and multiplication of callus followed by shoot or embryoid regeneration or direct embryogenesis. While meristem proliferation is the preferred technique for propagation of most plant species, this has not so far been proved possible with palms. Blake and Eeuwens (1980) obtained shoot like structures, with no intervening callus stage; but these structures did not develop into plantlets. Segments of

rachillae, when excised and grown on suitable medium, developed shoot like structures having active growing points which continued to form a series of leaves or bracts (Blake and Eeuwens, 1981). The number of shootlets was increased when the cultures were grown on a liquid medium. Addition of coconut water to the medium was beneficial. When coconut water was omitted, its effect could be replaced by 5×10^{-6}M BA, 2.5×10^{-7}M GA and 4-5% sucrose. Growth in the dark was better than in the light and a temperature of 30 - 32°C was optimal. Selection of segments of the rachillae from inflorescences when the outer spathe length ranged from 30 mm to 80 mm gave the maximum number of shootlets. After three weeks, the shootlets were placed on a root inducing medium containing 10^{-5}M to 10^{-6}M NAA. After a further three weeks, they were transferred to a low auxin medium to encourage the root initials to grow out. When root induction occurred, an organised shoot apex could not be maintained. Seedling apices were successfully cultured on Y3 medium (Eeuwens, 1976) by Blake and Eeuwens (1981). Cores were removed from the centre of coconut seedlings and cultured. Normal shoot growth was achieved by the addition of a low level of auxin (10^{-7}M). Much greater growth was obtained on addition of 0.25% activated charcoal to the medium when 10^{-5}M auxin gave the best growth. Roots were formed at the base of the shoots when transferred to a medium

containing 2.5×10^{-4}M NAA, with charcoal. The plantlets could be finally established in the soil.

B. Somatic organogenesis/embryogenesis

Generally, a high concentration of auxin and a low concentration of cytokinin in the medium promote abundant cell proliferation with the formation of callus (Skoog and Miller, 1957). On the other hand, low auxin and high cytokinin concentration in the medium result in the induction of shoot morphogenesis. Auxin, alone or in combination with a very low concentration of cytokinin, is important in the induction of root primordia. Somatic organogenesis can be direct or callus mediated (Evans *et al.*, 1981) and is useful in inducing genetic variability or to recover pre-existing natural genetic variability. In either case, regenerated variants can be used to complement the extant variability.

Somatic embryogenesis was first clearly described in carrot (Reinert, 1959; Halperin and Whetheral, 1964). It is seen limited to a few species; but results in the most rapid mode of plant regeneration (Evans *et al.*, 1981). There are two routes to somatic embryogenesis, as described by Sharp *et al.* (1980). The first is the direct embryogenesis where embryos initiate directly from tissue in the absence of callus proliferation. The second is the indirect

embryogenesis where some cell proliferation is required. A primary medium with an auxin source and a secondary medium devoid of growth substances, both containing a substantial supply of reduced nitrogen was found to be essential for inducing somatic embryoids (Ammirato, 1983). Reviews on somatic organogenesis and embryogenesis were published by Murashige (1978), Sharp et al. (1979), Vasil and Vasil (1980) and Styre and Chin (1983).

Rao et al. (1981 b) observed callus production when shoot tip explants from mature jack trees were cultured on MS medium containing IAA (0.1 ppm) alone or in combination with BA (2.0 ppm). However the callus failed to differentiate into shoot/root. Callussing could be obtained from inflorescence primordia also. Callus induced from hypocotyl segments differentiated into shoot buds when cultured on MS medium containing IBA or NAA (1.0 ppm) and BA (2.0 ppm).

Callus formation from different types of papaya explants has been reported by several workers (De Bruijne et al., 1974; Yie and Liaw, 1977; Litz and Conover, 1981). Callus induction from midrib explants and vein tissues occurred with 1.3-8.8 µM BA and 2.7-16.2 µM NAA (Litz et al., 1983) while callus induction from lamina explants required higher concentrations of NAA (6.5 - 27.0 µM) and BA (2.6 - 13.2 µM). Adventitious roots were formed from midrib callus of papaya cotyledons on MS medium with 0.5 - 81 µM NAA and 0 - 2.2 µM BA. Adventitious meristems

were formed from both lamina and midrib callus on culture media containing 0 - 1.1 /uM NAA and 0.2 - 4.4 /uM BA. Pandey and Rajeevan (1983) could induce good callussing from stem segments of papaya seedlings of the varieties Ranchi Selection 1-45D and Coorg Honey Dew (grown in the summer months) in B5 medium supplied with NAA 25 /uM and kinetin 10 /uM. Large quantity of callus was produced from the explants of the *in vitro* formed roots also in B5 medium supplemented with NAA 10 - 15 /uM and kinetin 10 /uM. Su and Tsay (1985) could induce callus from papaya anthers containing microspores in tetrads to early-binucleate stages by culturing on half-strength MS medium, with full-strength of Na Fe EDTA, supplemented with 2.0 ppm NAA, 1.0 ppm BA and 6.0% sucrose.

De Bruijne *et al.* (1974) described the formation of somatic embryoids from papaya callus derived from seedling petiole segments, following a three-stage procedure. Yie and Liaw (1977) demonstrated a two-stage procedure for induction of somatic embryogenesis in callus of seedling stem origin. Litz and Conover (1983) described the induction and control of high frequency somatic embryogenesis from ovular callus. Embryogenic ovular callus could be induced on MS medium containing 60 g/l sucrose and 2.0 mg/l 2,4-D. Maturation and germination of the embryoids occurred in the absence of growth substances. Su and Tsay (1985) reported the formation

of haploid plantlets and pollen-derived embryoids from anthers cultured at the uninucleate stage on 3.0% sucrose - containing MS medium without any growth substance, at low light intensity.

Grinblat (1972) using Citrus madurensis and Chaturvedi and Mitra (1974) using Citrus grandis obtained callus from stem explants taken from young seedlings grown under laboratory conditions. Grinblat regenerated plants from callus attached to the original explants while Chaturvedi and Mitra developed plantlets from subcultured callus. Plantlets were regenerated from callus, induced from juice vesicles (Kato, 1980) and root explants (Sauton et al., 1982) of Citrus spp. Maheswari and Rangaswamy (1958) described the induction of somatic embryos from polyembryonic citrus nucellar tissue in vitro. Rangan et al. (1968) observed that somatic embryogenesis could be induced in vitro from the nucellus of three monoembryonic Citrus cultivars. Plantlet formation via somatic embryoids induced from the nucellar callus of Citrus spp. was also reported by Esan (1973), Kochba et al. (1974) and Juarez et al. (1976).

In mango (Mangifera indica), Rao et al. (1981) reported the induction of callus from cotyledon tissue and root regeneration on MS medium containing NAA (5.0 mg/l), kinetin (2.5 - 5.0 mg/l) and coconut water (150 ml/l).

Callus induction occurred two weeks after inoculation. Growth was found to be better in the cultures maintained in the dark than in those under light. The callus was dark and compact. Distinct lobes were formed in one-month old cultures. Root initials developed during the second week and they were positively geotropic, somewhat flat and tapering at the top. Root caps were present; but root hairs were absent. Shoot development was not observed. Litz (1984) demonstrated the regeneration of somatic embryoids of monoembryonic mango cultivars from nucellar explants. Litz (1985) induced large number of somatic embryoids from the nucellar explants of polyembryonic mango cultivars. In the above two cases of somatic embryogenesis in mango cultivars, the nucellar explants were first cultured on a modified MS medium supplemented with 0.5 - 2.0 mg/l, 2,4-D for the induction of embryogenic callus and embryoids. They were then subcultured on MS medium without growth regulators for the germination of the embryoids formed.

In pineapple, Mathews and Rangan (1979, 1981) reported shoot formation from callus developed at the base of excised leaves and shoots. In the former study, callus initiation, followed by shoot bud regeneration, was observed on MS medium supplemented with 1.8 ppm NAA, 2.0 ppm IBA and 2.2 ppm BA. In the latter study, callus was initiated from

in vitro grown shoots on MS liquid medium containing 5.4 ppm NAA, 5.2 ppm IAA and 2.1 ppm kinetin. Shoot regeneration could be obtained on a number of media including MS, without growth substances or with various combinations of growth substances. Rao *et al.* (1981a) reported shoot and root regeneration from callus produced from hybrid seedlings grown *in vitro*.

Morel (1945) was the first to report on the production of callus from stem explants of grapes. Production of callus cultures from explants of stem tissues (Staudt *et al.*, 1972) and immature berries (Hawker *et al.*, 1973) has also been reported. Rajasekharan and Mullins (1979) produced viable somatic embryos with diploid genomes from callus. Somatic embryoids have been obtained from unfertilised ovules (Mullins and Srinivasan, 1976), immature stems, flower clusters and young leaves (Krul and Worley, 1977) of a *Vitis* spp. hybrid and petiole and leaf interveinal explants of *Vitis* spp. (Favre, 1977).

Callus production from several plant parts of apple has been reported (Mu *et al.*, 1977; Schneider *et al.*, 1978; Fukui *et al.*, 1981). Differentiation of roots and leaves from callus was observed by Mu *et al.* (1977). Chen *et al.* (1979) obtained plantlets regenerated from the callus of

explants from M.9 rootstocks. Callus was induced from stem segments on MS medium containing 2.2 /uM BA, 10.7 /uM NAA and 100 mg/l casein hydrolysate. When the callus was transferred to a similar medium without NAA, shoots differentiated. These shoots were then rooted *in vitro* on a medium containing IBA. Differentiation of callus (derived from seedling tissues of Golden Delicious) into leaves, shoots and roots was reported by Mehra and Sachdeva (1979) and Liu *et al*. (1981).

Nair *et al*. (1984) succeeded in inducing adventitious buds from excised leaf explants of custard apple (*Annona squamosa* L.) seedlings on Medium containing 0.5 mg/l BA and 0.5 mg/l kinetin. Various auxins in combination with the above medium produced callusing of the explants. Maximum number of shoots were obtained using the leaf base with petiole at a temperature of 27°C and a light intensity of 1000 lux. Roots were initiated erratically when individual shoots were treated with an auxin and then transferred to an auxin-free medium.

The pioneer work with coffee tissues was published by Staritsky (1970) who succeeded in inducing callus from shoot explants of *Coffea* spp. However, somatic embryos and plantlets were obtained only from *C. canephora*. Callus formation and organogenesis from various explants of *Coffea* spp.

cultured *in vitro* have been reported by several workers (Sharp *et al.*, 1973; Monaco *et al.*, 1974; Crocomo *et al.*, 1975). Somatic embryos were obtained at high frequencies from mature leaf cultures of *Coffea arabica* (Sondahl and Sharp, 1977). Somatic embryogenesis from cotyledonary leaves of *C. arabica* cv. Mundo Novo was described by Sondahl *et al.* (1985).

Callus has been induced from stem, petiole, leaf and root sections of cassava (Eskes *et al.*, 1974; Prabhudesai and Narayanaswamy, 1975; Parke, 1978; Rey and Fernandez, 1980). In general, stem sections seemed best suited for callus induction (Roca, 1984). Rapid growth of callus was achieved with a combination of 5.0 - 13.0 µM 2,4-D and a cytokinin (2.0 - 8.0 µM). Root formation in callus cultures was obtained when NAA was used as the auxin. Liu and Chen (1978) observed callus and root formation from anthers cultured on MS medium supplemented with BA and NAA. Somatic embryos and whole plants have been regenerated using cotyledonary explants from mature cassava seeds (Stamp and Henshaw, 1982). The highest frequency of embryo formation occurred at high concentration of 2,4-D (20 µM) while embryo development was enhanced at lower concentration (0.05 µM) and with the addition of BA (0.5 µM).

Callus cultures were established from tissues of coconut (Apavatjrut and Blake, 1977; Eeuwens, 1978; Fisher and Tsai, 1978). Blake and Eeuwens (1981) observed that development of coconut callus was dependent on the addition of activated charcoal (0.25%) to a medium with a high level of 2,4-D (10^{-4}M to 10^{-3}M). Branton and Blake (1983) reported success in producing a clonal plantlet from root callus cultures. Pannetier and Buffard-Morel (1982) could induce somatic embryos in the leaf callus. However, further development of the embryos was not possible. Raju et al. (1984) produced for the first time, clonal plantlets through direct somatic embryogenesis from leaf explants without the intervention of a callus phase. Gupta et al. (1984) observed globular embryo like structures, formed directly from leaf explants, on Y3 medium supplemented with 2,4-D. Monfort (1985) could obtain embryoids from coconut microspores. The embryoids were formed when the anthers were cultured for twenty weeks on Picard and de Buyser medium supplemented with activated charcoal, TIBA and glutamine.

II. Factors influencing success of *in vitro* propagation

A. Explant

1. Size

The size of the explant determines the survival of the culture. When tissues are cut, the cut surfaces turn

brown due to the oxidation of phenols to toxic quinones in the damaged cells (Monaco et al., 1977). When the explant size is small, the cut surface:volume ratio is high and there will be difficulty in the survival of the explant. In cassava Kartha and Gamborg (1975) demonstrated that only explants exceeding 0.2 mm length formed complete plants. Those less than 0.2 mm produced either callus or roots. Mellor and Stace - Smith (1977) observed that meristems of potato less than 0.3 mm long did not root and buds more than 0.7 mm long were prone to infection. In meristem culture for virus elimination, explants of 0.1 - 0.5 mm are used (Hussey, 1978). Tran Thanh Van and Trinh (1978) developed the concept and methodology for achieving in vitro morphogenesis in thin cell layers.

2. Bud location

In Chrysanthemum meristem culture, Hollings and Stone (1968) observed that the success rate of explants from terminal buds was 32% whereas that of explants from laternal buds was only 18%. According to them, the terminal buds had stronger growth potential, as they were in a younger stage of development, than the lateral ones. Hasegawa (1979) observed that a higher percentage of shoot tip explants of rose developed multiple shoots than the explants from lateral buds.

3. Age

During the maturation process of plant, several physiological changes take place that influence the *in vitro* behaviour of the explants (David, 1982). This effect has been demonstrated in the ability of the explants to form adventitious and axillary buds, in the rate of shoot elongation, and in rooting. Because of the decreased morphogenetic ability of mature material, it has not been generally possible to apply the techniques developed for juvenile material to mature plants. Bonga (1982) found it important to select the most juvenile tissues, as within the trees, there were tissues in which juvenility was better maintained than in other tissues. Somatic rejuvenation occurs naturally or can be experimentally induced in some tissues in mature trees. The rejuvenation may progress to a point where the tissues become capable of organogenesis or embryogenesis. Boulay (1979) propagated *Sequoia sempervirens* more than 50 years old, by using explants from stump sprouts. Tissues can be rejuvenated or at least invigourated by using stump sprouts at bole of the tree (Boulay, 1979), by grafting scion on to juvenile rootstocks (Goublay de Mantois, 1980), by adopting *in vitro* procedures using auxins, cytokinins and gibberellins, by a sequence of selected treatments (Bonga, 1981) or by acclimatizing plants to *in vitro* conditions before explants are taken (Durzan, 1984). Such rejuvenation or invigouration usually make the explants

to respond better. Durand - Cresswell at al. (1982) developed an elongation medium for shoot tip explants from the juvenile sprouts of Eucalyptus gunnii to stimulate elongation of the shoots and to obtain a favourable leaf morphology. Gibberellic acid (1.0 mg/l) and activated charcoal (15.0 g/l) were the components of the elongation medium.

B. Surface disinfection

Shoot tips, buds or nodal sections are usually cut to a size larger than that of the final explant, surface sterilised and trimmed to the final size before being transferred to the culture vessel (Hussey, 1979). The most commonly used surface sterilant is sodium hypochlorite. Buds can be sterilised with 5% sodium hypochlorite. For softer tissues, a dilution to lower strength may be needed; but anything below 0.5% may prove ineffective (Sommer and Caldas, 1981). The concentration as well as the duration of soaking may be reduced or increased according to the need. Forty five minutes of soaking in 0.5% NaClO was found beneficial by Kunisaki (1980). Concentrations ranging from 1% (Minocha, 1980) to 10% (Kuo and Tsay, 1977) have been used. Jones at al. (1977) observed that apple shoots, which were readily damaged by common surface sterilants became more resistant after a short period of incubation on a culture medium. Generally,

a drop of detergent is added to the surface sterilant. In the surface sterilisation of spruce shoot tips, the addition of detergent was found to increase the toxicity of NaClO to the explants. Alcohol alone or in combination with other surface sterilants has been used for disinfection (Bonga, 1982). Mercuric chloride is another commonly used surface sterilant. Several rinses with sterile distilled water are necessary to remove the sterilants before the excision of the explant (Hu and Wang, 1983).

C. Culture medium

1. Basal medium

A wide variety of media have been reported. The choice depends on the plant species and the intended use of the culture. The Murashige-Skoog medium, characterised by high concentrations of mineral salts has been widely used for general plant tissue culture and specifically for morphogenesis and plant regeneration (Murashige, 1974). The B5 medium which contains lower amounts of mineral salts was found to be preferred by cells of some species (Gamborg and Wetter, 1975).

2. Growth substances

Majority of stage I culture media contains a cytokinin. BA has been the most effective cytokinin for meristem, shoot tip and bud cultures, followed by kinetin

(Murashige, 1974). According to Nair *et al.* (1979), 2-isopentenyl adenine has been used less frequently. A small percent of stage I media has been reported to be without cytokinin (Hu and Wang, 1983). In such cases, they argued that cytokinin may be present in the explants, endogenously, at the required levels. For axillary shoot proliferation, cytokinin has been utilized to overcome the apical dominance of shoots and to enhance the branching of lateral buds from leaf axils (Murashige, 1974). According to Hu and Wang (1983), the concentration of cytokinin in the Stage II media range from 4.5 μM (in about 75% cases) to 25.0 μM or higher (in about 25% cases). A kind of synergism between two cytokinins, kinetin and BA, has been reported in the *in vitro* axillary bud proliferation of teak by Gupta *et al.* (1980). They further observed that at higher concentrations of BA (5.0 mg/l) as well as kinetin (5.0 mg/l), the explants of stage I died. Lo *et al.* (1980) reported that high cytokinin content was deleterious to the initiation and elongation of roots of both monocotyledonous and dicotyledonous plants. Sometimes the residual cytokinin from stage II cultures was found to be high enough to suppress root formation (Ancora *et al.*, 1981). For stage I cultures, exogenous auxin was not always needed. The young shoot apex has been described as an active site for auxin biosynthesis (Hu and Wang, 1983). Although exogenous

auxins do not promote axillary shoot proliferation, culture growth has been improved by their presence (Wang and Hu, 1980). One of the possible roles of auxin in Stage II medium is to nullify the suppressive effect of high cytokinin concentrations on axillary shoot elongation thereby restoring normal shoot growth (Lundergan and Janick, 1980). Too high a concentration of auxin may not only inhibit axillary bud branching but also induce callus formation (Hasegawa, 1980). When GA is supplemented in Stage I cultures its function is primarily bud elongation (Schnabdrauch and Sink, 1979).

D. Culture conditions

Not much studies have been carried out to reveal the optima for the culture conditions such as temperature, light, humidity etc. which influence the growth and development of plant materials in culture. Workers in meristem, shoot tip and bud cultures select a constant incubation temperature ranging between 24°C and 26°C. Higher temperature of 28°C favoured growth and development of coffee callus cells (Monaco et al., 1977). Favre (1977) found that for organogenesis from leaf blades of grapes, a temperature of 29°C and 12 hours photoperiod were more favourable than continuous light. In order to maximise in vitro growth, long photoperiods (12 to 24 hr per day) have been used (Hu and Wang, 1983). A photoperiod of 16 hr

and a light intensity of 1000 to 5000 lux were found to be the optima for the *in vitro* propagation of strawberry (Boxus *et al.*, 1979). Darkness was found to be favourable for callus growth in apple (Chong and Taper, 1974), coffee (Monaco *et al.*, 1977) and grapes (Rajasekharan and Mullins, 1981). Air humidity is infrequently controlled during incubation (Hu and Wang, 1983). When it is controlled, 70% has been found to be the most frequent setting.

E. Polyphenol oxidation

Polyphenolic compounds are present in many plants. When tissues of such plants are injured during dissection, phenolic compounds will be oxidised by polyphenol oxidases and the tissue will turn brown or black. The oxidation products are known to inhibit enzyme activity, kill the explant and darken the tissues and culture media. Such phenomena are serious setbacks in the establishment of primary cultures, especially in woody plants (Hu and Wang, 1983). Some of the procedures used to overcome this problem are addition of antioxidants to the culture medium, presoaking of the explants in an antioxidant before inoculation, incubating the primary cultures in reduced light or darkness for an initial period and frequent subculturing of the explants into fresh medium. Ichihashi and Kako (1977) could control the browning of *Cattleya* shoot tips by the addition of an antioxidant into a liquid medium in stationary

condition. Stevenson and Harris (1980) reported reduction in the discolouration of agar medium with PVP-10 (0.01%) in Fuchsia shoot tip cultures. Gupta et al. (1981) observed during the initial shoot tip culture of teak that the medium turned black and all the explants died. In order to reduce the blackening, the explants were agitated for 45 minutes in a solution of different antibrowning agents in 0.058 M sucrose. The chemicals tested were H_2O_2 (5%), ascorbic acid (0.28 mM), soluble PVP (0.7%) and polyclar AT (0.7%). Blackening was reduced by all the treatments. Multiple shoots, neverthless, were formed only from the explants treated with polyclar AT, an insoluble PVP. A less than 4% browning of the garlic meristem dome explants was obtained by Wang and Huang (1974) when the incubation light intensity during the first month was 150 lux. In order to reduce the accumulation of phenolic oxidates, the initial incubation period (1 to 6 weeks) has been carried out in darkness (Adams et al., 1979; Mc Comb and Newton, 1981; Monaco et al., 1977). The tissue and medium discolouration in thornless blackberry cultures were effectively controlled by Broome and Zimmerman (1978) when the shoot tip explants were transferred to fresh medium one or two days after initial culturing.

III. Rooting of shoots grown in vitro

The elongation of shoots in stage II is sometimes inhibited by high cytokinin level when an intermediate shoot

elongation stage becomes necessary (Hu and Wang, 1983). Wang as quoted by Hu and Wang (1983) reduced BA concentration from 264.0 to 22.0 μM to stimulate the elongation of Sassafras shoots from stage II cultures before transferring them to rooting medium. Stage III does not always have to be carried out in vitro (Mc Cown and Amos, 1979). Kusey et al. (1980) obtained 60% rooting of Gypsophila paniculata by planting shoots in Jiffy peat moss cylinders in green house under intermittent mist. Since auxin is essential for root initiation, majority of stage III media contains auxin as a supplement. Among the common auxins, NAA has been the most effective one for induction of rooting (Ancora et al., 1981; Kitto and Young, 1981). Sometimes, a combination of auxins may give better results (Gupta et al., 1980). According to Hu and Wang (1983), three phases are involved in rhizogenesis (induction, initiation and elongation). The root elongation phase has been found to be very sensitive to auxin concentration. High concentrations of auxin inhibited root elongation (Thimann, 1937). In the procedure developed for in vitro rooting of apple rootstocks, James and Thurbon (1981) cultured the shoots first in an auxin containing root induction - initiation medium for four to eight days. Then, the cultures were transferred to an auxin-free root developing medium. This two-phased procedure effectively prevented callus formation, resulted in 95% rooting and led to a three fold increase in

root number. A 48-hour soaking in an 11.0 μM NAA solution before transfer to White's medium induced root formation in Eucalyptus citriodora (Gupta et al., 1981). MS, B5 and LS are all high N, P and K salt media. Sometimes root initiation fails at such high salt concentration regardless of the types of hormone present. Abundant rooting was observed when the salt concentration in the medium was reduced to one-half, one-third or one-fourth of the standard strength (Kartha et al., 1974; Lane, 1979; Skirvin and Chu, 1979). But in such cases, poor top growth resulted sometimes (Wang, 1978; Gupta et al., 1981).

IV. In vitro cytological changes

Numerical or structural changes in chromosomes are reported to be associated with in vitro regeneration of plants (Larkin and Scowcroft, 1981). Numerical changes have been observed in callus cultures of tobacco (Sacristan and Melchers, 1969), sugarcane (Heinz et al., 1977), maize (Rice, 1982) and tomato (Evans and Sharp, 1983). This variability reflected either pre-existing cellular genetic differences or tissue-culture induced variability (Evans et al., 1984). According to Sacristan and Melchers (1969), longterm cultures resulted in tissue culture-induced variability in chromosome number in both the callus and in the plants regenerated from it. The origin of chromosomal instability of cultured tissue has been

attributed to endo-reduplication (Partanen, 1973), nuclear fusion (Mitra and Steward, 1961) as well as abnormal spindle formation (Sunderland, 1973).

Contrary to the above, Mitra et al. (1960) observed that only diploid plants were regenerated from the callus of carrot having a wide range of chromosome number.

With respect to apical meristem culture, such variations are rare (Ancora et al., 1981). According to Bonga (1982) the meristematic line, consisting of specific cells in more or less fixed position exercises a strict control over the mitotic events. Any mutated cell in a multicellular shoot apex will mostly form only limited areas of tissue which will finally be eliminated from the meristematic region (Hussey, 1978).

V. Planting out of plantlets

Physical, chemical and biological properties of the potting mixture and the atmospheric conditions during post-transfer growth are important in the establishment of *in vitro* regenerated plantlets which have been planted out. Kyte and Briggs (1979) found that a porous potting mixture of peat:perlite:composted bark (1:1:1) was the best for rooting tissue-cultured rhododendrons. They found the depth

of soil to be important, as the survival rate was much greater in 10.16 cm (4") pots than in shallow trays. Thorough washing of the plantlets to remove the traces of nutrient medium and sterilising the potting mixture eliminated serious problems of infection (Anderson, 1980).

Excessive water loss was observed from the leaves of apple plantlets immediately after transplanting (Zimmerman and Broome, 1980). High rate of water loss has been related to the reduced quantities of epicuticular wax (Grout and Aston, 1977), reduced layers of palisade cells (Fabbri et al., 1984) high volume of mesophyll intercellular spaces (Brainerd et al., 1981), and the slowness of stomatal response to water stress(Brainerd and Fuchigami, 1981).

Improper development of vascular connections between the shoot and the roots might also cause poor establishment of the plantlets (Grout and Aston, 1977).

A period of humidity acclimatization was suggested for the newly transferred plantlets to make them adapted to the outside environment (Hu and Wang, 1983). Sutter et al. (1985) found that survival of the plantlets depended as much on the vigorous growth of the newly produced leaves as on the adaptation of the leaves present at the time of planting out.

Barnes (1979) maintained high humidity for the newly transplanted watermelon plantlets under intermittent mist and found the explant survival rate to be poor. Subsequently, the plantlets could be successfully established in the green house by covering them with clear plastic cups to maintain high humidity. The cups were partially lifted for short durations during the second week and later kept removed for five to six hours daily. The cups were completely removed subsequently. Broome and Zimmerman (1978) obtained 60% survival rate in blackberry by growing the plantlets under inverted glass jars for one to three weeks.

MATERIALS AND METHODS

The investigations were carried out at the Plant Tissue Culture Laboratory attached to the Department of Plantation Crops & Spices, College of Horticulture, Vellanikkara during 1981-85 to standardise the micropropagation techniques in jack (*Artocarpus heterophyllus* Lam.) and mussaenda (*Mussaenda erythrophylla* Schum. & Thonn.). Studies were also made to understand the *in vitro* response of the explants of breadfruit (*Artocarpus altilis* L.), pepper (*Piper nigrum* L.) and nutmeg (*Myristica fragrans* Houtt.).

The general *in vitro* cloning procedure adopted in the studies is presented in Fig.1. The stages indicated are as per Murashige (1974).

The details of the procedures adopted for the different species are described in the following pages, stage-wise.

I. Jack (*Artocarpus heterophyllus* Lam.)

A. Culture establishment (Stage I)

Explants were taken from seedlings (4 to 6-week old), grafts (6-month old) and mature trees (5, 10 and 30-year old). They were subjected to the *in vitro* multiplication procedure described in Table 1.

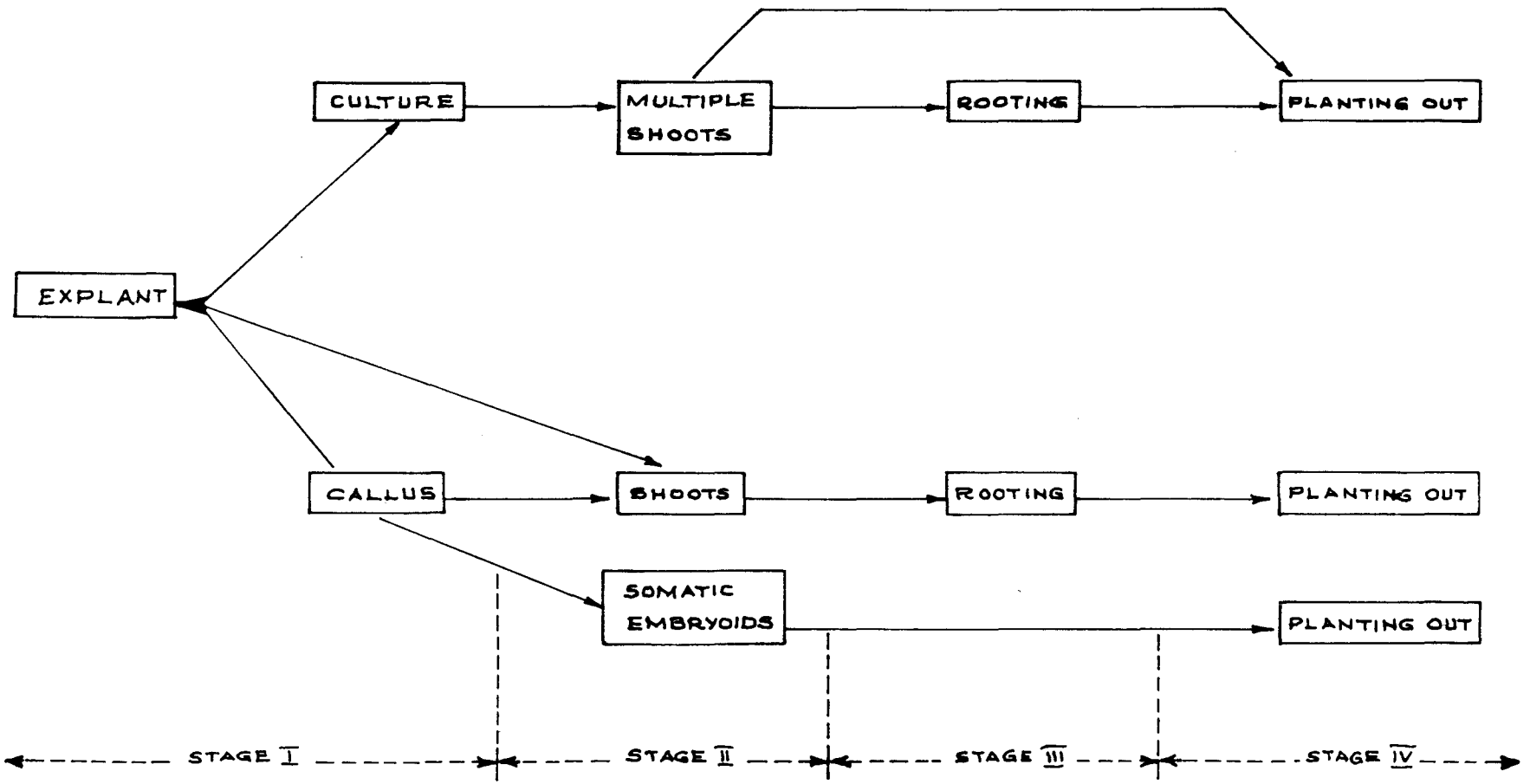


FIG: 1. IN VITRO CLONING PROCEDURE ADOPTED

Table 1. *In vitro* multiplication procedure

Method of multiplication	Explant
1. Enhanced release of axillary buds	1. Shoot tips from seedlings (4 to 6-week old)
	2. Shoot tips and nodal segments with axillary buds from fresh sprouts of 5-year old jack tree
	3. Shoot tips and nodal segments with axillary buds from fresh sprouts of 10-year old jack tree
	4. Shoot tips and nodal segments with axillary buds from fresh sprouts of 30-year old jack tree
	5. Shoot tips and nodal segments with axillary buds from 6-month old jack grafts
2. Somatic organogenesis	1. All the explants used for enhanced release of axillary buds
	2. Leaf segments
	3. Young inflorescences
	4. Root tips (from culture)
3. Somatic embryogenesis	1. Leaf segments
	2. Shoot tips
	3. Internodal segments
	4. Young inflorescences

The explants were dipped in 95% ethyl alcohol for 10 seconds before thorough washing with sterile water. Surface sterilisation was done by keeping them in 2% sodium hypochlorite solution for 30 minutes. Following thorough washing in sterile water, the explants were placed in a solution of 2% sucrose and 0.7% polyvinyl pyrrolidone (insoluble PVP) and agitated for 30 to 45 minutes. The explants were then either subjected to pre-culture treatment by keeping them for 24 hours in a solution containing cytokinin and GA under refrigerated conditions (4-5°C) or directly surface-sterilised with sodium hypochlorite followed by mercuric chloride solutions. A few drops of Teepol were added to the sterilants. The details of the surface sterilisation treatments are given in Table 2.

Table 2. Surface sterilisation treatments

Sterilant	Concentration (%)	Time (minutes)
Sodium hypochlorite	2.00 - 4.00	5 - 15
Mercuric chloride	0.05 - 0.20	5 - 15
Combinations of the best treatment of the above (Serial treatment)		

The explants, after surface sterilisation, were rinsed (at least five times) with sterile water. In the case of shoot tips, the sheaths were removed and the apices cut back to 1.5 cm length, aseptically. The leaves and internodal segments were also aseptically cut into 1.5 cm long segments. The explants were then transferred to the medium.

1. General *in vitro* culture techniques

The chemicals used were of analytical grade from British Drug House (BDH), Sisco Research Laboratories (SRL), Marck or Sigma. Standard procedures (Biondi and Thorpe, 1981) were followed for the preparation of the media. The pH of the media was adjusted to 5.7. Generally semisolid media containing 0.7 g/l agar (BDH) were used. Liquid medium (20.0 ml per test tube, with a filter paper platform) was tried in shoot multiplication studies. Corning brand test tubes and conical flasks were used. Sterilisation of media and conical flasks was done at 15 psi for 15 - 20 minutes. All aseptic manipulations were carried out in a Laminar air flow chamber. Cultures were incubated at 26 $\pm$ 2^{o}C with a 16-hour photoperiod (1000 lux), supplied by cool day light fluorescent tubes.

All the trials on Stage I and Stage III were carried out on Murashige and Skoog (1962) basal medium (MS medium). For the trials on Stage II MS and Anderson's (1980) media were used.

Details of the culture establishment trials (Stage I) are presented in Table 3.

2. Observations (Stage I)

a. Establishment of shoot tips and lateral buds

The observations on per cent survival (cultures which were alive) and per cent growing cultures were made on five explants per treatment after three weeks of inoculation and the means worked out.

b. Somatic organogenesis - callus production

Each treatment combination was tried on five explants and after three weeks of inoculation, observations were made on per cent explants initiating callus and growth of the callus.

Growth of the callus was assessed based on a visual rating (with score 1 to the smallest and score 4 to the largest callus). The mean score was expressed as the growth score (G).

Table 3. Culture establishment trials

Method of multiplication	Explant	Treatment
1. Enhanced release of axillary buds	1. Shoot apex (fresh)	1. 3x3 combination of NAA (0.1, 0.5, 1.0 μM) and kinetin (2.5, 5.0, 10.0 μM)
		2. 3x3 combination of NAA (0.1, 0.5, 1.0 μM) and BA (2.5, 5.0, 10.0 μM)
		3. 2x5 combination of NAA (0.2, 1.0 ppm) and BA (0.5, 1.0, 2.0, 10.0, 20.0 ppm)
		4. 3x3 combination of IAA (0.1, 0.5, 1.0 μM) and BA (2.5, 5.0, 10.0 μM)
		5. 3x3 diallel set of BA (0.1, 0.2, 0.3 ppm) and kinetin (0.1, 0.2, 0.3 ppm)
		6. 3x3 combination of GA (0.5, 1.0, 2.0 ppm) and activated charcoal (0.25, 0.5, 1.0%)
		7. GA 1.0 ppm (without activated charcoal)*
	2. Lateral bud (fresh)	1. 2x2 combination of GA (1.0, 2.0 ppm) and activated charcoal (0.5, 1.0%)

(Contd.)

* Since under treatment 6, GA 1.0 ppm gave promising results

Note: NAA : 1 μM = 0.182 ppm BA : 1 μM = 0.225 ppm
Kinetin: 1 μM = 0.215 ppm IAA : 1 μM = 0.175 ppm

Table 3 (continued)

Method of multiplication	Explant	Treatment
2. Somatic organogenesis (induction)		
a. Callus production	1. Shoot tip (from culture) 2. Internodal segment (from culture) 3. Leaf segments (from culture) 4. Root tip (from culture)	1. 4x3 combination of NAA (1.0, 2.0, 4.0, 8.0 ppm) and kinetin (1.0, 2.0, 4.0 ppm) 2. 4x3 combination of NAA (1.0, 2.0, 4.0, 8.0 ppm) and BA (1.0, 2.0, 4.0 ppm) 3. 4x3 combination of 2,4-D (0.1, 0.5, 1.0, 2.0 ppm) and BA (1.0, 2.0, 4.0 ppm)
b. Direct organogenesis (induction)	1. Shoot tip (from culture) 2. Internodal segment (from culture) 3. (a) Leaf segment (from culture) Lower half of lamina with petiole (b) Leaf segment (from culture) Upper half of lamina	1. Combination of kinetin (0.5 ppm) and BA (0.1, 0.5, 1.0, 2.0, 5.0 ppm) 2. Combination of BA (0.5 ppm) and kinetin (0.1, 0.5, 1.0, 2.0, 5.0 ppm)
3. Somatic embryogenesis (induction)	1. Shoot tip (from culture) 2. Internodal segment (from culture) 3. Leaf segment (from culture) 4. Young inflorescence (fresh)	1. 2,4-D (0.1, 0.5, 1.0, 2.0 ppm) 2. 4x3 combination of 2,4-D (0.1, 0.5, 1.0, 2.0 ppm) and kinetin (1.0, 2.0, 4.0 ppm)

Callus Index (CI) was computed by multiplying per cent explants initiating callus with the growth score. This was worked out to estimate the overall effectiveness of the treatments to induce and support the growth of callus.

c. Direct organogenesis

After five weeks of inoculation, observations were made on per cent explants initiating direct organogenesis and the multiplication rate (the number of shoots produced by an explant during the observation period).

The above observations were made on five explants per treatment and the means worked out.

d. Somatic embryogenesis

After four weeks of inoculation, observations were recorded (three explants per treatment) on percent explants initiating callus and the growth of the callus.

Growth score (G) and callus index (CI) were worked out as stated earlier.

B. Multiplication of propagules (Stage II)

1. Enhanced release of axillary buds

a. Standardisation of basic proliferation medium

A preliminary experiment with 24 treatment combinations involving BA (2.5, 5.0, 7.5, 10.0, 20.0, 40.0 ppm)

and NAA (0.1, 0.2, 0.4, 0.8 ppm) was conducted to standardise a basic proliferation medium (BPM) for the stimulation of axillary shoot production from the shoot tip cultures.

Proliferation of lateral bud was tested with the treatment combination identified as the most effective for proliferation in shoot apex culture.

Another experiment was conducted for the enhanced release of axillary buds from the shoot tip cultures of seedlings, 10-year old tree, 30-year old tree and 6-month old grafts. This experiment had treatment combinations involving BA (2.5, 5.0, 7.5, 10.0, 12.5, 20.0 and 40.0 ppm) and NAA (0.2 ppm) in semisolid medium and BA (5.0 ppm) and NAA (0.2 ppm) in liquid medium.

b. Shoot proliferation and growth of the shoot tip cultures

Studies were conducted to determine the effects of various compounds on the multiplication rate and growth of the shoot tip cultures. The basal media employed, the supplements and the different test compounds are indicated in Table 4. BA 5.0 ppm and NAA 0.2 ppm were used as supplements to the basal media for these trials based on the results of the standardisation of BPM. Single shoot apices from the proliferating cultures were used as explants.

Table 4. Various compounds (auxins; cytokinins; cytokinin related substances; gibberellin; amino acid supplement; carbon sources; inorganic and organic components of MS medium) and Anderson's medium tested on the multiplication rate and growth of shoot apex cultures from 5-year old jack trees

Basal medium	Supplemented with	Treatment		Level
1. MS	BA 5.0 ppm	Auxins	IAA, NAA, 2,4-D	0.1, 0.2, 0.4 ppm
2. (a) MS	NAA 0.2 ppm	Cytokinins	BA, kinetin	2.5, 5.0, 7.5, 10.0, 20.0, 40.0 ppm
(b) MS	"	"	"	3x3 diallel set of 0.1, 0.5, 1.0 ppm
3. MS	BA 5.0 ppm + NAA 0.2 ppm	Cytokinin related substances	Adenine sulphate, adenine	10.0, 20.0, 40.0, 80.0, 160.0 ppm.
4. MS	"	Gibberellin	GA_3	1.0, 2.0, 4.0, 8.0, 16.0 ppm
5. MS	"	Amino acid supplement	Casein hydrolysate	50.0, 100.0, 500.0, 1000.0, 2000.0 ppm
6. MS	"	Carbon source	Sucrose, glucose	1.0, 2.0, 3.0, 4.0%
7. MS organic growth factors + sucrose 3%	"	Inorganic salts of MS medium (macro and micro)		1/4, 1/2, 1.0, 2.0 times normal strength
8. MS inorganic salts + sucrose 3%	"	Organic growth factors of MS medium (inositol, pyridoxine HCl, thiamine HCl, Glycin, nicotinic acid)		"
9. Anderson's	"	—		—

2. Organogenesis callus mediated

Combinations of NAA (0.1, 0.5, 1.0 ppm) and kinetin (0.5, 1.0, 2.0, 4.0, 8.0 ppm) as well as NAA (0.1, 0.5, 1.0 ppm) and BA (0.5, 1,0, 2.0, 4.0, 8.0 ppm) were tried to enhance the production of shoots from callus of shoot tip cultures. The basal medium was MS. Callus from the first subculture was used for the study.

3. Somatic embryoid formation

The explants/calli from the induction medium were transferred to MS medium containing 3 x 3 diallel combination of BA (0, 0.5, 1.0 ppm) and kinetin (0, 0.5, 1.0 ppm) for the formation of somatic embryoids.

a. Observations

i. Basic proliferation medium.

There were four cultures per treatment combination. Observations on the number of shoots, the length of the longest shoot and the length of the longest leaf were recorded after five weeks of culture. The performance of each treatment was also visually rated in the range of 1 (small and compressed shoots) to 2 (fairly elongated shoots). The multiplication rate (the number of shoots produced per explant during an observation period) of the shoot cultures

in the BPM was also examined for 10 continuous subcultures done at four-week interval.

ii. Regulation of shoot proliferation and growth of shoot tip culture

Observations on the number of shoots, the length of the longest shoot and the length of the longest leaf were recorded after five weeks of culture. The performance of each treatment was also visually rated in the range of one to two as mentioned above.

iii. Organogenesis (Callus mediated)

Ten callus pieces were tried per treatment and organogenesis, if any, recorded after 60 days of culture.

iv. Somatic embryoid formation

Embryoid formation on three cultures per treatment was observed after 60 days of culture.

C. Standardisation of medium favouring shoot elongation

An experiment with treatment combinations involving BA (1.0, 2.0, 3.0 and 4.0 ppm) and IAA (0.1, 0.2 and 0.4 ppm) and BA (2.0 ppm) and NAA (0.1, 0.2 and 0.4 ppm) was conducted to standardise a medium which favoured the elongation of shoot in the cultures transferred from the proliferation medium.

1. Observations

There were four cultures per treatment. Observations (on the number of shoots, length of the longest shoot as well as the length of the longest leaf) were recorded after five weeks of culture.

D. In vitro rooting (Stage III)

The different factors (and their levels) tried for rooting of jack shoots of various origin are listed in Table 5. As a pre-treatment, the shoots from the medium favouring normal growth (elongation medium) were cultured for one to two weeks on MS medium supplemented with activated charcoal (0.1%) in reduced light intensity. From this culture, shoots > 1.0 cm in length were selected and used for the rooting experiment.

1. Observations

Number of days taken for root initiation was recorded. In addition, per cent cultures with roots, number of primary roots and intensity of root branching (based on a visual rating ranging from 1 to 3 (1 : poor; 2 : moderate; 3 : profuse) were recorded after 30 days of culture. Abnormalities on root and shoot growth were also recorded as and when observed.

Table 5. Compounds and physical conditions tried for the *in vitro* rooting of jack shoots of different sources.

Source of the original explant which gave rise to the shoots tested	Treatment	Basal medium
1. Six-week old seedling	a. Auxins 1. IBA (0.2 ppm) 2. IBA (0.8 ppm) 3. IBA (1.6 ppm) 4. IBA (8.0 ppm) 5. NAA (1.0 ppm) 6. IAA (10.0 ppm) 7. 2,4-D (0.1 ppm) 8. NAA (0.05 ppm) + IBA (0.4 ppm) 9. NAA (0.4 ppm) + IBA (0.4 ppm) 10. NAA (0.5 ppm) + IBA (0.2 ppm) 11. NAA (2.0 ppm) + IBA (0.4 ppm) 12. IAA (0.1 ppm) + IBA (0.1 ppm)	MS (with 1/2 concentration of mineral salts)
2. Five-year old tree	1. IBA (8.0 ppm) 2. NAA (1.0 ppm) 3. NAA (2.0 ppm) 4. IAA (10.0 ppm) 5. 2,4-D (0.1 ppm) 6. NAA (0.05 ppm) + (IBA 0.4 ppm) 7. NAA (0.4 ppm) + (IBA 0.2 ppm) 8. NAA (0.4 ppm) + (IBA 0.4 ppm) 9. NAA (0.4 ppm) + (IBA 0.8 ppm) 10. NAA (0.4 ppm) + (IBA 1.6 ppm) 11. NAA (0.5 ppm) + (IBA 0.2 ppm) 12. NAA (2.0 ppm) + (IBA 0.4 ppm) 13. NAA (2.0 ppm) + (IBA 2.0 ppm) for six days and then shoots transferred to 1/2 MS without growth substances 14. IAA (0.1 ppm) + IBA (0.1 ppm)	-do-
3. Ten-year old tree	1. NAA (0.4 ppm) + IBA (1.6 ppm) 2. NAA (2.0 ppm) + IBA (2.0 ppm) for six days and then shoots transferred to 1/2 MS without growth substances	-do-
4. Thirty-year old tree	-do-	-do-
5. Six-month old graft	-do-	-do-

(Contd.)

Table 5 (Contd.)

(1)	(2)		(3)
	b. MS medium		
1. Five-year old tree	1. Mineral salts (both macro and micro nutrients)	1/4, 1/2, 1 and 2 times of the normal MS salt concentration	MS organic growth factors + sucrose 3% + IBA (1.6 ppm) + NAA (0.4 ppm)
	2. Organic growth factors (inositol, pyridoxine HCl, thiamine HCl, glycine and nicotinic acid)	1/4, 1/2, 1 and 2 times of the normal MS organic growth factor concentration	1/2 MS salts + sucrose 3% + IBA (1.6 ppm) + NAA (0.4 ppm)
	3. Sucrose	1, 2, 3 and 4%	1/2 MS + IBA (1.6 ppm) + NAA (0.4 ppm)
	c. Physical conditions		
	1. Agar	0.4, 0.6, 0.8 and 1%	-do-

E. Planting out of plantlets/shoots to soil (Stage IV)

The influence of the following factors on the survival and establishment of the plantlets (shoots that had rooted *in vitro*) was assessed.

1. After rooting *in vitro*

a. Potting mixture

The following potting media were used for the study.

(i) Sand + soil (1:1 v/v) in mud pots
(ii) Sand + soil + cowdung (1:1:1 v/v) in mud pots
(iii) Vermiculite in mud pots
(iv) Coir dust in mud pots
(v) Sand + soil + coir dust (1:1:1 v/v) in mud pots
(vi) Trino-2 commercial pots (from Trico India Pvt.Ltd.
(vii) Sphagnum moss + soil (1:1 v/v) in mud pots

The potting mixtures were autoclaved at 15 psi for 20 minutes and used after 10 days. The cultures, immediately after visible root initiation (1-2 mm long roots) were exposed to higher light intensity (3500 lux) for a period of one week, after which transplanting was done to the potting media. Just before transplanting and at weekly intervals afterwards, the potting mixtures were drenched with Bavistin (0.06%).

b. Age of the plantlets at transplanting

The survival rate of the plantlets of different ages (0, 1, 2, 3 and 4 weeks after root initiation) were observed in vermiculite, which was identified as the best medium.

c. Light intensity

The plantlets just after the visible root initiation were subjected to different light intensities (250 lux, 1000 lux and 3500 lux) for one week. They were then transplanted to vermiculite to observe the effect of light intensity (before transplantation) on their survival.

d. Humidity control

Different methods were tried for maintaining high humidity around the transplanted plantlets for a period of three weeks after the transplanting. The methods consisted of covering the pots with glass beaker, placing the pots in petriplates containing sterile water and covering them with glass beaker, placing the pots, in plastic basins containing a thin layer of sand and covering them with microscope covers (thick transparent plastic covers having 60 cm height and 45 cm diameter at the base) and placing the pots under a pyramid shaped metallic structure (1.0 x 1.0 x 0.75 m) and

covering them with polythene sheet. Intermittent spraying of water with a handsprayer was done in the latter two cases.

e. Growth substances

Sterile solutions of ABA (0.03 ppm), BA (10.0 ppm), kinetin (10.0 ppm) and NAA (1.0 ppm) were sprayed on to the plantlets (transplanted to vermiculite in mud pots) to study their effects on the survival and establishment of the plantlets. The above solutions (containing a wetting agent, Teepol) were sprayed at weekly intervals for four weeks.

2. Direct transplanting of shoots from the elongation medium

The shoots grown in vitro (in elongation medium) were treated with IBA at five concentrations (5.0, 50.0, 100.0, 200.0 and 400.0 ppm) and transplanted to a standard potting mixture (sand:soil:cowdung 1:1:1 v/v) to see whether or not direct transplanting in a non-sterile medium would succeed. Shoots > 2.0 cm in length were selected and dipped in sterile IBA solution for 24 hours before transplanting. There were five shoots per treatment.

To maintain the humidity at the desired level, the pots were placed in plastic basins containing a thin layer of sand, and covered with microscope covers. Intermittent

spraying of water was also done. The other treatments were not attempted as they were not successful with plantlets.

3. Effect of nutrient starter solutions in enhancing the growth of the plantlets

The following starter solutions were tried.

i) MS nutrient solution (with macro and micronutrients) at half strength, having a pH of 5.7

ii) A fertilizer solution (NPK 10:52:10 g/l) having a pH of 5.7

iii) Tap water as the control

Watering of each pot with 5 ml of the above solutions was done at weekly intervals. Observations were made on four plantlets per treatment on the height of the plantlets, the number of leaves as well as the length and breadth of the leaves, three months after planting out.

4. Histological investigations

For the anatomical examination of the leaves of the plantlets (just before planting out and after hardening) and the field grown plants (to observe the difference, if any), the leaves were collected, and fixed in FAA (formalin 5 ml, acetic acid 5 ml and 70% ethanol 90 ml) for 24 hours. The fixed material was dehydrated in alcohol series, cleared

in xylol and embedded in paraffin. Sections (8 µ - 15 µ thick) were cut on a rotary microtome, stained in 0.5% Haematoxylin and mounted in canada balsam (Johansen, 1940).

5. Cytological examination of root tip squashes

Roots (2 to 3 mm in length) were excised, pretreated with 0.002 M 8-hydroxyquinoline for five hours and fixed in Carnoy's fluid (1:3 acetic acid: ethanol) for 48 hours at 10°C. The root tips were then hydrolysed for 12 minutes in IN HCl at 60°C and stained for 45 to 60 minutes with basic Fuchsin. Squashing of the root tips was done in 2% aceto-orcein. Metaphase plates were observed for numerical changes of chromosomes (Sharma and Sharma, 1980).

F. Statistical analysis

The data generated from the various experiments were subjected to statistical analysis in completely randomised design, wherever necessary, as per Panse and Sukhatme (1978).

G. Economics of production of jack plantlets

The cost of production of jack plantlets using explants from fresh stem sprouts of five-year old trees was worked out based on the facilities of the Tissue Culture Laboratory at the College of Horticulture, having a potential

of maintaining 4200 cultures for multiplication, 4200 cultures for shoot elongation, 4200 cultures for rooting and 7000 plantlets for hardening. One scientist (Rs.1400/= p.m.) and one Technician (Rs.1000/= p.m.) were considered necessary for the work. Based on the rate of culture establishment, rate of multiplication, rooting response of the shoots, the survival of the plantlets and the maximum capacity of the Laboratory, the number of plantlets which can be produced per year from 100 initial explants was estimated. The total cost involved per year was worked out based on the cost of building, equipment, glasswares, chemicals and miscellaneous items having been distributed over the years according to their potential/durability. The cost of production of one jack plantlet including one month's hardening was finally worked out.

II. Mussaenda

A. Culture establishment (Stage I)

The plants of Mussaenda erythrophylla Schum.& Thonn. available at the ornamental garden of the College of Horticulture, Vellanikkara were utilised for the study. The plants were quite mature and were more than five years old. The different explants were subjected to the in vitro multiplication procedures as described in Table 6.

Table 6. *In vitro* multiplication procedure

Method of multiplication	Explant
1. Enhanced release of axillary buds	1. Shoot tips 2. Axillary buds
2. Somatic organogenesis	1. Shoot tips 2. Segments of leaves (from culture) 3. Internodal segments 4. Segments of ovary wall
3. Somatic embryogenesis	1. Segments of leaves (from culture) 2. Shoot tips 3. Segments of ovary wall

The explants were collected and washed in sterile distilled water several times. They were then agitated in a solution of 50 ppm ascorbic acid for 30 minutes. It was followed by surface sterilisation with mercuric chloride solution (0.1% for 15 minutes). The summary of the surface sterilisation treatments is given in Table 7.

Table 7. Surface sterilisation treatments

Sterilant	Concentration (%)	Time (minutes)
Mercuric chloride	0.05 - 0.20	5 - 15
Sodium hypochlorite	2.00 - 4.00	5 - 15

The explants after surface sterilisation were rinsed (at least 5 times) with sterile water. In the case of shoot tips, the larger leaves were removed and the apices cut back to 1.0 cm length, aseptically. The leaves, internodal segments and ovary wall were also aseptically cut into 1.0 cm long segments. The explants were then transferred to the medium. Murashige and Skoog (1962) medium and Anderson's (1980) medium were used for the studies.

The general <u>in vitro</u> techniques were the same as adopted in the case of jack.

Details of the culture establishment trials are presented in Table 8.

1. Observations (Stage I)

a. Establishment of shoot tips and lateral buds

The observations on per cent survival (cultures which are alive) and per cent growing cultures were made on five explants per treatment after three weeks of culture.

b. Somatic organogenesis - callus production

Each treatment combination was tried on three explants and after four weeks of culture observations were made on per cent explants initiating callus and growth of callus.

Growth of callus was assessed based on a visual rating (with score 1 to the smallest and score 4 to the largest callus). The mean score was expressed as the growth score (G).

Callus Index (CI) was worked out by multiplying per cent explants initiating callus with the growth score for estimating the overall effectiveness of treatments to induce and support the growth of callus.

Table 8. Culture establishment tsial

Method of multiplication	Explant	Growth regulator treatments
1. Enhanced release of axillary buds	1. Shoot tip (fresh)	1. Kinetin (0.5, 1.0, 2.0 ppm) 2. BA (0.5, 1.0, 2.0 ppm) 3. 4x4 diallel set of BA (0.1, 0.3, 0.5, 1.0 ppm) and kinetin (0.1, 0.3, 0.5, 1.0ppm) 4. 3x3 combinations of kinetin (2.5, 5.0, 10.0) and NAA (0.1, 0.5, 1.0 M)
	2. Axillary bud (fresh)	Best treatment identified for shoot tip
2. Somatic organogenesis		
a) Callus production	1. Shoot tip (fresh)	1. 4x3 combinations of NAA (1, 2, 4 and 8 ppm) and kinetin (1, 2 and 4 ppm)
	2. Segments of leaves (from culture)	2. 4x3 set of NAA (1, 2, 4 and 8 ppm) and BA (1, 2 and 4 ppm)
	3. Segments of ovary wall (fresh)	3. 4x3 set of IAA (1, 2, 4 and 8 ppm) and BA (1, 2 and 4 ppm)
b) Direct organogenesis (induction)	1. Shoot tip (fresh)	1. Combinations of BA (0.1, 0.5, 1.0, 2.0, 5.0 ppm) and kinetin (0.5 ppm).
	2. Internodal segments (fresh)	2. Combinations of kinetin (0.1, 1.0, 2.0, 5.0 ppm) and BA (0.05 ppm).
	3. Leaf segments (from culture) Lower half of lamina with petiole	
3. Somatic embryo induction	1. Shoot tip (fresh)	1. 2,4-D (0.1, 0.5, 1.0, 2.0 ppm)
	2. Leaf segments (from culture)	2. 4x3 combinations of 2,4-D (0.1, 0.5, 1.0, 2.0 ppm) and kinetin (1.0, 2.0, 4.0 ppm)

c. Direct somatic organogenesis

After three weeks of culture observations were made on per cent explants initiating direct organogenesis and multiplication rate (the number of shoots produced by an explant during the observation period).

The above observations were made on three explants per treatment and the mean worked out.

d. Somatic embryogenesis

After four weeks of inoculation observations were recorded (three explants per treatment) on per cent explants initiating callus and growth of the callus.

Growth score (G) and Callus Index (CI) were worked out as stated earlier.

B. Multiplication of propagule (Stage II)

1. Enhanced release of axillary buds

a. Standardisation of basic proliferation medium (BPM)

A preliminary experiment with treatment combinations involving BA 0.1, 0.5, 1.0 ppm and kinetin 0.1, 0.5, 1.0 ppm was conducted to standardise a basic proliferation medium for the stimulation of axillary shoots from shoot tip cultures. Serial subculturing for five times at 4-week interval was done in the basic proliferation

medium to understand the effect on multiplication rate. Proliferation of lateral bud was tested with the best treatment combination identified as the most effective for the proliferation of shoot apex culture.

b. Shoot tip proliferation and growth of shoot tip cultures

Studies were conducted to determine the effect of various compounds on the multiplication rate and growth of shoot tips cultures. The basal media employed, the supplements and the different test compounds are indicated in Table 9.

BA 0.5 ppm and kinetin 0.5 ppm used in the basic media for these trials were fixed based on the results of the standardisation of BPM.

2. Organogenesis (callus mediated)

Combinations of kinetin (0.1, 0.3, 0.5, 1.0 ppm) and BA (0.1, 0.3, 0.5, 1.0 ppm); kinetin (1.0, 2.0, 4.0 ppm) and NAA (0, 0.5, 1.0, 2.0, 4.0, 8.0 ppm); BA (1.0, 2.0, 4.0 ppm) and NAA (0, 0.5, 1.0, 2.0 ppm) were tried to induce shoot/ root from callus of shot tip cultures. The basal medium was MS. Callus from the first subculture was used for the study.

Table 9. Various compounds (auxins; cytokinin related substances; carbon source; inorganic components of MS medium) and Anderson's medium tested on the multiplication rate and growth of musaenda shoot apex cultures

Basal medium	Supplemented with	Treatments	Level
1. MS	BA 0.5 ppm + kinetin 0.5 ppm	Auxins NAA, IAA	0.1, 0.2, 0.4 ppm
2. MS	BA 0.5 ppm + kinetin 0.5 ppm	Cytokinin-related substance Adenine sulphate	10, 20, 40, 80 ppm
3. MS	BA 0.5 ppm + kinetin 0.5 ppm	Carbon source Sucrose	2,3,4,5%
4. MS organic growth-factors + sucrose 3%	BA 0.5 ppm + kinetin 0.5 ppm	Inorganic salts of MS medium (macro and micro)	1/4, 1/2, 1.0, 2.0 times-normal strength
5. Anderson's	BA 0.5 ppm + kinetin 0.5 ppm	-	-

3. Somatic embryoid formation

The explants/calli from the induction medium were transferred to MS medium containing 3x3 diallel combinations of BA (0, 0.5 and 1.0 ppm) and kinetin (0,05 and 1.0 ppm).

4. Observations

a. Apical shoot multiplication

There were four cultures per treatment combination. Observations on the number of shoots and length of the longest leaf were recorded after four weeks of culture. Observations on five cultures per subculturing was made four weeks after subculture on the number of shoots produced, for the serial subculturing trial.

b. Regulation of shoot proliferation and growth of shoot tip culture

There were four to five cultures per treatment combination. Observations on the number of shoots, number of leaves and length of the longest leaf were recorded after four weeks of culture.

c. Organogenesis (Callus mediated)

Observations on organogenesis were taken after 60 days of culture on three cultures per treatment.

8. Somatic embryoid formation

Somatic embryoid formation on 15 cultures per treatment was observed after 80 days of culture.

C. *In vitro* rooting (Stage III)

The different factors (and their levels) tried for rooting of mussaenda shoots are listed in Table 10. Shoots of > 1.0 cm length were selected from the proliferation medium and used for the rooting experiment.

1. Observations

Number of days taken for root initiation was recorded on five shoots per treatment. In addition, per cent cultures with roots and the number of primary roots were recorded after 50 days of culture. Abnormalities on the root and shoot growth were also recorded as and when observed.

D. Planting out of plantlets/shoots to soil (Stage IV)

The influence of the following factors on the survival and establishment of the plantlets (shoots that had rooted *in vitro*) was assessed.

Table 10. Various factors tested (Auxins; inorganic components of MS medium; carbon source; agar, Anderson's rooting medium) on the *in vitro* rooting of mussaenda shoot cultures

Basal medium	Supplemented with	Treatment	Level
1. MS (with 1/2 concentration of inorganic salts)	-	3x3 combination of IBA and NAA	0.4, 0.8, 1.6 ppm
2. MS organic growth-factors	Sucrose 3% + IBA 0.4 ppm + NAA 0.4 ppm	MS inorganic salts (macro and micro)	1/4, 1/2, 1, 2 times the normal strength
3. MS	IBA 0.4 ppm + NAA 0.4 ppm	Carbon source Sucrose	1, 2, 3, 4%
4. MS	Sucrose 3% + IBA 0.4 ppm + NAA 0.4 ppm	Agar	0.4, 0.6, 0.8, 1.0%
5. Anderson's	Sucrose 3% + IBA 0.4 ppm + NAA 0.4 ppm	-	-

I. After rooting in vitro

a. Potting medium

The following potting media were used for the study.

(i) Sand + soil + cowdung (1:1:1 v/v) in mud pots

(ii) Vermiculite in mud pots

(iii) Trice-2 commercial pots (from Trice India Pvt.Ltd.).

There were seven plantlets per treatment.

The potting mixtures were autoclaved at 15 psi for 20 minutes and used after 10 days. After root initiation the cultures were exposed to higher light intensities (3500 lux) for one week, after which transplanting was done to the potting mixtures. Just before transplanting and at weekly intervals afterwards, Bavistin (0.06%) was drenched to the pots.

b. Humidity control

Different methods were tried for maintaing high humidity around the transplanted plantlets for a period of three weeks after transplanting. The methods consisted of covering the pots with glass beaker, placing the pots in petri plate containing sterile water and covering them with glass beaker and placing the pots in plastic basins containing a thin layer of sand and covering them with microscope covers (thick transparent plastic structure with 60 cm height and 45 cm

diameter at the base). In the latter case intermittent sprays of water with a handsprayer was done.

2. Direct transplanting of shoots from the proliferation medium.

The shoots grown in vitro were treated with IBA at 5 concentrations (5, 50, 100, 200 and 400 ppm) and transplanted to a standard potting mixture (sand:soil:dried, powdered cowdung 1:1:1 v/v) to observe whether or not direct transplanting in a non-sterile medium would be successful. Shoots 2.0 cm in length were selected and dipped in sterile IBA solution for 24 hours before transplanting. There were five shoots per treatment.

E. Statistical analysis

The data generated from the various trials were subjected to statistical analysis, in completely randomised design, wherever necessary, as per Panse and Sukhatme (1978).

III. Breadfruit (Artocarpus altilis L.)

Culture establishment (Stage I) studies were made, utilising mature breadfruit trees above 10 years old available at Mannuthy. Due to the problem of phenolics interference and weak explant response the studies were not continued beyond Stage I.

The different explants were subjected to culture establishment treatments as given in Table 11.

Table 11. *In vitro* multiplication procedures

Method of multiplication	Explant
1. Enhanced release of axillary buds	1. Shoot tips from mature breadfruit trees (above 10 years old)
2. Somatic organogenesis (Callus mediated)	1. Shoot tips 2. Young inflorescences
3. Somatic embryogenesis	1. Shoot tips

The explants were dipped in 95% ethanol for 10 seconds and then thoroughly washed with sterile water. They were agitated in a solution of 2% sucrose and 0.7% PVP 360 for 30 minutes. The explants were then surface sterilised with NaClO followed by mercuric chloride solutions. The details of the surface sterilisation treatments are given in Table 12.

Table 12. Surface sterilisation treatments of breadfruit explants

Sterilant	Concentration (%)	Time (minutes)
Sodium hypochlorite	1.5 - 3.0	10 - 20
Mercuric chloride	0.1 - 0.2	10 - 15
Combination of the best treatments of the above (Serial treatment)		

The explants after surface sterilisation were rinsed (at least 5 times) with sterile water. In the case of shoot tips the sheaths were removed and the apices cut back to 2.0 cm length aseptically. The root tips and young inflorescence were also aseptically cut into 2.0 cm long segments. The explants were then transferred to the media. Murashige and Skoog (1962) basic medium was used for all the trials. The general *in vitro* culture techniques adopted were as described for jack.

The details of the treatments of the culture establishment trial are given in Table 13.

A. Observations

I. Enhanced release of axillary buds

Observations were made on five explants per treatment after 3 weeks of culture period on per cent survival (cultures which are alive) and per cent growing cultures (fresh green and growing).

2. Somatic organogenesis (callus mediated)

Each treatment was tried on five explants and after 3 weeks of inoculation observations were made on per cent explants initiating callus and growth of the callus.

Table 13. Culture establishment trial

Method of multiplication	Explant	Treatment
1. Enhanced release of axillary buds	1. Shoot tip	1. 2x2 diallel set of kinetin (1.0 and 2.0 ppm) and NAA (1.0 and 2.0 ppm)
		2. BA (2.0 and 10.0 ppm)+ GA 1.0 ppm+ activated charcoal 1.0%
		3. BA (0.5 ppm) and kinetin (0.5 ppm) + GA 1.0 ppm+ activated charcoal 1.0%
		4. BA (2.0 ppm) and IAA (0.5 ppm) + GA 1.0 ppm + activated charcoal 1.0%
2. Somatic organogenesis (callus mediated)	1. Shoot tip	1. 2x2 diallel set of kinetin (1.0 and 2.0 ppm) and NAA (1.0 and 2.0 ppm)
	2. Young inflorescence	1. 2,4-D 1.0 ppm+ kinetin 1.0 ppm.
3. Somatic embryogenesis (induction)	1 Shoot tip	1. 2,4-D (1.0 ppm) + activated charcoal 1.0%

3. Somatic embryogenesis

After four weeks of inoculation observations were made on five explants per treatment on per cent explants initiating callus and growth of the callus.

IV. Pepper (*Piper nigrum* L.)

Stage I and Stage II studies were attempted utilising three year old pepper vines available at the College of Horticulture, Vellanikkara. Due to severe problem of systemic bacterial presence in the explants the study could not be continued.

Shoot apices and nodal segments of about 2.0 cm were used for culture establishment studies. The explants were dipped in 95% ethanol for 10 seconds and thoroughly washed with sterile water. The explants were agitated in a solution of 2% sucrose and 0.7% PVP 360 for 30 minutes and then surface sterilised. The details of the surface sterilisation treatments is given in Table 14.

Table 14. Surface sterilisation treatments of pepper explants

Sterilant	Concentration (%)	Time (minutes)
1. Sodium hypochlorite	1.5 - 3.0	10 - 20
2. Mercuric chloride	0.1 - 0.3	10 - 20
3. Combination of the best treatment of the above (serial treatment)		

The explants after surface sterilisation were rinsed (at least 5 times) with sterile water. The shoot tips and nodal segments were cut back to 2.0cm aseptically and transferred to the media. MS basal medium was used for the trials. The general *in vitro* techniques adopted were as described for jack. The details of the culture establishment trial are given in Table 15.

Table 15. Culture establishment trial on pepper explants

Method of multiplication	Explant	Treatment
1. Somatic organogenesis (callus mediated)	1. Shoot tip	1. 3x3 diallel set of kinetin (0, 1.0 and 2.0 ppm) and NAA (0, 1.0 and 2.0 ppm)
		2. 2,4-D (1.0 ppm)
		3. 2,4-D (1.0 ppm) and kinetin (1.0 ppm)
	2. Nodal segment	1. Kinetin (1.0 ppm) and NAA (2.0 ppm)

A. Observations

Each treatment was tried on four explants and after three weeks observations were made on per cent explants initiating callus and growth of the callus.

The details of stage II experiments are given in Table 16.

Table 16. Stage II trials

Method of multiplication	Explant	Treatment
1. Somatic organogenesis (Callus mediated)	1. Callus (from shoot tip)	1. 3x3 diallel set of kinetin (0,1 and 2 ppm) and NAA (0,1 and 2 ppm)
	2. Callus (from nodal segment)	2. Kinetin (0.5 ppm) and BA (0.5 ppm)

B. Observations

Each treatment was tried on four explants and after three weeks observations were made on per cent culture showing shoot differentiation, per cent cultures showing root differentiation and number of roots per explant.

V. Nutmeg (*Myristica fragrans* Houtt.)

Stage I studies were made utilising the mature nutmeg plants available at Mannuthy. Due to the problems of phenolics and weak explant response the studies were not continued.

Shoot tips (about 2.0 cm) were used as explants for micropropagation through enhanced release of axillary buds and callus mediated somatic organogenesis.

The explants were dipped in 95% ethanol for 10 seconds and then thoroughly washed with sterile water. The explants were agitated in a solution of 2% sucrose and 0.7% PVP 360 for 30 minutes. The explants were then surface sterilised with mercuric chloride solution (0.1%) for 20 minutes. The summary of the surface sterilant treatments is given in Table 17.

Table 17. Surface sterilisation treatments of nutmeg explants

Sterilant	Concentration (%)	Time (minutes)
1. Mercuric chloride	0.05, 0.10 and 0.20	10 - 20

The explants after surface sterilisation were rinsed (at least 5 times) with sterile water. The shoot tips were then cut back to 2.0 cm length aseptically and were transferred to the media. MS basal medium was used for the trial. The general *in vitro* techniques adopted were as described for jack.

The details of the treatments of the culture establishment trial are presented in Table 18.

Table 18. Culture establishment trial on nutmeg explants

Method of multiplication	Explant	Treatment
1. Enhanced release of axillary buds	1. Shoot tip	1. 2x2 diallel set of kinetin (1 and 2 ppm) and NAA (1 and 2 ppm) 2. BA (1, 2 and 3 ppm)
2. Somatic organogenesis (callus mediated)	-do-	1. 2x2 diallel set of kinetin (1 and 2 ppm) and NAA (1 and 2 ppm) 2. 2,4-D (1 ppm) and kinetin (1 ppm) + activated charcoal 1.0%

A. Observations

1. Enhanced release of axillary buds

Observations on four explants per treatment after three weeks were made on per cent survival (cultures which are alive) and per cent growing culture.

2. Somatic organogenesis (callus mediated)

Each treatment was tried on five explants and after three weeks observations were made on per cent explants initiating callus and growth of the callus.

RESULTS

The results generated in the investigation conducted at the Plant Tissue Culture Laboratory of the College of Horticulture, Vellanikkara are presented in the following pages, crop-wise.

I. Jack

A. Culture establishment (Stage I)

1. Establishment of shoot apices and lateral buds

Shoot apices and lateral buds excised from fresh stem sprouts of five-year old jack were subjected to various culture establishment treatments and the results are presented in Table 19. A combination of GA (1.0 ppm) and activated charcoal (1%) resulted in cent per cent survival and production of healthy, growing cultures of shoot apices under dark conditions. Gibberellic acid at 2.0 ppm in combination with activated charcoal (0.5 - 1.0%) also yielded similar results. But the cultures were less healthy and the leaves were fragile which fell off at the slightest shock. Though majority of the treatments had similar effects in terms of culture survival (60.0 - 100.0%), the resulting number of growing cultures was far from satisfactory (20.0 - 40.0%). Survival of lateral buds was 100% at GA

Table 19. Effect of different treatments on the survival and growth of the jack shoot apex cultures (via enhanced release of axillary buds)

Basal medium: MS

Treatment	Survival* (% cultures alive)	Cultures* exhibiting growth (%)
Kinetin 2.5 /uM + NAA 0.1 /uM	40	0
" + " 0.5 /uM	40	0
" + " 1.0 /uM	40	0
kinetin 5.0 /uM + NAA 0.1 /uM	60	0
" + " 0.5 /uM	40	0
" + " 1.0 /uM	60	0
kinetin 10.0 /uM + NAA 0.1 /uM	60	0
" + " 0.5 /uM	20	0
" + " 1.0 /uM	60	0
BA 2.5 /uM + NAA 0.1 /uM	100	20
" + " 0.5 /uM	80	0
" + " 1.0 /uM	60	20
BA 5.0 /uM + NAA 0.1 /uM	100	0
" + " 0.5 /uM	100	0
" + " 1.0 /uM	80	20
BA 10.0 /uM + NAA 0.1 /uM	100	0
" + " 0.5 /uM	60	0
" + " 1.0 /uM	80	0
NAA 0.2 ppm + BA 0.5 ppm	40	20
" + " 1.0 ppm	100	20
" + " 2.0 ppm	80	20
" + " 10.0 ppm	20	0
" + " 20.0 ppm	0	0
NAA 1.0 ppm + BA 0.5 ppm	80	0
" + " 1.0 ppm	80	0
" + " 2.0 ppm	40	0
" + " 10.0 ppm	60	0
" + " 20.0 ppm	0	0

(Contd.)

Table 19. (Continued)

Treatment	Survival* (% culture alive)	Culture* exhibiting growth (%)
IAA 0.1 µM + BA 2.5 µM	60	0
" + " 5.0 µM	100	20
" + " 10.0 µM	100	0
IAA 0.5 µM + BA 2.5 µM	80	0
" + " 5.0 µM	100	40
" + " 10.0 µM	80	0
IAA 1.0 µM + BA 2.5 µM	100	0
" + " 5.0 µM	60	20
" + " 10.0 µM	100	0
BA 0.1 ppm + kinetin 0.1 ppm	60	20
" + " 0.2 ppm	100	0
" + " 0.3 ppm	100	0
BA 0.2 ppm + kinetin 0.1 ppm	80	20
" + " 0.2 ppm	100	0
" + " 0.3 ppm	100	40
BA 0.3 ppm + kinetin 0.1 ppm	60	0
" + " 0.2 ppm	80	0
" + " 0.3 ppm	100	20
GA 0.5 ppm + Activated charcoal 0.25%	100	0
" + " 0.5%	100	20
" + " 1.0%	100	0
GA 1.0 ppm + Activated charcoal 0.25%	100	40
" + " 0.5%	100	40
" + " 1.0%	100	100
GA 2.0 ppm + " 0.25%	100	60
" + " 0.5%	100	100
" + " 1.0%	100	100
GA 1.0 ppm	60	0

* - Average of five observations
Culture period - three weeks

1.0 - 2.0 ppm with activated charcoal (0.5 - 1.0%); but growing cultures were too few (0 - 20.0%).

Slight elongation of shoot apices was observed in about three weeks of culture (Plate I). By this time, unfurling of the leaves commenced. The cultures were considered to have been established when the unfurling reached a satisfactory level (Plate 2). This stage was reached six weeks after inoculation in the case of shoot apices. The response of the lateral buds was slower and inferior to that of the shoot apices (Table 19a).

Though pre-culture treatments like shaking the explants in insoluble PVP (0.7%) for 30-45 min., trimming the explants at low temperature and culturing them at reduced light intensity were employed, the problem of oxidation of phenolics persisted. The severity of this problem was minimum when activated charcoal (1.0%) was incorporated to the medium especially in combination with GA_3 (Plate 3). During the multiplication of propagule (Stage II), incorporation of insoluble PVP in the medium was found to effectively minimise the problem (Plate 4).

2. Somatic organogenesis - callus production

a. Shoot apices

The effect of the treatments involving combinations of kinetin and NAA, BA and NAA, and BA and 2,4-D on initiation

Plate 1. Jack shoot apex from fresh stem sprout (source: five year old tree) in the establishment medium.

Magnification: x 1.36

Plate 2. Jack shoot apex from fresh stem sprout (source: five year old tree) after six weeks in the establishment medium, showing unfurling of leaves

Magnification: x 3.00


1


BOROSIL
2


3


4

Table 19a. Effect of the treatments identified as promising on the survival and growth of jack shoot apex and lateral bud cultures (via enhanced release of axillary buds)

Basal medium : MS

Treatment	Response of shoot apex*		Response of lateral bud	
	Survival** (% cultures alive)	Cultures** exhibiting growth (%)	Survival** (% cultures alive)	Cultures** exhibiting growth (%)
GA 1.0 ppm + Activated charcoal 0.5%	100	40	100	0
" + " 1.0%	100	100	100	20
GA 2.0 ppm + Activated charcoal 0.5%	100	100	100	0
" + " 1.0%	100	100	100	0

* - Results reproduced from previous experiment stated in Table 1

** - Average of five observations

Culture period - three weeks

of callus, callus growth expressed as growth score (G) and callus index (CI) was assessed and the results are presented in Table 20.

Among the treatment combinations involving kinetin and NAA, the highest CI value (180.0) was obtained at kinetin 1.0 ppm + NAA 2.0 ppm. The CI values of the rest of the treatment combinations were 100.0 or below (Table 20). A comparison of the mean CI values of the levels of NAA over the levels of kinetin (Appendix I-1) showed that NAA was more favourable at 2.0 ppm (CI = 106.66) than at the other levels tried. Among the levels of kinetin, a concentration of 1.0 ppm yielded the highest CI value (86.66). Callus index at kinetin 1.0 and 2.0 ppm decreased with NAA concentration above 2.0 ppm. Callus index registered by various levels of NAA at the highest concentration (4.0 ppm) of kinetin was negligible (0 - 20.0), as the treatments were poor in effecting the initiation (callus initiation 0 to 20%) and growth (G = 0 - 1.0) of the callus.

Among the treatment combinations involving BA and NAA, BA 1.0 ppm + NAA 2.0 ppm recorded the highest CI value (140.0). The CI values of the rest of the treatments were 120.0 or below (Table 20). A comparison of the mean CI values of the levels of NAA over the levels of BA showed that

Table 20. Effect of different treatments on the production and growth of calli from shoot apex and internodal segment cultures of jack (w.r.t. somatic organogenesis)

Basal medium : MS

Treatment	Shoot apex culture			Internodal segment culture		
	Cultures initiating* callus (%)	Growth score* (G)	Callus index* (CI)	Cultures* initiating callus (%)	Growth score* (G)	Callus* Index (CI)
kinetin 1.0 ppm + NAA 1.0 ppm	40	1.00	40.0	40	1.00	40.0
" + " 2.0 ppm	100	1.80	180.0	60	1.00	60.0
" + " 4.0 ppm	40	1.00	40.0	60	1.33	79.8
" + " 8.0 ppm	0	0	0	0	0	0
kinetin 2.0 ppm + NAA 1.0 ppm	0	0	0	0	0	0
" + " 2.0 ppm	40	1.00	40.0	0	0	0
" + " 4.0 ppm	80	1.25	100.0	20	2.00	40.0
" + " 8.0 ppm	40	1.00	40.0	0	0	0
kinetin 4.0 ppm + NAA 1.0 ppm	0	0	0	0	0	0
" + " 2.0 ppm	0	0	0	0	0	0
" + " 4.0 ppm	0	0	0	0	0	0
" + " 8.0 ppm	20	1.00	20.0	0	0	0
BA 1.0 ppm + NAA 1.0 ppm	80	1.00	80.0	40	1.00	40.0
" + " 2.0 ppm	100	1.40	140.0	60	1.00	60.0
" + " 4.0 ppm	100	1.20	120.0	20	1.00	20.0
" + " 8.0 ppm	20	1.00	20.0	60	1.33	79.8
BA 2.0 ppm + NAA 1.0 ppm	0	0	0	0	0	0
" + " 2.0 ppm	40	1.00	40.0	20	1.00	20.0
" + " 4.0 ppm	80	1.25	100.0	20	1.00	20.0
" + " 8.0 ppm	20	1.00	20.0	0	0	0
BA 4.0 ppm + NAA 1.0 ppm	20	1.00	20.0	0	0	0
" + " 2.0 ppm	20	1.00	20.0	0	0	0
" + " 4.0 ppm	20	1.00	20.0	20	1.00	20.0
" + " 8.0 ppm	40	1.50	60.0	0	0	0
BA 1.0 ppm + 2,4-D 0.1 ppm	20	1.00	20.0	0	0	0
" + " 0.5 ppm	100	1.20	120.0	60	1.00	60.0
" + " 1.0 ppm	100	1.40	140.0	60	1.33	79.8
" + " 2.0 ppm	40	1.00	40.0	0	0	0
BA 2.0 ppm + 2,4-D 0.1 ppm	0	0	0	20	1.00	20.0
" + " 0.5 ppm	40	1.00	40.0	20	1.00	20.0
" + " 1.0 ppm	60	1.00	60.0	20	1.00	20.0
" + " 2.0 ppm	0	0	0	40	1.00	40.0
BA 4.0 ppm + 2,4-D 0.1 ppm	20	1.00	20.0	0	0	0
" + " 0.5 ppm	40	1.00	40.0	20	1.00	20.0
" + " 1.0 ppm	20	1.00	20.0	0	0	0
" + " 2.0 ppm	20	1.00	20.0	0	0	0

* - Average of five observations
Culture period - three weeks

NAA was more favourable at 4.0 ppm (CI = 80.0) than at the other levels (Appendix I.2). Among the levels of BA, 1.0 ppm recorded the highest CI value (90.0). Callus index at BA 1.0 ppm was found to decrease with NAA above 2.0 ppm. CI values of NAA 1.0, 2.0 and 4.0 ppm were found to be reduced at higher levels of BA (2.0 and 4.0 ppm), while NAA 8.0 ppm registered a slight increase with increasing concentrations of BA.

Among the CI values of the treatment combinations involving BA and 2,4-D, the highest value (140.00) was recorded by BA 1.0 ppm + 2,4-D 1.0 ppm. The CI values of the rest of the treatments were 120.0 or below (Table 20). From a comparison of the mean CI values of the levels of 2,4-D over the levels of BA (Appendix I.3), it could be seen that, 2,4-D was more favourable at 1.0 ppm (CI = 73.33) than at the other levels tried. Among the levels of BA, 1.0 ppm recorded the highest CI value (80.0). Callus indices of the levels of BA were found to decrease with 2,4-D above 1.0 ppm.

Among the 36 treatment combinations tried, the highest number of cultures initiating callus (100.0%), the best growth score (G = 1.8) and the best callus index (CI = 180.0) were recorded by the treatment combination

Plate 3. Jack shoot apex from fresh stem sprout (Source: five year old tree) showing culture establishment. The problem of polyphenol interference minimum with activated charcoal (1.0%) incorporated in MS medium containing GA_3 (1.0 ppm)

Magnification: x 1.46

Plate 4. Jack shoot apex from fresh stem sprout (Source: five year old tree) in the proliferation medium. The problem of polyphenol interference minimum on addition of insoluble PVP (500 ppm) to the BPM

Magnification: x 1.38

kinetin 1.0 ppm + NAA 2.0 ppm (Plate 5). Only eight treatments recorded callus initiation in more than 50.0 per cent of the cultures. Two treatments effected growth score above 1.5. Seven treatments recorded CI values above 75.0. The initiation and growth of callus was poor in most of the other treatments. BA 1.0 ppm + NAA 2.0 ppm and BA 1.0 ppm + 2,4-D 1.0 ppm (with 100.0 per cent callus initiation) recorded a lower growth score of 1.4 and CI value of 140 as compared to the best combination identified.

b. Internodal segments

The data presented in Table 20 show that among the treatment combinations involving kinetin and NAA, the highest CI value (79.8) was recorded by kinetin 1.0 ppm + NAA 4.00 ppm. CI values of the rest of the treatments were 60.0 or below.

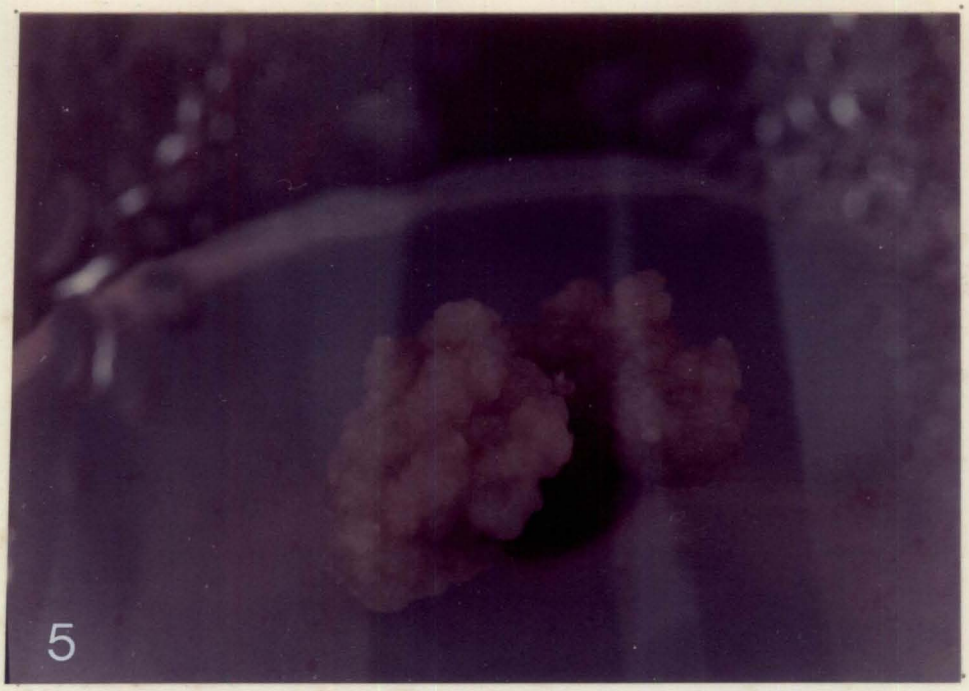
The treatment combinations involving higher levels of NAA (8.0 ppm) and kinetin (4.0 ppm) were totally ineffective for the production of callus from internodal segments (Appendix I.4). Only one treatment combination viz. kinetin/NAA combination 1.0 + 4.0 ppm recorded CI value (79.8) above 75.0. CI values of the rest of the treatments were 60.0 or below. The mean CI values of kinetin showed declining trend with increasing levels, whereas NAA

Plate 5. Callus production from jack shoot apex culture (Source: five year old tree) on MS medium + kinetin 1.0 ppm + NAA 2.0 ppm. Callus Index = 180.0

Magnification: x 1.93

Plate 6. Callus production from young inflorescence explant of jack (Source: ten year old tree) on MS medium + 2,4-D 0.5 ppm + kinetin 1.0 ppm. Callus Index = 100.0

Magnification: x 1.24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responded favourably from 1.0 to 4.0 ppm. The highest mean CI values of kinetin (44.95) and NAA (39.93) were recorded at 1.0 ppm and 4.0 ppm, respectively.

Among the combinations involving BA and NAA, the maximum CI value (79.8) was recorded by BA/NAA combination 1.0 + 8.0 ppm. The remaining treatments registered CI values of 60.0 and below (Table 20). The highest mean CI values of BA (49.95) and NAA (33.33) were recorded at 1.0 ppm and 2.0 ppm, respectively. The mean CI value of BA decreased with increasing levels, whereas NAA registered an increasing trend except at 4.0 ppm (Appendix I.5).

BA 1.0 ppm + 2,4-D 1.0 ppm registered the highest CI value (79.8) among the treatment combinations of BA and 2,4-D. The CI values of the rest of the treatments were 60.0 or below (Table 20). A comparison of the mean CI values of the levels of 2,4-D over the levels of BA showed that 2,4-D was more favourable at 0.5 and 1.0 ppm (33.33) than at the other two levels. Among the levels of BA, 1.0 ppm recorded the highest CI value (34.95). CI values decreased with increasing concentrations of BA. The response of 2,4-D decreased drastically with concentrations above 1.0 ppm (Appendix I.6).

In general, the effectiveness of the 36 treatment combinations was poor with regard to initiation and growth

of the callus. Only six treatment combinations recorded above 50.0 per cent callus initiation, the highest value (60.0%) being recorded by kinetin/NAA combination 1.0 + 2.0 ppm and 1.0 + 4.0 ppm; BA/NAA combination 1.0 + 2.0 and 1.0 + 8.0 ppm; and BA / 2,4-D combination 1 + 0.5 ppm and 1.0 + 1.0 ppm. Moderate rate of callus growth (G = 2.0) was observed only in kinetin/NAA combination 2.0 + 4.00 ppm. In all the other treatments, callus growth was poor (G = 1.33 or below). The highest CI value (79.8) was recorded by kinetin/NAA combination 1.0 + 4.0 ppm, BA/NAA combination 1.0 + 8.0 ppm and BA/2,4-D combination 1.0 + 1.0 ppm. The CI values of the rest of the treatments were below 60.0.

c. Leaf segments

The data on callus production from leaf segments are presented in Table 21. The only treatment combinations which recorded callus initiation from more than 50.0 per cent of the explants were BA/2,4-D combination 2.0 + 0.1 ppm (60.0%) and 2.0 + 0.5 ppm (60.0%). Only 16 of the 36 treatments were able to initiate callus. Even in these, the growth of the callus was slow and poor (G = 1.00). The maximum CI value (60.0) was registered by BA/2,4-D combination 2.0 + 0.1 ppm and 2.0 + 0.5 ppm. The CI values of the other fourteen treatments were 40.0 or below.

Table 21. Effect of different treatments on the production and growth of calli from leaf segment and root tip cultures of jack (w.r.t. somatic organogenesis)

Basal medium : MS

Treatment	Leaf segment			Root tip		
	Cultures* initiating callus (%)	Growth* score (G)	Callus* Index (CI)	Cultures* initiating callus (%)	Growth* score (G)	Callus* Index (CI)
kinetin 1.0 ppm + NAA 1.0 ppm	0	0	0	80	1.00	80.0
" + " 2.0 ppm	40	1.00	40.0	60	1.33	79.8
" + " 4.0 ppm	40	1.00	40.0	60	1.33	79.8
" + " 8.0 ppm	40	1.00	40.0	0	0	0
kinetin 2.0 ppm + NAA 1.0 ppm	0	0	0	20	1.00	20.0
" + " 2.0 ppm	20	1.00	20.0	40	1.00	40.0
" + " 4.0 ppm	0	0	0	20	1.00	20.0
" + " 8.0 ppm	0	0	0	20	1.00	20.0
kinetin 4.0 ppm + NAA 1.0 ppm	0	0	0	0	0	0
" + " 2.0 ppm	0	0	0	0	0	0
" + " 4.0 ppm	0	0	0	0	0	0
" + " 8.0 ppm	0	0	0	40	1.50	60.0
BA 1.0 ppm + NAA 1.0 ppm	0	0	0	20	1.00	20.0
" + " 2.0 ppm	40	1.00	40.0	40	1.00	40.0
" + " 4.0 ppm	40	1.00	40.0	40	1.50	60.0
" + " 8.0 ppm	0	0	0	0	0	0
BA 2.0 ppm + NAA 1.0 ppm	0	0	0	40	1.00	40.0
" + " 2.0 ppm	20	1.00	20.0	0	0	0
" + " 4.0 ppm	0	0	0	0	0	0
" + " 8.0 ppm	20	1.00	20.0	20	1.00	20.0
BA 4.0 ppm + NAA 1.0 ppm	20	1.00	20.0	0	0	0
" + " 2.0 ppm	0	0	0	0	0	0
" + " 4.0 ppm	0	0	0	0	0	0
" + " 8.0 ppm	0	0	0	40	1.00	40.0
BA 1.0 ppm + 2,4-D 0.1 ppm	20	1.00	20.0	20	1.00	20.0
" + " 0.5 ppm	40	1.00	40.0	0	0	0
" + " 1.0 ppm	40	1.00	40.0	60	1.33	79.8
" + " 2.0 ppm	40	1.00	40.0	80	1.25	100.0
BA 2.0 ppm + 2,4-D 0.1 ppm	60	1.00	60.0	0	0	0
" + " 0.5 ppm	60	1.00	60.0	40	1.00	40.0
" + " 1.0 ppm	0	0	0	60	1.33	79.8
" + " 2.0 ppm	0	0	0	20	1.00	20.0
BA 4.0 ppm + 2,4-D 0.1 ppm	20	1.00	20.0	20	1.00	20.0
" + " 0.5 ppm	0	0	0	0	0	0
" + " 1.0 ppm	0	0	0	20	1.00	20.0
" + " 2.0 ppm	0	0	0	0	0	0

* - Average of five observations

Culture period - three weeks

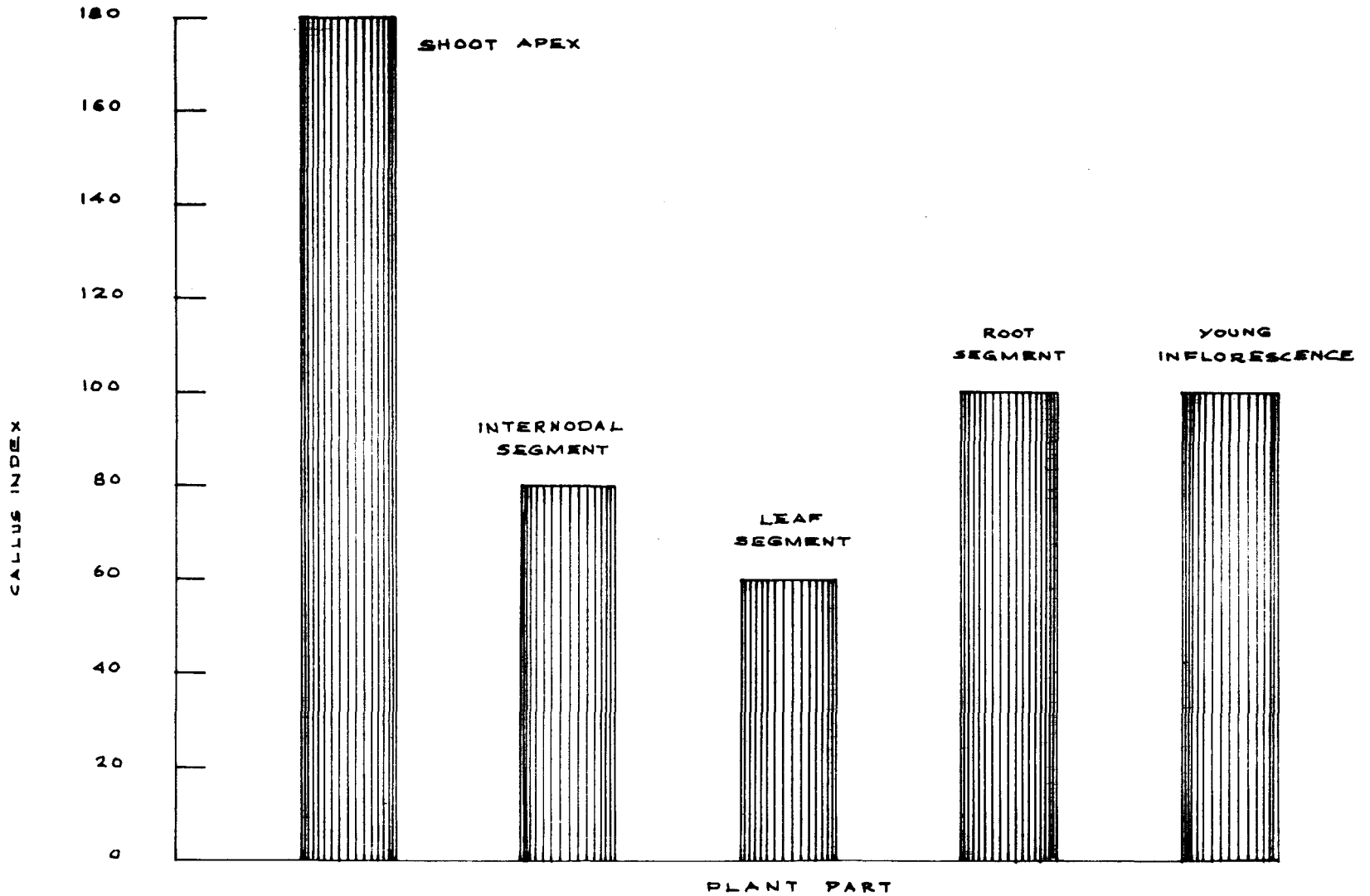

FIG: 2. CALLUS PRODUCTION BY EXPLANTS FROM DIFFERENT PLANT PARTS OF JACK AT THE BEST TREATMENTS IDENTIFIED

d. Root tips

The results of growth substance treatments on callus production from *in vitro* formed root tips are presented in Table 21.

Among the treatment combinations involving kinetin and NAA, the highest CI value (80.0) was obtained at kinetin/NAA combination 1.0 + 1.0 ppm (Table 21). The mean CI values of kinetin showed decreasing trend with increasing levels, the most favourable level being 1.0 ppm (CI 59.9). The favourable level of NAA was 2.0 ppm (CI = 39.93), above which the CI values decreased (Appendix I.7).

The CI value (60.0) recorded by BA/NAA combination 1.0/4.0 ppm was the highest among the combinations involving BA and NAA. The CI values of the rest of the treatments were 40.0 or below (Table 21).

Among the treatment combinations involving BA and 2,4-D, the highest value was recorded by BA/2,4-D combination 1.0 + 2.0 ppm (100.0). The CI values of the remaining treatments were below 80.0 (Table 21). Comparison of mean CI values of BA over the levels of 2,4-D showed that BA effected a decreasing trend with increasing levels. It was observed that 2,4-D was most favourable at 1.0 ppm (Appendix I.8).

The initiation and growth of the callus was poor in majority of the treatments when explants from root tips were used. Only six treatments recorded callus initiation in above 50.0 per cent cultures, two treatments effected growth score of above 1.5 and six treatments recorded CI values of above 75.0. The maximum callus initiation (80.0%) was recorded by kinetin/NAA combination 1.0 + 1.0 ppm and BA / 2,4-D combination 1.0 + 2.0 ppm. The rest of the treatments registered callus initiation of 60.0% or below. The highest growth score of callus (G = 1.5) was recorded by kinetin/NAA combination 1.0 + 8.0 and BA/NAA combination 1.0 + 4.0 ppm, while the maximum CI value (100.0) was registered by BA/2,4-D combination 1.0 + 2.0 ppm. CI values of the remaining treatments were 80.0 or below.

3. Direct somatic organogenesis

Combinations of BA and kinetin were tried for the induction of direct organogenesis from <u>in vitro</u> formed shoot apices, internodal segments, lower half of leaves with petiole and upper half of leaves. None of the treatments tried were successful in inducing direct organogenesis from the explants used.

4. Somatic embryoid induction - callus production

The results obtained on the induction of somatic embryoids from shoot apices, internodal segments, leaf segments, and young inflorescences, with the different combinations of 2,4-D and kinetin are presented in Table 22. No direct somatic embryoid formation was observed. Callus was induced by the treatments from the different explants tried.

a. Shoot apices

Majority of the treatments tried were not efficient in inducing callus from shoot apices cultured. Responses were observed only for five of the 12 treatments tried. Best callus initiation (100.0), growth score (G = 1.67) and callus index (CI = 167) were recorded by 2,4-D/kinetin combination 1.0 + 1.0 ppm and 1.0 + 2.0 ppm (Table 22). A comparison of the mean CI values of kinetin over the different levels of 2,4-D showed that kinetin was most favourable at 1.0 ppm (CI = 66.75). The CI value of 2,4-D showed an increasing trend upto 1.0 ppm after which there was a drastic reduction (Appendix I.9).

b. Internodal segments

The response of the internodal segments to the various treatments was also poor. Only one treatment

Table 22. Effect of different treatments on the induction of somatic embryoids (callus induction) from shoot tip, internodal segment, leaf segment and young inflorescence cultures of jack

Basal medium : MS

Treatment	Shoot apex			Internodal segment			Leaf segment			Young inflorescence		
	Cultures* initiating callus (%)	Growth* score (G)	Callus* Index (CI)	Cultures* initiating callus (%)	Growth* Score (G)	Callus* Index (CI)	Cultures* initiating callus (%)	Growth* score (G)	Callus index (CI)	Cultures* initiating callus (%)	Growth* score (G)	Callus* index (CI)
2,4-D 0.1 ppm + kinetin 0 ppm	0	0	0	33.3	1.0	33.3	0	0	0	100.0	1.0	100.0
" 0.5 ppm + "	0	0	0	0	0	0	0	0	0	0	0	0
" 1.0 ppm + "	33.3	1.00	33.3	0	0	0	0	0	0	66.7	1.0	66.7
" 2.0 ppm + "	33.3	1.00	33.3	33.3	1.0	33.3	33.3	1.0	33.3	33.3	1.0	33.3
2,4-D 0.1 ppm + kinetin 1.0 ppm	0	0	0	33.3	1.0	33.3	33.3	1.0	33.3	100.0	1.0	100.0
" + " 2.0 ppm	0	0	0	100.0	1.0	100.0	33.3	1.0	33.3	0	0	0
2,4-D 0.5 ppm + kinetin 1.0 ppm	100.0	1.00	100.0	33.3	1.0	33.3	66.7	1.0	66.7	100.0	1.0	100.0
" + " 2.0 ppm	0	0	0	33.3	1.0	33.3	0	0	0	0	0	0
2,4-D 1.0 ppm + kinetin 1.0 ppm	100.0	1.67	167.0	0	0	0	100.0	1.0	100.0	33.3	1.0	33.3
" + " 2.0 ppm	100.0	1.67	167.0	0	0	0	33.3	1.0	33.3	0	0	0
2,4-D 2.0 ppm + kinetin 1.0 ppm	0	0	0	0	0	0	0	0	0	33.3	1.0	33.3
" + " 2.0 ppm	0	0	0	0	0	0	0	0	0	0	0	0

* - Average of three observations

Culture period - four weeks

(2,4-D/kinetin combination 0.1 + 2.0 ppm) registered moderate callus index value of 100.0. The CI values of the remaining five treatments initiating callus were 33.33 (Table 22).

c. Leaf segments

The leaf segments subjected to the various treatments also exhibited poor performance. Only a single treatment (2,4-D/kinetin combination 1.0+1.0 ppm) registered a CI value of 100.0. Callus was seen initiated in 100.0 per cent of the cultures receiving this treatment. The CI values of the rest of the treatments were 33.33 except in the case of 2,4-D/kinetin combination 0.5+1.0 ppm where the CI value was 66.7. The growth score recorded by all the effective treatments were the same (Table 22).

d. Young inflorescence

Only three of the twelve treatments (2,4-D/kinetin combination 1.0 + 0 ppm, 0.1 + 1.0 ppm and 0.5 + 1.0 ppm and 0.5 + 1.0 ppm) recorded a moderate CI value of 100.0 (Plate 6). In these cases, callus initiation was observed in all the cultures (100%). The callus index values of the rest of the treatments were 66.7 and below. These treatments recorded a lower per cent callus initiation (33.3 - 66.7).

Callus growth was poor (G = 1.0) in all the effective treatments. Five treatments did not register any response (Table 22).

B. Multiplication of the propagules (Stage II)

1. Enhanced release of axillary buds

a. Standardisation of basic proliferation medium

The shoot apices, placed in the establishment medium and incubated for four weeks in darkness and two weeks in light, were transferred to 24 treatment combinations involving BA (2.5, 5.0, 7.5, 10.0, 20.0 and 40.0 ppm) and NAA (0.1, 0.2, 0.4 and 0.8 ppm). The data on the number of shoots produced per initial explant in these media after five weeks of culture are given in Table 23. The results of the analysis of variance are presented in Appendix II.

The maximum number of shoots per explant (8.75) was achieved at BA/NAA combination 7.5/0.2 ppm. This treatment was statistically on par with BA/NAA combination 7.5 + 0.1 ppm (8.0 shoots) and BA/NAA combination 10.0 + 0.2 ppm (7.75 shoots). Among the shoots produced in the above treatments, a large proportion was highly compressed and small shoots (Plate 7). The percentage of such shoots showed an increasing trend with increase in the level of BA. The treatments BA/NAA 5.0 + 0.2 ppm and BA/NAA 5.0 + 0.1 ppm,

Plate 7. Production of highly compressed shoots from jack shoot apex (source: five year old tree) cultured on MS medium containing high level of cytokinin

Magnification: x 1.24

Plate 8. Production of fairly elongated multiple shoots from jack shoot apex (source: five year old tree) cultured on MS medium + BA 5.0 ppm + NAA 0.2 ppm. Multiplication rate after five weeks of culture = 4.5x

Magnification: x 1.28

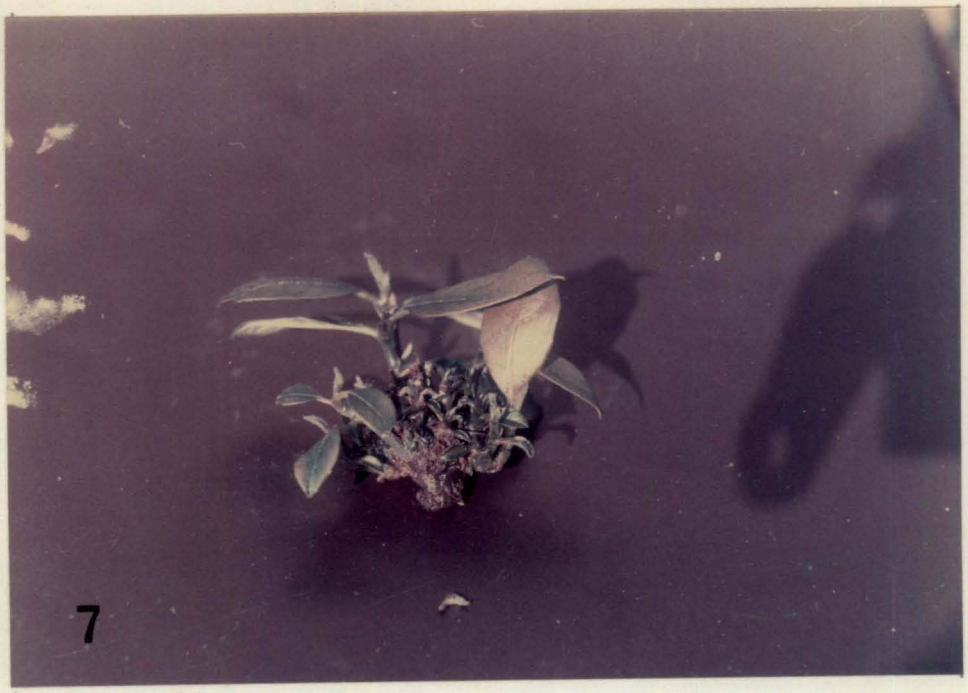
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Plate 9. Production of fairly elongated multiple shoots from jack shoot apex (source: five year old tree) on MS medium + BA 5.0 ppm + NAA 0.1 ppm

Magnification: x 0.86

Plate 10. Multiple shoot production from jack shoot apex (source: five year old tree) after serial subculturing for ten times on the BPM. Increase in multiplication rate due to sequential subculturing: 26.79%

Magnification: x 0.89

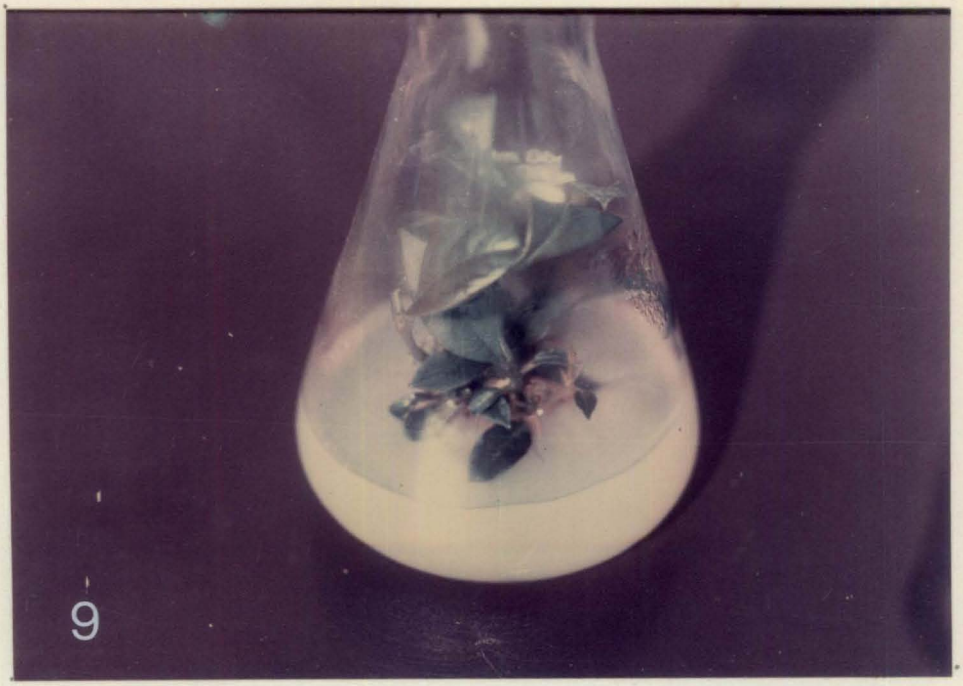

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Table 23. Effect of combinations of BA and NAA on multiple shoot formation from jack shoot apex cultures

Basal medium : MS

Treatment	Shoots* / initial explant	Fairly* elongated shoots/ initial explant
NAA 0.1 ppm + BA 2.5 ppm	1.00	1.00
" + " 5.0 ppm	4.00	3.75
" + " 7.5 ppm	8.00	3.50
" + " 10.0 ppm	7.00	3.25
" + " 20.0 ppm	0	0
" + " 40.0 ppm	0	0
NAA 0.2 ppm + BA 2.5 ppm	1.25	1.25
" + " 5.0 ppm	4.75	4.50
" + " 7.5 ppm	8.75	3.25
" + " 10.0 ppm	7.75	3.00
" + " 20.0 ppm	5.75	2.50
" + " 40.0 ppm	0	0
NAA 0.4 ppm + BA 2.5 ppm	1.00	1.00
" + " 5.0 ppm	5.00	3.50
" + " 7.5 ppm	6.50	3.50
" + " 10.0 ppm	5.25	2.00
" + " 20.0 ppm	0	0
" + " 40.0 ppm	0	0
NAA 0.8 ppm + BA 2.5 ppm	1.00	1.00
" + " 5.0 ppm	3.00	3.00
" + " 7.5 ppm	4.00	3.00
" + " 10.0 ppm	5.25	2.00
" + " 20.0 ppm	0	0
" + " 40.0 ppm	0	0
CD (5%)	1.75	1.10
SEM ±	0.61	0.38

* - Average of four observations
Culture period - five weeks

which were statistically at par, produced a high proportion of fairly elongated shoots (Plates 8 and 9). Lateral buds when used as the explants failed to produce multiple shoots.

For further multiplication, the shoots were excised and placed in fresh medium of the composition selected. Subculturing the proliferating shoot cultures was done at five-week intervals.

The results of the studies conducted to assess the improvement in the rate of multiplication of the shoots consequent on continuous subculturing are presented in Table 24. The cultures which produced 4.25 shoots initially registered a decrease (by 5.88%) during the first subculture and an increase (ranging from 5.88 to 52.94%) during the subsequent subcultures. On the average, the multiplication rate increased by 26.79% as a result of subculturing (Plate 10). No apparent reduction was observed in the growth, and vigour of the cultures due to continuous subculturing.

b. Regulation of proliferation and growth of the shoots in the cultures

The results of the studies conducted on the effect of cytokinins, cytokinin - related substances (adenine and adenine sulphate), auxins, gibberellic acid, amino acid

Table 24. Multiplication rate per explant (from 5-year old jack trees) on subculturing at 4-week interval

(Medium: MS + BA 5.0 ppm + NAA 0.2 ppm)

Culture	Shoots* per culture	% increase in number of shoots over the initial culture
1	4.25	-
2	4.00	**
3	4.75	11.76
4	4.50	5.88
5	5.75	35.29
6	6.00	41.17
7	4.75	11.76
8	6.50	52.94
9	5.75	35.29
10	6.50	52.94
Mean		26.79
CD (5%)	1.97	
SEM ±	0.57	

** - A decrease of 5.88% was observed
* - Average of four observations

supplement (casein hydrolysate), MS inorganic and organic components and carbon sources (sucrose and glucose) on the multiplication and growth of the shoots from shoot tip cultures are presented in Tables 25 to 28.

i. Cytokinins

The data on the influence of BA and kinetin (basal medium containing NAA 0.2 ppm) on the number of shoots, length of the longest shoot and length of the longest leaf are presented in Table 25, and the analysis of variance, in Appendix II.

Number of shoots

There was significant variation among the treatments for the number of shoots produced. Maximum number of shoots (8.75) was recorded by BA 7.5 ppm which was on par with BA 10.0 ppm (7.75 shoots). Shoot production was observed to decrease with increase in the concentration of BA above 10.0 ppm. Highly compressed and small shoots showed an increasing trend with increase in the levels of BA. Maximum number of fairly elongated shoots (4.5) were recorded by BA 5.0 ppm. With respect to kinetin, the effects at 10.0 ppm (4.0 shoots) and at 7.5 ppm (3.5 shoots) were on par and were superior to the lowest

Table 25. Effect of cytokinins on multiple shoot formation from jack shoot apices

Basal medium : MS + NAA 0.2 ppm

Treatment	Shoots* per explant	Length of	
		Longest* shoot (cm)	Longest* leaf (cm)
BA 2.5 ppm	1.25	3.03	3.33
" 5.0 ppm	4.75	2.15	2.90
" 7.5 ppm	8.75	1.35	1.68
" 10.0 ppm	7.75	1.08	1.28
" 20.0 ppm	5.75	0	0
" 40.0 ppm	0	0	0
kinetin 2.5 ppm	1.25	2.23	2.53
" 5.0 ppm	2.50	1.58	2.65
" 7.5 ppm	3.50	1.18	2.50
" 10.0 ppm	4.00	1.08	1.45
" 20.0 ppm	3.00	0.90	0.60
" 40.0 ppm	2.00	0.83	0.63
BA 0.1 ppm + kinetin 0.1 ppm	1.00	1.05	1.78
" + " 0.5 ppm	1.25	0.98	1.83
" + " 1.0 ppm	1.00	1.40	2.33
BA 0.5 ppm + kinetin 0.1 ppm	1.00	1.00	2.08
" + " 0.5 ppm	1.50	1.48	2.00
" + " 1.0 ppm	1.75	1.48	2.50
BA 1.0 ppm + kinetin 0.1 ppm	1.50	1.50	2.18
" + " 0.5 ppm	1.50	1.43	2.60
" + " 1.0 ppm	1.50	1.43	2.65
CD (5%)	1.62	0.40	0.21
SEM ±	0.57	0.14	0.07

* - Average of four observations

Culture period - five weeks

(2.50 ppm) and the highest levels (40.0 ppm). However, the maximum number of fairly elongated shoots (2.5) was produced by kinetin 5.0 ppm. The effects of combinations of BA and kinetin did not statistically differ from each other. The performance was generally poor, the maximum number of shoots being 1.75, registered at BA/kinetin combination 0.5 + 1.0 ppm.

Length of the longest shoot

The responses to the various treatments were statistically significant. The length of the longest shoot (3.03 cm) recorded by BA 2.5 ppm, was superior to that recorded by the rest of the treatments. The length of the longest shoot showed decreasing trend with increasing levels of BA. Among the levels of kinetin, 2.5 ppm effected the maximum length (2.23 cm). The performance of the rest of the kinetin treatments was inferior and showed a declining trend with increasing levels. Among the combinations of BA and kinetin tried, the maximum response (1.5 cm length) was recorded by BA/kinetin combination 1.0 + 1.0 ppm. This treatment was on par with majority of the other treatments.

Length of the longest leaf

The length of the longest leaf produced by the shoots subjected to the various treatments differed

significantly. The highest value (3.33 cm length) was recorded by BA 2.5 ppm, which was significantly superior to the rest of the treatments. The length of the longest leaf decreased with increasing concentrations of BA. Kinetin was most favourable at 5.0 ppm, registering a leaf length of 2.65 cm. The length was decreased at the higher levels of kinetin, the least being 0.6 cm at 20.0 ppm. The BA/ kinetin combination 1.0 + 1.0 ppm registered the maximum leaf length of 2.65 cm.

ii. Cytokinin-related substances

The effects of adenine and adenine sulphate on the multiplication and growth of the shoot cultures are presented in Table 26 and the analysis of variance in Appendix II.

Number of shoots

Significant improvement in the rate of shoot multiplication was observed by the addition of adenine and adenine sulphate. Among the levels of adenine tried, 80.0 ppm registered the maximum value (8.0). This was 45.45 per cent higher than that obtained in the control (BPM) which registered a multiplication rate of 5.5. Adenine 80.0 ppm was on par with those of 160.0 ppm (7.8) and 40.0 ppm (6.5) Adenine 40.0 ppm, 20.0 ppm and 10.0 ppm did not significantly differ from the BPM in influencing

Table 26. Effects of adenine, adenine sulphate, casein hydrolysate and GA_3 on multiple shoot formation from jack shoot apices

Basal medium : MS+BA 5 ppm+NAA 0.2ppm

Treatment		Shoots per explant	Length of the	
			Longest shoot (cm)	Longest leaf (cm)
Adenine	10 ppm	5.50*	2.43*	3.08*
"	20 ppm	6.00	2.50	2.93
"	40 ppm	6.50	2.38	1.95
"	80 ppm	8.00	1.85	2.05
"	160 ppm	7.75	1.90	1.43
"	0 ppm	5.50	2.15	2.90
	CD (5%)	1.56	0.30	0.45
	SEM ±	0.52	0.10	0.15
Adenine sulphate	10 ppm	6.00*	2.65*	2.95*
"	20 ppm	7.00	2.45	2.90
"	40 ppm	8.75	1.95	1.98
"	80 ppm	8.50	1.83	1.63
"	160 ppm	8.00	1.35	1.73
"	0 ppm	5.50	2.15	2.90
	CD (5%)	1.25	0.32	0.49
	SEM ±	0.42	0.11	0.16
Casein hydrolysate	50 ppm	6.2**	1.92**	2.24**
"	100 ppm	7.8	1.42	1.62
"	500 ppm	8.4	1.16	1.26
"	1000 ppm	8.0	1.00	0.86
"	2000 ppm	7.4	1.00	0.58
"	0 ppm	6.0	2.44	3.18
	CD (5%)	1.13	0.48	0.64
	SEM ±	0.39	0.16	0.22
GA_3	1.0 ppm	5.2**	2.58**	3.56**
"3	2.0 ppm	5.6	1.92	2.82
"	4.0 ppm	4.6	2.22	3.16
"	8.0 ppm	5.0	2.30	2.40
"	16.0 ppm	5.2	2.70	3.44
	0 ppm	5.2	2.44	3.16
	CD (5%)	NS	NS	0.63
	SEM ±	0.37	0.28	0.22

* - Average of four observations
** - Average of five observations
Culture period - five weeks

the number of shoots. Levels of adenine sulphate significantly differed from each other. Adenine sulphate 40.0 ppm which registered the maximum number of shoots (8.8) was on par with 80.0 and 160.0 ppm. The response obtained at adenine sulphate 40.0 ppm was 60.0 per cent more than that obtained in the control.

Length of the longest shoot

Marked differences were observed in the length of the shoots produced at the different levels of adenine as well as adenine sulphate. In both cases the highest concentrations reduced the shoot length whereas the lowest levels increased it (compared to the control). Among the levels of adenine, 20.0 ppm registered the maximum shoot length (2.5 cm) whereas 50.0 ppm caused the least (1.85 cm). The effects of adenine 10.0 ppm, 40.0 ppm, 80.0 ppm and 160.0 ppm were on par with the BPM, which recorded a shoot length of 2.15 cm. The effect of adenine sulphate in increasing the shoot length was the maximum at 10.0 ppm (2.65 cm length) which was on par with that of 20.0 ppm. Adenine sulphate at 40.0 ppm, 80.0 ppm and 160.0 ppm exhibited a reduction in the shoot length. The reduction was the maximum at 160.0 ppm (1.35 cm length). In the case of adenine sulphate the levels that increased the

number of shoots reduced the length of the shoots. In the case of adenine also, a similar but less pronounced influence could be observed.

Length of the longest leaf

The length of the longest leaf was also significantly influenced by the levels of adenine and adenine sulphate. In both cases significant reduction in the length was observed at the higher levels (40.0, 80.0 and 160.0 ppm). The lower levels were on par with the BPM which recorded a leaf length of 2.90 cm. Leaf growth was affected by those levels of adenine and adenine sulphate (80.0 to 160.0 ppm) which favoured increase in the number of shoots.

Adenine and adenine sulphate at the levels tried, showed positive influence on enhancing the shoot multiplication rate. At the optimum levels, adenine (at 80.0 ppm) and adenine sulphate (at 40.0 ppm) recorded increase of 45.45% and 60.0% respectively, over the control (BPM), which recorded a multiplication rate of 5.5x. However, these levels along with most of the other favourable levels affected the growth of the cultures, by reducing the length of the longest shoot and the length of the longest leaf. Only adenine sulphate 20.0 ppm registered an increase in shoot multiplication rate (27.27%) over the control (multiplication rate 5.5x) without significantly affecting the growth of the cultures.

iii. Auxins

The effects of the levels of IAA, NAA and 2,4-D (basal medium containing BA 5.0 ppm) on the number of shoots, the length of the longest shoot and the length of the longest leaf are presented in Table 27 and the analysis of variance, in Appendix II.

Number of shoots

Auxins did not significantly influence the number of shoots produced. The effects of the levels of IAA, NAA and 2,4-D were not statistically different from each other. The differences in shoot number effected by the levels within each auxin were also not significant.

Length of the longest shoot

The influence of the levels of auxins exhibited significant variation. NAA 0.2 ppm which recorded the maximum shoot length (2.55 cm) was on par with IAA 0.2 ppm and 0.4 ppm, 2,4-D 0.1 ppm and NAA 0.4 ppm. All these treatments were significantly superior to the zero level. Increasing concentrations of 2,4-D registered a depressing effect on the shoot length.

Table 27. Effect of auxins on multiple shoot formation from jack shoot apices

Basal medium : MS+BA 5.0 ppm

Treatment	Shoots* per explant	Length of the	
		Longest* shoot (cm)	Longest* leaf (cm)
IAA 0.1 ppm	4.75	1.83	2.50
" 0.2 ppm	5.25	2.53	2.95
" 0.4 ppm	5.25	2.43	2.90
NAA 0.1 ppm	4.00	1.98	2.88
" 0.2 ppm	4.75	2.55	3.10
" 0.4 ppm	5.00	2.38	3.18
2,4-D 0.1 ppm	5.00	2.45	3.00
" 0.2 ppm	5.00	2.08	2.63
" 0.4 ppm	5.25	2.05	2.48
Control (MS+BA 5.0 ppm)	5.00	1.93	2.43
CD (5%)	NS	0.40	0.38
SEM ±	0.53	0.14	0.13

* - Average of four observations

Culture period - five weeks

Length of the longest leaf

Majority of the levels of auxins tried significantly increased the length of the longest leaf. In this respect, NAA 0.4 ppm was on par with NAA 0.2 ppm, NAA 0.1 ppm, 2,4-D 0.1 ppm, IAA 0.2 ppm and IAA 0.4 ppm. While a decreasing trend with increasing concentration was registered by 2,4-D, NAA brought about an increasing trend with increasing concentrations.

iv. Gibberellic acid (GA_3)

The responses to the levels of GA_3 tried with respect to the number of shoots, the length of the longest shoot and the length of the longest leaf are presented in Table 26 and the analysis of variance, in Appendix II.

The number of shoots and the length of the longest shoot were not significantly altered by the levels of GA_3. However, the length of the longest leaf was significantly reduced by GA_3 8.0 ppm (2.4 cm). No beneficial effect was observed when GA_3 was included in the BPM.

v. Amino acid supplement, casein hydrolysate (CH)

The number of shoots, the length of the longest shoot and the length of the longest leaf as influenced by the levels of CH are presented in Table 26 and the analysis of variance in Appendix II.

The highest number of shoots was recorded at 500.0 ppm (8.4) which was 40% greater than that of the BPM, which recorded 6.0 shoots. Casein hydrolysate 500.0 ppm was on par with 1000.0 ppm (8.0), 100.0 ppm (7.8) and 2000.0 ppm (7.4).

All the levels of CH significantly reduced the length of the longest shoot and that of the longest leaf. The resultant small sized shoots were observed to have slow rate of growth during further culturing. The addition of CH in the BPM was, thus, not found to be beneficial.

vi. MS inorganic and organic components

The multiplication rate and growth of the cultures as influenced by the strength of the inorganic salts and organic growth factors of the MS medium are presented in Table 28 and the analysis of variance, in Appendix II.

The concentration of the inorganic salts in the MS medium exhibited significant influence on the multiplication rate and growth of the jack shoot cultures. The number of shoots, the length of the longest shoot and the length of the longest leaf were considerably reduced by lowering the amount of mineral salts below the normal strength. When the salt concentration was reduced to half strength, the number of shoots, the length of the

Table 28. Effect of MS inorganic salts, MS organic growth factors, sucrose and glucose on multiple shoot formation from jack shoot apices

Basal medium containing BA 5 ppm + NAA 0.2 ppm

Treatment		Shoots* per explant	Length of the	
			Longest* shoot (cm)	longest* leaf (cm)
MS Inorganic salts	1/4 conc.	2.2	1.08	1.12
"	1/2 conc.	3.6	1.24	2.06
"	1 conc.	5.2	2.42	3.06
"	2 conc.	5.4	1.82	2.04
	CD (5%)	0.95	0.45	0.72
	SEM ±	0.32	0.15	0.24
MS Organic growth factors	1/4 conc.	3.0	1.28	1.76
"	1/2 conc.	3.0	2.08	2.12
"	1 conc.	5.0	2.48	2.46
"	2 conc.	5.4	2.20	2.26
	CD (5%)	0.90	0.69	NS
	SEM ±	0.30	0.23	0.24
Sucrose 1%		3.6	0.92	1.60
" 2%		4.6	1.26	2.76
" 3%		5.4	2.24	2.92
" 4%		6.0	1.80	2.70
	CD (5%)	0.79	0.62	0.59
	SEM ±	0.27	0.21	0.20
Glucose 1%		3.4	1.52	2.28
" 2%		5.0	2.14	3.06
" 3%		4.6	2.00	2.70
" 4%		3.4	1.58	2.54
	CD (5%)	1.04	0.50	0.51
	SEM ±	0.35	0.17	0.17

* - Average of five observations

Culture period - five weeks

longest shoot and the length of the longest leaf were reduced by 30.77, 48.87 and 32.68 per cent, respectively as compared to those under the normal strength. Further reduction in the multiplication rate and growth of the shoots was observed when the inorganic salt concentration was reduced to quarter strength. The increase obtained in the number of shoots consequent as increasing the salt concentration to double strength was not statistically significant (as compared to that in normal strength). Significant reduction in the length of the longest shoot (25.0%) and the length of the longest leaf (33.33%) was effected by the double strength MS medium.

When the strength of the organic growth factors of MS medium was reduced to half or quarter of the normal strength the production of shoots was significantly affected. The reduction was 40.0 per cent in both the cases. The double concentration of the organic factors (5.4 shoots) was on par with the normal strength (5.0 shoots). Quarter strength of organic factors significantly reduced the shoot length (48.39% reduction) compared the full strength (2.48 cm). Half and double concentrations of the organic factors also caused a reduction in shoot length (16.12 and 11.29%, respectively). However, these were statistically on par with the full strength. The responses of the different concentrations on the length of the longest leaf was not significant.

The above results showed that the normal strength of the inorganic salts and organic growth factors as present in the MS medium was more effective for multiplication and growth of the jack shoot cultures.

vii. Carbon sources

The effects of sucrose and glucose as carbon/energy sources on the shoot multiplication rate and growth of the cultures is given in Table 28 and the analysis of variance in Appendix II.

Reduction in the quantity of sucrose, below the normal level (3%) resulted in reduced number of shoots. Reduction (33.33%) recorded by sucrose 1% was statistically significant while that by 2% (14.81%) was on par with the effect of the full strength. Though the effect of sucrose 4% on the number of shoots was slightly greater (11.11%) than that of the normal level it was not statistically significant. Sucrose at 1 and 2% levels significantly reduced the length of the shoots. The reduction was 58.93 and 43.75 per cent, respectively. Sucrose at 4% was not significantly different from the normal strength. Sucrose 1% significantly reduced the length of the longest leaf also. The reduction in length was 45.2 per cent, compared to that under the full strength and was significantly different from the rest of the treatments which were on par with each other.

The maximum favourable effects of glucose with respect to the number of shoots (5.0), the length of the longest shoot (2.14 cm) and the length of the longest leaf (3.06 cm) were realised at 2.0% concentration. Glucose 3.0% was less effective than the 2.0% level but the difference was not statistically significant. The lowest and highest levels of glucose were unfavourable for the multiplication and growth of the shoots.

Sucrose (3 - 4%) and glucose (2 - 3%) were thus identified as ideal sources of carbon supporting the multiplication and growth of the jack shoot cultures.

viii. Influence of Anderson's medium

When the Anderson's medium was supplemented with BA/NAA combination 5.0 + 0.2 ppm, only 3.3 shoots per culture were produced. This multiplication rate was 30.53 per cent less than that obtained with the MS proliferation medium. The length of the longest shoot (2.17 cm) and the length of the longest leaf (1.88 cm) registered by the Anderson's medium were less (by 14.90% and 39.35% respectively) than those of the MS proliferation medium. The Anderson's medium, containing quarter strength of ammonium nitrate and potassium nitrate and double

strength of FeSO4 EDTA, was found to be unsuitable for jack shoot proliferation.

2. Somatic organogenesis - differentiation of callus

The treatment combinations of NAA (0.1, 0.5 and 1.0 ppm) and kinetin (0.5, 1.0, 2.0, 4.0 and 8.0 ppm) as well as NAA (0.1, 0.5 and 1.0 ppm) and BA (0.5, 1.0, 2.0, 4.0 and 8.0 ppm) were tried for inducing organogenesis from callus. None of the treatments were found to be effective.

3. Somatic embryogenesis (callus mediated)

The callus from the induction medium was transferred to MS medium containing combinations of BA (0, 0.5 and 1.0 ppm) and kinetin (0, 0.5 and 1.0 ppm) for inducing somatic embryoid formation. Embryoid formation was not observed in any of the treatments.

C. Medium for shoot elongation

With the MS medium as the base, combinations of BA and IAA as well as BA and NAA were tried to bring about elongation of shoots. The results are presented in Table 29. The BA/NAA combination 2.0 + 0.2 ppm was found to be significantly superior to the rest of the treatments with respect to the length of the shoots (3.83 cm). The treatment recorded a leaf length of 3.7 cm which was on par with the

Table 29. Effect of combinations of BA and auxins on the elongation of jack shoots from the shoot proliferation medium

Basal medium : MS

Treatment	Shoots* per explant	Length of the longest* shoot (cm)	Length of the longest* leaf (cm)
BA 1.0 ppm + IAA 0.1 ppm	1.00	2.40	3.13
" + " 0.2 ppm	1.00	2.70	3.18
" + " 0.4 ppm	1.25	3.03	1.78
BA 2.0 ppm + IAA 0.1 ppm	1.00	2.95	3.28
" + " 0.2 ppm	1.25	3.45	3.78
" + " 0.4 ppm	1.00	3.15	3.58
BA 3.0 ppm + IAA 0.1 ppm	3.75	2.75	3.00
" + " 0.2 ppm	2.25	2.98	2.93
" + " 0.4 ppm	1.75	2.95	2.95
BA 4.0 ppm + IAA 0.1 ppm	2.75	2.80	3.18
" + " 0.2 ppm	3.00	3.05	3.00
" + " 0.4 ppm	2.25	2.98	3.18
BA 2.0 ppm + NAA 0.1 ppm	1.0	2.58	3.20
" + " 0.2 ppm	1.0	3.83	3.70
" + " 0.4 ppm	1.0	2.55	3.43
Control (MS medium)	1.0	2.18	2.93
CD (5%)	0.55	0.33	0.41
SEM ±	0.19	0.12	0.15

* - Average of four observations

Culture period - five weeks

effects of the BA/IAA combinations (2.0 + 0.2 ppm and 2.0 + 0.4 ppm) and the BA/NAA combination 2.0 + 0.4 ppm. The treatment combination of BA/NAA 2.0 ppm + 0.2 ppm was found to be suitable for the elongation of shoots from the proliferation medium, before subjecting them to in vitro rooting treatments (Plates 11 and 12).

D. In vitro rooting (Stage III)

1. Auxins

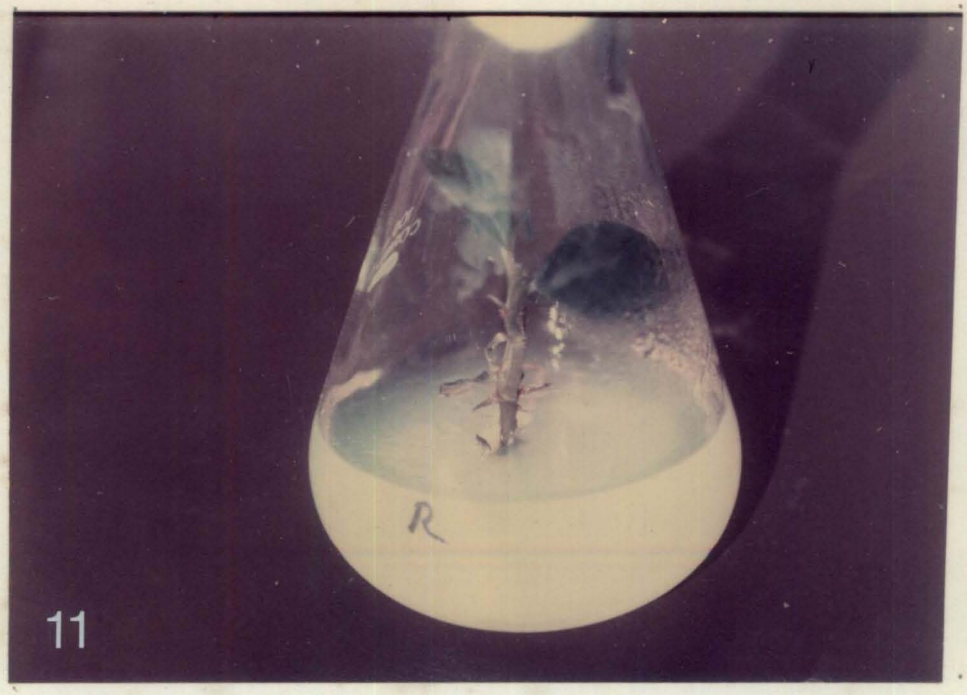
The effects of the levels of auxins either alone or in combination, on the in vitro rooting of jack shoot cultures were observed and the results are presented in Table 30. Of the thirteen treatments tried, only two treatments, namely (i) IBA/NAA combination 1.6 + 0.4 ppm and (ii) IBA/NAA combination 2.0 + 2.0 ppm (for six days followed by transferring the shoots to 1/2 MS medium without growth substances) were effective in inducing roots from jack shoot cultures from 5-year old trees (Plate 13). The root initiation was slightly greater in the former (80.0%) than in the latter (70%). However, the latter treatment produced more number of roots per shoot (5.43) and took less time for root initiation (13.43 days) as compared to the former (4.17 roots per shoot and 24.67 days). In all the treatments, callussing (to varying degrees) at the cut end of the shoots was observed before the initiation of

Plate 11. Elongation of jack shoot on transfer to MS medium + BA 2.0 ppm + NAA 0.2 ppm, from the BPM

Magnification: x 0.92

Plate 12. Elongation of jack shoot on transfer to MS medium + BA 2.0 ppm + NAA 0.2 ppm, from the BPM

Magnification: x 0.97



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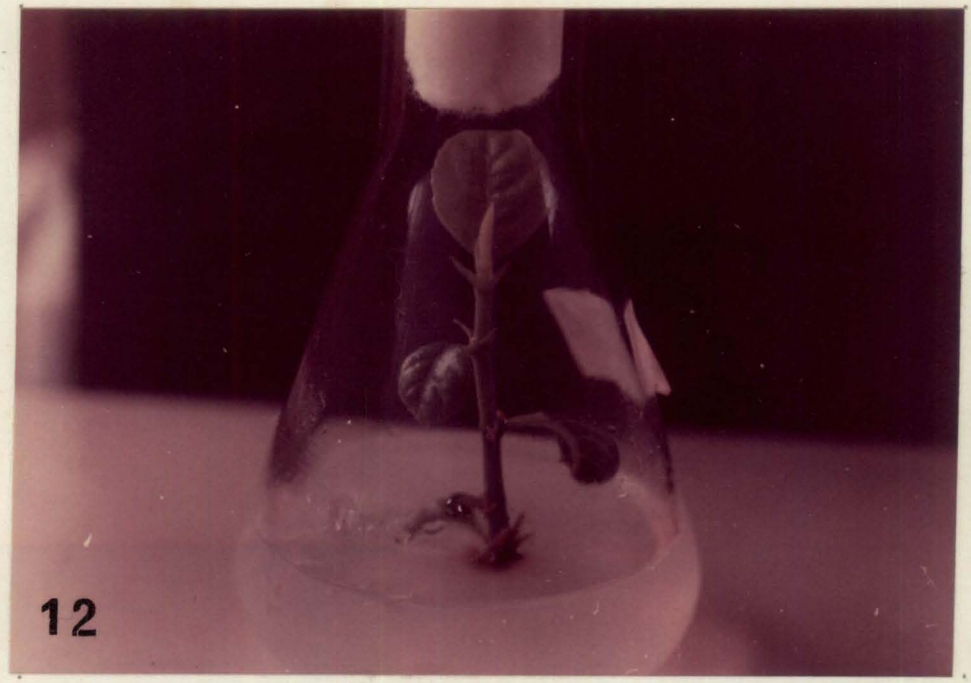

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Plate 13. In vitro rooting of jack shoot apex culture (source: five year old jack tree) in "1/2 MS +IBA 2.0 ppm + NAA 2.0 ppm for six days and then 1/2 MS without growth substances" resulted in 70.0% rooting, with 5.43 roots per shoot initiated in 13.43 days

Magnification: x 0.97

Plate 14. Callussing at the cut end of shoot, preceding the in vitro rooting of jack shoot culture

Magnification: x 2.3

13

14

Table 30. Effect of auxins on the in vitro rooting of jack shoots cultures from various age groups

Basal medium : ½ concentration of MS inorganic salts and full concentration of MS organic growth factors

Treatment	Explant from seedlings			Explant from 5-year old tree			Explant from 10-year old tree			Explant from 30-year old tree			Explant from 6-month old grafts		
	Root[a] initiation (%)	Roots per explant	Days for root initiation	Root initiation (%)	Roots per explant	Days for root initiation	Root initiation (%)	Roots per explant	Days for root initiation	Root initiation (%)	Roots per explant	Days for root initiation	Root initiation (%)	Roots per explant	Days for root initiation
1. IBA 0.2 ppm	100	4.25	28.00	-	-	-	-	-	-	-	-	-	-	-	-
2. IBA 0.8 ppm	100	5.00	29.25	-	-	-	-	-	-	-	-	-	-	-	-
3. IBA 1.6 ppm	75	7.33	29.00	-	-	-	-	-	-	-	-	-	-	-	-
4. IBA 8.0 ppm	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
5. NAA 1.0 ppm	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
6. NAA 2.0 ppm	-	-	-	0	0	0	-	-	-	-	-	-	-	-	-
7. IAA 10.0 ppm	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
8. 2,4-D 0.1 ppm	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
9. NAA 0.05 ppm + IBA 0.4 ppm	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-
10. NAA 0.4 ppm + IBA 0.2 ppm	-	-	-	0	0	0	-	-	-	-	-	-	-	-	-
11. NAA 0.4 ppm + IBA 0.4 ppm	100	6.00	20.75	0	0	0	-	-	-	-	-	-	-	-	-
12. NAA 0.4 ppm + IBA 0.8 ppm	-	-	-	0	0	0	-	-	-	-	-	-	-	-	-
13. NAA 0.4 ppm + IBA 1.6 ppm	-	-	-	80.0[b]	4.17	24.67	40.0[d]	2.50	44.00	0	0	0	0	0	0
14. NAA 0.5 ppm + IBA 0.2 ppm	100	5.75	46.25	0	0	0	-	-	-	-	-	-	-	-	-
15. NAA 2.0 ppm + IBA 0.4 ppm	75	2.67	38.33	0	0	0	-	-	-	-	-	-	-	-	-
16. NAA 2.0 ppm + IBA 2.0 ppm (for 6 days and then without growth substances)	-	-	-	70.0[c]	5.43	13.43	40.0	2.50	24.00	15.00[e]	1.0	46.70	50.0[f]	2.00	20.50
17. IAA 0.1 ppm + IBA 0.1 ppm	50	2.50	42.50	0	0	0	-	-	-	-	-	-	-	-	-

a - Average of four observations
b - Average of 15 observations
c - Average of 10 observations
d - Average of five observations
e - Average of 20 observations
0 - No response
- - No treatment

roots (Plates 14 and 15). Callussing was least for "IBA/NAA combination 2.0 + 2.0 ppm followed by transferring the shoots into 1/2 MS medium without growth substances". The root growth in this case was visually observed to be more vigorous (Plate 13). Root initiation was generally followed by new leaf production and shoot elongation. The roots produced were thick and light yellow to deep yellow in colour (Plates 13 and 16).

2. Concentration of MS medium

a. Inorganic salts

There was marked influence of the strength of the inorganic salts in the percentage of rooting and the number of roots per shoot. The most favourable effect was at half strength. Eighty per cent rooting and four roots per shoot were recorded by this level, while the normal concentration recorded only 40 per cent rooting and 2.5 roots per shoot (Table 31). Quarter and double strengths of the MS inorganic salts affected root initiation having produced only 20% rooting and one to two roots per shoot.

b. Organic growth factors

Alteration of the strength of the MS organic growth factors from the normal, appreciably affected root initiation. The root initiation was 20%, 60% and 40% at quarter, half

Plate 15. Slight callussing, observed at the cut end of shoot, preceding the *in vitro* rooting of jack shoot culture

Magnification: x 1.27

Plate 16. Three-week old jack plantlet showing thick, deep yellow roots

Magnification: x 1.34

15

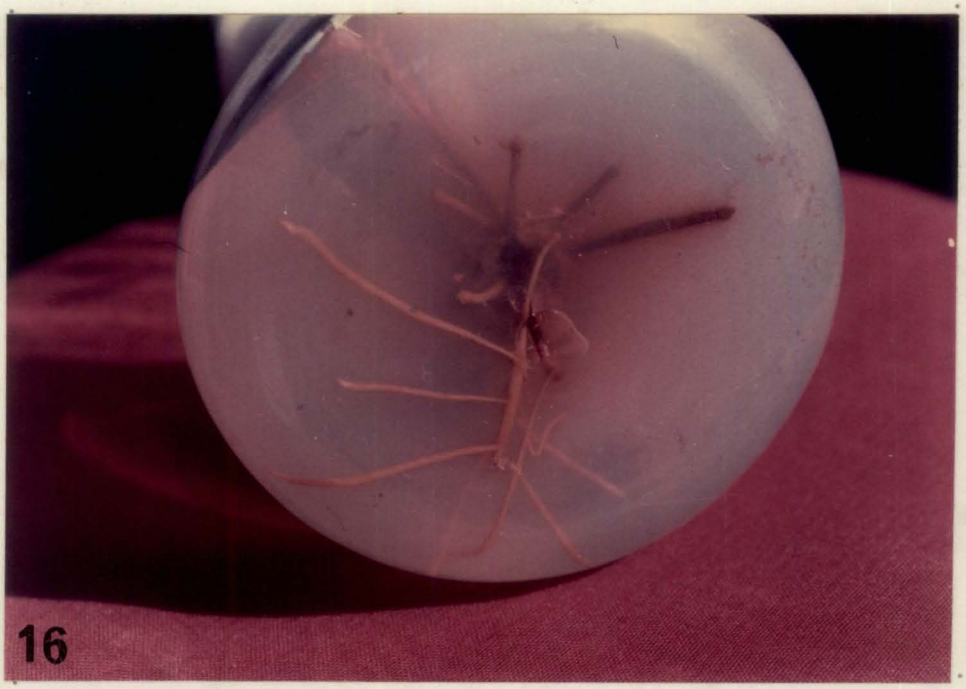
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Table 31. Effect of MS inorganic salts, MS organic growth factors, sucrose and agar on the in vitro rooting of jack shoot cultures

Basal medium containing IBA 1.6 ppm and NAA 0.4 ppm

Treatment	Cultures* initiating roots (%)	Roots per explant
MS Inorganic salts 1/4 conc.	20.0	1.00
" 1/2 conc.	80.0	4.00
" 1 conc.	40.0	2.50
" 2 conc.	20.0	2.00
MS Organic growth factors 1/4conc.	20.0	3.00
" 1/2 conc.	60.0	2.67
" 1 conc.	100.0	5.60
" 2 conc.	40.0	2.50
Sucrose 1%	20.0	2.00
" 2%	60.0	3.67
" 3%	80.0	4.00
" 4%	80.0	3.80
Agar 0.4%	80.0	5.25
" 0.6%	80.0	4.75
" 0.8%	60.0	4.00
" 1.0%	20.0	2.00

* - Average of five observations

Culture period - five weeks

and double strength of the organic factors, respectively (Table 31). The corresponding values for the number of roots produced per shoot were 3.00, 2.67 and 2.50 respectively. Hundred per cent rooting with 5.6 roots per shoot was achieved only at the normal concentration of MS organic factors.

c. Sucrose

The optimum sucrose concentration was found to be 3% for obtaining the highest rooting percentage (80%) and the largest number of roots per shoot (4.00). Though the higher concentration of sucrose (4%) also effected 80% rooting, only 3.75 roots per shoot were produced (Table 31). The per cent cultures initiating roots and the number of roots per shoot decreased with decrease in the sucrose content below 3%.

3. Physical condition of the medium

Solid medium with agar in the range of 0.4 - 0.6% was observed to effect 80% rooting and 4.75 - 5.25 roots per shoot (Table 31). However, 0.6% agar was found to be better for easiness of handling the cultures. Agar 0.8% recorded only 60% rooting and 4.0 roots per shoot. Per cent rooting and number of roots per shoot were observed to be poor at 1% agar.

The *in vitro* rooting was better at half strength of the MS inorganic salts, full strength of the MS organic growth factors, 3% sucrose and 0.6% agar.

E. Planting out the plantlets/shoots to soil (Stage IV)

1. After *in vitro* rooting (Plantlets)

The effects of potting mixtures, age of the plantlets, light intensity prior to planting out, humidity maintenance methods, growth substance sprays and nutrient starter solutions on the survival of the plantlets were assessed and the results are presented in Tables 32 to 33.

a. Potting mixture

The most suitable potting medium was found to be vermiculite. It registered the maximum survival (55.6%) of the plantlets. The potting mixture consisting of sand, soil and coir dust (1:1:1 v/v) was also found to be useful; but recorded only 40% survival of the plantlets (Table 32). The other potting mixtures tried failed to support the survival and growth of the plantlets.

b. Age of the plantlets

Age of the plantlets at the time of planting out was found to influence their survival (Table 32). The survival rate of 55.6% was obtained when the plantlets,

Table 32. Influence of potting mixtures, humidity maintenance methods, age of the plantlets, light intensity one week prior to planting out and growth substance sprays on the survival of the plantlets after planting out.

		Survival of plantlets planted out (%)
A. Potting mixture		
1. Sand + soil (1:1 v/v mixture)		0[b]
2. Sand + soil + cowdung (1:1:1 v/v mixture)		0[b]
3. Vermiculite		55.6[a]
4. Coir dust		0
5. Sand + soil + coir dust (1:1:1 v/v mixture)		40.0[b]
6. 'Trice-2' commercial peat pot		0[b]
7. Sphagnum moss + soil (1:1 v/v) mixture		0[b]
B. Humidity maintenance devices		
1. Glass beaker		0[c]
2. Petri plate + glass beaker		33.3[c]
3. Microscope cover		55.6[a]
4. Pyramid shaped polythene cover		0[c]
C. Age of the plantlets at the time of planting out		
Age of the plantlets	Hardening	
1. Root initiation	Nil	0[b]
2. "	One week	55.6[a]
3. One week after root initiation	One week	0[b]
4. Two weeks after root initiation	One week	40.0[b]
5. Three weeks after root initiation	One week	0[b]

(Contd.)

Table 32. (Contd.)

	Survival of plantlets planted out (%)
D. Light intensity one week prior to planting out	
1. 250 lux	0[b]
2. 1000 lux	40.0[b]
3. 3500 lux	58.3[d]
E. Growth substance sprays after planting out	
1. BA 10.0 ppm	50.0[e]
2. kinetin 10.0 ppm	50.0[e]
3. NAA 1.0 ppm	25.0[e]
4. ABA 0.03 ppm	50.0[e]

a - Average of 18 observations
b - Average of 5 observations
c - Average of 6 observations
d - Average of 12 observations
e - Average of 4 observations

just after the root initiation (Plate 17), were subjected to one week of *in vitro* hardening with respect to light and temperature before transfer to the potting mixture. Planting out immediately after root initiation (without hardening) failed to give success. Delaying the planting out till the roots are sufficiently elongated was found to be unfavourable. Plantlets, three weeks after root initiation (Plate 16) and subjected to one week's hardening when planted out failed to survive.

c. Light intensity

When the cultures were subjected to hardening with respect to light at 3500 lux for one week, the plantlets survived better (58.3%) than when they were exposed to lower light intensity of 1000 lux (40.0% survival). When the hardening process was totally eliminated, the plantlets failed to survive (Table 32).

d. Humidity maintenance methods

Humidity during the initial period after planting out was observed to influence the survival of the plantlets. Microscope covers (Plate 18) were found to be the most suitable, maintaining 90 - 100% R.H. and supporting 55.6% of plantlet survival (Table 32). Placing the pots in petriplates containing water and covering the same with

Plate 17. Jack plantlet, just after root initiation

Magnification: x 1.5

Plate 18. Microscope cover for maintaining the relative humidity at the required level (90-100%) plantlet survival = 55.6%

Magnification: x 0.07


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glass beaker (Plate 19) was found to be less successful, accounting for only 33.3% survival. The rest of the treatments were unsuccessful.

e. Growth substance sprays

The various growth substance sprays given to the plantlets planted out did not improve their survival rate (Table 32).

f. Nutrient starter solution application

Two starter solutions were tried for boosting up the growth of the established plantlets. When the nutrient solution of NPK 10:52:10 ratio was applied to the potting mixture, all the plantlets dried up. Growth of the plantlets was markedly superior when 1/2 MS inorganic salt solution was applied (Plates 20 and 21). . The plantlets registered a height of 14.75 cm with six leaves having a length of 9.75 cm and breadth of 5.30 cm, three months after planting out (Table 33).

2. Direct transplanting of shoots without roots

Shoots from the elongation medium were taken out, immersed in different concentrations of IBA solution for 24 hours and then transplanted to a potting mixture (sand, soil, cowdung 1:1:1 v/v). Rooting was not observed in any

Plate 19. Jack plantlet in pot placed in petri plates containing water and covered with glass beaker. Plantlet survival = 33.3%

Magnification: x 1.03

Plate 20. Jack plantlets, three months after transfer to ordinary potting medium

Magnification: x 0.22


19


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Plate 21. Jack plantlet, three months after transfer to ordinary potting medium

Magnification: x 0.39

Plate 22. Multiple, shoot production from jack shoot apex (source: six-week old seedling) on MS medium + BA 10.0 ppm + NAA 0.2 ppm. Multiplication rate after five weeks: 17.4x

Magnification: x 1.52


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Table 33. Effect of nutrient starter solutions on the growth of the plantlets, planted out.

Treatment	Height of the plantlets (cm)	Leaves/ plant-let	Length of the longest leaf (cm)	Breadth of the longest leaf (cm)
1. Tap water	7.00	3.75	3.85	2.83
2. 1/2 MS salt solution	14.75	6.00	9.75	5.30
3. NPK 10:52:10 nutrient solution	0	0	0	0

*Average of four observations made 3 months after planting out

of the above treatments. The shoots started wilting after 10 days with symptoms of decaying from the basal region.

F. *In vitro* shoot proliferation and rooting of explants from seedling, 10-year old tree, 30-year old tree and six-month old graft.

In the studies to ascertain the influence of the source of the explants on the *in vitro* shoot multiplication and rooting, the explants of seedling origin gave very good response, whereas those of mature trees and grafts exhibited low response, even though fresh stem sprouts were used and repeated subculturing was done (Table 34).

In the case of seedling explants, maximum shoot proliferation (17.4) was achieved at BA 10.0 ppm (Plate 22). This level of BA was significantly superior to the rest of the levels. The response to the higher and the lower levels varied from 4.4 to 14.0 shoots per explant.

Excellent rooting of shoots (100.0%) with 4.25 to 6.00 roots per shoot was obtained by treatment with IBA/NAA combination 0.2 + 0.5 ppm and 0.4 + 0.4 ppm as well as IBA 0.2 ppm and 0.8 ppm. The IBA/NAA combination 0.4 + 0.4 ppm recorded the maximum number of roots (6.0) in 20.75 days (Table 30). Fifty to seventyfive per cent rooting (with 2.5 to 7.33 roots per shoot) was obtained in about 29.00-46.25

Table 34. Effect of BA on the *in vitro* multiplication rate and growth of jack shoots of different age groups

Basal medium: MS + NAA 0.2 ppm

Treatment	Explant from seedlings	Explant from 10-year old trees			Explant from 30-year old trees			Explant from 6-month old grafts		
	shoots[a] per explant	Shoots[a] per explant	Length of the longest shoot (cm)[a]	Length of the longest leaf (cm)[a]	Shoots per explant	Length of the longest shoot (cm)	Length of the longest leaf (cm)	Shoots[e] per explant	Length of the longest shoot (cm)[e]	Length of the longest leaf (cm)[e]
BA 2.5 ppm	4.4	-	-	-	-	-	-	-	-	-
BA 5.0 ppm	8.8	2.80	2.26	2.52	2.09[b]	1.97[b]	1.32[b]	1.0	1.90	1.77
BA 5.0 ppm (Liquid medium)	-	-	-	-	1.25[c]	2.39[c]	1.84[c]	-	-	-
BA 7.5 ppm	11.8	1.00	2.00	1.42	1.00[d]	2.25[d]	1.25[d]	1.0	1.15	0.80
BA 10.0 ppm	17.4	1.00	0	0	0	0	0	1.0	0	0
BA 12.5 ppm	-	1.00	0	0	-	-	-	1.0	0	0
BA 20.0 ppm	14.0	-	-	-	0	0	0	-	-	-
BA 40.0 ppm	13.8	-	-	-	0	0	0	-	-	-

a - Average of five observations
b - Average of 11 observations
c - Average of eight observations
d - Average of two observations
e - Average of four observations

Culture period - five weeks

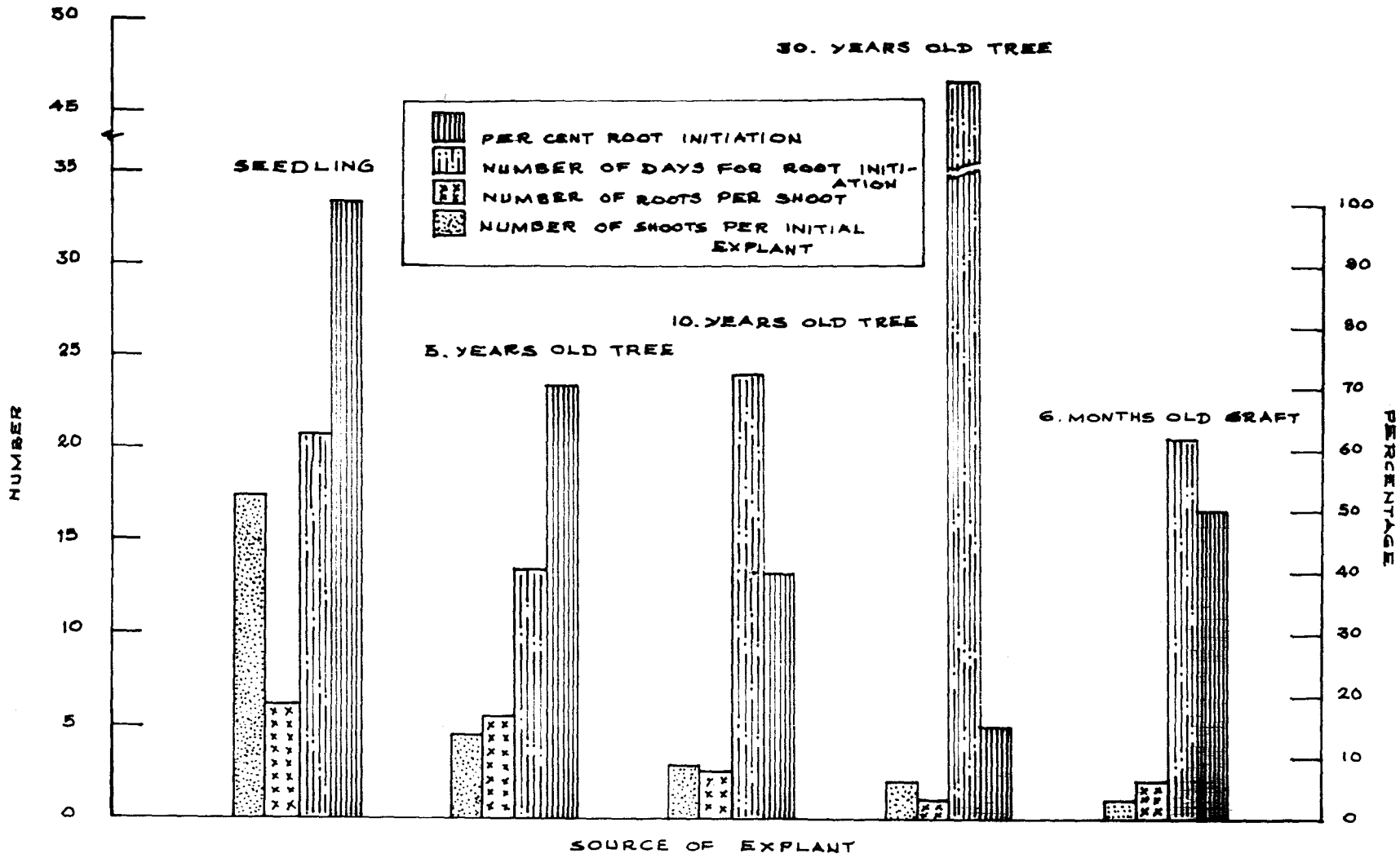


FIG: 3 PERFORMANCE OF JACK SHOOT EXPLANTS FROM DIFFERENT SOURCES AT THE BEST TREATMENTS IDENTIFIED

days with IAA/IBA combination 0.1 + 0.1 ppm, IBA 1.6 ppm and NAA/IBA combination 2.0 + 0.4 ppm (Table 30). The rest of the 12 treatments tried were found to be ineffective.

Fresh stem sprouts from 10-year old trees, after culture establishment, were subjected to shoot proliferation treatments. Multiplication of the shoots was observed only at the lowest level (5.0 ppm) of BA tried (Table 34). The rate of multiplication was very low (2.8 shoots). Higher levels restricted the growth of the shoots resulting in single shoot per culture. At BA 5.0 ppm, the length of the longest shoot was 2.26 cm. The longest leaf measured 2.52 cm. BA 7.5 ppm recorded 2.0 cm as the shoot length and 1.42 cm as the leaf length. The shoots and the leaves were very small at the higher levels of BA. When the shoots from the proliferation medium were transferred to an elongation medium (BA 2 ppm + NAA 0.2 ppm), no marked improvement in shoot growth was observed. The shoots attained a length of only 2.5 cm, with a leaf length of 2.5 cm in about six weeks. Rooting of shoots was attempted with "1/2 MS + IBA 1.6 ppm + NAA 0.4 ppm" and "1/2 MS + IBA 2.0 ppm + NAA 2.0 ppm (for 6 days) and then 1/2 MS without growth substances". Forty per cent rooting was obtained in both treatments, with 2.5 roots per shoot (Table 30). However, the latter treatment took only 24 days for root initiation, while the former took 44 days.

Multiple shoot formation was examined with explants from fresh stem sprouts of 30-year old trees, with different levels of BA in solid medium (containing NAA 0.2 ppm). Multiple shoots were obtained (Table 34) only at BA 5.0 ppm (2.09 shoots per culture). Shoots turned brown and died at the higher levels of BA tried (10.0 ppm, 20.0 ppm and 40.0 ppm), except at 7.5 ppm. In liquid medium, BA at 5 ppm recorded only 1.25 shoots per culture. This treatment was inferior to BA 5 ppm in solid medium with respect to shoot proliferation. However, the shoot and leaf growth were better in the liquid medium (2.39 cm and 1.84 cm, respectively) than in the solid medium (1.97 cm and 1.32 cm, respectively). At BA 7.5 ppm a shoot length of 2.25 cm and a leaf length of 1.25 cm were observed (Table 34).

The shoots were subjected to two rooting treatments "1/2 MS + IBA/NAA combination 1.6 + 0.4 ppm" and "1/2 MS + IBA/NAA combination 2.0 + 2.0 ppm (for 6 days) and then 1/2 MS without growth substances". Rooting was not observed in the former case, even after three sub cultures. The latter treatment produced 15.0 per cent rooting after 46.7 days, with three subcultures (Table 30).

Explants from six-month old jack grafts were subjected to shoot proliferation trials, expecting better

performance resulting from partial rejuvenation. However, the treatments with BA (5.0 to 12.5 ppm) + NAA (0.2 ppm) were not effective in inducing multiplication of the shoot apices and axillary buds, although 100% survival of shoots and fairly satisfactory shoot growth were observed (Table 34).

Rooting of the shoots were tried with two treatments "1/2 MS + IBA/NAA combination 1.6 + 0.4 ppm" and "1/2 MS + IBA/NAA combination 2.0 + 2.0 ppm (for six days) and then 1/2 MS without growth substances". The rooting percentage after one subculture was 50%. Only two roots per shoot (Plate 23) were produced after 20.5 days (Table 30).

G. Cytological examination by root tip squash technique

The influence of *in vitro* culture techniques on the chromosome stability of the plantlets raised from explants of five-year old trees and seedlings has been examined by the conventional root tip squash technique. The metaphase observations are presented in plates 24 and 25.

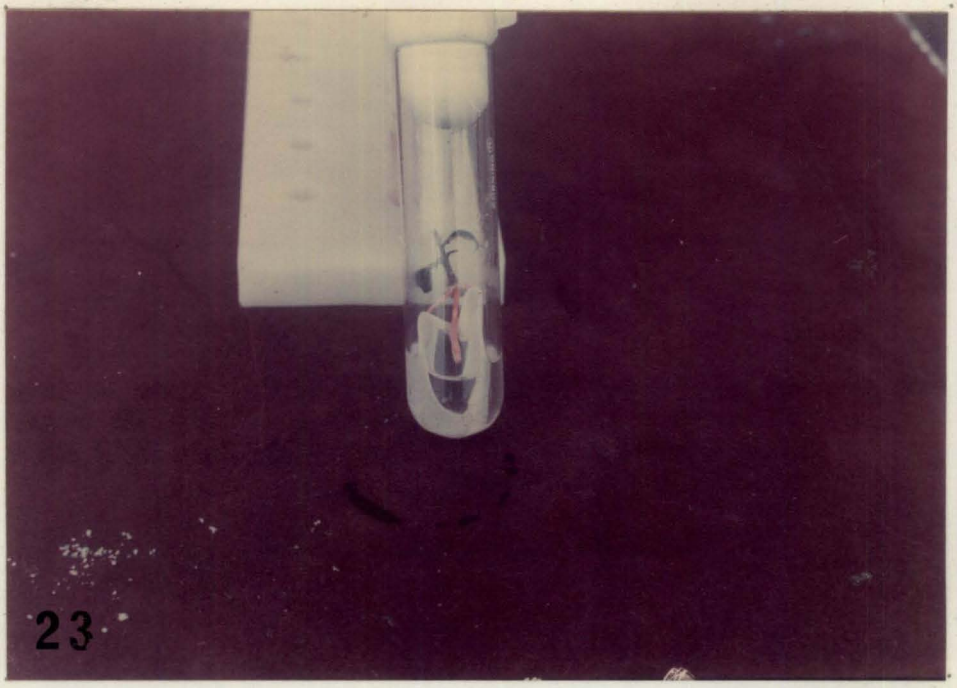
The plantlets recovered from the shoot apices of five year old trees and seedlings maintained the diploid nature with the normal chromosome count of 2n = 56.

Plate 23. *In vitro* rooting of jack shoot apex culture (source: six-month old jack graft) in "1/2 MS + IBA 2.0 ppm + NAA 2.0 ppm for six days and then 1/2 MS without growth substances"

Magnification: x 0.52

Plate 24. Metaphase plate of a cell from the root tip squash of jack plantlet, showing a chromosome number of 2n = 56

Magnification: x 1600


23

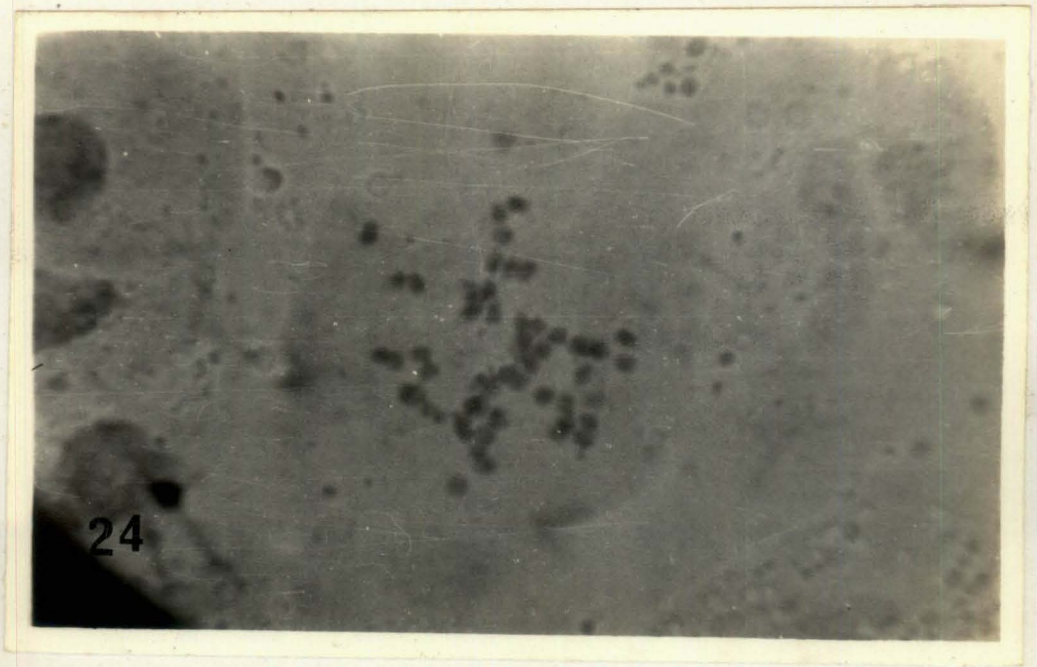
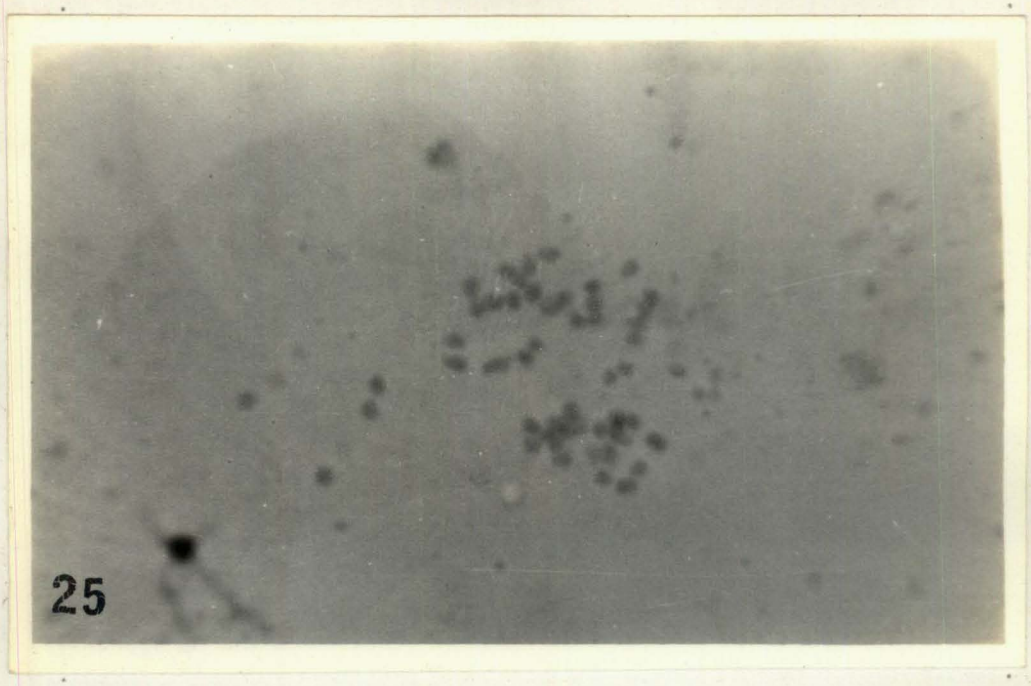

24

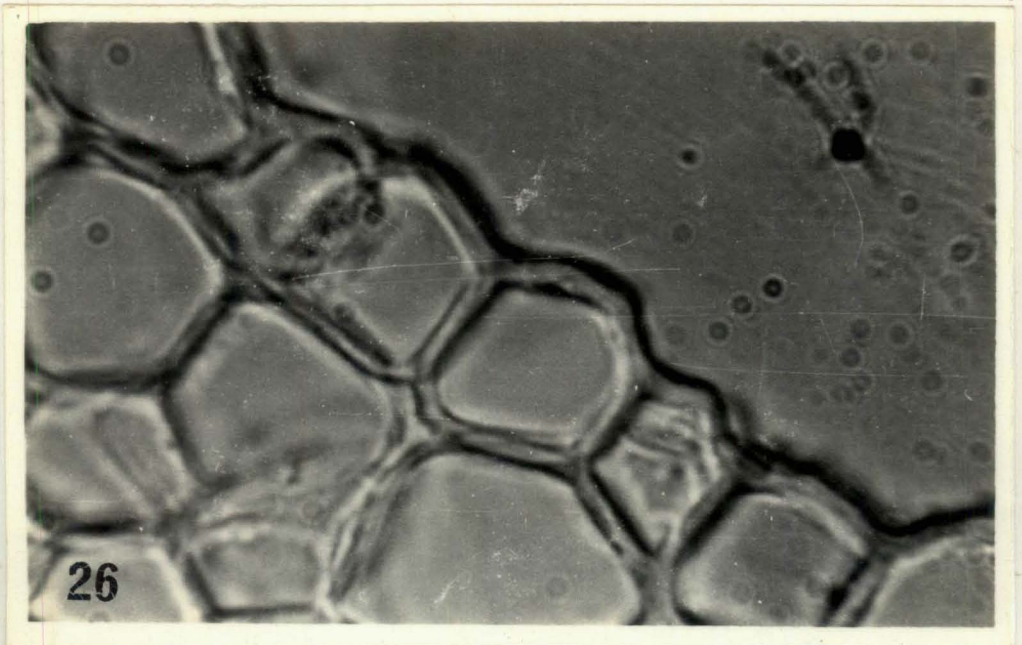
Plate 25. Metaphase plate of a cell from the root tip squash of jack seedling, showing a chromosome number of 2n = 56

Magnification: x 1600

Plate 26. C.S. of leaf produced by jack plantlet, showing thin cuticle

Magnification: x 700


25


26

H. Histological investigations

The leaves from the seedlings (used as the source of explants) and the new plantlets (prior to and after hardening) were examined to understand the anatomical differences, if any, that could contribute to better survival.

The leaves of the new plantlets were observed to have thinner cuticle (Plate 26) as compared to those of the seedling leaves (Plate 27). The new leaves produced by the hardened plantlets after establishment outside (Plate 28) also had thicker cuticles.

Economics of production of jack plantlets

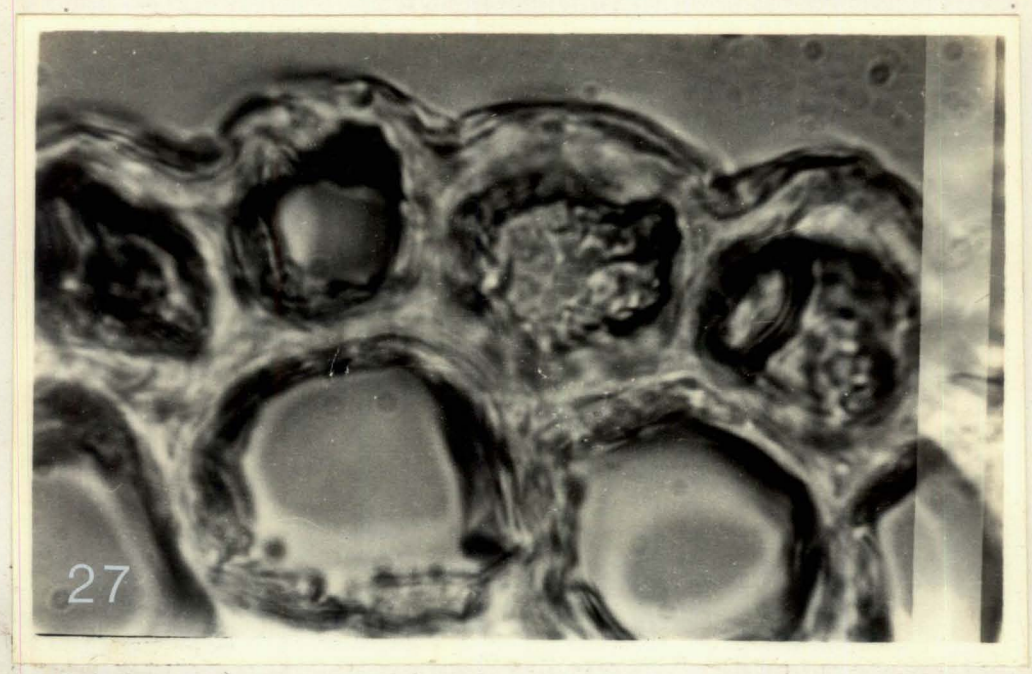
The cost of production of jack plantlets using explants from fresh stem sprouts of five-year old trees was worked out based on the facilities of the Tissue Culture Laboratory at the College of Horticulture, having a potential of maintaining 4200 cultures for multiplication, 4200 cultures for shoot elongation, 4200 cultures for in vitro rooting and 7000 plantlets for hardening (Table 35). One scientist (Rs.1400/= p.m.) and one Technician (Rs.1000/= p.m.) were considered necessary for the work. The number of initial explants was 100. The duration for culture establishment was six weeks; for shoot proliferation, five weeks; for shoot elongation, four weeks; for in vitro rooting, four

Plate 27. C.S. of leaf produced by jack seedling, showing normal cuticle

Magnification: x 1100

Plate 28. C.S. of leaf produced by jack plantlet after establishing outside, showing normal cuticle

Magnification: x 1100


27

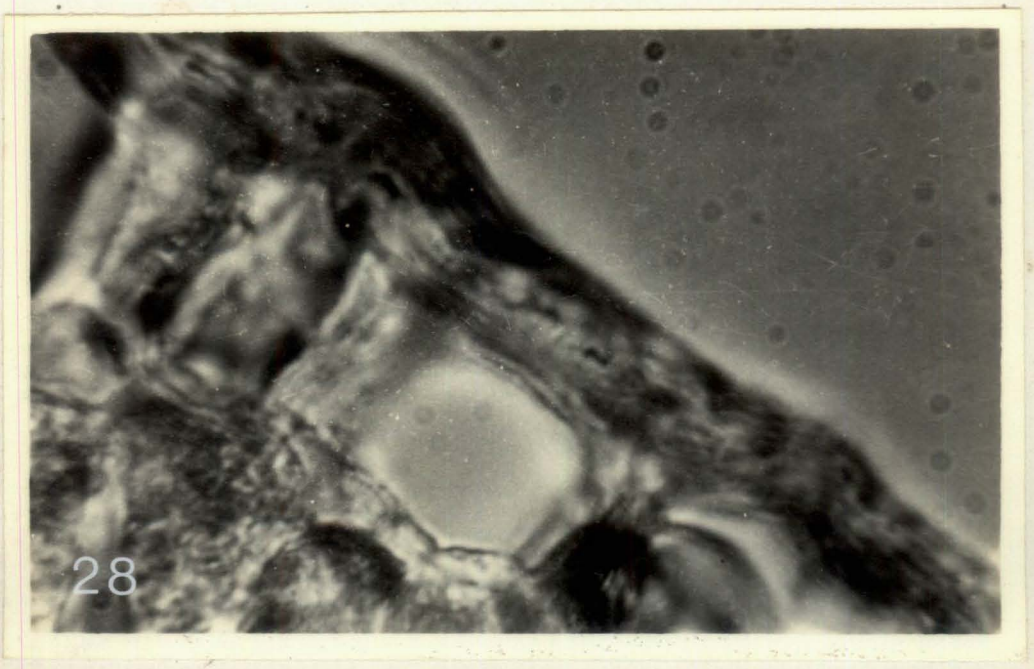

28

Table 35. Economics of production of jack plantlets through *in vitro* culture

I. Cost of production

Maximum capacity for the production of plantlets under the existing physical facilities of the T.C. lab at the College of Horticulture	:	4200 cultures for shoot multiplication, 4200 cultures for shoot elongation, 4200 cultures for rooting and 7000 plantlets for hardening

	A. Physical			Expenditure per year (Rs.)
1.	Glasswares	Rs.	30000/5 yrs.	6000.00
2.	Chemicals	Rs.	10000/5 yrs.	2000.00
3.	Wooden racks - 7 Nos.	Rs.	6300/20 yrs.	315.00
4.	Fluorescent tubes (63 Nos.) and fittings	Rs.	6000/5 yrs.	1200.00
5.	Autoclave - 1 No.	Rs.	25000/20 yrs.	1250.00
6.	Laminar airflow chamber - 1 No.	Rs.	25000/10 yrs.	2500.00
7.	Air conditioner - 2 Nos.	Rs.	30000/15 yrs.	2000.00
8.	Shaker - 1 No.	Rs.	8500/10 yrs.	850.00
9.	Hot air oven - 2 Nos.	Rs.	10000/10 yrs.	1800.00
10.	Balances - 2 Nos.	Rs.	10000/20 yrs.	500.00
11.	pH meter - 1 No.	Rs.	4000/10 yrs.	400.00
12.	Refrigerator - 1 No.	Rs.	6000/15 yrs.	400.00
13.	Water bath - 1 No.	Rs.	5000/20 yrs.	250.00
14.	Heating mantles - 1 No.	Rs.	400/5 yrs.	80.00
15.	Double glass distillation unit - 1 set	Rs.	6000/10 yrs.	600.00
16.	Cotton, inoculation aids, foils, alcohol etc.	Rs.	1000/yr.	1000.00
17.	Mudpot and potting mixture	Rs.	4000/yr.	4000.00
18.	Humidity maintenance devices	Rs.	3000/10 yrs.	300.00
19.	Electricity charges, maintenance charges and miscellaneous items	Rs.	4000/yr.	4000.00
20.	Building and furnishings	Rs.	100000/50 yrs.	2000.00
	B. Salary			
1.	Salary of one scientist (Pay Rs.1400/- p.m.)	Rs.	16800/yr.	16800.00
2.	Salary of one technician (Pay Rs. 1000/- p.m.)	Rs.	12000/yr.	12000.00
			Total	Rs. 59445.00

(Contd.)

Table 35.(Contd.)

II. Number of plantlets produced per year

No.of weeks	Culture establi-shment	Shoot proli-feration	Shoot elonga-tion	Rooting	Plantlets after 1-month har-dening
6	100	100			
11		573			
16		3283.3			
21		18813.3			
25			4200		
26		24066			
29				2940	
30			4200		
31		24066			
34				2940	1634.64
35			4200		
36		24066			
39				2940	1634.64
40			4200		
41		24066			
44				2940	1634.64
45			4200		
46		24066			
49				2940	1634.64
50			4200		
				Total	6538.56

III. Cost of production of single plantlet

Total cost involved per year	Rs.	59445.00
Number of plantlets produced per year		6538.56
Cost of production of one plantlet	Rs.	9.09

weeks and for planting out and hardening the plantlets, five weeks. After 21 weeks, two cycles can be introduced into the production system. A portion (4200 shoots) of the multiple shoots produced (18813.3 shoots) can be diverted for in vitro rooting, the remaining portion to be recycled for the shoot proliferation. Based on the capacity of the Laboratory, rate of culture establishment (100.0%), rooting response of the shoots (70.0%) and the survival of the plantlets (55.6%), 6538.56 plantlets per year was estimated to be produced. The total cost involved per year worked out to Rs. 59445.00, the cost of building, equipment, glasswares, chemicals and miscellaneous items having been distributed over the years according to their potential/ durability. The cost of production of one jack plantlet including one month's hardening was found to be Rs.9.09. A further period of upto six months may be required in the nursery which could increase the cost by about Rs.0.50.

II. Mussaenda

A. Culture establishment (Stage II)

1. Establishment of shoot apices and lateral buds

Shoot apices and lateral buds of mussaenda were subjected to various culture establishment treatments and the results are presented in Table 36. Hundred per cent culture survival and actively growing cultures of the shoot apices were observed in MS medium supplemented with BA

Table 36. Effect of different treatments on the survival and growth of mussaenda shoot apex cultures (via enhanced release of axillary buds)

Basal medium : MS

Treatment	Survival[a] (% cultures alive)	Cultures[a] exhibiting growth (%)
kinetin 0 ppm	60	0
" 0.5 ppm	80	20
" 1.0 ppm	60	40
" 2.0 ppm	20	0
BA 0.5 ppm	80	40
" 1.0 ppm	80	40
" 2.0 ppm	60	0
kinetin 2.5 µM + NAA 0.1 µM	100	0
" + " 0.5 µM	80	0
" + " 1.0 µM	100	20
kinetin 5.0 µM + NAA 0.1 µM	80	20
" + " 0.5 µM	100	0
" + " 1.0 µM	100	20
kinetin 10.0 µM + NAA 0.1 µM	60	0
" + " 0.5 µM	100	0
" + " 1.0 µM	60	0
BA 0.1 ppm + kinetin 0.1 ppm	60	0
" + " 0.3 ppm	80	0
" + " 0.5 ppm	60	0
" + " 1.0 ppm	100	0
BA 0.3 ppm + kinetin 0.1 ppm	60	0
" + " 0.3 ppm	100	0
" + " 0.5 ppm	80	0
" + " 1.0 ppm	40	0
BA 0.5 ppm + kinetin 0.1 ppm	80	20
" + " 0.3 ppm	100	40
" + " 0.5 ppm	100	100
" + " 1.0 ppm	80	40
BA 1.0 ppm + kinetin 0.1 ppm	80	20
" + " 0.3 ppm	60	20
" + " 0.5 ppm	80	20
" + " 1.0 ppm	60	0

a - Average of five observations Culture period - 2 weeks

0.5 ppm + kinetin 0.5 ppm. Majority of the other treatments also supported the survival of the cultures fairly well (60.0 - 100.0 per cent). However, the proportion of growing cultures in these treatments was low (0 to 40%). Out of the 32 treatments involving combinations of BA, kinetin and NAA, only 14 favoured the growth of the cultures. When lateral buds were subjected to the best treatment identified, namely, BA/kinetin combination 0.5 + 0.5 ppm, 73.3 per cent survival of the cultures and 26.1 per cent growing cultures resulted.

In about two weeks after the inoculation of the shoot apices, the outer leaves started growing at a fast rate (Plate 29). By the time they expanded fully, growth of the new leaves was initiated. The cultures were considered established when the above changes reached a satisfactory level (Plate 30). This stage was attained three weeks after inoculation, in the case of shoot apices. Lateral buds needed about five weeks to attain this stage, as their growth was slower. The problem of phenolics was sparse in both the cultures.

2. Somatic organogenesis - callus production

a. Shoot apices

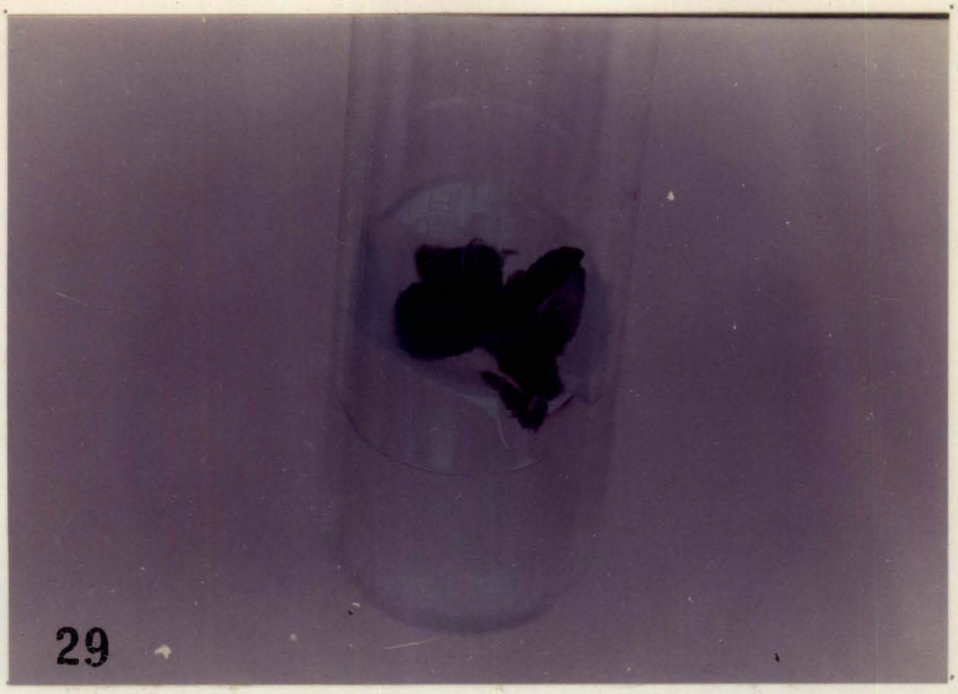
The effect of the 36 treatments involving combinations of kinetin and NAA, BA and NAA as well as BA and

Plate 29. Mussaenda shoot apex after two weeks in the establishment medium (MS + BA 0.5 ppm + kinetin 0.5 ppm)

Magnification: x 1.56

Plate 30. Mussaenda shoot apex after three weeks in the establishment medium

Magnification: x 1.77


29


30

IAA on the production and growth of callus from the shoot apices of mussaenda are presented in Table 37.

Among the treatment combinations involving kinetin and NAA, the highest values for callus index (CI = 367.0), growth score (G = 3.67) and per cent callus initiation (100.0%) were registered by kinetin/NAA combination 1.0 + 2.0 ppm (Table 37). Fifty per cent of the treatments recorded CI values ranging from 133.0 to 367.0, growth score from 1.33 to 3.67 and hundred per cent callus initiation. A comparison of the mean CI values of the levels of NAA over the levels of kinetin showed that NAA was more favourable at 2.0 ppm (CI = 233.33) than at the other levels (Appendix III.1). Mean CI values of kinetin over the levels of NAA slightly increased with increasing concentrations, the maximum being at 4.0 ppm (CI = 75.18).

Among the BA/NAA combinations, maximum callus index of 233.0 and maximum number of cultures initiating callus (100.0%) were obtained in BA/NAA combinations 2.0 + 2.0 ppm and 4.0 + 8.0 ppm (Table 37). BA/NAA combinations 1.0 + 2.0 and 2.0 + 8.0 ppm registered the maximum growth score (G = 2.5). On the whole fifty per cent treatments registered callus initiation of 66.7 per cent or above, while one third of the treatments proved ineffective. Moderate to good callus index (CI = 166.8 to 233.0) and growth score

Table 17. Effect of different treatments on the production and growth of callus from shoot apex, ovary wall segment and leaf segment cultures of muskmelon (w.r.t. somatic organogenesis)

Basal medium : MS

Treatment	Shoot apex culture			Leaf segment culture			Ovary wall segment culture		
	Cultures[a] initiating callus (%)	Growth[a] score (G)	Callus[a] Index (CI)	Cultures[a] initiating callus (%)	Growth[a] score (G)	Callus[a] Index (CI)	Cultures[a] initiating callus (%)	Growth[a] score (G)	Callus[a] Index (CI)
kinetin 1.0 ppm + NAA 1.0 ppm	33.3	1.00	33.30	66.7	1.00	66.70	100.0	1.00	100.00
" + " 2.0 ppm	100.0	3.67	367.00	100.0	2.00	200.00	100.0	4.00	400.00
" + " 4.0 ppm	33.3	1.00	33.30	33.3	1.00	33.30	66.7	1.00	66.70
" + " 8.0 ppm	66.7	1.00	66.70	0	0	0	100.0	1.00	100.00
kinetin 2.0 ppm + NAA 1.0 ppm	0	0	0	33.3	1.00	33.30	33.3	1.00	33.30
" + " 2.0 ppm	100.0	2.33	233.00	100.0	1.67	167.00	100.0	3.00	300.00
" + " 4.0 ppm	100.0	1.33	133.00	100.0	1.33	133.00	100.0	4.00	400.00
" + " 8.0 ppm	100.0	1.67	167.00	100.0	2.00	200.00	100.0	1.67	167.00
kinetin 4.0 ppm + NAA 1.0 ppm	66.7	1.00	66.70	66.7	1.50	100.00	66.7	1.00	66.70
" + " 2.0 ppm	66.7	1.50	100.00	66.7	1.00	66.70	100.0	2.00	200.00
" + " 4.0 ppm	100.0	2.67	267.00	100.0	2.67	267.00	100.0	2.67	267.00
" + " 8.0 ppm	100.0	2.67	267.00	100.0	2.00	200.00	100.0	1.33	133.00
BA 1.0 ppm + NAA 1.0 ppm	33.3	1.00	33.30	-	-	-	-	-	-
" + " 2.0 ppm	66.7	2.55	166.75	-	-	-	-	-	-
" + " 4.0 ppm	0	0	0	-	-	-	-	-	-
" + " 8.0 ppm	33.3	2.00	66.60	-	-	-	-	-	-
BA 2.0 ppm + NAA 1.0 ppm	0	0	0	-	-	-	-	-	-
" + " 2.0 ppm	100.0	2.33	233.00	-	-	-	-	-	-
" + " 4.0 ppm	66.7	1.00	66.70	-	-	-	-	-	-
" + " 8.0 ppm	66.7	2.50	166.80	-	-	-	-	-	-
BA 4.0 ppm + NAA 1.0 ppm	0	0	0	-	-	-	-	-	-
" + " 2.0 ppm	0	0	0	-	-	-	-	-	-
" + " 4.0 ppm	100.0	1.67	167.00	-	-	-	-	-	-
" + " 8.0 ppm	100.0	2.33	233.00	-	-	-	-	-	-
BA 1.0 ppm + IAA 1.0 ppm	33.3	1.00	33.30	-	-	-	-	-	-
" + " 2.0 ppm	66.7	1.50	100.00	-	-	-	-	-	-
" + " 4.0 ppm	100.0	1.00	100.00	-	-	-	-	-	-
" + " 8.0 ppm	100.0	1.33	133.00	-	-	-	-	-	-
BA 2.0 ppm + IAA 1.0 ppm	33.3	1.00	33.30	-	-	-	-	-	-
" + " 2.0 ppm	66.7	1.00	66.70	-	-	-	-	-	-
" + " 4.0 ppm	100.0	1.00	100.00	-	-	-	-	-	-
" + " 8.0 ppm	100.0	1.67	167.00	-	-	-	-	-	-
BA 4.0 ppm + IAA 1.0 ppm	100.0	1.00	100.00	-	-	-	-	-	-
" + " 2.0 ppm	0	0	0	-	-	-	-	-	-
" + " 4.0 ppm	100.0	1.33	133.00	-	-	-	-	-	-
" + " 8.0 ppm	100.0	1.00	100.00	-	-	-	-	-	-

a - Average of three observations. - No callus formed
Culture period - four weeks - No treatment

(G = 1.67 to 2.50) were recorded by five out of the 12 treatments tried. The mean CI values of NAA when compared over the levels of BA showed that NAA was more favourable at 8.0 ppm (CI = 155.47) than at other levels (Appendix III.2). BA was more effective at 2.0 ppm (CI = 116.63).

Treatments involving combinations of various levels of BA and IAA recorded lower values for callus index and growth. However, callus initiation was fairly good (Table 37). While seven out of the twelve treatments induced callus in hundred per cent cultures, only two treatments recorded growth scores above 1.5. Callus index value above 150.0 was obtained in only one treatment. Maximum CI value (CI = 167.0) and growth score (G = 1.67) coupled with hundred per cent callus initiation were recorded by BA/IAA combination 2.0 + 8.0 ppm. The mean CI values of IAA over the levels of BA showed that IAA was more favourable at 8.0 ppm (CI = 133.33) than at the other levels (Appendix III.3). The effect of IAA was found to increase with increasing levels. Benzyl adenine was equally effective at 1.0 and 2.0 ppm (CI = 91.75).

Majority of the treatments were effective in inducing profuse callus growth from the shoot apices (Plate 31). Sixteen treatments recorded hundred per cent

Plate 31. Callus production from mussaenda shoot apex culture on MS medium + kinetin 1.0 ppm + NAA 2.0 ppm. Callus Index = 367.0

Magnification: x 2.0

Plate 32. Callus production from ovary wall segment culture of mussaenda on MS medium + kinetin 1.0 ppm + NAA 2.0 ppm. Callus index = 400.0

Magnification: x 2.15


31


32

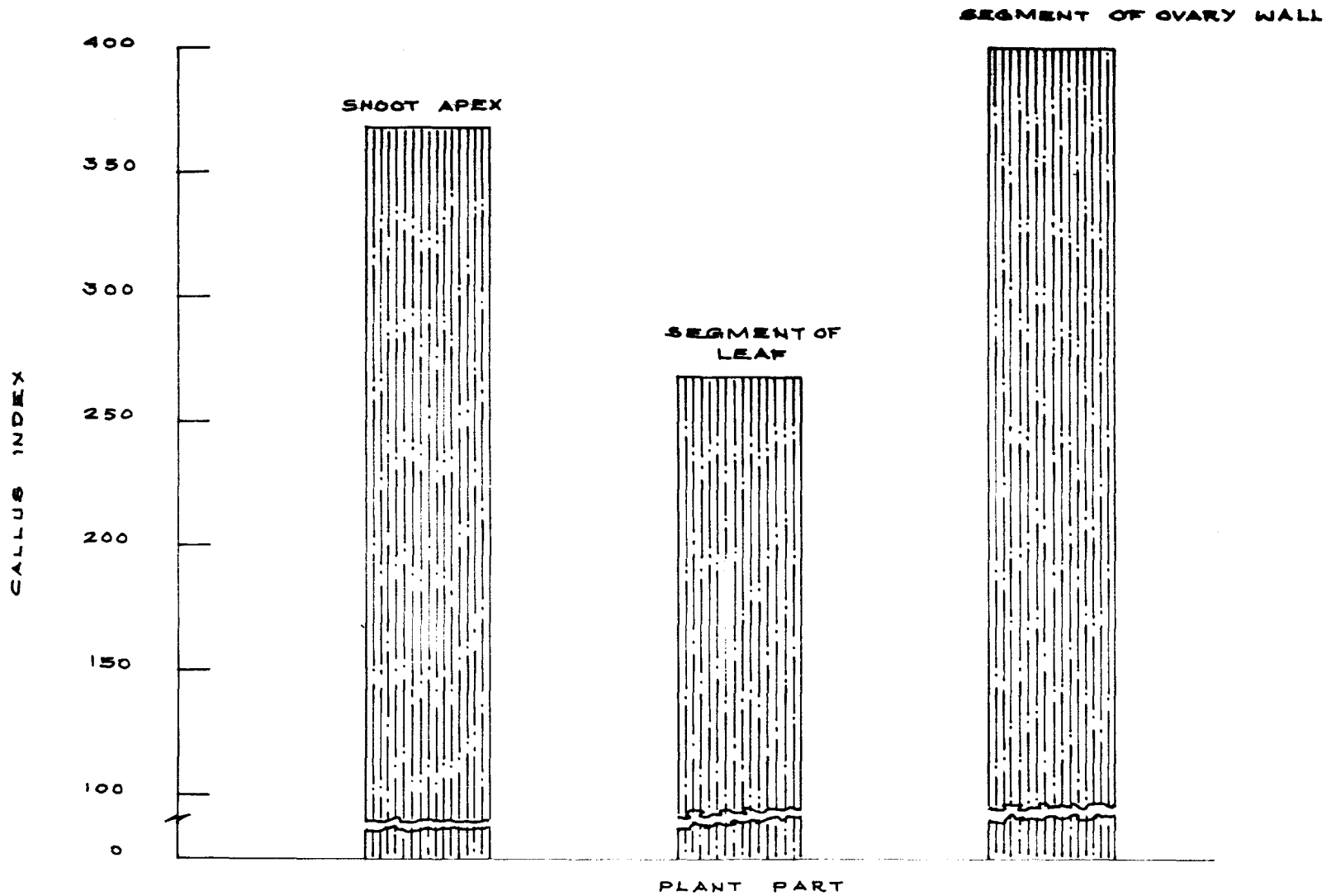


FIG: 4. CALLUS PRODUCTION BY EXPLANTS FROM DIFFERENT PLANT PARTS OF MUSSAENDA AT THE BEST TREATMENT IDENTIFIED

callus initiation, while the remaining recorded 0% to 66.7%. The kinetin/NAA combination 1.0 + 2.0 ppm registered the maximum callus index (CI = 367.0), growth score (G = 3.67) and per cent cultures initiating callus (100.0%). Six treatments recorded CI values above 100.0. The kinetin/NAA combinations 4.0 + 4.0 ppm (CI = 267.0), 4.0 + 8.0 ppm (CI = 267.0), 2.0 + 2.0 ppm (CI = 233.0), BA/NAA combinations 2.0 + 2.0 ppm (CI = 233.0) and 4.0 + 8.0 ppm (CI = 233.0) were among the treatments showing good performance. All these treatments induced hundred per cent callus initiation with growth score ranging from 2.33 to 2.67.

b. Segments of leaf

Twelve combinations of kinetin and NAA were evaluated for their capacity to induce callus initiation and growth from the segments of the in vitro formed leaves of mussaenda. The results are presented in Table 3%.

Callus index values of 200.0 and above coupled with hundred per cent callus initiation were recorded by four treatments. Growth scores ranged from 2.00 to 2.67 in all these treatments. The maximum values for callus index (CI = 267.0), growth score (G = 2.67) and per cent cultures initiating callus (100.0) were registered by kinetin/NAA combination 4.0 + 4.0 ppm. Comparison of the mean CI values

of the levels of NAA over the levels of kinetin showed that the effect was maximum at 2.0 ppm (CI = 144.57) and 4.0 ppm (CI = 144.43). The effect of kinetin when compared over the levels of NAA was found to increase with increasing concentrations and reached the maximum at 4.0 ppm (CI = 158.43).

c. Segments of ovary wall

Response of segments of ovary wall of mussaenda to treatments involving kinetin and NAA are presented in Table 37.

All the twelve treatments tried were effective in inducing profuse callussing from segments of ovary wall (Plate 32). Except one, all the treatments registered 66.7 to 100.0 per cent cultures initiating callus. The growth score ranged from 1.0 to 4.0 and five treatments recorded values 2.0 or above. In five treatments the CI values were 200.0 or above. Kinetin/NAA combinations 1.0 + 2.0 ppm and 2.0 + 4.0 ppm were the most effective treatments, recording CI values of 400.0, growth score of 4.0 and hundred per cent callus initiation. Mean CI values of NAA when compared over the levels of kinetin was found to be the maximum at 2.0 ppm (CI = 300.0). Higher levels of NAA recorded lower CI values. Kinetin was more favourable at 2.0 ppm when

compared over the levels of NAA (CI = 225.08). Mean CI values of kinetin 1.0 ppm and 4.0 ppm were lower (CI = 166.68).

The callus initiation started in about 10 days in all the above cases. It was preceeded by swelling at the cut ends of the shoot apices and swelling all over the segments of ovary wall. In the case of leaves, callus initiation started along the veins at first. In all the cases, finally the entire explant was covered by white/light cream, friable to slightly compact callus which turned light green at times.

3. Direct somatic organogenesis

Induction of direct organogenesis from <u>in vitro</u> formed shoot apices, internodal segments and lower halves of leaves with petiole, was tried with treatment combinations involving BA and kinetin. However, all the treatments failed to induce direct organogenesis from the explants.

4. Somatic embryoid induction - callus production

Responses of shoot apices and <u>in vitro</u> formed leaf segments to the treatment combinations of 2,4-D and kinetin are presented in Table 38. Direct somatic embryoid formation was not observed. All the treatments, except two, induced callus from the explants.

Table 38. Effect of different treatments on the induction of somatic embryoids (callus induction) from shoot apex and leaf segment cultures of musaenda.

Basal medium: MS

Treatment	Shoot apex culture			Leaf segment culture		
	Cultures[a] initiating callus (%)	Growth[a] score (G)	Callus[a] Index (CI)	Cultures[a] initiating callus (%)	Growth[a] score (G)	Callus[a] Index (CI)
2,4-D 0.1 ppm	66.7	1.00	66.7	66.7	1.50	100.0
" 0.5 ppm	100.0	1.00	100.0	66.7	1.00	66.7
" 1.0 ppm	33.3	1.00	33.3	66.7	1.00	66.7
" 2.0 ppm	0	0	0	33.3	1.00	66.7
2,4-D 0.1 ppm + kinetin 1.0 ppm	66.7	1.00	66.7	66.7	1.00	66.7
" + " 2.0 ppm	100.0	1.00	100.0	100.0	1.00	100.0
" + " 4.0 ppm	66.7	1.00	66.7	33.3	1.00	33.3
2,4-D 0.5 ppm + kinetin 1.0 ppm	100.0	1.00	100.0	66.7	1.00	66.7
" + " 2.0 ppm	33.3	1.00	33.3	66.7	1.00	66.7
" + " 4.0 ppm	0	0	0	33.3	1.00	33.3
2,4-D 1.0 ppm + kinetin 1.0 ppm	66.7	3.00	200.1	100.0	2.33	233.0
" + " 2.0 ppm	100.0	1.67	167.0	100.0	1.00	100.0
" + " 4.0 ppm	100.0	1.00	100.0	33.3	1.00	33.3
2,4-D 2.0 ppm + kinetin 1.0 ppm	100.0	3.67	367.0	66.7	2.50	166.8
" + " 2.0 ppm	66.7	2.50	166.8	100.0	1.33	133.0
" + " 4.0 ppm	100.0	1.00	100.0	66.7	2.50	166.8

a - Average of three observations

Culture period - four weeks

a. Shoot apices

Hundred per cent callus initiation was effected by seven out of the 16 treatments tried (Table 38). Two treatments (2,4-D 2.0 ppm and 2,4-D/kinetin combination 0.5 + 4.0 ppm) proved ineffective. Twelve treatments induced 66.7 per cent or above callus initiation. Maximum callus index (CI = 367.0) and growth score (G = 3.67) and hundred per cent callus initiation were recorded by 2,4-D/kinetin combination 2.0 + 1.0 ppm. There were nine treatments registering CI values of 100.0 or above. Comparison of the mean CI values of the levels of 2,4-D (0.1, 0.5, 1.0 and 2.0 ppm) over the levels of kinetin (0, 1.0, 2.0 and 4.0 ppm) showed that 2,4-D was more favourable at 2.0 ppm (CI = 158.45). Mean CI values of kinetin compared over the levels of 2,4-D, were found to decrease with increasing concentrations, the maximum being at 1.0 ppm (CI = 183.45).

b. Segments of leaf

Twelve out of the 16 treatments effected 66.7 per cent or above callus initiation (Table 38). However, only four treatments recorded growth score of 1.5 or above and only three treatments registered CI values of 166.8 or above. Maximum CI value (CI = 233.0) and growth score (G = 2.33) and hundred per cent callus initiation were

observed in the case of 2,4-D/kinetin combination 1.0 + 1.0 ppm. Mean CI values of the levels of kinetin compared over the levels of 2,4-D, decreased with increasing concentrations, the maximum being at 1.0 ppm (CI = 133.3). 2,4-D was more favourable at 2.0 ppm (Mean CI = 124.98).

B. Multiplication of propagule (Stage II)
1. Enhanced release of axillary buds
a. Standardisation of basic proliferation medium

After three weeks of culture in the establishment medium, the single shoots were subjected to nine treatment combinations involving BA (0.1, 0.5 and 1.0 ppm) and kinetin (0.1, 0.5 and 1.0 ppm). The number of shoots produced per culture, number of leaves and length of the longest leaf, after four weeks of culture were recorded and are presented in Table 39. The analysis of variance is given in Appendix II.

i. Number of shoots

The maximum number of shoots (2.75) was observed in the case of BA/kinetin combination 0.5 + 0.5 ppm (Plate 33). However, the differences in the number of shoots recorded by the various treatments were not statistically significant. Effect of the rest of the treatments ranged from 1.00 to 1.50 shoots.

Plate 33. Multiple shoot production from mussaenda shoot apex culture on MS medium + BA 0.5 ppm + kinetin 0.5 ppm. Multiplication rate = 2.75x

Magnification: x 1.48

Plate 34. White/cream callus produced from mussaenda shoot apex culture

Magnification: x 1.04

33

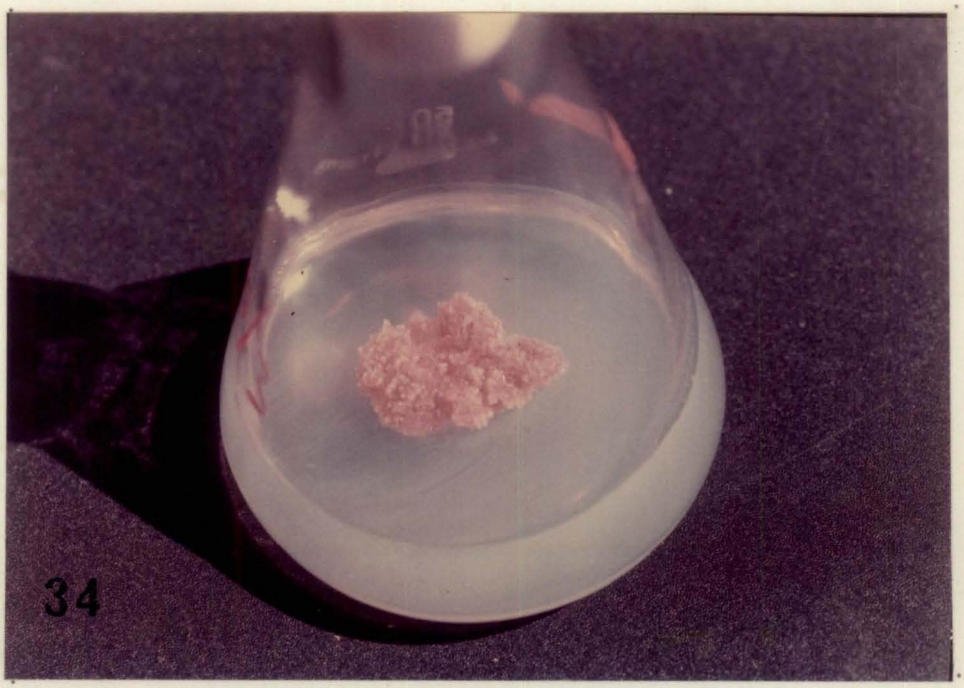


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Table 39. Effect of combinations of BA and kinetin on multiple shoot formation from mussaenda shoot apices

Basal medium : MS

Treatment	Shoots per[a] initial explant	Leaves[a] per explant	Length[a] of the longest leaf (cm)
BA 0.1 ppm + kinetin 0.1 ppm	1.00	2.50	1.20
" + " 0.5 ppm	1.00	3.00	1.78
" + " 1.0 ppm	1.00	2.50	1.78
BA 0.5 ppm + kinetin 0.1 ppm	1.50	5.00	2.20
" + " 0.5 ppm	2.75	10.25	3.10
" + " 1.0 ppm	1.25	4.45	3.00
BA 1.0 ppm + kinetin 0.1 ppm	1.50	4.25	1.93
" + " 0.5 ppm	1.25	4.50	2.03
" + " 1.0 ppm	1.50	4.00	1.55
CD (5%)	NS	3.59	0.66
SEM ±	0.38	1.24	0.23

a - Average of four observations

Culture period - four weeks

ii. Number of leaves

There was significant differences in the number of leaves produced by the various treatments. The BA/kinetin combination 0.5 + 0.5 ppm having produced the maximum number of leaves per culture (10.25) was significantly superior to the rest of the treatments which were on par with each other.

iii. Length of the longest leaf

The effects of the different treatments tried were significantly different. BA/Kinetin combination 0.5 + 0.5 ppm and 0.5 + 1.0 ppm were on par with respect to the length of the longest leaf (3.1 and 3.0 cm, respectively). These were significantly superior to the rest of the treatments.

After about four weeks, the shoots could be separated and placed in fresh medium of the same composition. The rate of multiplication of the shoots was observed for five continuous subcultures of four weeks each and the results are presented in Table 40. In the basic proliferation medium, the shoot apices produced an average of 2.6 shoots per initial explant over a period four weeks. During the subsequent subcultures, the multiplication rate increased and remained to an average of 13.46%. Lateral buds subjected to BA/kinetin combination 0.5 + 0.5 ppm failed to form multiple shoots.

Table 40. Multiplication rate per explant of mussaenda on subculturing at 4-week interval

Medium : MS + BA 0.5 ppm + kinetin 0.5 ppm

	Shoots* per culture	Per cent increase in number of shoots over the initial culture
1	2.6	
2	2.8	7.69
3	3.4	30.77
4	2.6	0
5	3.0	15.39
Mean		13.46

* Average of five observations

b. Regulation of shoot proliferation and growth of the shoot apex cultures

Effects of auxins (IAA and NAA), adenine sulphate, MS inorganic salts, and sucrose on the multiplication and growth of the shoots was assessed and the results are presented in Tables 41 to 43.

i. Auxins

Response of shoot apices to the treatments of IAA and NAA (basal medium containing BA 0.5 ppm + kinetin 0.5 ppm) are given in Table 41. The analysis of variance is presented in Appendix II.

Compared to the basic proliferation medium, IAA (0.1, 0.2 and 0.4 ppm) and NAA (0.1, 0.2 and 0.4 ppm) did not effect significant difference in the number of shoots, number of leaves and length of the longest leaf. The number of shoots and number of leaves were observed to be slightly reduced by the levels of the two auxins. However, a slight improvement in the length of the longest leaf was observed. Callus formation from the base of the shoots was observed in fifty per cent of the treatments.

ii. Adenine sulphate

The number of shoots, the number of leaves and the length of the longest leaf as influenced by the different

Table 41. Effect of IAA and NAA on multiple shoot formation from mussaenda shoot apices

Basal medium : MS + kinetin 0.5 ppm+ BA 0.5 ppm

Treatment		Shoots per[a] explant	Leaves per[a] explant	Length of[a] the longest leaf (cm)
IAA	0.1 ppm	2.75	8.25	3.65
	0.2 ppm	1.75	4.75	3.40
	0.4 ppm	2.50	6.00	3.50
NAA	0.1 ppm	2.25	5.50	3.25
	0.2 ppm	1.75	5.00	3.70
	0.4 ppm	2.00	4.75	3.40
Control (kinetin 0.5 ppm+BA 0.5 ppm)		2.75	10.25	3.10
	CD (5%)	NS	NS	NS
	SEM ±	0.45	1.34	0.25

a - Average of four observations

Culture period - four weeks

levels of adenine sulphate are presented in Table 42. The analysis of variance is given in Appendix II.

Inclusion of adenine sulphate (10.0, 20.0, 40.0 and 80.0 ppm) in the basic proliferation medium did not significantly alter the number of shoots, the number of leaves and the length of the longest leaf. The difference in response of the various levels were not significant.

iii. MS inorganic salts

The multiplication rate and growth of the cultures as modified by the strength of inorganic salts in MS medium are presented in Table 43, and the analysis of variance, in Appendix II.

The number of shoots produced per culture was significantly reduced by the double as well as the quarter strength of MS inorganic salts. However, the difference in the number was not significant at the full and half strengths (2.4 and 1.6 shoots, respectively). The number of leaves and the length of the longest leaf were significantly reduced by altering the normal salt concentration. The normal concentration of MS inorganic salts was found to be the optimum for supporting multiplication and growth of mussaenda shoot cultures.

Table 42. Effect of adenine sulphate on multiple shoot formation from mussaenda shoot apices

Basal medium : MS + BA 0.5 ppm+ kinetin 0.5 ppm

Treatment	Shoots[a] per explant	Leaves[a] per explant	Length of[a] the longest leaf (cm)
Adenine sulphate 10 ppm	2.6	6.8	3.28
" 20 ppm	2.6	6.8	3.04
" 40 ppm	2.6	5.5	3.28
" 80 ppm	2.2	6.0	3.44
CD (5%)	NS	NS	NS
SEM ±	0.49	1.7	0.21

a - Average of five observations

Culture period - four weeks

iv. Sucrose

The effect of sucrose (2.0, 3.0, 4.0 and 5.0 per cent) on the multiplication rate and growth of mussaenda shoot cultures is given in Table 43 and the analysis of variance, in Appendix II.

The number of shoots was not significantly altered by the levels of sucrose incorporated in the basic proliferation medium. However a slight reduction in the number was observed at 2.0 and 5.0 per cent levels. The number of shoots produced at sucrose 3.0% and 4.0% were identical. Effect of the levels of sucrose on the number of leaves per culture and length of the longest leaf were not significant. Sucrose 3.0 per cent produced the highest number of leaves (7.6) while 4.0 per cent effected the greatest length of the leaf (3.46).

v. Influence of Anderson's medium

The Anderson's medium was found to be unfavourable for the multiplication of mussaenda shoot cultures (Table 44). The number of shoots produced per culture was only 1.55. The number of leaves (3.82) was also very much reduced than those of the basic proliferation medium, though the length of the longest leaf (3.66 cm) was found to be slightly increased.

Table 43. Effect of MS inorganic salts and sucrose on multiple shoot formation from mussaenda shoot apices

Basal medium containing BA 0.5 ppm+ kinetin 0.5 ppm

Treatment		Shoots[a] per explant	Leaves[a] per explant	Length[a] of the longest leaf (cm)
MS inorganic salts	1/4 conc.	1.0	2.0	0.92
"	1/2 conc.	1.6	3.6	1.66
"	1 conc.	2.4	6.0	3.14
"	2 conc.	1.0	2.0	2.30
	CD (5%)	1.08	2.40	0.71
	SEM ±	0.36	0.80	0.24
Sucrose 2%		1.8	4.0	2.78
" 3%		3.0	7.6	3.38
" 4%		3.0	6.8	3.32
" 5%		2.8	6.8	3.46
	CD (5%)	NS	NS	NS
	SEM ±	0.52	0.15	0.20

a - Average of five observations

Culture period - four weeks

Table 44. Effect of Anderson's medium on multiple shoot formation from mussaenda shoot apices

Treatment	Medium containing BA 0.5 ppm + kinetin 0.5 ppm		
	Shoots[a] per explant	Leaves per explant	Length of the longest leaf (cm)
Anderson's medium	1.55	3.82	3.66
MS basic proliferation medium*	2.75	10.25	3.10

* Results reproduced from previous experiment in Table 38

a - Average of 11 observations

2. Somatic organogenesis - differentiation of callus

Treatment combinations of kinetin (0.1, 0.3, 0.5 and 1.0 ppm) and BA (0.1, 0.3, 0.5 and 1.0 ppm); kinetin (1.0, 2.0 and 4.0 ppm) and NAA (0, 0.5, 1.0, 2.0, 4.0 and 8.0 ppm) and BA (1.0, 2.0, and 4.0 ppm) and NAA (0, 0.5, 1.0 and 2.0 ppm) were tried to regenerate shoot/root from callus, produced from shoot apex cultures. The results are presented in Table 45 to 46.

Three out of the 46 treatments tried were effective in inducing shoot regeneration. The per cent cultures initiating shoot regeneration was 33.3 in all the three treatments. The effective treatments were BA/kinetin combinations 0.5 + 0.3 ppm (two shoots per culture), 0.5 + 0.5 ppm (five shoots per culture) and BA 2.0 ppm (four shoots per culture). The white/cream coloured callus (Plate 34) in about 30 days of culture period, organised into meristematic protuberances (Plate 35). Greening of the callus was simultaneously initiated as a result of chlorophyll synthesis. Greening of a few points was observed to become more intesne and shoot differentiation was initiated from such points. The shoot regeneration process was slow and the new shoots formed were tiny with a shoot apex surrounded by miniature green leaves (Plate 36). Shoots were formed only from the upper surface of the callus. Root formation was not observed in the cultures.

Plate 35. Meristematic protuberences in the callus from mussaenda shoot apex cultures on MS + BA 0.5 ppm + kinetin 0.5 ppm, 30 days after culture

Magnification: x 2.15

Plate 36. Shoot differentiation in the callus of mussaenda on MS medium + BA 0.5 ppm + kinetin 0.5 ppm

Magnification: x 1.92

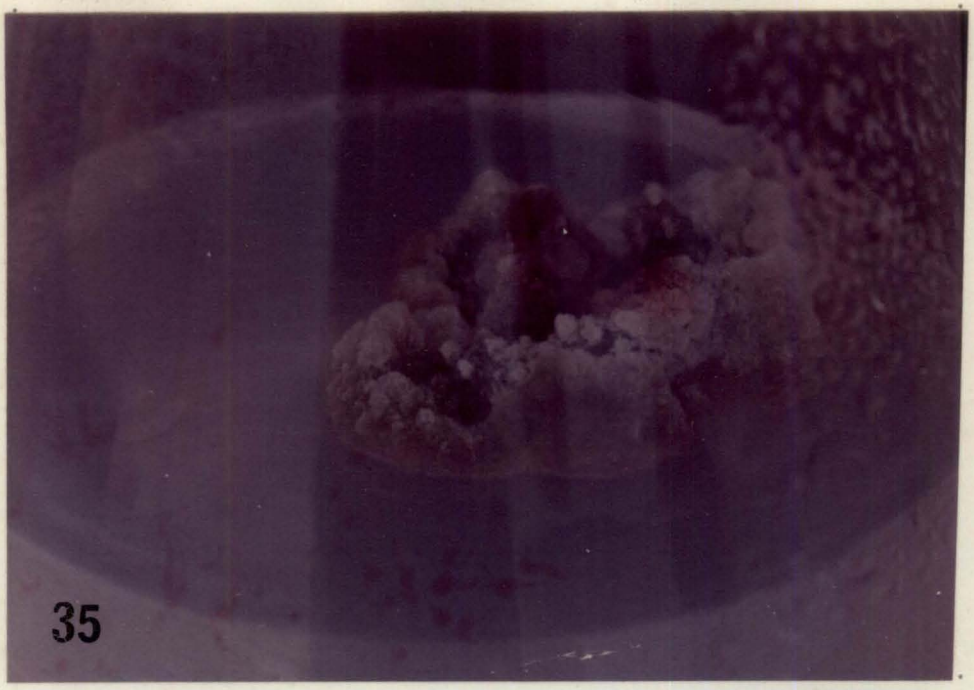

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Table 45. Effect of combinations of BA and kinetin, and BA and NAA on somatic organogenesis (shoot differentiation) from the calli of mussaenda.

Basal medium : MS

Treatment	Cultures[a] initiating shoots (%)	Shoots per culture
kinetin 0.1 ppm + BA 0.1 ppm	0	0
" + " 0.3 ppm	0	0
" + " 0.5 ppm	0	0
" + " 1.0 ppm	0	0
kinetin 0.3 ppm + BA 0.1 ppm	0	0
" + " 0.3 ppm	0	0
" + " 0.5 ppm	33.3	2.0
" + " 1.0 ppm	0	0
kinetin 0.5 ppm + BA 0.1 ppm	0	0
" + " 0.3 ppm	0	0
" + " 0.5 ppm	33.3	5.0
" + " 1.0 ppm	0	0
kinetin 1.0 ppm + BA 0.1 ppm	0	0
" + " 0.3 ppm	0	0
" + " 0.5 ppm	0	0
" + " 1.0 ppm	0	0
BA 1.0 ppm + NAA 0 ppm	0	0
" + " 0.5 ppm	0	0
" + " 1.0 ppm	0	0
" + " 2.0 ppm	0	0
BA 2.0 ppm + NAA 0 ppm	33.3	4.0
" + " 0.5 ppm	0	0
" + " 1.0 ppm	0	0
" + " 2.0 ppm	0	0
BA 4.0 ppm + NAA 0 ppm	0	0
" + " 0.5 ppm	0	0
" + " 1.0 ppm	0	0
" + 2.0 ppm	0	0

a - Average of three observations

Compared to shoot differentiation, rhizogenesis was more frequent. Nine out of the 58 treatments tried were effective in inducing moderate to profuse root regeneration from the callus. In seven out of the nine effective treatments, 66.7 per cent or above cultures exhibited root initiation. Maximum number of roots was produced at the kinetin/NAA combination 2.0 + 8.0 ppm. Among the effective treatments were kinetin/NAA combinations 4.0 + 8.0 ppm (9.67 roots), 2.0 + 2.0 ppm (8.67 roots) and BA/NAA combination 1.0 + 2.0 ppm (6.50 roots). The number of roots produced by the various treatments ranged from 2.00 to 13.33. The roots were formed from all over the surface of the callus (Plates 37 and 38). They were initiated after about 60 days of culture as white outgrowths from the callus and rapidly elongated with tufts of snow white root hairs. Some of the roots turned light green due to the presence of chlorophyll. The roots in contact with the medium exhibited a faster rate of growth with the production of primary and secondary branches.

Results of the above trials indicated the organogenetic potential of callus from mussaenda shoot apices.

3. Somatic embryogenesis (Callus mediated)

The callus from the somatic embryoid induction medium was transferred to MS medium containing combinations of BA

Plate 37. Root differentiation in the callus of mussaenda on MS medium + kinetin 2.0 ppm + NAA 8.0 ppm

Magnification: x 1.92

Plate 38. Root differentiation in the callus of mussaenda on MS medium + kinetin 2.0 ppm + NAA 8.0 ppm

Magnification: x 1.51

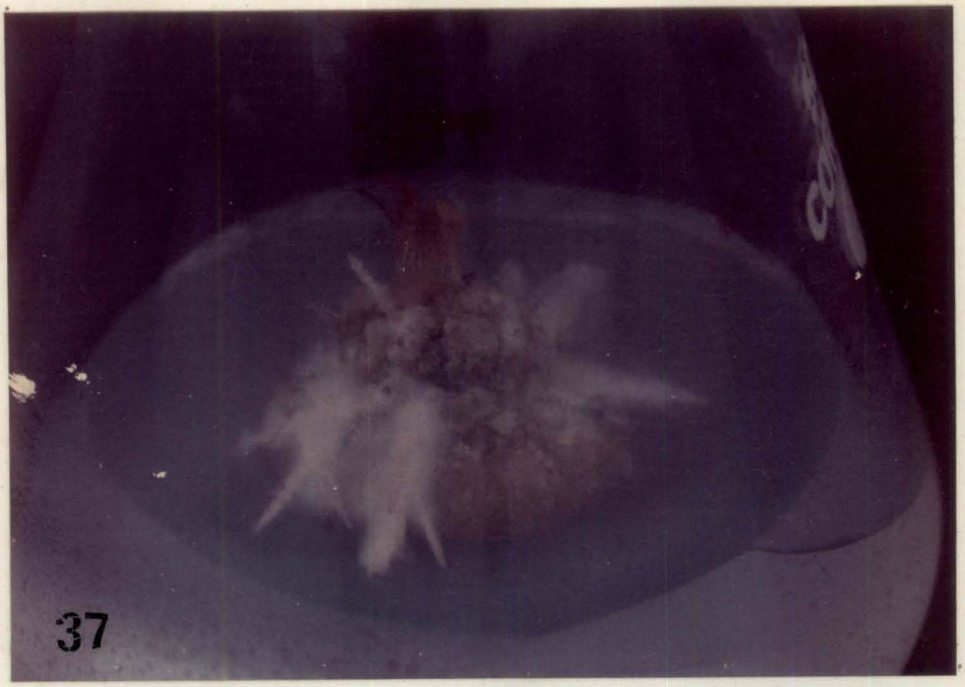
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Table 46. Effect of combinations of NAA and kinetin, and NAA and BA on somatic organogenesis (root differentiation) from the calli of mussaenda.

Basal medium : MS

Treatment	Cultures[a] initiating roots	Roots per culture
kinetin 1.0 ppm + NAA 0 ppm	0	0
" + " 0.5 ppm	0	0
" + " 1.0 ppm	0	0
" + " 2.0 ppm	0	0
" + " 4.0 ppm	66.7	6.50
" + " 8.0 ppm	0	0
kinetin 2.0 ppm + NAA 0 ppm	0	0
" + " 0.5 ppm	0	0
" + " 1.0 ppm	0	0
" + " 2.0 ppm	100.0	8.67
" + " 4.0 ppm	0	0
" + " 8.0 ppm	100.0	13.33
kinetin 4.0 ppm + NAA 0 ppm	0	0
" + " 0.5 ppm	0	0
" + " 1.0 ppm	33.3	3.0
" + " 2.0 ppm	0	0
" + " 4.0 ppm	100.0	6.67
" + " 8.0 ppm	100.0	9.67
BA 1.0 ppm + NAA 0 ppm	0	0
" + " 0.5 ppm	0	0
" + " 1.0 ppm	33.3	2.00
" + " 2.0 ppm	66.7	6.50
BA 2.0 ppm + NAA 0 ppm	0	0
" + " 0.5 ppm	0	0
" + " 1.0 ppm	0	0
" + " 2.0 ppm	100.0	3.00
BA 4.0 ppm + NAA 0 ppm	0	0
" + " 0.5 ppm	0	0
" + " 1.0 ppm	0	0
" + " 2.0 ppm	0	0

a - Average of three observations

(0, 0.5 and 1.0 ppm) and kinetin (0.05 and 1.0 ppm). The results of the trials are presented in Table 47. Globular structures resembling somatic embryoids (Plate 39) were formed after 70-73 days of culture in BA/kinetin combinations 0.5 + 0.5 ppm and 1.0 + 0.5 ppm. These structures then exhibited simultaneous root and shoot development (Plate 40). The frequency of development of the structures was low and was observed in 26.7 per cent cultures of BA/kinetin combination 0.5 + 0.5 ppm and in 6.7 per cent cultures of BA/kinetin combination 1.0 + 0.5 ppm. In the former treatment, 4.5 shoots and in the latter, 2.0 shoots were formed finally. In both the cases, tufts of snow-white miniature roots were formed at the base of the shoots. The shoots developed were small white and with miniature light green leaves (Plate 40). Both the shoots and the roots were observed to have a slow rate of growth.

C. In vitro rooting (Stage III)

1. Auxins

Combinations of the auxins IBA (0.4, 0.8 and 1.6 ppm) and NAA (0.4, 0.8 and 1.6 ppm) were tried for in vitro rooting of mussaenda shoots and the results are presented in Table 48. Root initiation was observed in five treatments. The per cent root initiation in these ranged from 20.0 to 60.0 with 3.0 to 7.0 roots per culture, initiated in 27.0 to 42.5 days. Maximum

Table 47. Effect of combinations of kinetin and BA on somatic embryoid formation from the calli of mussaenda

Basal medium : MS

Treatment	Cultures[a] initiating somatic embryoids (%)	Somatic embryoids formed per culture	Days taken for initiation of somatic embryoids
BA 0 ppm + kinetin 0 ppm	0	0	0
" + " 0.5 ppm	0	0	0
" + " 1.0 ppm	0	0	0
BA 0.5 ppm + kinetin 0 ppm	0	0	0
" + " 0.5 ppm	26.7	4.5	70
" + " 1.0 ppm	0	0	0
BA 1.0 ppm + kinetin 0 ppm	0	0	0
" + " 0.5 ppm	6.7	2.0	73
" + " 1.0 ppm	0	0	0

a - Average of 15 observations

Plate 39. Development of globular structures (somatic embryoids?) in the callus of mussaenda transferred from the induction medium to MS + BA 0.5 ppm + kinetin 0.5 ppm

Magnification: x 1.54

Plate 40. Simultaneous development of tiny shoots and tufts of snow white roots from the globular structures (somatic embryoids?) emerged from the callus of mussaenda transferred from the induction medium to MS + BA 0.5 ppm + kinetin 0.5 ppm

Magnification: x 2.92

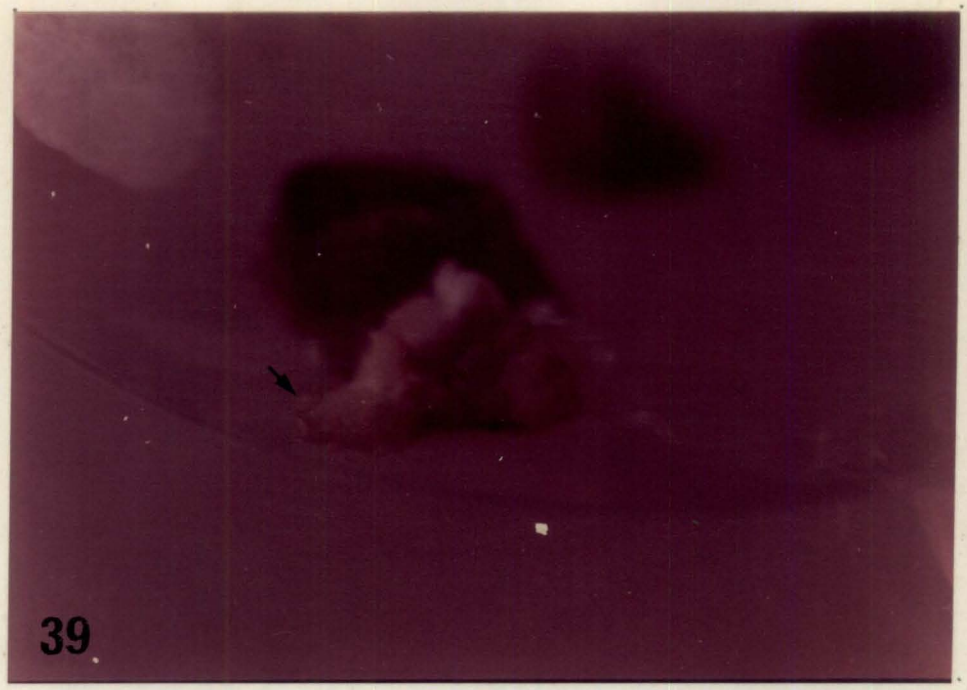

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Table 48. Effect of combinations of NAA and IBA on the *in vitro* rooting of Musaaenda shoot cultures

Basal medium: 1/2 conc. of MS inorganic salts and full conc. of MS organic growth factors

Treatment	Root[a] initiation (%)	Roots per explant	Days for root initiation
IBA 0.4 ppm + NAA 0.4 ppm	60.0	7.0	37.33
" + " 0.8 ppm	0	0	0
" + " 1.6 ppm	0	0	0
IBA 0.8 ppm + NAA 0.4 ppm	40.0	3.0	42.50
" + " 0.8 ppm	20.0	7.0	27.00
" + 1.6 ppm	0	0	0
IBA 1.6 ppm + NAA 0.4 ppm	60.0	3.0	41.33
" + " 0.8 ppm	20.0	3.0	39.00
" + " 1.6 ppm	0	0	0

a - Average of five observations

number of roots (7.0) initiated in minimum number of days (27.0) was observed at IBA/NAA combination 0.8 + 0.8 ppm. However, the per cent root initiation in this case was only 20.0. The IBA/NAA combination 0.4 + 0.4 ppm induced 7.0 roots and recorded 60.0 per cent root initiation; but took 37.31 days for the initiation. The other treatments recorded only 3.0 roots per culture.

In general, the rooting response was poor, with low number of roots per culture, more days for root initiation and low percentage of root initiation. The roots produced were slender and not as healthy as those differentiated from the callus. They produced a few primary branches only. Rooting was invariably associated with good amount of callussing from the base of shoots. In most of the cases, including the best treatment, the shoots turned pale yellow with symptoms of withering, by the time the roots were initiated, as the half strength of MS inorganic salts used for the rooting appeared to be insufficient to support the shoot growth for long. At the full salt strength, the number of roots per culture and per cent root initiation were considerably reduced.

2. MS inorganic salts

Concentration of the MS inorganic salts had marked influence on the number of roots per culture and per cent

Table 49. Effect of MS inorganic salts, sucrose and agar on the *in vitro* rooting of mussaenda shoot cultures

Treatment		Basal medium containing IBA 0.4 ppm and NAA 0.4 ppm	
		Cultures[a] initiating roots (%)	Roots per explant
MS Inorganic salts	1/4 conc.	40.0	2.5
"	1/2 conc.	60.0	8.5
"	1 conc.	40.0	3.5
"	2 conc.	0	0
Sucrose	1%	0	0
"	2%	40.0	5.5
"	3%	80.0	5.5
"	4%	60.0	4.5
Agar	0.4%	40.0	3.0
"	0.6%	80.0	3.0
"	0.8%	20.0	3.0
"	1.0%	0	0

a - Average of five observations

root initiation as evident from the data presented in Table 49. Half strength of the salts recorded the maximum number of roots per culture (8.5) and per cent root initiation (60.0). Quarter as well as full strength registered lower per cent root initiation (40.0 each) and reduced number of roots per culture (2.5 and 3.5 respectively). Double strength of the salts inhibited root initiation. Half strength of MS inorganic salts could be seen to be the most favourable for the *in vitro* rooting of mussaenda shoots.

3. Sucrose

The influence of sucrose on the *in vitro* root initiation of mussaenda shoots was studied with different levels (1.0, 2.0, 3.0 and 4.0 per cent) of sucrose included in 1/2 MS medium supplemented with IBA/NAA combination 0.4 + 0.4 ppm. The results are presented in Table 49. Sucrose at 1.0 per cent was unfavourable for root initiation. The effects of 2.0 and 3.0 per cent sucrose on the number of roots produced were equal (5.5 roots each), though the per cent root initiation differed (40.0 and 80.0 per cent respectively). Sucrose at 4.0 per cent level recorded 60.0 per cent root initiation with 4.5 roots per culture. Sucrose at 3.0 per cent level was found to be ideal for root initiation.

4. Agar

Agar at 1.0 per cent level inhibited root initiation, as the shoot growth was affected (Table 49). The other levels tried (0.4, 0.6 and 0.8 per cent) did not differ in the number of roots produced. However agar 0.6 per cent recorded the maximum per cent root initiation (80.0%).

5. Anderson's rooting medium

Anderson's rooting medium was observed to be unsuitable for the in vitro root initiation from mussaenda shoots, as only 2.5 roots per culture were produced, with 40.0 per cent root initiation (Table 50).

D. Planting out of plantlets/shoots to soil (Stage III)

1. After in vitro rooting (Plantlets)

Attempts for planting out mussaenda plantlets, with different potting mixtures and humidity maintenance methods did not succeed. Weak and slender roots which were low in number, presence of abundant callus at the shoot-root junction which got easily decayed after planting out and the partial withering of shoots by the time the roots were initiated, might have contributed to the lack of establishment of the plantlets.

Table 50. Effect of Anderson's rooting medium on the *in vitro* rooting of mussaenda shoot cultures

Treatment	Medium containing IBA 0.4 ppm + NAA 0.4 ppm	
	Cultures[a] initiating roots (%)	Roots per culture
Anderson's rooting medium	40	2.50
MS rooting medium*	60	7.00

* Results reproduced from previous experiment stated in Table 48

a - Average of five observations

2. Direct transplanting of shoots without roots

The *in vitro* grown shoots were treated with IBA (5.0, 50.0, 100.0, 200.0 and 400.0 ppm) and transplanted to a potting mixture (soil:sand: coir dust 1:1:1 v/v) to see the effect on root initiation and establishment of the shoots. However root initiation was not observed in any of the treatments tried and the shoots started decaying in about two weeks.

III. Breadfruit

A. Culture establishment (Stage I)

1. Establishment of shoot apices

Response of shoot apices of mature breadfruit trees (above 10 year-old) to various culture establishment treatments are presented in Table 51. The treatments tried were combinations of kinetin (1.0 and 2.0 ppm) and NAA (1.0 and 2.0 ppm); BA (2.0 and 10.0 ppm)+ GA (1.0 ppm) + activated charcoal (1.0%); BA (0.5 ppm)+ kinetin (0.5 ppm), GA (1.0 ppm) + activated charcoal (1.0%) and BA (2.0 ppm) + IAA (0.5 ppm) + GA (1.0 ppm) + activated charcoal (1.0%).

The per cent survival of the cultures was fairly good in all the treatments (80.0 to 100.0) except BA 2.0 ppm + IAA 0.5 ppm + GA 1.0 ppm + activated charcoal 1.0%

Table 51. Effect of different treatments on the survival and growth of breadfruit shoot cultures (via enhanced release of axillary buds)

Basal medium : MS

Treatment	Survival[a] (% cultures alive)	Cultures[a] exhibiting growth (%)
kinetin 1.0 ppm + NAA 1.0 ppm	100.0	0
" + " 2.0 ppm	80.0	0
kinetin 2.0 ppm + NAA 1.0 ppm	80.0	0
" + " 2.0 ppm	90.0	0
BA 2.0 ppm + GA 1.0 ppm + activated charcoal 1%	80.0	20.0
BA 10.0 ppm + GA 1.0 ppm + activated charcoal 1%	80.0	40.0
BA 0.5 ppm + kinetin 0.5 ppm + GA 1.0 ppm + activated charcoal 1%	80.0	20.0
BA 2.0 ppm + IAA 0.5 ppm + GA 1.0 ppm + activated charcoal 1%	60.0	0

a - Average of five observations

which recorded 60.0 per cent survival. The problem of browning of the medium and explant due to phenolics oxidation was severe in almost all the treatments tried. The least problem was observed at kinetin 1.0 ppm + NAA 2.0 ppm (20.0 per cent cultures affected) and at BA 0.5 ppm + kinetin 0.5 ppm + GA 1.0 ppm + activated charcoal 1.0% (40.0 per cent cultures affected). In the rest of the treatments, 60.0 - 100.0 per cent cultures were severely affected by the browning due to phenolics oxidation (Plate 41). However, killing of the explants due to browning was observed to be very slow. Subculturing of the explants to medium of the same composition was done after three weeks. The per cent cultures showing signs of growth were very much limited in the above treatments. Only three treatments supported the growth of the cultures. The most favourable treatment was BA 10.0 ppm + GA 1.0 ppm + activated charcoal 1.0%, supporting 40.0 per cent growing cultures. Twenty per cent growing cultures were observed in the case of BA 2.0 ppm + GA 1.0 ppm + activated charcoal 1.0% and BA 0.5 ppm + kinetin 0.5 ppm + GA 1.0 ppm + activated charcoal 1.0%. Eventhough very slight growth of the explants was observed (Plate 42) during the early stages, the cultures ceased to grow after a stage. The growth

Plate 41. Shoot apex culture of breadfruit (source: ten-year old tree) showing browning due to polyphenol oxidation

Magnification: x 2.60

Plate 42. Shoot apex culture of breadfruit (source: ten-year old tree) in the establishment medium (MS + BA 100 ppm + GA 1.0 ppm + activated charcoal 1.0%) after repeated subculturing

Magnification: x 2.80

41

42

response could not be improved even after repeated subculturing in medium of the same composition. Hence attempts could not be made for stage II studies.

2. Somatic organogenesis - callus production

Shoot apices failed to initiate callus under the influence of the various treatments tried. However, young inflorescence responded weakly to the treatment with 2,4-D 1.0 ppm + kinetin 1.0 ppm (Table 52). The light green callus produced (Plate 43) recorded a poor growth score of 1.0, with a CI value of 60.0. The per cent culture initiating callus was 60.0. Callus initiation was closely followed by browning of the cultures due to phenolics oxidation. The problem remained even after repeated subculturing and finally the callus turned brown and died. As a result the callus could not be subjected to treatments for differentiation.

3. Somatic embryogenesis - callus production

The cultures turned brown and died when subjected to the treatment.

IV. Pepper

a. Culture establishment (Stage I)

Response of shoot apices and nodal segments of pepper to various culture establishment treatments were

Plate 43. Callus production from young inflorescence explant of breadfruit (source: ten-year old tree) on MS medium + 2,4-D 1.0 ppm + kinetin 1.0 ppm

Magnification: x 1.75

Plate 44. Nodal segment culture of pepper affected by bacterial contamination. Slight swelling/callussing visible

Magnification: x 0.74


43


44

Table 52. Effect of different treatments on the production and growth of calli from shoot apex and young inflorescence cultures of breadfruit (w.r.t. somatic organogenesis)

Basal medium : MS

Treatment	Shoot apex cultures			Young inflorescence cultures		
	Cultures[a] initiating callus (%)	Growth[a] score (G)	Callus[a] Index (CI)	Cultures[a] initiating callus (%)	Growth[a] score (G)	Callus[a] Index (CI)
kinetin 1.0 ppm + NAA 1.0 ppm	0	0	0	-	-	-
" + 2.0 ppm	0	0	0	-	-	-
kinetin 2.0 ppm + NAA 1.0 ppm	0	0	0	-	-	-
" + 2.0 ppm	0	0	0	-	-	-
kinetin 1.0 ppm + 2,4-D 1.0 ppm	-	-	-	60.0	1.0	60.0

a - Average of five observations

observed and the data are presented in Table 53.

The shoot apices were subjected to treatments involving combinations of NAA (0, 1.0 and 2.0 ppm) and kinetin (0, 1.0 and 2.0 ppm), 2,4-D 1.0 ppm and 2,4-D 1.0 ppm+ kinetin 1.0 ppm. The nodal segments were subjected to NAA 2.0 ppm + kinetin 1.0 ppm.

Severe contamination problem (50.0 to 75.0% initially) due to the presence of systemic bacteria was observed (Plate 44). Even in the case of explants/callus, found to be apparently free from contamination, the problem appeared in advanced stage of organogenesis, indicating the systemic nature of the bacteria. Treating the explant with streptomycin 250 ppm in addition to the normal disinfection process did not help. As a result of the problem, advanced stage of organogenesis could not be realised. Browning of the medium and explants was another problem. However this could be effectively tackled by subculturing.

In the case of shoot apices NAA/kinetin combination 2.0 + 1.0 ppm recorded the greatest response with respect to callus growth (G = 3.0) and CI value (CI = 300.0), followed by NAA/kinetin combination 1.0 + 1.0 ppm registering G = 2.5 and CI = 250. The CI values of the rest of the treatments were 100.0 or below. NAA/kinetin combination

Table 53. Effect of different treatments on the production and growth of calli from shoot apex and nodal segment cultures of pepper (w.r.t. somatic organogenesis)

Basal medium : MS

Treatment	Shoot apex culture			Nodal segment culture		
	Cultures initiating callus (%)	Growth score (G)	Callus Index (CI)	Cultures initiating callus (%)	Growth score (G)	Callus Index (CI)
kinetin 0 ppm + NAA 0 ppm	0	0	0	-	-	-
" + " 1.0 ppm	50.0	1.0	50.0	-	-	-
" + " 2.0 ppm	50.0	1.0	50.0	-	-	-
kinetin 1.0 ppm + NAA 0 ppm	0	0	0	-	-	-
" + " 1.0 ppm	100.0	2.5	250.0	-	-	-
" + " 2.0 ppm	100.0	3.0	300.0	80.0	2.0	160.0
kinetin 2.0 ppm + NAA 0 ppm	0	0	0	-	-	-
" + " 1.0 ppm	100.0	1.0	100.0	-	-	-
" + " 2.0 ppm	100.0	1.0	100.0	-	-	-
2,4-D 1.0 ppm	50.0	1.0	50.0	-	-	-
2,4-D 1.0 ppm + kinetin 1.0 ppm	100.0	1.0	100.0	-	-	-

0 - no response

- - no treatment

Culture period - three weeks

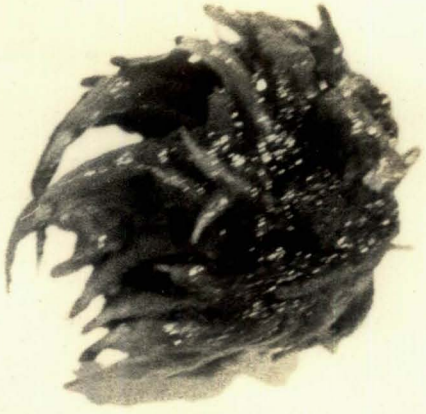
Table 54. Effect of combinations of NAA and kinetin on shoot/root differentiation from the calli from shoot apices and nodal segments of pepper

Treatment	Callus from shoot apex		Callus from nodal segment	
	Shoots differentiated per culture	Roots differentiated per culture	Shoots differentiated per culture	Roots differentiated per culture
kinetin 0 ppm + NAA 0 ppm	0	0	0	0
" + " 1.0 ppm	0	0	0	1.00
" + " 2.0 ppm	0	2.50	0	0
kinetin 1.0 ppm + NAA 0 ppm	1.00	0	0.33	0
" + " 1.0 ppm	0	1.67	0	2.33
" + " 2.0 ppm	0	0.50	0	2.33
kinetin 2.0 ppm + NAA 0 ppm	0.33	0	1.00	0
" + " 1.0 ppm	0.33	0	0	1.00
" + " 2.0 ppm	0	0	0	0
kinetin 0.5 ppm + BA 0.5 ppm	0	0.75	0	1.50

Culture period - three weeks

Plate 45. Root differentiation in the callus of nodal explant culture of pepper (source: three-year old vine) on MS medium + NAA 2.0 ppm

Magnification: x 2.08



45

2.0 + 1.0 ppm effected growth score of 2.0 and CI value of 160.0 in the case of nodal segments.

B. Multiplication of the propagule (Stage II)

When the callus was subjected to redifferentiation treatments roots and shoots were formed (Table 54). Root regeneration was more common. Callus from nodal segments had more regenerative capacity. In the case of callus from shoot apices roots were formed at NAA/kinetin combinations 2.0 + 0 ppm (2.5 roots/explant) 1.0 + 1.0 ppm (1.67 roots/explant), 2.0 + 1.0 ppm (0.5 roots/explant) and at kinetin/BA combination 0.5 + 0.5 ppm (0.75 root/explant). Shoot formation was observed at kinetin 1.0 ppm (1 shoot/explant) and 2.0 ppm (0.33 shoots/explant) and NAA/kinetin combination 1.0 + 2.0 ppm (0.33 shoots/explant). In the case of nodal explants roots were formed at NAA 1.0 ppm (1.0 root/explant), NAA/kinetin combinations 1.0 + 1.0 ppm (2.33 roots/explant), 2.0 + 1.0 ppm (2.33 roots/explant), 1.0 + 2.0 ppm (1 root/explant) and at kinetin/BA combination 0.5 + 0.5 ppm (1.5 roots/explant). Shoot formation was observed at kinetin 1.0 ppm (0.33 shoots/explant) and 2.0 ppm (1.0 shoot/explant).

The roots regenerated were cylindrical, long and quite healthy with white colour and a rapid rate of growth. However the shoots formed were rudimentary and appeared as

green growing points. Further development did not take place in any of the cultures due to the problem of bacterial contamination.

V. Nutmeg

A. Culture establishment (Stage I)

1. Establishment of shoot apices

Response of shoot apices from mature female nutmeg plants to various culture establishment treatments are presented in Table 55. Majority of the cultures were severely affected the problem of polyphenol oxidation (75.0 - 100.0%). The per cent survival of the cultures recorded by various treatments were low. Maximum per cent survival (50.0) was observed at NAA/kinetin combinations 2.0 + 1.0 ppm, 1.0 + 2.0 and 2.0 + 2.0 ppm. However none of the treatments were effective in supporting growth of the cultures, even when subcultured.

2. Somatic organogenesis - callus production

Initiation and growth of callus from mature shoot apices of nutmeg were tried with treatment combinations involving NAA (1.0 and 2.0 ppm) and kinetin (1.0 and 2.0 ppm), and 2,4-D (1.0 ppm) and kinetin (1.0 ppm) along with activated charcoal 1.0%. All the treatments except 2,4-D/

Table 55. Effect of different treatments on the survival and growth of shoot cultures and the production and growth of callus from shoot apex cultures of nutmeg

Treatment	Survival[a] (% cultures alive)	Cultures exhibiting growth (%)	Cultures[b] initiating callus (%)	Growth score (G)	Callus Index (CI)
kinetin 1.0 ppm + NAA 1.0 ppm	0	0	0	-	-
" + " 2.0 ppm	50.0	0	0	-	-
kinetin 2.0 ppm + NAA 1.0 ppm	50.0	0	0	-	-
" + " 2.0 ppm	50.0	0	0	-	-
BA 1.0 ppm	25.0	0	-	-	-
BA 2.0 ppm	25.0	0	-	-	-
BA 3.0 ppm	0	0	-	-	-
2,4-D 1.0 ppm + kinetin 1.0 ppm + activated charcoal 1.0%	-	-	40.0	1.0	40.0

a - Average of four observations

b - Average of five observations

kinetin combination 1.0 + 1.0 ppm + activated charcoal 1.0% failed to initiate callus from the explants. However the response of this effective treatment was poor. Slight callus growth, with growth score of 1.0 and callus index of 40.0 was observed in 40.0 per cent cultures. The callus did not survive, due to the problem of polyphenol oxidation, even when subcultured.

DISCUSSION

A number of important horticultural crops are commercially propagated through seeds. Most of them are cross pollinated and as such heterozygous. The variability associated with the heterozygosity is seen expressed in yield, quality and disease/pest resistance. The horticultural qualities of superior cultivars are normally governed by complexes of genes. During sexual propagation, the gene combinations are altered.

Conventional methods of vegetative propagation like air layering, rooting of cuttings, budding and grafting are possible in several horticultural species. However, efforts to standardise them to a commercially feasible level have not been successful, except in the case of mango, jack, cashew and pepper. During the past decade, tissue culture has emerged as a possible alternative in the crops where conventional methods have posed problems. Even in cases where conventional methods of vegetative propagation has reached commercial acceptability, tissue culture techniques have been shown to have definite advantages. Tissue culture ensures an extremely rapid rate of multiplication which is not season-dependent and requires only a

small amount of plant tissue as the initial explant. "Filtering off of the pathogens" (Morel and Martin, 1952), cryopreservation of germplasm (Kartha *et al.*, 1979), *in vitro* secondary plant metabolite production (Yamada and Hashimoto, 1982) etc. are some of the other advantages of tissue culture.

With respect to perennial horticultural crops, the commercial applicability of tissue culture methods has been demonstrated only in oil palm (George and Sherrington, 1984), apple (Zimmerman, 1979), plum, peach (Zimmerman, 1985) and strawberry (Boxus *et al.*, 1977). Difficulties in culture establishment, influence of the physiological age of the explant, polyphenol interference, systemic presence of pathogens, problems during planting out, necessity of long-term evaluation of field performance etc. have been documented as the hurdles to be overcome. In the case of the various horticultural crops of Kerala, namely, jack, breadfruit, pepper, nutmeg, mussaenda etc. studies covering these aspects are scanty. The present investigations aimed at standardising tissue culture techniques in some of the above crops. The salient findings have been discussed in the following pages.

I. Jack

Being cross pollinated, seed propagation in jack, *Artocarpus heterophyllus* Lam. causes considerable variation

among the progenies, particularly in the shape, size and quality of the fruits. The firm flaked Varikka and the soft flaked Koozha are the two predominant types in cultivation. Consumer preference is for the Varikka type. It is not possible to predict the fruit form or quality at the seedling stage, based on any plant characteristics, because of the absence of strong correlations. Conventional methods of vegetative propagation like inarching (Srinivasan, 1970), epicotyl grafting (Nagabhushanam, 1983), air layering (Srinivasan, 1970; Dhua and Sen, 1984) and rooted stem cuttings (Chatterjee and Mukherjee, 1982; Dhua et al., 1983) are possible. However, the rate of multiplication is low and the methods, rather cumbersome. It is in this context that the in vitro cloning methods are considered advantageous.

There are three possible routes available for in vitro propagule multiplication, namely, shoot tip culture, somatic organogenesis and somatic embryogenesis (Murashige, 1974). The first and the third pathways are ideal for clonal propagation, whereas the second is largely employed for recovering desirable variants. All the three routes were attempted in the present studies, with explants from seedlings, young grafts, fresh stem sprouts of immature and mature trees, and young inflorescences.

Murashige (1974) recognised four stages in tissue culture propagation, namely, Stage I - culture establishment, Stage II - multiplication of propagules, Stage III - rooting and hardening for transfer to soil and Stage IV - planting out to soil and special treatments for initiating rapid growth and development. As each of these stages requires specific chemical and physical environments, attempts were made to standardise them with reference to jack.

Methods were standardised for enhanced release of axillary buds from the explants of fresh stem sprouts of five-year old jack trees. The procedure for the in vitro clonal propagation of jack through enhanced release of axillary buds involved agitating the surface sterilised shoot apices from fresh stem sprouts in a solution of 0.7% insoluble PVP + 2.0% sucrose for 30 minutes and keeping them in sterile water at 4-5°C for 24 hours, followed by disinfection and culture in an establishment medium (GA 1.0 ppm + activated charcoal 1.0%) in darkness for a period of four weeks, with repeated subculturing (Stage I). The cultures were then exposed to light for a period of two weeks, after which the growing shoot apices were transferred to a proliferation medium consisting of BA 5.0 ppm + NAA 0.2 ppm + insoluble PVP 500.0 ppm. In about five weeks a number of axillary shoots were seen to grow from the shoot

apices (Stage II). The process of multiple shoot production from each of the new shoots could be continued until the desired number of shoots was obtained. The shoots were then transferred to an elongation medium (BA 2.0 ppm + NAA 0.2 ppm + insoluble PVP 500.0 ppm) to have a favourable shoot morphology before rooting. Incubating the shoots for two weeks on MS medium containing activated charcoal 1.0 per cent was found to be beneficial for in vitro rooting. The shoots were then cultured in darkness on a root induction medium (1/2 MS + IBA 2.0 ppm + NAA 2.0 ppm) for six days and subcultured in another medium (1/2 MS without growth substances) for root elongation (Stage III). Just after the appearance of the roots, the plantlets were hardened by exposure to high light intensity for one week. The plantlets were then transplanted to vermiculite medium under high relative humidity (90.0 to 100.0%) and watered with a solution of MS inorganic salts at half concentration (Stage IV). After another gradual hardening process and as new leaves were produced, the plantlets were transferred to garden pots filled with a mixture of sand:soil:cowdung (1:1:1 v/v). The potted plants were then transferred to open field conditions.

The culture establishment medium is useful for conditioning of the explant and for stimulating its initial growth. In establishing the shoot apices from fresh sprouts

(from the basal portions of the stem) of five, ten and thirty-year old jack trees, difficulty was experienced due to browning of the tissues and media, and callussing at the base of the cultured shoots. Browning of the explants and media has been reported to be common with use of media containing cytokinins especially BA as observed in Pistacia vera (Barghchi and Alderson, 1985). Pretreatment of the explants with insoluble PVP (0.7%), keeping them in sterile water at reduced temperature (4-5°C) for 24 hours, trimming the explants at low temperature (4-5°C), inclusion of activated charcoal (1.0%) in the establishment medium, providing reduced light intensity during the initial period of culture and/or frequent subculturing helped to reduce the browning and subsequent necrosis of the tissue. By these treatments, the oxidation of polyphenols (Hu and Wang, 1983) might have been reduced or the oxidation products (Gupta et al., 1981) might have been adsorbed. In either case beneficial effects can be logically expected. Following subculture of shoots, less callus was seen produced at the base of the shoots (as in the case of seedling explants). The subculture of shoots might have brought about a decrease in the production of endogenous auxins or reduced their availability (Barghchi and Alderson, 1985) thus decreasing callus production. The (juvenile) leaf morphology exhibited

by the subcultured shoots may be an indication of a reversion to a more juvenile state. Decrease in tissue browning and callussing, and the appearance of juvenile morphology have been reported in the case of 'mature explants' from several species as a consequence of serial subculturing (Vietez et al., 1985; Barghchi and Alderson, 1985). In the present studies, inclusion of GA 1.0 ppm and activated charcoal 1.0% in the establishment medium was found to be ideal for conditioning of the explants and for stimulating their initial growth. The function of GA is primarily bud elongation (Chalupa, 1977; Schnabdrauch and Sink, 1979). However at the higher level of GA tried, the cultures became less healthy, with fragile leaves. Unfavourable effects of higher concentrations of GA have been reported in eucalyptus (Durand-Cresswell et al., 1982). Enhanced release of axillary buds was realised in the proliferation medium. The high concentration of cytokinin might have broken the apical dominance of shoots and enhanced the branching of lateral buds from leaf axils (Hu and Wang, 1983). The rate of multiplication of shoots in the proliferation medium was only 4.25 x, in a period of four weeks; but during subsequent subcultures, the rate increased to 5.39 x. This increase in multiplication rate may be due to the modification of the physiological state of the plant material. Examples are

available which indicate that the recalcitrant state of many species can be gradually modified through serial subculturing (Franclet, 1979; David, 1982). Litz and Conover (1978) obtained increase in shoot multiplication rate of papaya as a result of subculturing.

Distinct differences in the rate of multiplication was observed vis-a-vis the physiological age of the explants. While a multiplication rate of 17.4 x was obtained for the seedling explants, the rates were only 4.5 x, 2.8 x and 2.09 x for the explants from fresh stem sprouts of 5-year old, 10-year old and 30-year old trees, respectively. In spite of taking explants from fresh basal sprouts and adopting suitable pre-culture treatments like shaking (to remove the endogenous inhibitors present), using activated charcoal and insoluble PVP (to reduce the problem of phenolics oxidation) and supplementing the culture medium with exogenous growth substances, only partial rejuvenation (Bonga, 1982; Durzan, 1984; Zimmerman, 1985) of the explants resulted. The use of explants from young grafts also did not help to make the response favourable. This could be attributed to the 'residual memory' of the explants. Explants from mature trees are known to retain the memory and as a result, major problems have been encountered in the micropropagation of woody perennials (Durzan, 1984). The limited

success obtained in teak (Gupta et al., 1980) and eucalyptus (Gupta et al., 1981) has been ascribed as due to the use of explants from old trees. However the problem has been solved to some extent by serial subculturing and pre-treating the explants with insoluble PVP in teak and incubating the explants at low temperature (15°C) with continuous illumination followed by the use of liquid medium, in eucalyptus. In order to have better response of mature explants, detailed investigations are warranted to understand the physiological and biochemical bases favouring juvenility in jack.

After assessing the influence of cytokinins, adenine, adenine sulphate and auxins, a proliferation medium was standardised. BA (5.0 ppm) was observed to provide the maximum number of fairly elongated shoots which facilitated convenient handling during subsequent culturing. Higher concentrations of BA had a suppressive effect on axillary shoot elongation. However, Rao et al. (1981 b) used 30.0 ppm BA or 2 iP for inducing multiple shoots from shoot apices of mature jack trees. In their study, the details of the rate of multiplication and shoot growth were not mentioned. BA has been considered as the most effective cytokinin for stimulating axillary shoot proliferation in a number of species like apple (Lundergan and Janick, 1980), strawberry (Kartha et al., 1980) and pistachio (Barghchi and Alderson, 1985). Adenine as well as adenine sulphate had significant influence in increasing the rate of production of multiple shoots in jack.

However, the levels which recorded maximum multiplication rates had adverse effects on the growth of the cultures. Only adenine sulphate 20.0 ppm registered an increase in multiplication rate (27.3%) without affecting the growth of the cultures. Adenine and related substances are precursors of endogenous cytokinins (Nitsch et al., 1967), and hence exhibit promotive effects on shoot formation and help to counteract the inhibitory action of auxins. Adenine was included in the proliferation medium used by Nemeth (1981) for Malus spp. Increased multiplication rate by the action of adenine sulphate has been reported in Phlox subulata (Schnabdrauch and Sink, 1979) and Carrizo citrange (Kitto and Young, 1981).

The proliferation rate and growth of jack shoot cultures as influenced by auxins (IAA, NAA and 2,4-D) were observed. The growth of the cultures, rather than the rate of multiplication, was improved by the auxins. Among the three, NAA (0.2 ppm) significantly improved the growth of the cultures. Auxins may be useful to nullify the suppressive effect of high cytokinin concentrations on axillary shoot elongation and to restore normal shoot growth (Lundergan and Janick, 1980). Too high a concentration of auxin may inhibit axillary bud branching and induce callus formation (Hasegawa, 1980; Hamatt and Evans, 1985). Similar

response to auxin application of shoot cultures has been reported in apricot (Skirvin and Rukan, 1979), rose (Hasegawa, 1980) and coffee (Kartha et al., 1981).

Inclusion of casein hydrolysate (CH) at concentrations ranging from 500.0 to 2000.0 ppm in the proliferation medium significantly enhanced the proliferation rate. The growth of the cultures was, however, severely affected. Casein hydrolysate is a non-specific organic nitrogen source and serves as an amino acid supplement (Skoog and Miller, 1957) and has been known to increase the rate of in vitro proliferation in pineapple (Mathews and Rangan, 1981) and pomegranate (Mascarenhas et al., 1981). Mascarenhas et al. (1981) could obtain elongation of the pomegranate multiple shoots only after transferring them to a medium with lower CH content or without CH.

According to Schnabdrauch and Sink (1979) GA, if included in the proliferation medium may have a role in axillary bud elongation. However, in the present studies, no favourable effect of GA on shoot elongation was observed. Similar lack of response has been observed in apple (Lundergan and Janick, 1980) and globe artichoke (Ancora et al., 1981).

Explants of certain fruit crops like red raspberries and blueberries do not exhibit their maximum potential if grown on normal strength of the MS medium, which is considered as a high salt medium (Anderson, 1980a). However in the present studies, the full strength MS medium (supplying the required quantities of inorganic nutrients, vitamins, amino acids and carbon source) was found to be the optimum for supporting the growth of the jack shoot cultures. Media with lower salt concentrations (1/2 strength; 1/4 strength) were found to be inferior with respect to the rate of multiplication and growth of the cultures. Unlike in rhododendron (Anderson, 1980), the Anderson's medium (which has reduced concentrations of ammonium nitrate and potassium nitrate and increased concentrations of ferrous sulphate and Na_2 EDTA) proved inefficient to support the proliferation and growth of jack shoot cultures. Sucrose (30.0 - 40.0 g/l) and glucose (20.0 g/l) was found to be ideal carbon/energy sources and osmoticum for supporting the growth of the jack shoot cultures. This requirement may be related to the specific carbohydrate metabolism through which water relations and endogenous phytohormones are regulated (Chong and Pua, 1985).

In general, conditions that stimulate abundant shoot induction inhibit continued shoot growth (David, 1982; Amerson et al., 1985). As the elongation of the majority of the

jack shoots in Stage II was not satisfactory, an intermediate shoot elongation stage, prior to Stage III, became necessary. Intermediate stage has been considered necessary in Sassafras (Hu and Wang, 1983). A medium containing NAA (0.2 ppm) and reduced concentration of BA (2.0 ppm) was found to be suitable for the elongation of the jack shoots from the proliferation medium.

In the case of explants from mature trees, a further intermediate stage involving culturing for two weeks in a medium containing activated charcoal 1.0% was found to be beneficial for the in vitro rooting of jack shoots. The ability of activated charcoal to absorb the toxic substances and the residual cytokinin from Stage II medium (Fridborg et al., 1978) may have brought about the rooting response. The process of root initiation is an auxin-mediated event in contrast to the cytokinin dependent shoot initiation. Higher levels of endogenous BA have been reported to have inhibitory effect on in vitro rooting (Takayama and Misawa, 1980; Ancora et al., 1981).

Stage III involves de novo regeneration of adventitious roots from the shoots obtained in Stage II or the intermediate stage. In the present studies, various auxins, either alone or in combination, were tried with the objective of increasing the number of roots per shoot cultured and for

enhancing the percentage rooting. Only two treatments were found to be effective in the case of explants from 5-year old trees. One of these treatments was that reported by Rajmohan and Mohanakumaran (1983) which involved incubating the jack shoot cultures on 1/2 MS medium containing IBA/NAA combination 1.6 + 0.4 ppm to yield 80.0% rooting with 4.17 roots per shoot in 24.67 days. However, 70.0% rooting, maximum number of roots per shoot (5.43), minimum days for root initiation (13.43) and minimum callusing at the cut end of the shoots were recorded when the shoots were cultured for six days on 1/2 MS + IBA (2.0 ppm) + NAA (2.0 ppm) followed by transfer to 1/2 MS without the growth substances. Similar two-phase procedure helped in giving a strong root induction stimulus to the hard-to-root apple rootstocks, avoiding callusing and root growth inhibition (Snir and Erez, 1980; James and Thurbon, 1981) and to loblolly pine (Amerson et al., 1985). The induction and initiation of roots take place in about four to eight days in the first phase. The first phase is unfavourable for root elongation because of the high auxin content. The auxin free second phase is ideal for root elongation. As observed in the present investigations, synergism between two auxins to yield better results in in vitro rooting has been reported earlier (Gupta et al.,1980; Skirvin et al., 1982).

The seedling explants responded to different auxin treatments for *in vitro* rooting. Among the promising treatments, NAA (0.4 ppm) + IBA (0.4 ppm) gave hundred per cent rooting of shoots with six roots per shoot in 20.75 days. This increased response of seedling explants may be the result of the association of juvenile phase with high degree of regenerative potential (Bonga, 1982).

Response of the explants from 10 and 30-year old trees and young grafts were poor even after serial subculturing. A low percentage of rooting (40.0, 15.0 and 50.0 for 10-year old tree, 30-year old tree and young graft of jack, respectively) with less than 2.5 roots per shoot was observed after serial subculturing upto three times. Physiological and biochemical changes associated with the developmental age of the source of explant may be the reason for reduced response (Durgan, 1984). Use of fresh basal sprouts of the trees seems to have effected only partial rejuvenation. Subculturing process is reported to change the physiological state of the explants and gradually rejuvenate them to make *in vitro* root induction progressively easier. Increased response of explants from mature plants to *in vitro* rooting treatments, due to serial subculturing has been recorded in grapes (Mullins *et al.*, 1979), hundred-year old teak trees

(Gupta et al., 1980) and twenty-year old eucalyptus trees (Gupta et al., 1981).

Use of grafts as source of explants has given encouraging results in Pseudotsuga menziesii (Goublay de Nantois, 1980), suggesting a degree of rejuvenation on grafting. However, in the present investigations, use of grafts as source of explant did not yield increased response. With respect to response vis a vis age, the scions behaved as the original tree from which they were taken. Thus grafting seems to have not brought about rejuvenation to a level favouring increase in response.

The concentration of inorganic salts in the basal medium influence the in vitro rooting regardless of the growth substances present. When the salt concentration in the medium was lowered to one-half, one-third or one-fourth of the standard strength, rooting became abundant (Kartha et al., 1974; Skirvin and Chu, 1979). Better root induction was observed in coffee with half strength MS medium (Kartha et al., 1981). In the present studies also, half concentration of MS medium gave the maximum favourable effect on in vitro rooting (80.0% rooting) without affecting the shoot growth unlike in certain species (Wang, 1978; Gupta et al., 1981). Full strength of organic growth factors of MS medium was found to be most favourable for root initiation.

Sucrose was optimum at 3.0 per cent for the production of normal plantlets from jack shoots. As a source of energy as well as a factor for osmoregulation for optimising the rooting response, sucrose has already been recognised (Chong and Pua, 1985).

Agar is used as the carrier material to solidify the medium for supporting root initiation and growth in vitro. Physiologicelly agar is not a completely inert material and is a source of various types of substances which may influence growth in sensitive species (Hu and Wang, 1983). Kitto and Young (1981) observed an inverse relationship between the rooting ability and the agar concentration in Carrizo citrange cultures. In the present studies, agar 0.6 per cent was found to be the optimum for the in vitro rooting of jack shoot cultures.

Rooting of cultured shoots need not always be carried out in vitro. Kusey et al. (1980) obtained 60.0 per cent rooting of Gypsophila Paniculata by planting the cultured shoots in 'Jiffy 7' peat moss cylinders in green house under intermittent mist. However in the present studies attempts for direct rooting of shoots from the proliferation medium in a non-sterile medium (sand:soil: cowdung 1:1:1 v/v) under high humidity (after pretreating with IBA) were not successful.

Attempt was made to induce callus mediated somatic organogenesis from various explants of 5-year old jack. The expression of morphogenetic potential was found to be limited to the de-differentiation stage. The callus proved totally recalcitrant to re-differentiation at the various treatments tried. Rao et al. (1981 b) also reported the recalcitrant nature of the callus from shoot tip explants of mature jack trees. The potential for callus production from the different explants was observed to be poor to moderate. Shoot apices were found to have the maximum response, registering a callus index of 180.0 at the best treatment. The level as well as the type of auxin or cytokinin to establish callus varied with the explants (shoot apices, internodal segments, leaf segments and root apices). The basic phenomena involved in the induction of callus from plant parts are still unresolved. Hence, the reasons for the observed difference in the requirement of growth substances by various parts of the same plant can only be conjectured as due to the differences in physiological conditions like level of endogenous phytohormones, nutrients and metabolites, presence of heretofore unidentified growth substances, interaction between the various growth factors, etc. According to Skoog and Miller (1957), quantitative interaction between diverse growth factors may have decisive role in organogenesis. The initiation of organised development in the callus involves a shift in metabolism (Thorpe, 1980).

Identification of the areas of metabolism involved in this shift as well as determination of the role of various phytohormones and other interacting metabolites may help to change the recalcitrant nature of the callus from jack explants.

Somatic organogenesis was also attempted through direct differentiation from *in vitro* formed shoot apices, internodal segments and leaf segments. However, all the treatments tried proved ineffective to induce direct organogenesis. Successes reported of using leaf explants for plantlet production through direct *in vitro* organogenesis in pineapple (Mathews and Rangan, 1979) and custard apple (Nair *et al*., 1984) suggest that further work on these lines may yield promising results in jack.

Attempts to induce somatic embryogenesis in jack explants in the presence of an auxin like 2,4-D and to develop them in an auxin-free medium also proved unsuccessful in the present studies. Successful instances of somatic embryoid formation have been reported in species like *Coffea arabica* (Sondahl and Sharp, 1977), *Vitis vinifera* (Srinivasan and Mullins, 1980) and *Carica papaya* (Litz and Conover, 1982). These suggest that detailed investigations involving different levels and types of auxins, reduced

nitrogen, different culture methods and conditions may prove successful.

The determinants in the establishment of the jack plantlets in soil (like potting mixture, age of the plantlets, light intensity before transplantation, application of nutrient solutions, humidity maintenance etc.) were standardised in these investigations. A satisfactory percentage of survival (55.6) of the plantlets could be achieved by exposing the cultures just after root initiation to high light intensities of about 3500 lux for one week and transferring the plantlets to vermiculite medium (pre-soaked for 24 hours with Bavistin 0.06%) contained in small mud pots. At this stage, agar could be easily removed as the roots were not long. Transfer of older plantlets gave poor survival. This may be due to their low root regeneration potential (after transplantation). The possibility of greater injury and subsequent decay of the injured roots also exist. Another cause for the low establishment of older plantlets could be the difficulty in making the roots completely free from agar. Durand - Cresswell et al. (1982) recommended the planting out of plantlets just after the appearance of the root tips, in the case of eucalyptus. In the present studies, vermiculite medium supported better survival of the plantlets. This may be due to its ability to maintain optimum moisture

status at the same time providing sufficient aeration. The other potting mixtures tried, except sand:soil:coir dust (1:1:1 v/v) failed to give satisfactory level of establishment. According to Murashige (1974) and Hussey (1978) subjecting the plantlets to high light intensities before transplantation helps in better establishment. Enhanced photosynthesis under the influence of high light intensities helps in building up a high food reserve to be utilised during the transform period from partially heterotrophic to autotrophic growth of the plantlets after transplantation (Murashige, 1978).

Hardening the plantlets to make them adapt to the outside environment is a critical process due to the anatomical and physiological peculiarities of the plantlets. From the leaves of the plantlets after transplanting excessive water loss has been recorded which was ascribed as due to improper development of cuticle and slowness of stomatal response to water stress (Brainered and Fuchigami, 1981; Fabbri et al., 1984). The problem may be aggravated by improper vascular connections between the roots and the shoot during the initial stages of development. Anatomical studies of the jack plantlets illustrated the defective nature of the leaf cuticle. A period of humidity acclimatization was considered

necessary for the newly transferred plantlets to adapt to the outside environment, during which the plantlets undergo morphological and physiological adaptation enabling them to develop typical terrestrial plant water control mechanism (Grout and Aston, 1977; Sutter et al., 1985). In the present studies, high relative humidity (90.0 to 100.0%) was maintained during the initial period of planting out with the help of microscope covers and intermitant water sprays. After two to three weeks, the covers were lifted for short intervals to make the plantlets hardened with respect to lower relative humidity. By this method, they could be hardened for their life in the open, in about four to six weeks.

Addition of inorganic nutrients to the potting mixture is essential for the normal growth of the potted plantlets (Brown and Sommer, 1982; Amerson, et al., 1985). Application of 5.0 ml nutrient solution containing MS inorganic salts at half concentration and having a pH of 5.7, at weekly intervals enhanced the survival and promoted normal growth of the plantlets. The liquid fertilizer solution (NPK 10:52:10 g/l) which yielded good results on the growth of cassava plantlets (Roca, 1984) was not found to be beneficial to the jack plantlets.

A tissue culture method, to become commercially acceptable, has to assure genetic stability in terms of the number and structure of the chromosomes. In the present investigations, the stability with respect to the number of chromosomes alone could be examined. A normal chromosome count of 2n = 56 was observed on cytological examination of the root tip squashes of the jack plantlets. It may be recalled that the plantlets had resulted from the enhanced release of axillary buds. Genetic stability has been observed in the case of axillary shoots and this has been attributed to the properties of the meristematic line involved in their origin. The meristematic line, consisting of specific cells in more or less fixed position, exercises a strict control over the mitotic events (Bonga, 1982; Vasil, 1985). According to Hussey (1978) mutated cells, if any, in a multicellular shoot apex will form only limited areas of tissue which eventually remain suppressed in the meristematic region. The vast majority of the plantlets regenerated from meristem/shoot apex culture have been found to be genetically stable (Ancora et al., 1981). Mutated single cells in a callus, on the other hand, can multiply and give rise to a mutant adventitious plant.

The cost of producing jack plantlets using explants from fresh stem sprouts of five-year old trees was worked out

based on the facilities of the Tissue Culture Laboratory at the College of Horticulture, having a potential of maintaining 4200 cultures for multiplication 4200 cultures for shoot elongation, 4200 cultures for rooting and 7000 plantlets for hardening. One scientist (Rs.1400/= p.m.) and one Technician (Rs.1000/= p.m.) were considered necessary for the work. Based on the rate of culture establishment, rooting response of the shoots and the survival of the plantlets at various stages, 6538.56 plantlets per year can be produced from 100 explants. The total cost involved per year worked out to Rs. 60845.00, the cost of building, equipment, glassware and chemicals having been distributed over the years according to their potential/durability. Under the above conditions, the cost of production of one jack plantlet including one month's hardening was found to be Rs.9.09. A further period of six months may be required in the nursery which could increase the cost by about Rs. 0.50. The cost can be reduced by augmenting the physical facilities, by improving the rate of multiplication of the propagule, by reducing the mortality of the plantlets using mist facilities etc. The current cost of jack grafts in the KAU farms is Rs.8.00 per graft and therefore, the method reported herein offers promise.

In the foregoing discussion, the salient findings of the present investigations have been critically evaluated which indicate possibilities for developing the apical meristem culture technique as a commercial method in jack. For successful commercial exploitation and for unravelling some of the puzzles thrown out by these investigations, intensified work in specific areas seems necessary.

Explants from fresh stem sprouts of mature jack trees (10 and 30 year old) and shoot apices of young grafts gave comparatively low multiplication rate and limited rooting response. Repeated subculturing and special pre-culture and culture treatments yielded only a partial rejuvenation of such explants. Detailed investigations are warranted to understand the physiological and biochemical bases of juvenility and ageing so as to evolve techniques that would bring about a total rejuvenation of the mature explants. Only then can we make the protocal developed in these studies applicable to mature trees.

As somatic organogenesis is useful to generate variants and is essential for haploid culture and production of homodiploids, somatic hybrids etc. further efforts may be made to improve the callus production and to induce callus re-differentiation to obtain plantlets.

Eventhough induction of direct somatic organogenesis or direct/callus mediated somatic embryogenesis proved unsuccessful, attempts should be made with different culture media, culture conditions and explant source in the light of success reported in other crops (Litz, 1985).

Studies on vigour, final tree size, cropping capacity and phenotypic stability of the trees resulting from shoot apex culture are necessary before commercialising the method.

Influence of different genotypes and seasons on the response of the explants has to be studied. The review presented in chapter 2 indicates that genotypes in apple (Zimmerman, 1984),grapes (Krul and Mowbray, 1984),banana (de Guzman et al., 1976) etc. and seasons in papaya (Pandey and Rajeevan, 1983), coconut (Raju et al., 1984) etc. influence the response of the explants.

II. Mussaenda

Mussaenda erythrophylla Schum & Thonn is propagated by vegetative means, cutting and layering being commonly employed. As such strict true-to-type nature with respect to the colour of the foliaceous sepals exist. Being an ornamental plant, variability in colour will have economic relevance. The present studies were undertaken with a view of developing an in vitro cloning procedure through shoot

apex culture. Standardisation of techniques for somatic organogenesis and callus mediated embryogenesis, which are reported to yield variants in other crops was also attempted.

Standardisation of the different stages (Mureshige, 1974) in the *in vitro* clonal propagation of mussaenda through shoot apex culture was attempted. The procedure involved agitating the shoot apices in a solution of ascorbic acid (50 ppm) for 30 minutes, surface sterilisation with mercuric chloride solution (0.1%) for 15 minutes and culture in an establishment medium (kintein/BA combination 0.5+0.5 ppm) for a period of three weeks (Stage I). The growing shoot apices were then transferred to a proliferation medium containing BA 0.5 ppm + kinetin 0.5 ppm and cultured for four weeks, during which period enhanced release of axillary buds occurred (Stage II). A multiplication rate of 2.75x only could be obtained, which is considered low as compared that in jack (4.5x). During subsequent subculture (for five times), the multiplication rate increased slightly to an average of 2.95x. The shoots from the proliferation medium after separation were made to root in 1/2 MS medium supplemented with IBA 0.4 ppm and NAA 0.4 ppm under dark conditions in about 37 days (Stage III). Good amount of callus was seen developed at the root-shoot junction.

Partial withering and yellowing of the shoots were observed by the time the roots were initiated. These seem to have impared the success in Stage IV.

The MS medium supplemented with BA 0.5 ppm and kinetin 0.5 ppm was found to be the most suitable one for conditioning the explant and for initiating growth in the culture. It may be recalled that the establishment medium for jack was MS medium supplemented with GA 1.0 ppm and activated charcoal 1.0 per cent. Such differential requirement has been recorded in a number of crops (Hu and Wang, 1983). Further, while jack exhibited differential requirement between Stage I and II, mussaenda showed good response to the culture establishment medium even at Stage II. Hence subculturing to medium of the same composition was sufficient for shoot proliferation. The rate of multiplication was only 2.75x. Serial subculturing at four-week interval in the same proliferation medium increased the multiplication rate by 7.27 per cent indicating the return of the shoots to a more juvenile state as a result of sequential subculturing. Similar results have been observed in species like teak (Gupta et al., 1980), eucalyptus (Gupta et al., 1981), pistachio (Barghchi and Alderson, 1985) and jack (in the present studies).

The composition of the proliferation medium was worked out based on the results of the studies involving cytokinins (BA and kinetin), adenine sulphate and auxins (IAA and NAA). BA/kinetin combination 0.5 + 0.5 ppm was found to favour multiplication and growth of the shoot cultures. Similar kind of synergism between kinetin and BA has been reported in the in vitro axillary shoot proliferation of teak and eucalyptus (Gupta et al., 1980; 1981).

Auxins were not found beneficial in mussaenda (Stage II), as they did not influence the multiplication and growth of the shoots. In addition, callus formation was also evident in the treatments involving auxins. The presence of endogenous auxins at the required level as described by Hasegawa (1980) in rose shoot cultures, may have brought about these responses.

Inclusion of adenine sulphate in the basic proliferation medium did not significantly influence the multiplication rate or growth of the cultures. This observation is contrary to the results reported in Phlox subulata (Schnabdrauch and Sink, 1979), Carrizo citrange (Kitto and Young, 1981) and jack (in the present studies).

The MS inorganic salts at normal strength supported the growth of mussaenda shoots and favoured their

multiplication. Unlike in rhododendron (Anderson, 1980), the Anderson's medium with reduced concentration of ammonium nitrate and potassium nitrate and increased concentration of ferrous sulphate and Na_2 EDTA, proved inefficient to support the proliferation and growth of mussaenda shoot cultures. As in the case of jack, sucrose at 30.0 to 40.0 g/l was found to be a suitable carbon/energy source and osmoticum.

For the induction of *in vitro* rooting, mussaenda shoots from the proliferation medium were subjected to various treatment combinations of IBA and NAA. IBA/NAA combination 0.4 + 0.4 ppm gave 60.0% rooting with seven roots per shoot in about 37 days after inoculation. Gupta *et al*. (1980) also obtained better results of *in vitro* rooting in teak with a combination of auxins. In general, the roots produced in the case of mussaenda were slender and weak, with only a few primary branches. Good amount of callussing was observed at the base of the shoots. The callus may bring about poor vascular connection between the roots and the shoot. These responses point either to high levels of the auxins tried (Lane, 1979) or to the need for treatment with other auxins. Nemeth (1981) reported that CDPPM, a rare synthetic auxin, produced 90% more roots than IBA in apple rootstocks.

Concentration of the MS inorganic salts influenced the per cent root initiation and the number of roots per culture. Half strength of the salts recorded the most favourable influence. The inorganic salt concentration of the basal medium has been found to be critical for root initiation in a number of species, regardless of the type of growth regulator present. A reduction to one-half, one-third or one-fourth of the standard strength favoured rooting in many species (Kartha et al., 1974, 1981; Skirvin and Chu, 1979). In the present studies, by the time the roots were initiated (37.0 days), the mussaenda shoots cultured at reduced level of MS inorganic salts turned pale yellow with symptoms of whithering. This indicated the insufficiency of inorganic salts for supporting shoot growth for long periods. On the contrary at full salt strength, while shoots were supported well for longer periods the number of roots per culture (3.5) and the root initiation (40.0%) were reduced. Poor shoot growth in media favouring in vitro rooting has been observed in other species also (Wang, 1978; Gupta et al., 1981). The Anderson's rooting medium, though reported to be good for rhododendron (Anderson, 1980) did not favour in vitro rooting of mussaenda. Sucrose 30.0 g/l was found to be the ideal carbon source and osmoticum for rooting. Agar, as a carrier material, supported maximum root initiation (80.0%) at 0.6 per cent concentration.

In the present investigations, efforts were made for the direct establishment in potting mixture of shoots from the proliferation medium, pre-treated with IBA solution. Such attempts were not successful. It may be recalled that in jack also the method proved a failure. However, there are successful reports of direct planting out of the shoots from the proliferation medium (Mc Cown and Amos, 1979; Kusey et al., 1980).

Attempts on callus mediated somatic organogenesis from various explants of mussaenda indicated shoot apices, leaf segments and segments of the ovary wall as suitable for callus production. Segments of the ovary wall yielded profuse callussing, registering a callus index value of 400.0 at the best treatment. Among the combinations of auxins and cytokinins, those involving NAA and kinetin gave the maximum response in the different explants tried. However, the optimum levels varied with the explants as in the case of jack. The difference in physiological conditions of the explants like level of endogenous phytohormones, nutrients, metabolites, heretofore unidentified growth substances etc. might have been the responsible for this variation. According to Skoog and Miller (1957) quantitative interaction between diverse growth factors may have decisive role in organogenesis. Changes in the

total and relative amounts of cytokinins and auxins may promote dis-organised callus growth, shoot formation or root formation.

Instances of shoot regeneration from the callus of shoot apices were observed in mussaenda, though at a rather low frequency of 33.3%. The regeneration occurred only after long period of culture on MS medium supplemented with BA 2.0 ppm or BA/kinetin combination 0.5 + 0.5 ppm and 0.5 + 0.3 ppm. BA/kinetin combinations gave better results than BA alone. The shoot regeneration process was slow and the new shoots formed were tiny with the apex surrounded by miniature green leaves. The results clearly reveal the regenerative potential of mussaenda callus. Further improvement in the frequency of shoot differentiation and the growth of the differentiated shoots may be possible by optimising the mode of culture (liquid culture), culture environment and culture medium. Plantlet regeneration from the callus leads to high incidence of bud differentiation (Brown and Sommer, 1982) and enables generation of desirable variants.

The callus from shoot apices of mussaenda was observed to have great potential for rhizogenesis. Differentiation of roots was more frequent than shoots.

Maximum number of roots per culture (13.33 with 66.7% root initiation) was observed at kinetin/NAA combination 2.0 + 8.0 ppm after 60 days of culture. The roots differentiated from the callus (in contrast to those produced when the shoots were subjected to in vitro rooting treatments) were healthy and exhibited a faster rate of growth, producing primary and secondary branches. Skoog and Miller (1957) have opined that high cytokinin/low auxin favours shoot differentiation whereas the reverse, root formation. It may be possible that mussaenda callus contains a high level of endogenous auxin, inhibiting shoot differentiation and promoting root differentiation.

In an attempt to induce direct somatic organogenesis, the in vitro formed shoot apices, internodal segments and lower halves of leaves (with petiole) were cultured in media supplemented with combinations of BA and kinetin. Although direct indication of organogenesis has been reported from leaf explants of many species (Behki and Lesley, 1976; Nair et al., 1984), the treatments failed to induce direct organogenesis from the explants of mussaenda.

The potential of the mussaenda explants for direct and callus mediated somatic embryogenesis was also examined in the present studies. Direct somatic embryogenesis was not observed. However, globular structures resembling

somatic embryoids were formed when the callus from the induction medium was transferred to medium containing BA/kinetin combination of 0.5 + 0.5 ppm and 1.0 + 0.5 ppm, after 70 to 73 days of culture. These structures then exhibited simultaneous root and shoot development. The frequency of occurrence of the structures was 26.7%. Only limited number of these structures were observed (which gave rise to 4.5 plantlets finally, at the best treatment), contrary to numerous reported in other species (Ammirato, 1983). The shoots developed were small and light green with miniature light green leaves and a tuft of snow white, small roots. The plantlets exhibited a slow rate of growth. The morphology and growth rate of the roots were strikingly different from those of the roots developed by somatic organogenesis. Proliferation of roots/shoots in other parts of the callus was totally absent. These observations seem to indicate that the globular structures resulted from somatic embryogenesis and not from somatic organogenesis. However, since histological examination to ascertain the lack of vascular connection between the plantlets and the mother callus (which is one of the proofs to confirm somatic embryoid formation) was not done, this argument cannot be forcefully made. Somatic embryogenesis has been observed in a number of species (Raghavan, 1976; Ammirato, 1983; Litz, 1985). The possibility exists that cells from any plant,

given the appropriate stimuli and conditions, could be fostered to embark on the embryogenetic pathway (Ammirato, 1983) rather than following organogenetic pathway. Failure to demonstrate this capacity has been largely due to faulty techniques and/or inhibiting conditions within the system (Raghavan, 1983). Improvement in response may be effected by optimising conditions to completely eliminate the inhibitory factors, by trying different modes of culture (like liquid culture), by providing optimum culture conditions and by altering the composition of the culture media.

Planting out of the mussaenda plantlets was attempted with different potting mixtures and under varying humidity levels, as done in the case of jack. However, none of the treatments were effective in supporting the survival of the plantlets. The weak and slender roots which were low in number, the callus present at the shoot-root junction which decayed easily after planting out and the partial withering of shoots by the time the roots were initiated, could be considered as the reasons for the lack of survival of the plantlets.

The protocol for <u>in vitro</u> formation of multiple shoots and plantlets, standardised for mussaenda in the

present studies need further refinement with respect to optimisation of the mode of culture and culture environment. Emphasis must be given to increase the shooting and rooting potential. The interference of callus observed in the studies need to be eliminated.

Further refining of the process of somatic organogenesis, observed in the present investigations, is needed to increase the frequency and to improve the growth of the plantlets.

Histological studies to ascertain the status of the globular structures (resembling somatic embryoids) showing simultaneous shoot and root development, efforts to increase the frequency of somatic embryogenesis and attempts to improve the growth of the resulting plantlets are also warranted.

Since attempts to induce multiple shoots to exploit them as sources of plantlets did not yield encouraging results, the course open seems to be direct/callus mediated somatic embryogenesis at the desired frequencies. Promising results on these lines have been reported in the case of crops like coffee (Sondahl and Sharp, 1977) and papaya (Litz and Conover, 1983). The method will have added advantage

in mussaenda where colour/type mutants would be very valuable.

III. Breadfruit

Breadfruit (Artocarpus altilis L.) is commonly grown in the homesteads of Kerala for its fruits which are used for vegetable purpose. It is propagated through vegetative means via rooted root cuttings and root suckers (Purseglove, 1974). This method offers a low percentage of success and is especially cumbersome. Standardisation of effective tissue culture techniques may be rewarding for the multiplication of the species. At present there are no reports on tissue culture of breadfruit.

The present preliminary studies, aimed at understanding the in vitro behaviour of the explants from mature trees pointed to two primary problems, namely, polyphenol oxidation and reduced response of the explants. The explants from the shoot apices could be effectively surface sterilised by sequential treatments with 1.5% sodium hypochlorite (for 10 minutes) and 0.1% mercuric chloride (for 10 minutes) solutions. The MS medium proved suitable to support the growth of the cultures, as indicated by the growth (although slight) registered and the prolonged survival of the cultures which were not severely affected by browning due to polyphenol

oxidation. While the shoot apices failed to initiate callus, the young inflorescences responded weakly to the callus induction treatment with 2,4-D 1.0 ppm + kinetin 1.0 ppm. The callus produced recorded a low callus index value (CI = 60.0) and turned light green before the discolouration due to polyphenol oxidation set in.

The studies indicated the possibility of somatic organogenesis by optimising the mode of culture, preculture treatments and culture conditions.

IV. Pepper

Pepper (Piper nigrum L.) is a leading cash crop of Kerala. It is propagated by vegetative means, using rooted stem cuttings. The recently developed 'bamboo method' (CPCRI, 1985) for the rapid vegetative propagation of pepper via rooted single node cuttings is estimated to generate 1.5 to 2.0 million rooted cuttings in an year from one hectare. This method may be successfully adopted for the rapid multiplication and early establishment of new genotypes evolved.

The present productivity of the crop is reported to be comparatively low (Bavappa, 1985). Improvement of the productivity and production of pepper is being given much emphasis at present. The pepper breeding programmes

at the various centres of Kerala concentrate on evolving varieties with higher yield, better quality and disease/ pest resistance/tolerance.

Plants recovered from tissue culture, especially via callus mediated somatic organogenesis have been reported to exhibit high amount of variability (Carlson, 1985). Somaclonal variation helps to widen the natural genetic spectrum and is useful for the selection and hybridisation programmes. It was in this context that the present preliminary attempt on the somatic organogenesis of pepper was undertaken.

Tissue culture procedure was not previously tried in this crop. In the present studies, profuse callussing could be induced from the nodal segments and shoot apices by culturing them on MS medium supplemented with NAA 2.0 ppm + kinetin 2.0 ppm. The MS medium could, therefore, be assumed to be suitable for supporting the survival and growth of the pepper explants. Discolouration of the cultures due to polyphenol oxidation was observed. However, the problem could be effectively solved by serial subculturing. Frequent subculturing has been demonstrated to reduce the incidence of culture discolouration in many species (Hu and Wang, 1983). The surface sterilisation technique employed

in the present studies failed to eliminate the systemic presence of bacteria in the culture. Bacterial contamination appeared even at advanced stages of organogenesis, making it difficult to have a systematic study of the somatic organogenesis in pepper. Systemic presence of bacteria has been reported to contaminate the cultures at the time of culture establishment or subsequently during proliferation, in many species (Zimmerman, 1985). In the limited instances of initial freedom from contamination, profuse root regeneration in the calli of shoot apices and nodal explants was observed at a wide range of NAA/kinetin combinations, indicating the high morphogenetic potential of pepper explants. Shoots were observed to form rudimentary green growing points from the calli. Use of explants from excised embryo or from seedlings raised under sterile conditions may solve the problem caused by systemic bacteria and aid in somatic organogenesis in pepper.

V. Nutmeg

Nutmeg (Myristica fragrans Houtt.), one of the popular tree spices and a member of the multi-storeyed cropping system of Kerala. It is commonly propagated through seeds. Being cross pollinated, seed propagation in nutmeg leads to variability among the progeny. Nutmeg is a dioecious plant and recognition of the sex type is possible

only when the reproductive phase sets in. Vegetative propagation through grafting (inarching) is possible (Purseglove, 1974); but is cumbersome and yields only a low rate of multiplication. Further, the grafts exhibit a slow growth rate during the initial years. Mass clonal propagation through tissue culture appears to be a priority area for tackling these set backs.

Previous reports on the tissue culture of nutmeg are lacking. The attempts made to understand the in vitro response of nutmeg explants were seriously marred by the problem of polyphenol oxidation. The callussing observed at 2,4-D/kinetin combination of 1.0 + 1.0 ppm (although slight) indicated the potential of the explants for somatic organogenesis and the suitability of MS medium to support the survival and growth of the explants. The explants could be successfully made aseptic by treating with mercuric chloride (0.1%) for 15 minutes. The in vitro response of nutmeg, initially with juvenile explants, needs further studies.

Summary

SUMMARY

Attempts were made in the Plant Tissue Culture Laboratory of the College of Horticulture, Vellanikkara during 1981-85 to standardise tissue culture techniques in some of the important horticultural crops of Kerala. Detailed investigations on the micropropagation of jack (*Artocarpus heterophyllus* Lam.) and mussaenda (*Mussaenda erythrophylla* Schum. & Thonn.) were made. Studies were also conducted to understand the *in vitro* response of the explants from breadfruit (*Artocarpus altilis* L.), pepper (*Piper nigrum* L.) and nutmeg (*Myristica fragrans* Houtt.).

In the case of jack, the explants from plants of different physiological age were subjected to a variety of culture media and culture conditions for observing the enhanced release of axillary buds and their subsequent rooting (direct as well as *in vitro*) and for inducing direct/callus mediated somatic organogenesis/embryogenesis. Standardisation of methods for hardening the plantlets and their planting out was also attempted.

In the case of mussaenda, attempts were made for the *in vitro* propagation via enhanced release of axillary buds and direct/callus mediated somatic organogenesis/ embryogenesis.

Preliminary studies on culture establishment and propagule multiplication were made in the case of breadfruit, pepper and nutmeg.

The salient findings of the above studies are summarised in this chapter.

I. Jack

1. Jack explants from the shoot apices of five-year old trees successfully established in MS medium supplemented with GA 1.0 ppm and activated charcoal 1.0%. The medium supported cent per cent survival and healthy, growing cultures. Survival of the lateral buds was hundred per cent in MS medium with GA 1.0 to 2.0 ppm and activated charcoal 0.5 to 1.0%; but growing cultures were too few (0 to 20.0%).

2. The problem of phenolics was effectively minimised by adopting suitable pre-culture treatments like shaking the explants in a solution of insoluble PVP (0.7%) and sucrose 2.0% for 30-45 minutes, trimming the explants at low temperature and culturing them at reduced light intensity, frequent subculturing, incorporation of activated charcoal (1.0%) in the culture establishment medium and inclusion of insoluble PVP 500 ppm in the multiplication medium.

3. In the case of explants from fresh stem sprouts of five-year old jack trees, MS medium containing BA/NAA combination 5.0 + 0.2 ppm and 5.0 + 0.1 ppm recorded the maximum number of fairly elongated shoots per culture (4.5 and 4.0, respectively). However, the length of the longest shoot was significantly greater (2.55 cm) in the former treatment.

4. The highest number of shoots per culture in the above case was recorded in MS + BA/NAA combination 7.5 + 0.2 ppm (8.75 shoots) which was on par with MS + BA/NAA combination 7.5 + 0.1 ppm (8.0 shoots) and MS + BA/NAA combination 10.0 + 0.2 ppm (7.75 shoots). However, among the shoots produced in the above treatments, a large proportion was highly compressed and small.

5. Auxins (IAA, NAA and 2,4-D) did not influence the shoot multiplication rate. However, the growth of the cultures (in terms of the length of the longest shoot and the length of the longest leaf) was significantly improved by majority of the levels of auxins tried.

6. Significant improvement in the shoot multiplication rate was obtained by the inclusion of adenine or adenine sulphate in the basic proliferation medium. Among the levels of adenine tried, 80.0 ppm registered the maximum

increase of 45.45%. Adenine sulphate 40.0 ppm registered the greatest increase of 59.09%. However, majority of the favourable levels of adenine and adenine sulphate affected the growth of the cultures by reducing the length of the longest shoot and the length of the longest leaf. Only adenine sulphate 20.0 ppm recorded an increase in multiplication rate (27.27%) without significantly affecting the growth of the cultures.

7. No beneficial effect was observed when GA_3 was included in the basic proliferation medium, in terms of the rate of shoot multiplication and growth of the cultures.

8. Inclusion of casein hydrolysate 500.0 ppm in the basic proliferation medium increased the rate of shoot multiplication by 40.0%. However, all the favourable levels of CH tried, severely affected the growth of the cultures.

9. The number of shoots per culture, the length of the longest shoot and the length of the longest leaf were significantly reduced by lowering the content of MS inorganic salts to half strength (reduction of 30.77%, 48.87% and 32.68%, respectively) and quarter strength (reduction of 57.69%, 55.37% and 63.39%, respectively) than the normal level. Doubling the salt concentration caused, reduction in the length of the longest shoot and the length of the longest

leaf (25.00% and 33.33%, respectively) and did not improve the shoot multiplication rate.

10. Reducing the quantity of sucrose below the normal level (3.0%) was unfavourable in terms of the shoot multiplication rate and growth of the cultures. Increasing the quantity to 4.0% did not significantly improve the proliferation rate and growth of the cultures. Sucrose 3.0 to 4.0% was found to be ideal as carbon/energy source.

11. Maximum favourable effects of glucose as carbon/energy source (in terms of shoot proliferation and growth of the cultures) were observed at 2.0 to 3.0% level.

12. The Anderson's medium (supplemented with BA 5.0 ppm and NAA 0.2 ppm) was found to be unfavourable for shoot multiplication and growth of the cultures, recording a reduction of 30.53% in the number of shoots per culture, 14.9% in the length of the longest shoot and 39.35% in the length of the longest leaf as compared to the MS proliferation medium.

13. The multiplication rate of the shoots was increased by 26.79%, on an average, as a result of ten serial subculturing at four-week interval.

14. The MS medium supplemented with BA 2.0 ppm and NAA 0.2 ppm was found to be the most favourable for the elongation of shoots transferred from the proliferation medium, recording a shoot length of 3.83 cm and a leaf length of 3.7 cm.

15. As for the *in vitro* rooting of jack shoot cultures, the treatment "1/2 MS + IBA/NAA combination 2.0 + 2.0 ppm (for six days) and then 1/2 MS without growth substances" was found to be the most favourable, giving 70.0% root initiation with 5.43 roots in 13.43 days.

16. Half strength of the MS inorganic salts, full strength of the MS organic growth factors, 3.0% sucrose and 0.6% agar were identified as the most favourable levels for the *in vitro* rooting of jack shoot cultures.

17. Physiological age of the explants exhibited significant influence on the *in vitro* shoot proliferation and rooting of jack. The response was maximum for the seedling explants, followed by the explants of fresh stem sprouts from five-year old trees. A drastic reduction was apparent in the response of the explants from fresh stem sprouts of ten and thirty-year old trees and explants from six-month old grafts.

18. Seedling explants registered a multiplication rate of 17.4 x at MS + BA 10.0 ppm + NAA 0.2 ppm and demonstrated excellent rooting of shoots (100.0%)with 6.00 roots per shoot when treated with 1/2 MS + IBA/NAA combination 0.4 + 0.4 ppm in 20.75 days. In vitro rooting was possible in a wide range of auxin treatments.

19. Even with repeated subculturing, multiplication rates of 2.80x, 2.09x and 1.00x only were obtained for the explants from fresh stem sprouts of 10-and 30-year old trees and for the explants from shoot apices of 6-month old grafts, respectively. The corresponding percentage of in vitro rooting was 40.0 (with 2.5 roots in 24.0 days), 15.0 (with 1.0 root in 46.7 days) and 50.0 (with 2.0 roots in 20.5 days).

20. Attempts for direct planting out of the shoots taken from the proliferation medium (pretreated with IBA solutions) were not successful.

21. Vermiculite was identified as the best medium for planting out the plantlets, supporting 55.6% survival. The potting mixture consisting of sand: soil: coir dust (1:1:1 v/v) recorded only 40.0% survival.

22. The highest percentage (55.6) of survival of the plantlets was observed when the plantlets, just after root

initiation, were subjected to one-week of *in vitro* hardening at a high light intensity (3500 lux) at room temperature.

23. Microscope covers were found to be suitable as humidity maintenance devices, maintaining 90 to 100% R.H. and supporting 55.6% of plantlet survival.

24. Spraying the planted out plantlets with growth substances did not improve their survival.

25. When 1/2 MS inorganic salt solution was applied, the plantlets registered a height of 14.75 cm with six leaves, three months after planting out.

26. The tissue cultured plants could be successfully transferred to garden pots filled with ordinary potting mixture (sand: soil: cowdung 1:1:1 v/v) and kept in open field conditions for six months.

27. Callus production was obtained from explants of shoot apices, internodal segments, leaf segments and root apices of five-year old trees as well as from young inflorescence explants of 10 year old trees.

28. Explants from shoot apices were the best, recording the highest callus production (Callus Index = 180.0).

29. The highest percentage of callus initiation (100.0) and the best callus index (CI = 180.0) were obtained when the shoot apices were treated with MS + NAA 2.0 ppm + kinetin 1.0 ppm. This was significantly superior to the rest of the treatments involving combinations of auxins and cytokinins.

30. Attempts for inducing regeneration in the callus were not successful.

31. Attempts for inducing direct somatic organogenesis were not successful.

32. Attempts for inducing callus mediated somatic embryogenesis were not successful.

33. Cytological examination revealed the stability of the chromosome number (2n = 56) in the plantlets produced.

34. Histological studies demonstrated the presence of thin cuticle in the leaves of the new plantlets and the presence of normal cuticle in the new leaves produced by them after the hardening.

35. The cost of production of one jack plantlet, including one month's hardening was worked out to Rs.9.09.

II. Mussaenda

1. Mussaenda explants from shoot apices were successfully established in MS medium supplemented with BA 0.5 ppm and kinetin 0.5 ppm, which supported cent per cent survival and production of healthy growing cultures. In the same medium, the lateral buds recorded 73.3% survival and 26.1% growing cultures.

2. The best medium for inducing enhanced release of axillary buds and growth of the cultures was identified as MS + BA 0.5 ppm + kinetin 0.5 ppm, recording 2.75 shoots per explant with 10.25 leaves, 3.1 cm long.

3. Serial subculturing in the proliferation medium (for five times at 4-week interval) increased the rate of shoot multiplication to 2.95x for the explants from shoot apices.

4. Auxins (IAA and NAA) were found to be not beneficial for the multiplication of shoots.

5. Inclusion of adenine sulphate in the basic proliferation medium did not improve the shoot multiplication rate and growth of the cultures.

6. The shoot multiplication rate as well as growth of the cultures (in terms of the number of leaves produced

and the length of the longest leaf) were markedly reduced by reducing the quantity of the MS inorganic salts to quarter or half strength. Doubing the salt concentration not only did not improve the multiplication rate, but also led to poor growth of the cultures.

7. The rate of shoot multiplication and the growth of the cultures were not significantly altered by varying the sucrose content in the medium.

8. The Anderson's medium (supplemented with BA 0.5 ppm and kinetin 0.5 ppm) was found to be unfavourable for the multiplication and growth of the mussaenda shoot cultures, registering a multiplication rate of 1.55x with 3.82 leaves having 3.66 cm length.

9. In vitro rooting of the cultured mussaenda shoots was found to be better in 1/2 MS + IBA/NAA combination 0.4 + 0.4 ppm, inducing 60 per cent rooting with 7.0 roots per culture in 37.31 days.

10. Half strength of the MS inorganic salts, 3.0% sucrose and 0.6% agar were identified as the most favourable levels for the in vitro rooting of mussaenda shoot cultures.

11. The Anderson's rooting medium was observed to be unsuitable for the in vitro rooting of mussaenda shoots,

as only 2.5 roots per shoot were initiated with 40.0 per cent root initiation.

12. Attempts for the direct planting out of the mussaenda shoots taken from the proliferation medium (pretreated with IBA solutions) were not successful.

13. Attempts for planting out the mussaenda plantlets were unsuccessful. The limited number of weak and slender roots, the abundant callussing observed at the root-shoot junction and the partial withering and yellowing of the shoots by the time the roots were initiated, could be ascribed as the possible reasons.

14. Appreciable quantity of callus could be produced from various explants (shoot apices, leaf segments and segments of ovary wall) of mussaenda. Ovary wall has been identified as the best tissue for the production of callus, recording a callus index value of 400.0 at the best treatment.

15. The MS medium supplemented with kinetin/NAA combination 1.0 + 2.0 ppm and 2.0 + 4.0 ppm was the most effective treatment for inducing callussing, recording a callus index value of 400.0 (explant: segments of ovary wall).

16. Attempts for inducing direct somatic organogenesis were not successful.

17. Shoot regeneration from the callus was observed at a low frequency (33.3%). At the most effective treatment (MS + BA/kinetin combination 0.5 + 0.5 ppm), five shoots per culture were produced.

18. Root regeneration from the callus was observed at a frequency of 66.7%. At the most effective treatment (MS + kinetin/NAA combination 2.0 + 8.0 ppm), 9.67 roots per culture were produced after 60 days of culture.

19. Structures resembling somatic embryoids, showing simultaneous root and shoot development, were observed in the callus subcultured from the embryoid induction medium. At MS + BA/kinetin combination 0.5 + 0.5 ppm, these structures developed at a frequency of 26.7% after 70 days of culture. Finally, 4.5 small shoots per culture with tufts of miniature roots were developed.

III. Bread fruit

1. The explants from shoot apices successfully established in MS + BA 10.0 ppm + GA 1.0 ppm + activated charcoal 1.0%, supporting 80.0% survival and 40.0% growing cultures.

2. Slight callus production (callus index = 60.0) was observed when explants from young inflorescences were

subjected to treatment with MS + 2,4-D/kinetin combination 1.0 + 1.0 ppm.

3. Due to the problem of polyphenol interference and reduced response of the explants tried, the studies could not be continued.

IV. Pepper

1. Good callus production was observed from the explants of shoot apices, recording a callus index value of 300.0, at the best treatment (MS medium supplemented with NAA 2.0 ppm and kinetin 1.0 ppm).

2. Callus from the nodal explants exhibited more regenerative capacity than the calli from other explants. At MS + NAA/kinetin combination 1.0 + 1.0 ppm and MS + NAA 1.0 ppm, 2.33 roots per culture were formed. Formation of rudimentary shoots, which appeared as green growing points was observed at MS + kinetin 1.0 ppm (0.33 shoots per culture) and MS + kinetin 2.0 ppm (1.0 shoot per culture).

3. Due to the systemic presence of bacteria, the study could not be continued.

V. Nutmeg

From the explants of shoot apices, slight callussing (callus index = 40.0) was observed at MS + 2,4-D 1.0 ppm + kinetin 1.0 ppm + activated charcoal 1.0%. Due to the problem of polyphenol interference, the callus did not survive, even after subculturing.

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* Originals not seen

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Research Notes

IN VITRO ROOTING OF JACK SHOOT CULTURES

Fresh sprouts from the basal portion of the stem of a five year old jack tree were collected. The shoot tips (5 cm) were cut, dipped in 95% ethyl alcohol for 10 seconds, washed with sterile water containing teepol, washed again with sterile water and surface sterilised by keeping in 2.0% sodium hypochlorite solution for 30 min. Following thorough washing in sterile water, the shoot tips were placed in a solution of 2.0% sucrose and 0.7% polyvenyl pyrrolydon (soluble PVP) for 30 min. The basal ends were again cut and dipped in a sterile solution containing BA 10.0 ppm and GA 1.0 ppm for 24 hours under refrigerated conditions (4-5°C). Shoot apices (2 cm length) were cut under low temperature and low intensity of light. They were surface sterilised with sodium hypochlorite (3.0% for 5 min) and mercuric chloride (0.1% for 10 min) solutions. After rinsing with sterile water, the sheaths covering the shoot tips were removed and the apices cut back to 1.5 cm length. They were placed on semisolid MS medium containing GA (1.0 ppm) and activated charcoal (1.0%). The cultures were incubated at 25-30°C in darkness for four weeks and then exposed to light for two weeks. They were sub-cultured in a medium containing BA and IAA for inducing multiple shoot formation. After the induction, the shoots were separated and sub-cultured in a medium with reduced BA and IAA for favouring normal development of shoots. They were then transferred to a medium containing activated charcoal(1.0%) and GA (1.0 ppm) and grown for two weeks. The cultures were transferred to a modified MS medium (major and minor nutrients reduced to half strength) supplemented with IBA 1.6 ppm, NAA 0.4 ppm, sucrose 3.0%, agar 0.7% and with a pH of 5.6. The cultures were incubated in darkness at 25-30°C. Due to the production of slight amounts of phenolics, sub-culturing was found to be necessary. Very slight callussing preceded root initiation. Roots were seen initiated in about 24 days. On an average, six roots were produced. They attained 1.0 cm length in about 20 days. The roots were reddish yellow and cylindrical. Just after the initiation of roots, the shoots elongated rapidly.

When shoot apices of two-month old seedlings were used for the study, roots were initiated in about 18 days in the same medium with 0.4 ppm IBA and 0.4 ppm NAA.

സംഗ്രഹം

അഞ്ചുവർഷം പ്രായമുള്ള പ്ലാവിന്റെ അഗ്രമുകുളം കത്തിച്ചു ചെയ്തശേഷം അനവധി മുകുളങ്ങൾ ഉത്തേജിപ്പിക്കുകയും അതിനുശേഷം ഒരോ മുകുളവും വേർപെടുത്തിയെടു

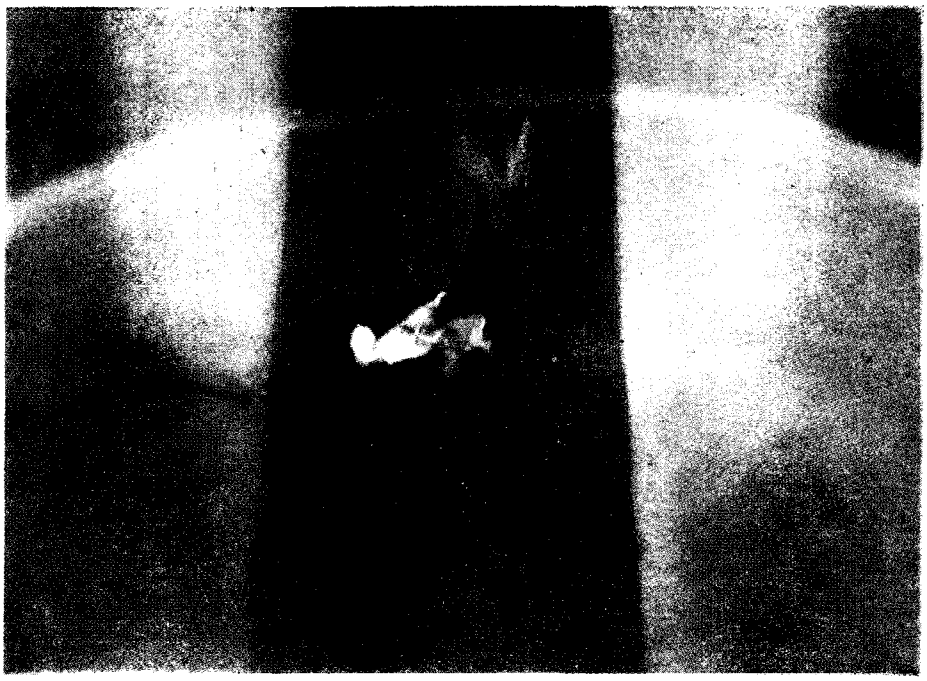

Fig. 1 *In vitro* rooting of jack shoot culture

APPENDIX I

Two way comparison of the mean Callus Index values of jack explants in response to different combinations of growth substances

Sl.No.	Explant	Combination of growth substances		Callus index values		Mean
1.	Shoot apex	NAA	kinetin	1.0 ppm	2.0 ppm	4.0 ppm	
		1.0 ppm		40.0	0	0	13.33
		2.0 ppm		180.0	140.0	0	106.66
		4.0 ppm		40.0	100.0	0	46.66
		8.0 ppm		0	40.0	20.0	20.00
		Mean		86.66	70.0	5.0	
2.	Shoot apex	NAA	BA	1.0 ppm	2.0 ppm	4.0 ppm	
		1.0 ppm		80.0	0	20.0	33.33
		2.0 ppm		140.0	40.0	20.0	66.66
		4.0 ppm		120.0	100.0	20.0	80.00
		8.0 ppm		20.0	20.0	60.0	33.33
		Mean		90.00	40.00	30.0	
3.	Shoot apex	2,4-D	BA	1.0 ppm	2.0 ppm	4.0 ppm	
		0.$ ppm		20.0	0	20.0	13.33
		0.5 ppm		120.0	40.0	40.0	66.66
		1.0 ppm		140.0	60.0	20.0	73.33
		2.0 ppm		40.0	0	20.0	20.00
		Mean		80.00	25.00	25.00	

(Contd.)

Appendix I (Contd.)

Sl.No.	Explant	Combination of growth substances		Callus index values		Mean
4.	Internodal segment	NAA	kinetin	1.0 ppm	2.0 ppm	4.0 ppm
		1.0 ppm		40.0	0	0	13.33
		2.0 ppm		60.0	0	0	20.00
		4.0 ppm		79.8	40.0	0	39.93
		8.0 ppm		0	0	0	0
		Mean		44.95	10.0	0	
5.	Internodal segment	NAA	BA	1.0 ppm	2.0 ppm	4.0 ppm	
		1.0 ppm		40.0	0	0	13.33
		2.0 ppm		60.0	20.0	0	33.33
		4.0 ppm		20.0	20.0	20.0	20.00
		8.0 ppm		79.8	0	0	26.60
		Mean		49.95	10.00	5.00	
6.	Internodal segment	2,4-D	BA	1.0 ppm	2.0 ppm	4.0 ppm	
		0.1 ppm		0	20.0	0	6.66
		0.5 ppm		60.0	20.0	20.0	33.33
		1.0 ppm		79.8	20.0	0	33.27
		2.0 ppm		0	40.0	0	13.33
		Mean		34.95	25.0	5.0	

(Contd.)

Appendix I (Contd.)

Sl.No.	Explant	Combination of growth substances	Callus index values			Mean
7.	Root tip	NAA \ kinetin	1.0 ppm	2.0 ppm	4.0 ppm	
		1.0 ppm	80.0	20.0	0	33.33
		2.0 ppm	79.8	40.0	0	39.93
		4.0 ppm	79.8	20.0	0	33.27
		8.0 ppm	0	20.0	60.0	33.33
		Mean	59.9	33.33	15.0	
8.	Root tip	2,4-D \ BA	1.0 ppm	2.0 ppm	4.0 ppm	
		0.1 ppm	20.0	0	20.0	20.0
		0.5 ppm	0	40.0	0	13.33
		1.0 ppm	79.8	79.8	20.0	59.87
		2.0 ppm	100.0	20.0	0	40.0
		Mean	49.95	34.95	10.0	
9.	Shoot apex	2,4-D \ kinetin	0 ppm	1.0 ppm	2.0 ppm	
		0.1 ppm	0	0	0	0
		0.5 ppm	0	100.0	0	33.3
		1.0 ppm	33.3	167.0	167.0	122.43
		2.0 ppm	33.3	0	0	11.1
		Mean	16.7	66.75	41.75	

APPENDIX II

Analysis of variance for different characters

Treatment/character	Means squares	
	Treatment	Error
1	2	3
I. Jack		
A. Enhanced release of axillary buds		
1. Combinations of BA/NAA		
a. Number of shoots	26.94	1.50 **
b. Number of fairly elongated shoots	4.72	0.59 **
2. Serial subculturing in BPM		
a. Number of shoots	3.47	1.29 *
3. BA; kinetin; BA/kinetin		
a. Number of shoots	18.60	1.32 **
b. Length of the longest shoot	1.16	0.08 **
c. Length of the longest leaf	1.96	0.02 **
4. Adenine		
a. Number of shoots	4.84	1.10 **
b. Length of the longest shoot	0.31	0.04 **
c. Length of the longest leaf	1.80	0.09 **
5. Adenine sulphate		
a. Number of shoots	7.24	0.71 **
b. Length of the longest shoot	0.86	0.05 **
c. Length of the longest leaf	1.62	0.11 **
6. Casein hydrolysate		
a. Number of shoots	4.86	0.75 **
b. Length of the longest shoot	1.68	0.14 **
c. Length of the longest leaf	4.61	0.24 **
7. GA_3		
a. Number of shoots	0.53	0.70 NS
b. Length of the longest shoot	0.38	0.40 NS
c. Length of the longest leaf	0.90	0.24 *

(Contd.)

Appendix II (Contd.)

1	2	3
8. IAA; NAA; 2,4-D		
a. Number of shoots	0.56	1.13 NS
b. Length of the longest shoot	0.30	0.08 **
c. Length of the longest leaf	0.30	0.07**
9. MS inorganic salts		
a. Number of shoots	11.27	0.50 **
b. Length of the longest shoot	1.86	0.11 **
c. Length of the longest leaf	3.14	0.29 **
10. MS organic factors		
a. Number of shoots	8.20	0.45 **
b. Length of the longest shoot	1.32	0.26 *
c. Length of the longest leaf	0.44	0.29 NS
11. Sucrose		
a. Number of shoots	5.40	0.35 **
b Length of the longest shoot	1.70	0.21 **
c. Length of the longest leaf	1.82	0.19 **
12. Glucose		
a. Number of shoots	3.40	0.60 **
b. Length of the longest shoot	0.47	0.14 *
c. Length of the longest leaf	0.53	0.15 *
B. Shoot elongation		
1. IAA/BA; NAA/BA		
a. Number of shoots	1.92	0.15 **
b. Length of the longest shoot	0.63	0.05 **
c. Length of the longest leaf	0.81	0.08 **

(Contd.)

Appendix II (Contd.)

1	2	3
II. Mussaenda		
A. Enhanced release of axillary buds		
1. Combinations of BA/kinetin		
a. Number of shoots	1.19	0.56 NS
b. Number of leaves	21.97	6.12 *
c. Length of the longest leaf	1.58	0.21 **
2. IAA; NAA		
a. Number of shoots	0.75	0.80 NS
b. Number of leaves	17.74	7.14 NS
c. Length of the longest leaf	0.18	0.24 NS
3. Adenine sulphate		
a. Number of shoots	0.16	1.18 NS
b. Number of leaves	13.60	14.48 NS
c. Length of the longest leaf	0.11	0.22 NS
4. MS inorganic salts		
a. Number of shoots	2.20	0.65 *
b. Number of leaves	17.87	3.20 *
c. Length of the longest leaf	4.45	0.28 **
5. Sucrose		
a. Number of shoots	1.65	1.35 NS
b. Number of leaves	12.47	11.30 NS
c. Length of the longest leaf	0.48	0.20 NS

* Significant at 5% level

** Significant at 1% level

NS Not significant

APPENDIX III

Two way comparison of the mean Callus Index values of mussaenda explants in response to different combinations of growth substances

Sl.No.	Explant	Combination of growth substances		Callus Index values			Mean
1.	Shoot apex	kinetin	NAA	1.0 ppm	2.0 ppm	4.0 ppm	8.0 ppm	
		1.0 ppm		33.3	367.0	33.3	66.7	125.08
		2.0 ppm		0	233.0	133.0	167.0	133.25
		4.0 ppm		66.7	100.0	267.0	267.0	175.18
		Mean		33.33	233.33	144.43	166.90	
2.	Shoot apex	BA	NAA	1.0 ppm	2.0 ppm	4.0 ppm	8.0 ppm	
		1.0 ppm		33.3	167.8	0	66.6	66.93
		2.0 ppm		0	233.0	66.7	166.8	116.63
		4.0 ppm		0	0	167.0	233.0	100.00
		Mean		11.10	133.60	77.90	155.47	
3.	Shoot apex	BA	IAA	1.0 ppm	2.0 ppm	4.0 ppm	8.0 ppm	
		1.0 ppm		33.3	100.0	100.0	133.0	91.65
		2.0 ppm		33.3	66.7	100.0	167.0	91.75
		4.0 ppm		100.0	0	133.0	100.0	83.75
		Mean		55.53	55.53	111.00	133.33	

(Contd.)

Appendix III (Contd.)

Sl.No.	Explant	Combination of growth substances		Callus Index values				Mean
4.	Leaf segment	kinetin	NAA	1.0 ppm	2.0 ppm	4.0 ppm	8.0 ppm	
		1.0 ppm		66.7	200.0	33.3	0	75.00
		2.0 ppm		33.3	167.0	133.0	200.0	133.33
		4.0 ppm		100.0	66.7	267.0	200.0	158.43
		Mean		46.63	144.57	144.43	133.33	
5.	Segments of ovary wall	kinetin	NAA	1.0 ppm	2.0 ppm	4.0 ppm	8.0 ppm	
		1.0 ppm		100.0	400.0	66.7	100.0	166.68
		2.0 ppm		33.3	300.0	400.0	167.0	225.08
		4.0 ppm		66.7	200.0	267.0	133.0	166.68
		Mean		66.67	300.0	244.57	133.33	
6.	Shoot apex	2,4-D	kinetin	0 ppm	1.0 ppm	2.0 ppm	4.0 ppm	
		0.1 ppm		66.7	66.7	100.0	66.7	75.03
		0.5 ppm		100.0	100.0	33.3	0	58.33
		1.0 ppm		33.3	200.1	167.0	100.0	125.03
		2.0 ppm		0	367.0	166.8	100.0	158.45
		Mean		50.0	183.45	116.78	66.68	
7.	Leaf segment	2,4-D	kinetin	0 ppm	1.0 ppm	2.0 ppm	4.0 ppm	
		0.1 ppm		100.0	66.7	100.0	33.3	75.00
		0.5 ppm		66.7	66.7	66.7	33.3	58.35
		1.0 ppm		66.7	233.0	100.0	33.3	108.25
		2.0 ppm		33.3	166.8	133.0	166.8	124.98
		Mean		66.67	133.3	99.93	66.68	

STANDARDISATION OF TISSUE/MERISTEM CULTURE TECHNIQUES IN IMPORTANT HORTICULTURAL CROPS

By

K. RAJMOHAN

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the

requirements for the degree of

Doctor of Philosophy in Horticulture

Faculty of Agriculture

Kerala Agricultural University

Department of
Plantation Crops and Spices
COLLEGE OF HORTICULTURE
Vellanikkara, Trichur
1985

ABSTRACT

Attempts were made in the Plant Tissue Culture Laboratory of the College of Horticulture, during 1981-85 to standardise tissue culture techniques for the propagation of some of the important horticultural crops of Kerala.

Explants from shoot apices of fresh stem sprouts of five-year old jack (*Artocarpus heterophyllus* Lam.) trees registered a multiplication rate of 4.5 x when cultured for five weeks and produced 70% rooting (in 13.43 days) with 5.43 roots per shoot. The MS medium supplemented with GA 1.0 ppm and activated charcoal 1.0% was identified as suitable for culture establishment, supporting the survival and initial growth of the explants. Benzyl adenine 5.0 ppm was found to be the optimum cytokinin level for the production of fairly elongated multiple shoots and NAA 0.2 ppm was identified as the optimum auxin level for supporting the growth of the cultures. The normal strength of the inorganic salts and organic growth factors of the MS medium, with 3 - 4% sucrose or 2 - 3% glucose was found to support the multiplication and growth of jack shoot cultures. GA_3 did not influence the shoot proliferation or growth. Adenine sulphate at 20 ppm was found to increase

the multiplication rate by 27.3%, without affecting the growth of the cultures. Adenine as well as casein hydrolysate were found to be not beneficial. The Anderson's medium was found to be unsuitable to support the proliferation and growth of jack shoot cultures. Serial subculturing for 10 times at 4-week interval was found to increase the multiplication rate to 5.39x. The MS medium supplemented with BA 2.0 ppm, NAA 0.2 ppm and insoluble PVP 500 ppm was found to be suitable for shoot elongation. Half strength of the MS inorganic salts, full strength of the MS organic factors, 3% sucrose and 0.6% agar were found to be the optimum for the in vitro rooting of jack shoot cultures. When planted out, the plantlets were observed to have 55.6% survival.

Callus production was made possible from explants of shoot apices, internodal segments, leaf segments and root apices. Efforts to induce re-differentiation in the callus, direct organogenesis and direct/callus mediated somatic embryogenesis were not successful.

Shoot apices from the seedlings registered a multiplication rate of 17.4x. In this case, the percentage of rooting was 100, with 6.0 roots formed in 20.75 days. Explants from fresh stem sprouts of ten-year old and thirty-year old trees recorded a shoot multiplication rate

of 2.8 and 2.09, respectively in five weeks. In the former, the rooting percentage was 40 with 2.5 roots produced in 24 days, after 2-3 subcultures. In the latter, there was 15% rooting with 2.0 roots formed in 46.7 days, after 2-3 subcultures. Explants from six-month old jack grafts failed to produce multiple shoots and exhibited 50% rooting with 2.0 roots formed in 20.5 days. Cytological examination revealed the stability of chromosome number in the plantlets. Anatomical studies revealed the presence of thin cuticle in the leaves of the young plantlets.

The procedure for the <u>in vitro</u> clonal propagation of jack through the enhanced release of axillary buds involved agitating the surface sterilised shoot apices in a solution of 0.7% insoluble PVP + 2% sucrose for 30-45 minutes and keeping them in sterile water at 4-5^{o}C for 24 hours followed by disinfection (3% sodium hypochlorite solution for 5 minutes and 0.1% mercuric chloride solution for 10 minutes) and culture in the establishment medium (MS + GA 1.0 ppm + activated charcoal 1.0%) in darkness for four weeks with repeated subculturing. The cultures were then exposed to light for two weeks, after which the growing shoot apices were transferred to the proliferation medium (MS + BA 5.0 ppm + NAA 0.2 ppm + adenine sulphate 20 ppm + insoluble PVP 500 ppm). Shoots from the proliferation

medium were transferred after five weeks to an elongation medium (MS + BA 2.0 ppm + NAA 0.2 ppm + insoluble PVP 500 ppm). The shoots were then cultured on MS medium containing activated charcoal 1.0% for two weeks. For the *in vitro* root induction, the shoots were cultured in darkness in 1/2 MS + IBA 2.0 ppm + NAA 2.0 ppm + sucrose 3% + agar 0.6% (for 6 days) and then transferred to 1/2 MS without growth substances for root elongation.

Just after the appearance of the roots, the plantlets were hardened by exposure to high light intensity (3500 lux) for one week. The plantlets were then transferred to vermiculite medium, under high relative humidity (90-100%) provided by microscope covers. The plants were watered with a solution of the MS inorganic salts at half strength. After another gradual hardening process and as new leaves were produced, the plantlets were transferred to garden pots and kept in the open field conditions. The cost of production of one jack plantlet, including one month's hardening was worked out to Rs.9.09

Explants of mussaenda (*Mussaenda erythrophylla* Schum. & Thonn.) were effectively surface sterilised by treating with 0.1% mercuric chloride solution for 15 minutes. The suitable culture establishment medium was identified as

MS + BA 0.5 ppm + kinetin 0.5ppm. A shoot multiplication rate of 2.75x was realised in a period of four weeks on MS medium supplemented with BA 0.5 ppm and kinetin 0.5 ppm. Subculturing was found to increase the multiplication rate to 2.95x. Full strength of the MS inorganic salts was found to support the proliferation and growth of mussaenda shoot cultures. Adenine sulphate, auxins and Anderson's medium were found to be not beneficial. The shoots were made to root on MS medium containing half strength of the inorganic salts and full strength of the organic growth factors, 3% sucrose, 0.6% agar, 0.4 ppm IBA and 0.4 ppm NAA, under dark conditions in 37 days. Anderson's rooting medium was found to be inefficient for the <u>in vitro</u> rooting of mussaenda shoot cultures. Attempts for planting out were not successful, probably due to the low number of roots (which were weak and slender), the development of callus at the root-shoot junction and the partial withering and yellowing of the shoots by the time the roots were initiated. Attempts for the direct planting out of the shoots pretreated with IBA solution were not successful.

Segments of ovary wall was identified as the best source of explant for callus production, registering a callus index value of 400, at the best treatment (NAA/ kinetin combinations 2.0 + 1.0 ppm and 4.0 + 2.0 ppm).

Shoot regeneration from the callus occurred at a frequency of 33.3% on MS medium supplemented with BA 2.0 ppm or BA/kinetin combinations 0.5 + 0.5 ppm and 0.5 + 0.3 ppm. Root regeneration was observed at a frequency of 66.7% with 13.33 roots per culture on MS medium containing kinetin/NAA combination 2.0 + 8.0 ppm after 60 days' culture. Attempts for direct organogenesis were not successful.

Globular structures resembling somatic embryoids having simultaneous root and shoot development were observed when the callus from the induction medium (MS + 2,4-D 2.0 ppm + kinetin 1.0 ppm) was transferred to MS medium containing BA/kinetin combinations 0.5 + 0.5 ppm or 1.0 + 0.5 ppm after 70 to 73 days' culture. About 4.5 shoots with a tuft of miniature roots were formed per culture, at the best treatment. Attempts for inducing direct somatic embryogenesis were not successful.

Preliminary studies on culture establishment were made for breadfruit (Artocarpus altilis L.) and nutmeg (Myristica fragrans Houtt.). Slight callus production was made possible in both the cases. Preliminary studies on somatic organogenesis were made in the case of pepper (Piper nigrum L.). Callus production and redifferentiation were observed.