ENHANCING THE *IN VITRO* RESPONSE OF EXPLANTS FROM MATURE JACK (*Artocarpus heterophyllus* Lam.) TREES
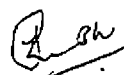
By

REENA PHILIP B.Sc. (Ag.)

THESIS
SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENT FOR THE DEGREE
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1993

DECLARATION

I hereby declare that this thesis entitled "Enhancing the _in vitro_ response of explants from mature jack (_Artocarpus heterophyllus_ Lam.) trees", 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.

College of Agriculture,
Vellayani,
15 -11-1993.

REENA PHILIP

CERTIFICATE

Certified that this thesis entitled. "Enhancing the _in vitro_ response of explants from mature jack (<u>Artocarpus heterophyllus</u> Lam.) trees", is a record of research work done independently by Ms. REENA PHILIP, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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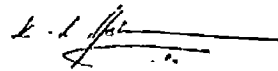Vellayani,
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Dr. K. RAJMOHAN
Chairman,
Advisory Committee

Associate Professor,
Department of
Horticulture.

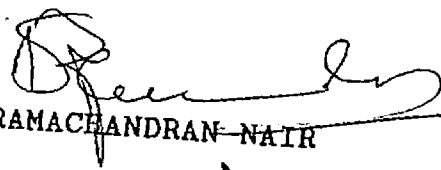APPROVED BY:

CHAIRMAN:

Dr. K. RAJMOHAN

MEMBERS:

1. Dr. N. MOHANAKUMARAN

2. Dr. S. RAMACHANDRAN NAIR

3. Dr. N. SAIFUDEEN

EXTERNAL EXAMINER:

(N.G. Nair)

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INTRODUCTION

and have low rates of multiplication. Tissue culture techniques could be a possible alternative for rapid clonal propagation of jack. Cloning of mature tree is essential for the purpose, as it is not possible to determine the genetic potential of seedlings to develop the desired qualities later in their life period.

Explants from several tree species exhibit reduced response *in vitro* with the transition from juvenile to mature phase. This maturity barrier is primarily expressed as the reduced ability of explants to form adventitious and axillary buds, slow rate of shoot elongation and poor extent of rooting (Bonga, 1982). In fact, *in vitro* techniques often offer no advantage over traditional methods because the capacity of a plant to propagate vegetatively *in vitro* is often lost at an age when the plant can still be propagated by rooting of cuttings (Bonga and Durzan, 1982). Rajmohan (1986) observed a significant reduction in the *in vitro* shoot proliferation and rooting response with the increase in ontogenetic age of jack. This might be due to several physiological and biochemical changes taking place during the maturation process of plants (Bonga, 1982), which in turn influenced the *in vitro* behaviour of explants.

In several tree crops the maturity barrier problem could partially by overcome by selecting the most juvenile

tissue as the explant and by special rejuvenation/ invigouration treatments before or during micropropagation (Bonga, 1981). The pretreatments suggested include application of plant growth substances (David *et al.*, 1978), serial rooting of cuttings (Goublay de Nantois, 1980), serial grafting on to juvenile rootstocks (Bonga, 1981), adopting suitable *in vitro* procedures involving plant growth substances and subculturing at frequent intervals (Sriskandarajah *et al.*, 1982).

The maturity barrier problem in jack and the methods to overcome this has not been studied enough. The present investigations aimed at identifying suitable pretreatments to enhance the *in vitro* response of mature jack explants.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Tissue culture techniques have considerably helped the improvement of propagation efficiency of several tree crops. However, propagation of a proven genotype of a tree necessitates the use of mature tree explants (Haissig *et al.*, 1987). It has been found that explants from mature trees exhibit reduced response *in vitro* with the increase in ontogenetic age of the trees. Several physiological and biochemical changes take place during the maturation process of plants, which influence the *in vitro* behaviour of explants (Bonga, 1982). These changes will influence the formation of adventitious and axillary buds, the rate of shoot elongation and the extent of rooting. Further, microbial contamination and oxidation of phenolic substances in the primary cultures are also major hurdles in the *in vitro* propagation of tree species.

The problems in the micropropagation of tree species and the different treatments adopted to surpass them are reviewed in the following pages.

2.1. Lack of response of mature tree tissues/explants

As trees mature, complex changes takes place in the nucleus and cytoplasm, as a result of which the regenerative

ability of the cell is progressively repressed. In many cases, the capacity of a plant to propagate vegetatively *in vitro* is lost at an age when the plant can still be propagated by cuttings (Wohok and Abo-El-Nil, 1977; Yang, 1977; Berlyn and Beck, 1980). No buds are formed in explants taken from branches of <u>Sequoia</u> trees, which are more than 20 years old (Boulay, 1979). Since maturation is believed to occur in meristems (Muckadell, 1959), explants from mature plants are physiologically older than those from juvenile plants and will often display undesirable characteristics as plagiotropic and reduced growth of resultant plants, poor rooting capacity, etc. (Bonga, 1980).

In the case of jack, distinct difference in the *in vitro* response <u>vis</u>-<u>a</u>-<u>vis</u> ontogenetic age of the explants was observed by Rajmohan and Mohanakumar (1983) and Rajmohan (1986). The *in vitro* proliferation and rooting responses were found to be the maximum for the (two-month old) seedling explants (a shoot multiplication rate of 17.4 x and cent per cent rooting with 6.00 roots formed in 20.75 days). Explants from fresh stem sprouts of five year old trees registered a multiplication rate of 4.5 x when cultured for five weeks and produced 70 per cent rooting (in 13.43 days) with 5.43 roots per shoot. Explants from fresh stem sprouts of ten-year and thirty-year old trees recorded a shoot multiplication rate of 2.80 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 per cent rooting with 2.0 roots formed in 46.7 days, after 2-3 subcultures.

Better understanding of juvenility and maturation will pave the way for successful culture of mature tree tissue. It is essential that tree genotypes are assessed for their performance at maturity and then used for propagation, so that the progeny has the desired genetic traits (Thorpe and Biondi, 1984). Preferential cloning is also desirable when establishing plantations of *dioecious* species like *Phoenix dactylifera* since the gender can be determined only after the trees have reached maturity (Reynolds, 1982).

Franclet (1979) observed that, tissues from shoots of severely pruned trees are amenable to graft and tissue culture rejuvenation, and pointed out the importance of physiological invigouration or rejuvenation, for further ontogenetic rejuvenation. Thorpe and Biondi (1984) also suggested that rejuvenation may be the key to success with mature trees. Methods are available now which can rejuvenate the adult trees and make them responsive to *in vitro* methods (Durzan, 1984).

2.2. Rejuvenating treatments

2.2.1. <u>Ringing</u> <u>and</u> <u>etiolation</u>

Bassuk and Maynard (1987) reported that stock plant etiolation led to the initiation of new plant growth. Partial etiolation of branches was proposed as a suitable pre culture treatment for *in vitro* propagation of selected mature trees, when physiologically juvenile materials such as stump sprouts, epicormic shoots or root suckers are available (Ballester *et al.*, 1989). They found that localized etiolation of branches in the crown of a 30-year old chestnut tree gave plant materials that responded much better to establishment and multiplication *in vitro* than unetiolated plant material.

2.2.2. <u>Grafting</u> <u>onto</u> <u>juvenile</u> <u>seedlings</u>:

There are reports that grafting on juvenile rootstocks in a few species causes rejuvenation. Doorenbos (1954) reported that in <u>Hedera</u> <u>helix</u>, such grafting caused complete rejuvenation based on morphological and physiological characteristics. Rejuvenation was promoted by the presence of juvenile leaves on stocks and inhibited by the adult leaves on the scions. With <u>Hedera</u> <u>canariensis</u>, greater rejuvenation occurred when scions consisted of a single bud with a small segment of stem than when the scions

consisted of a single bud with a large piece of stem, successful rejuvenation in either case requiring temperature above a certain minimum (Stoutemeyer and Britt, 1961).

In Hevea brasiliensis, rooting potential of mature scions has been enhanced by repeated grafting in serial fashion at short intervals on successive young juvenile seedlings (Muzik and Cruzado, 1958).

Monselise (1973) reported greater vegetative vigour and delay of flowering when mature calamondin scions were grafted on young nucellar cloned sour orange rootstocks. Franclet (1979) reported that on Eucalyptus camaldulensis aged 15 to 75 years, three serial micrograft onto seedling rootstocks, with two month periods between, were required to obtain increased rooting potential, whereas increased rooting potential was obtained after a single graft cycle, followed by growth, with Cupressus dupreziana. However, he noted that the Cupressus dupreziana rejuvenated clone began to flower eight years after rejuvenation, when a seedling plant can be expected to flower only after 40 years. Franclet (1979) suggested that small size of the scion is important, in agreement with the findings of other workers. (Doorenbos, 1954; Stoutemeyer & Britt, 1961). Many other workers also reported the importance of serial grafting on juvenile rootstocks in overcoming the maturity problems in tree

species (Arnold and Erikson, 1981; Cauvin, 1981; Clair *et al.*, 1985; Franclet, 1983; Monteuuis, 1984).

In the cases where a degree of juvenility was reestablished by grafting, this presumably was caused by changes in correlative controls. The most prominent change in control probably involves increased availability of hormones and other chemical factors produced by the root. Cauvin (1981) reported that by grafting the shoot meristems are placed closer to the roots. Liu and Tilliberg (1984) reported that by placing so, they are moved to the source of hormones and other factors.

Tusa *et al.* (1979) reported the technology of the micrografting of citrus species. Young seedling of Troyer citrange and <u>Poncirus trifoliata</u> when grafted with meristems of clementine, orange, grapefruit, citron, lemon and mandarin cultivars got success rates from 23 to 87 per cent.

Alfaro and Toshino (1987) suggested possible rejuvenation in mature avocado by grafting onto juvenile rootstocks *in vitro*. About 50 per cent of the shoots that emerged from the scions showed restored rooting competence. They observed that the restored rooting competence reflected a developmental phase, reversal from adult to juvenile than a simple transfer of rooting cofactor from rootstock to scion.

INTRODUCTION

The major problem with sexual propagation of many woody species is their heterozygous nature, which in turn reduces the chance of getting true to type plants. Since they have a long gestation period to start yielding and a long period for the yield to get stabilised, it takes years to test whether the plants have the desired characteristics or not. Hence it is essential to develop methods of vegetative propagation of mature tree crops with known superior traits.

In the case of trees which are old enough to observe the potential, clonal propagation by traditional methods like rooting of cuttings and grafting are most often difficult. Cloning by means of tissue culture is a potential alternative to the traditional methods and has been successfully employed to obtain propagules from mature plants of some tree species.

Jack (Artocarpus heterophyllus Lam.) is a perennial fruit crop grown in most of the households in Kerala. It is valued for its timber as well. A wide variety of cultivars with different fruit qualities are available. It is almost impossible to get true to type plants through seed propagation. Traditional methods of vegetative propagation like rooting of cuttings, budding and grafting are difficult

reversion of mature <u>Hedera</u> <u>helix</u> to the juvenile form. Dose response experiments indicated that GA_3 stimulated reversion over a 50-100 fold range with a half maximal response at approximately 0.5 microgram GA_3 per plant. According to Jameson and Horrell (1988) GA_3 promoted the development of juvenile traits and leaf forms in adult <u>Hedera</u> <u>helix</u>, whereas ABA promoted growth in juvenile ivy plants. Metzger (1988) also reported the role of gibberellin in juvenility and phase change.

The effect of cytokinin on rejuvenation was reported in <u>Pinus</u> <u>pinaster</u> (David *et al.*, 1978), in some coniferous trees (Abo-El-Nil, 1982) and in woody species (Bouriquet *et al.*, 1984). Preece *et al.* (1984) observed that application of BA plus GA_3 as foliar spray two weeks before removing the explants from stock plants was inhibitory to rooting when explants were in a low cytokinin medium; and it was inhibitory to shoot formation from explants on a high cytokinin medium.

Clark and Hackett (1980) also showed the significance of BA treatment and defoliation in assimilate translocation of juvenile-adult grafts of <u>H·.helix.</u> According to Mohamed *et al.* (1991) BA and GA induced

juvenile characters while ABA and a GA biosynthesis inhibitor paclobutrazol, resulted in a more adult phenotype in strawberry cultures.

2.2.4. Propagation by rooted cuttings

Mature characteristics in tree species are generally stable when propagated by cuttings. However, Jacobs (1939) stated that root cuttings of pine appeared to undergo a certain degree of rejuvenation and that in woody pines primary leaves as well as occasional juvenile buds are produced. Black (1972) reported similar results for Pseudotsuga menziesii and found that propagation of trees by three year old cuttings had increased rooting potential, more juvenile morphological characteristics and greater vigour than the original ortet.

Franclet (1979) used pruning techniques with several conifer species to obtain cuttings with higher rooting potential from the mature crowns of trees of seed origin. He observed that tissue from shoot of severely pruned trees is more amenable to graft and tissue culture rejuvenation which indicated that physiological invigouration or partial ontogenetic rejuvenation was important for further ontogenetic rejuvenation.

Vermeer (1991) reported that partial rejuvenation could be induced by rooting and expressed in their rate of multiplication and performance *in vitro*. Rejuvenated material was behaving like a seedling derived material.

Chatterjee and Mukherjee (1980) observed that dipping leafy cuttings of jack in 5000 and 10000 mg/l IBA for 30 sec resulted in 90 per cent and 60 per cent rooting, respectively.

2.2.5. Selecting the most juvenile tissues as explant

The terminal and axillary buds from mature trees yielded good results as explants in Ulmus, Quercus and Fagus (Chalupa, 1979). Gupta *et al.* (1981) succeeded in propagating 100 year old teak trees using terminal and axillary buds. Mascarenhas *et al.* (1982) described conditions for obtaining high rate of multiplication of Eucalyptus sp. using the axillary buds. They also used root explants of mature trees in the case of Dalbergia. Another source of juvenile material is floral parts. In eucalyptus callus was obtained from filaments of stamens (Bennet and McComb, 1982).

2.2.6. Stress treatments

Stafford (1974) observed that heat treatment as well as chilling treatment removed the constrains of maturity in woody species. Heat treatment was effective in strawberry plants (Macovei, 1979).

Nitsch and Norreel (1972) reported the cold treatments gave a greater number of androgenic anthers of Datura innoxia. Later Norreel (1976) has found that cold treatment followed by centrifugation is the best androgenic stimulating treatment in the anther and isolated pollen grain culture of Datura innoxia.

Bonga (1982) reported that by starvation, cold treatment and centrifugation treatment, partial damaging of cell organelles and their DNA could be achieved, thereby rejuvenating the mature explants. Fabijanski et al. (1991) found that initial incubation of Broccoli (Brassica oleracea var Italica) at elevated temperatures followed by subjecting to low temperature enhanced the somatic embryo formation in anther culture. They reported that it was due to the formation of heat shock proteins in cultured anthers.

2.2.7. *In vitro* treatments

2.2.7.1. Effects of phloroglucinol, cobalt chloride and silver nitrate on *in vitro* rejuvenation

Phloroglucinol (PG) in the nutrient medium is found to produce a two to three fold increase in the proliferation and rooting of shoots of the rootstock M.7 apple cultures (Jones, 1986). There are other reports of the effects of PG on apple shoots. Apart from apple, PG was found to hasten rejuvenation in a wide range tree species. Baleriola-Lucas and Mullins (1984) reported the effectiveness of phloroglucinol in overcoming the difficulty in establishing initial explants of woody species. But this effect of PG is found to be dependent on both the cultivar tested and on the physiological state of the tissues (Jones, 1986). Hammatt and Grant (1993) reported the effect of this phenolic substance in hastening the process of rejuvenation in *in vitro* culture of mature wild cherry. PG when added to the rooting medium significantly improved the production of roots.

The importance of cobalt chloride and silver nitrate as ethlyene inhibitors in overcoming the poor response of initial cultures has been reported by various authors. Biddington and Robinson (1991) reported that incorporation of silver nitrate overcame the poor responses

of anther culture of brussels sprouts by reducing the ethlyene produced during the initial stages of the culture. In case of rice callus cultures Adkins (1992) observed that addition of silver nitrate improved the rice callus growth by 54 per cent whereas addition of 1-amino cyclopropane-1-carboxylic acid (ACC) an ethylene precursor, decreased the rice callus growth by 15 per cent.

In rose shoot cultures Kevers *et al.* (1992) observed that an ethylene trap in the flask atmosphere reduced shoot proliferation rate. Cobalt chloride acted as an inhibitor of ethylene biosynthesis and increased the multiplication rate by providing a higher number of axes of suitable size for further subculture. The action of cobalt chloride as an ethylene inhibitor was reported in pearl millet by Pius *et al.* (1993). Incorporation of cobalt chloride and silver nitrate has found to delay the loss of regeneration potential in embryogenic cultures of pearl millet by reducing the ehylene content in the atmosphere of the culture vessel.

2.2.7.2. Serial subculturing *in vitro*

Recent studies indicate that even though mature meristems are quite stable both *in vivo* and *in vitro*, their phase related characteristics can be modified as a result of

in vitro culture. Both the length of culture and the number of subculture involved seem to be related to such changes.

Passecker (1973) reported that adventitious shoots, even when they arose from shoots of the adult phase, belonged to the juvenile phase of plant development.

Boulay (1979) found that in Sequoia sempervirens when the primary explant was of plagiotropic orientation, the number of orthotropic shoot produced increased with the number of subcultures, and orthotropic shoot formation was enhanced by inclusion of activated charcoal in the medium. Further these increased root formation after a variable number of subcultures suggesting that conditions in the culture produce ontogenetic rejuvenation.

Mullins *et al.* (1979) observed that the juvenile form of "Cabernet Sauvignon" an ancient grape clone, reappeared *in vitro* in shoots from serially subcultured mature shoot tips. Juvenility was indicated by lack of tendrils (the flower bearing structures) and spiral phyllotaxy, both being juvenile structures in grape.

The possibility of progressively improving the degree of rejuvenation in clones by repetitive subculturing was first studied with Salyx babylonica (Febvre, 1981). The effect of two different subculture regimes were determined and quantitative changes in nucleotides in the tissues during

the subcultures were assessed. A succession of long subculture periods caused dormancy of the clones, while short subculture periods resulted in their invigouration.

Apple cultivars cultured from shoot tips also showed some reversion in leaf shape in tissue cultures (Zimmermann, 1981; Sriskandarajah et al., 1982). This reversion is manifested by lobed leaves, deep, irregular serration, and thinner leaf blades which might also explain the increased rooting of shoots after a certain number of subcultures (Sriskandarajah et al., 1982). Takeno et al. (1983) found that this increase in rooting ability with increasing number of subcultures was associated with a reduction in endogenous gibberellin and cytokinin levels. Although this evidence suggested a rejuvenation of shoots in vitro, apple trees propagated by these in vitro methods did not show delayed flowering in the field (Zimmermann, 1981).

Lyrene (1981), Gupta et al. (1981) and Economou (1982) on their work with blueberry, eucalyptus and deciduous azalea, respectively, reported that shoot production and rooting ability increased with subcultures. In all the three cases rejuvenation was measured in terms of increased rooting potential and changes in morphological characteristics. The importance of serial subculturing in vitro on the rejuvenation of mature explants was observed by Franclet

(1983), Boulay (1984) and Debergh (1988). Webster and Jones (1989) reported that shoot production and rooting ability increased with subcultures, thereby promoting rejuvenation.

2.3. Variation in response to the explant

Variation in *in vitro* response was seen not only among species but also between half-sib and full-sub families. Results often varied when the explants were taken even from the same tree (Jacquiot, 1951). Results were found to vary from year to year even when explants were collected at the same time each year (David, 1982).

In apple the optimum period for obtaining explants was reported to be during spring or summer (Monsion and Dunez, 1971). In chrysanthemum, culture establishment decreased for explants taken after October, reaching a low in January and then rose to almost 100 per cent for explants taken in March-April (Kodytek, 1985).

2.4. Culture contamination

A major difficulty in perennial crop tissue culture is to make aseptic materials (Kuniaski, 1977) especially when using shoot tips as explants. As branches are exposed in the field for a longer time, they harbour various micro-

organisms, many of which grow into the plant tissue. Cultures established from such tissues are thus easily contaminated (Chen and Evans, 1990). Jea and Farrell (1993) reported that city water could contaminate tissue culture stock plants. Contamination rate of stem explant tissue cultures excised from greenhouse-grown stock plants irrigated with city water was 50 to 100 per cent. He found that watering greenhouse grown plants with filtered city water decreased contamination of stem explant culture from 30 to 50 per cent. Thus the rate of contamination could be overcome by maintaining stock plants in a healthy state (Debergh and Maene, 1981) and by suitable *in vitro* techniques (Pierik, 1988). Explants must be free from micro-organisms when placed on nutrient media and this was usually achieved by surface sterilisation with solutions of calcium or sodium hypochlorite (Rajmohan and Mohanakumaran 1988) or mercuric chloride (Ramesh, 1990). However, such treatments have failed with some adult trees, presumably because of penetration of tissues by micro-organisms (Franclet, 1979). There are inhibitory effects of antibiotics and fungicides to bacteria and fungi during culture. Pollock *et al.* (1983) found that two derivatives of penicillin G and three kinds of cephalospoxime were relatively nontoxic as well as

broadspectrum antibiotics to plant cells even up to 100 µg/ml.

2.5. Phenolics interference

During the initial culture, explant browning is often a serious problem which prevents successful culture establishment and further maintenance of cultures.

Sondahl *et al.* (1981) reported that unsuitable incubation conditions, such as too high temperatures or too strong light intensity, can increase polyphenol oxidase activity and thus accelerate the browning in Coffea arabica cultures. Physiological state of the material was also important. In Castanea sativa, cultures of juvenile materials contained less quinonic compounds than those from mature explants (Chevre, 1983). According to Chen and Evans (1990) genotype had also a a great influence on browning. They found that in rubber tree anther culture, only a few anther of the cultivar Haiken 2 turned brown and calli were readily induced. In some other strains, anthers browned easily, but callus induction was difficult.

There are several reports regarding the methods to overcome browning of explants. Addition of antioxidants could prevent formation of quinonic compounds. In mature palm tissue cultures, ascorbic acid reduced browning to a

great extent, whereas addition of PVP showed no beneficial effect (Reynolds and Murashige, 1979). Gupta *et al.* (1980) reported that browning of <u>Tectona</u> <u>grandis</u> explant could be remarkably reduced when 0.7 per cent PVP, 0.28 mM ascorbic acid and 5 per cent hydrogen peroxide were added together to 0.58 per cent sucrose solution and agitated on a rotary shaker for 45 min. Baleriola-Lucas *et al.* (1982) reported that addition of 2.0 per cent polyvinyl pyrrolidone to MS medium containing 10 μM BA successfully controled browning of explants in apple. The beneficial influence of activated charcoal for the micropropagation of mature clones of <u>Sesquoiadendron</u> <u>gigantium</u> was reported by Claudebon *et al.* (1988). In chestnut, addition of ascorbic acid partially solved the phenolics problem (Chauvin and Salesses, 1988).

Frequent transfer of easily browning explants to fresh medium reduced the damage by quinonic components. Broome and Zimmermann (1978) reported that in shoot tip culture of blackberry, transferring the shoot tip explants to fresh medium one to two days after initial culturing considerably reduced browning.

2.6. Low root initiation from mature explants

Induction of rooting in woody species is a problem, especially in mature explants. Remarkable difference was noticed in root initiation in cultured shoot apices of the

juvenile and adult forms of <u>Hedera</u> <u>helix</u> under the influence of auxin and catechol (Hackett, 1970). Welander and Huntrieser (1981) reported that the rooting ability of shoots raised *in vitro* from juvenile apple rootstock was more than those from adult rootstocks. Bergmann *et al.* (1988) compared the rootability of stem cutting from seedlings of <u>Aesculus</u> <u>sp</u>. and mature <u>Aesculus</u> x <u>Arnoldiana</u> and it was found that rooting was 100 per cent in cuttings taken from two week-old-seedlings. But those taken from the crown of the mature tree did not root at all.

Thorpe and Biondi (1984) reported that hormonal treatment was a critical factor in rooting. In apple IBA alone (Welander and Huntrieser, 1981) or IBA and dark treatments alone or together (Amitrani *et al.*, 1989), given after separation of proliferated shoot tip cultures, gave high rooting percentages.

Notably, callus may be formed at the basal part of the plantlet which block the direct contact between root and stem vascular tissue. Such plantlets cannot survive transplantation. This problem could be overcome by root induction outside the test tube (Hu and Wang, 1983). Zimmermann (1988) reported that direct rooting micropropagated shoots under mist or in high humidity was usually more economical than rooting *in vitro* in the case of woody plants.

MATERIALS AND METHODS

MATERIALS AND METHODS

Investigations on improving the *in vitro* response of explants from mature jack trees were carried out at the Plant Tissue Culture Laboratory, Department of Horticulture, College of Agriculture, Vellayani from March 1991 to July 1992.

The materials and methods used for the *in vitro* production of jack plantlets <u>via</u> various rejuvenating treatments to the stock plants and to the explants as well as <u>via</u> manipulation of the culture media are described below.

3.1. Stock plant treatments

3.1.1. <u>Induction of fresh stem sprouts</u>

Ringing was done on healthy stems at about 30 cm from the shoot tip either by removing a ring of bark (size 2 cm), using a knife, or by tying a metallic wire around shoot. Shoot tip above the ringed portion became dead and fresh sprouts were found to emerge below the ringed portion. These sprouts were used as explants for the study. Observations on the number of surviving cultures were recorded. There were 10 replicates.

3.1.2 Etiolation of the fresh stem sprout

The shoot tip (15 cm long) was covered with aluminium foil and etiolated for one month. Etiolation lead to the initiation of new sprouts which were used as explants for *in vitro* culture. Observations were recorded on the number of surviving cultures.

3.1.3. Grafting onto juvenile rootstocks

Mature scions were grafted onto juvenile rootstocks by approach grafting method. Scions were from mature 'Varikka' trees of about 40 - 50 years old. Rootstocks were of one year old.

Branches of scions were forced to bend down towards the ground and *in situ* approach grafting was done onto one year old juvenile seedlings raised from seeds. Fresh sprouts from the grafts were used as explants for *in vitro* culture. Observations on the number of surviving cultures, growing cultures and the number of multiple shoots were recorded.

3.1.4. Application of plant growth substances

Solutions of BA and NAA 10, 20 and 40 mg/l were prepared by dissolving 2.5, 5.0 and 10.0 mg BA and NAA in small quantities of dilute HCl and ethanol, respectively, and then making them up to 250 ml with distilled water. The

prepared solutions were sprayed using a hand sprayer at weekly intervals on the fresh sprouts of six different mature jack trees. These sprouts were used as explants for *in vitro* culture. Observations on the number of live cultures were recorded. Six replications were tried.

3.1.5. <u>Use</u> <u>of</u> <u>rooted</u> <u>cuttings</u>

Solutions of IBA 2000, 5000 and 10,000 mg/l were prepared by dissolving 200, 500 and 1000 mg IBA, respectively in 50 ml alcohol and making up the volume to 100 ml with distilled water. Mature jack cuttings with atleast two nodes were dipped in these solutions for 10 and 30 seconds (five replications). They were potted in sand and were irrigated daily.

3.2. Pre – culture treatment of the explants

3.2.1. <u>Stress</u> <u>treatments</u>

Simulation of meiotic rejuvenation was attempted by giving the following stress treatments.

3.2.1.1. Cold shock

Excised mature shoot apices were rinsed in running tap water and were washed with distilled water containing a few drops of the wetting agent, 'Teepol'. Leaf sheaths were removed and were rinsed in sterile double glass distilled

water. These explants of about 3 cm size were kept in a petri dish smeared with 'Glycerol'. These petri dishes were then kept in the freezer (-20°c). Cold shock was given for 5, 10 and 20 minutes. The cold shock treated explants were then surface sterilised with mercuric chloride (0.1%) for 13 minutes and then rinsed in sterile double glass distilled water (5 to 6 times). These explants were then inoculated onto the establishment medium. Cold shock treatment for 20 minutes was found to be lethal. Hence the cold shock treatment was again tried for 5, 7 and 9 minutes. Observations on the percentage of surviving cultures were then recorded.

3.2.1.2. Heat treatment

Excised mature shoot apices were used as explants for heat treatments. They were washed first in running tap water and then with distilled water containing a few drops of wetting agent, 'Teepol'. Leaf sheaths of these explants were removed and then rinsed in sterile double glass distilled water. Heat treatment was tried at 55°C for 15, 30 and 60 seconds by dipping them in test tubes containing water at 55°C. Since this heat shock was found to be lethal, the experiment was repeated at 42°C for 2,4 and 6 minutes. The explants were then surface sterilised using mercuric chloride (0.1%) for 13 minutes, followed by five to six rinsing in sterile double glass distilled water. The

explants were then cultured in the establishment medium. Observations on the number of live cultures and growing cultures were recorded at weekly intervals.

3.2.1.3. Cetrifugation

Mature explants were subjected to centrifugation . The explants were first washed in running tap water and then in distilled water containing a few drops of the wetting agent, 'Teepol'. They were then rinsed in sterile double glass distilled water for several times after removing the leaf sheaths. They were then centrifuged at 2,000 rpm in a table top centrifuge for 10, 20 and 30 minutes. The explants were surface sterilised using mercuric chloride (0.1%) for 13 minutes, followed by five to six rinsing in sterile double glass distilled water. The explants were then cultured in the establishment medium. Observations on the number of surviving cultures were recorded.

3.2.2. Treatment with plant growth substances

Explants were dipped in plant growth substances as pretreatment for promoting rejuvenation of mature tree explants. Collected explants were first washed in running tap water and then with distilled water containing a few drops of the wetting agent, "Teepol". Leaf sheaths were then removed and the explants were rinsed in sterile double

distilled water. They were then dipped in GA 1.0 mg/l for 24 hours. Then they were surface sterilised with mercuric chloride (0.1%) for 13 minutes and rinsed in double distilled water. These explants were finally inoculated into the culture establishment medium. The same procedure was followed for BA 10.0 mg/l. Observation on the number of live cultures were recorded.

3.2.3. <u>Treatment with antioxidants to reduce the maturity associated phenolics interference</u>

Phenolics interference is a serious problem in mature jack cultures. The explants were washed first in running tap water for 5-6 hours and then in water containing the wetting agent, 'Teepol'. After removing the leaf sheaths, explants were rinsed in double distilled water for several times. They were treated with ascorbic acid 200 mg/l for 20 minutes. Then they were surface sterilised with mercuric chloride (0.1%) for 13 minutes, rinsed in double distilled water and were inoculated onto the establishment medium. Observations on the number of live cultures were recorded. The same procedure was followed with (a) cystein HCl 0.1% for 20 minutes and (b) PVP 0.7% + 2.0% sucrose for 45 minutes.

3.3. *In vitro* treatments

3.3.1. Effect of incorporation of phloroglucinol, silver nitrate and cobalt chloride onto the establishment media

Studies were conducted to determine the effects of various ethylene inhibitors and phloroglucinol on the establishment of shoot tip cultures. Cobalt chloride @ 13, 130 and 650 mg/l; silver nitrate @ 1.0, 5.0 and 10.0 mg/l and phloroglucinol @ 1.0, 5.0 and 10.0 mg/l were added to the cultures establishment media. The explants used (mature shoot apices) were first washed in running tap water for 5-6 hours and then in water containing the wetting agent, "Teepol". After removing the leaf sheaths, explants were rinsed in double distilled water for several times. Then they were surface sterilised with mercuric chloride (0.1%) for 13 minutes, rinsed in double distilled water and were then inoculated on to the culture establishment medium. Observations on the number of live cultures were recorded after on month.

In vitro production of jack plantlets

The protocol developed by Rajmohan (1986) was adopted with necessary modifications for the *in vitro* production of jack plantlets. Shoot apices (1-2 cm) from mature jack trees were excised, with and without giving the

3. Shoot elongation

MS medium supplemented with BA 2.0 mg/l, NAA 0.2 mg/l, calcium pantothenate 0.1 mg/l, insoluble PVP 500.0 mg/l, sucrose 30.0 g/l abd agar 7.0 g/l.

4a. *In vitro* rooting
    Stage 1

MS medium (with half strength inorganic salts) supplemented with IBA 2 mg/l and NAA 2 mg/l, calcium pantothenate 0.1 mg/l, sucrose 30.0 g/l and agar 7.0 g/l.

4b. *In vitro* rooting
    Stage 2

Medium 4(a) without IBA and NAA

The chemicals used were of analytical grade from BDH, Qualigens and Sigma. Standard procedures (Thorpe and Biondi, 1984) were followed for the preparation of the media.

The pH of the media was adjusted to 5.7. Erlenmeyer flasks (Borosil) of 100 ml capacity and test tubes of 150 x 125 mm were used . Sterilisation of the media was done at 15 PSI for 20 minutes.

All aseptic manipulations were carried out in a laminar air flow chamber. The cultures for establishment were kept in darkness and the proliferation cultures were incubated at 26 $\pm$ 2^{O}C with a 16 hour photoperiod and 40 microE m^{-2} s^{-1} light intensity supplied by cool white fluorescent tubes. The cultures for *in vitro* rooting were incubated in darkness.

stock plant treatments mentioned before. They were then used as explants for *in vitro* multiplication. The explants were washed first in running tap water for 10 to 15 minutes and then in water containing the wetting agent, 'Teepol'. Leaf sheaths were then removed and the explants were rinsed in double distilled water several times. After giving the pre-culture treatments to the explants as mentioned before, they were dipped in Norfloxacin (0.1%) for 30 minutes. These explants were then surface sterilised with 0.1% HgCl for 13 minutes. A few drops of the wetting agent "Laboline" were added to the sterilants. The explants were then rinsed five to six times with sterile distilled water and were then inoculated onto the establishment medium.

The composition of the media used for the various stages of *in vitro* production of jack plantlets is given below.

No.	Stages of *in vitro* propagation	Composition of medium
1.	Culture establishment	MS medium supplement with GA 1.0 mg/l, AC 0.1% calcium pantothenate 0.1 mg/l, sucrose 30.0 g/l and Agar 7.0 g/l.
2.	Shoot multiplication	MS medium supplemented with BA 5.0 mg/l, NAA 0.2 mg/l, calcium pantothenate 0.1 mg/l, insoluble PVP 500.0 mg/l, sucrose 30.0 mg/l and agar 7.0 g/l

The multiple shoot formed were subcultured at 30 days interval. Part of these subcultures were transferred to the medium for producing elongated shoots. The elongated shoots were then transferred to the rooting medium. The rooting process was done in two stages. First, the shoot were transferred to the medium containing auxin and were then incubated in darkness for seven days. They were transferred to a medium without the auxins and incubated in darkness for seven to ten days. Root initiation was accomplished by this period.

The rooted plantlets were then grown under high light intensity (50 microE m^{-2} s^{-1}) for 20 days. By this period, the roots were well developed. The plantlets were then carefully removed from the agar medium using forceps. The roots were washed with sterile tap water until they were free of agar. The plantlets were then planted in plastic pots of 5.0 x 5.0 x 7.5 cm^3 size (having small holes for drainage) filled with sterile sand.

RESULTS

RESULTS

A study was conducted for improving *in vitro* propagation efficiency of mature phase of jack trees at the Tissue Culture Laboratory of College of Agriculture, Vellayani, Thiruvananthapuram in March 1991 to July 1992. The result of the study are presented below.

4.1. *In vitro* propagation of mature jack trees

Since microbial contamination was found to be a major hurdle in culture establishment, surface sterilization with $HgCl_2$ was first standardised. The results are presented in Table 1. Sterilization with $HgCl_2$ for 13 minutes gave the highest percentage of contamination free culture (63.6 %) followed by treatment for 10 minutes (57.1 %). Survival was only 12.5 per cent when the duration of treatment was 20 minutes. No systematic pattern between the duration of treatment and the percentage of live cultures could be observed, possibly due to presence of dead cultures.

In order to study the effect of media on culture establishment, shoot apices and lateral buds excised from mature jack trees were subjected to four different treatments (Table 2). Combination of GA (1.0 mg/l) and activated charcoal (1.0 %) incorporated with Murashige & Skoog medium

Table 1. Effect of duration of $HgCl_2$ (0.1%) treatment on microbial contamination of explants

Explant : Mature shoot apices

Duration of treatment (minutes)	Live cultures (%)
10	57.10
13	63.60
17	27.27
20	12.50
Control	0.00

Data represent the mean values of 7 to 11 replicates

supported 92.85 per cent survival and produced healthy growing cultures of shoot apices under dark conditions. While treatments with BA (2.0 mg/l) and NAA (0.2 mg/l) resulted 63.66 per cent survival, BA (5 mg/l) with NAA (0.2 mg/l) supported 60 per cent survival. BA (2 mg/l) with IBA (0.5 mg/l) gave the lowest survival rate (57.14 %).

The cultures were periodically observed for development. Slight elongation of shoot apices was observed in about three weeks after culturing (Plate 1). The effect of proliferation media on response of mature explants was studied by counting the number of shoots, large leaves, small leaves and total number of leaves from the fortieth day onwards at ten days interval (Table 3). The first leaf was formed in 40 days. The cultures developed on an average of two shoots, three large leaves and two small leaves in about two months after culturing. The cultures were healthy in appearance (Plate 2).

In order to study the effect of rooting media on response of explants, MS was used as the basal medium. The shoots used were 2-3 cm long with 2-3 leaves. *In vitro* rooting was observed in 14.43 days, with 4.86 roots per shoot (Plate 3).

Table 2.   Effect of media on the culture establishment of explants

Explant   :   Mature shoot apices

Medium	Live cultures (%)
J_1 (MS + BA 2mg/l + NAA 0.2 mg/l)	63.66
J_2 (MS + BA 2mg/l + IBA 0.5 mg/l)	57.14
J_3 (MS + GA 1mg/l + AC 1 %)	92.85
J_4 (MS + BA 5mg/l + NAA 0.2 mg/l)	60.00

Data represent the mean values of 7 to 15 replications

Table 3. Effect of incubation time on response of mature explants

Explant : Shoot apices   Basal media : MS

Days after culturing	No.of shoots	No.of large leaves	No.of small leaves	Total No.of leaves
40	1.00	1.00	0	1.00
50	1.00	1.83	2.00	3.83
60	2.00	3.33	2.16	5.49
70	2.66	4.16	1.00	5.16

Data represent the mean values of 6 replicates

Plate 1. Elongation of shoot apex from mature jack
          tree, three weeks after culture in the
          establishment medium

Plate 2. Multiple shoot formation from shoot apex of
          mature jack tree cultured in proliferation
          medium

Various methods were tried to rejuvenate the mature phase of jack trees. Results of the treatments are given below.

4.2. Stock plant treatment

4.2.1. Ringing, etiolation and grafting on to juvenile rootstocks

The stock plant treatments were, grafting on to juvenile rootstocks, ringing and etiolation. The results are presented in Table 4. Among the above three treatments, grafting on to juvenile rootstocks produced the highest percentage of live cultures (75 per cent) followed by ringing (57.14 per cent). Etiolation gave least rejuvenation, the percentage establishment of live cultures being 10 only.

4.2.2. Application of plant growth substances

Solutions of BA and NAA each at the rate of 10 mg/l and 40 mg/l were prepared and sprayed on the fresh sprouts of mature jack shoots. A control was maintained without applying any plant growth substances. The results of this experiment are presented in Table 5. The percentage survival was the highest (50 per cent) when BA 10 mg/l was used followed by NAA (40 mg/l). BA 20 mg/l, BA 40 mg/l and NAA 10 mg/l did not yield any live culture due to necrosis of

Table 4.   Effect of different stock plant treatments on the
response of mature explants

Explants : Shoot apices   Basal media   : MS

Treatment	Live cultures (%)	Growing culture with multiple shoots (%)
Grafting on to juvenile root stocks	75.00	10.00
Ringing	57.14	0
Etiolation	10.00	0
Control	25.00	0

Data represent the mean values of ten replicates

Table 5.   Effect of application of plant growth substances on the
response of mature explants

Explants : Shoot apices   Basal media : MS

Treatment	Live cultures (%)
BA 10 ppm	50.00
BA 20 ppm	0
BA 40 ppm	0
NAA 10 ppm	0
NAA 20 ppm	25.00
NAA 40 ppm	33.33
Control	25.00

Data represent the mean values of six replicates

explants. NAA 20 mg/l and the control produced the same percentage of survival (50).

4.2.3. Rooting of cutting

Efforts to induce rooting of cutting as per standard procedures (Chatterjee and Mukherjee, 1980) were not successful.

4.3. Explant treatment

4.3.1. Stress treatments

4.3.1.a. Cold treatments

Cold shock was given to mature explants by subjecting them to -20°C for 5, 10 and 20 minutes, the results of which are given in Table 6.1.a. Treatment for 20 minutes was found to be lethal. The treatment for five minutes yielded maximum number of live cultures (83.33 per cent) (Plate 4) followed by treatment for 10 minutes (40 per cent). Since cold shock for 5 minutes yielded the best results, the experiment was repeated with shorter ranges of duration around the neighborhood of five minutes. The results presented in Table 6.1.b. shows that the treatment for the five minutes produced the maximum rejuvenation (88.88 per cent) followed by treatment for seven minutes (50 per cent). Cultures subjected to cold shock for five minutes and

Table 6.   Effect of stress treatments on the response of mature
            explants

Explants : Shoot apices   Basal media : MS

6.1   Cold shock

(a)

Treatments (min)	Live cultures (%)
5	83.33
10	40.00
20	0
Control	25.00

(b)

Treatment (min)	Live culture (%)	Growing culture with multiple shoot (%)
5	88.88	20
7	50.00	0
9	33.33	0
Control	25.00	0

Data represent the mean values of six replicates

Plate 3. Rooted shoot culture of mature jack tree

Plate 4. Elongated shoot apex of mature jack tree
         subjected to cold shock at $-20^{\circ}C$ for five
         minutes, three weeks after culture in the
         establishment medium

got established produced multiple shoots when transferred to shoot proliferation media.

4.3.1.b.   Heat treatment

Explants from mature jack trees were first subjected to heat treatment at 55°C for 15, 30 and 60 seconds. Since treatments at 55°C was found to be lethal. A lower temperature of 42°C was tried for 2, 4 and 6 minutes. The results of the above experiment is presented in Table 6.2. Heat treatment at 42° C for 2 minutes yielded the best result with 66 per cent cultures surviving. Treatment for 4 and 6 minutes gave equal rates of survival (33.33 per cent).

4.3.1.c. Centrifugation

Explants from mature jack trees were centrifuged at 2000 rpm for 10, 20 and 30 minutes, the results of which are presented in Table 6.3. Centrifugation for 10 minutes gave the highest percentage of live cultures (57.14 per cent). When the duration of the treatment was increased to 20 and 30 minutes the survival rate remained the same (28.57 per cent).

Among the stress treatments tried cold shock for five minutes at -20° gave the best result (88.88 per cent survival) followed by heat treatment at 42° C for two minutes (66.66 per cent survival). Centrifugation at 2000 rpm for 10 minutes recorded 57.14 per cent culture establishment.

Table 6.2.   Heat treatment

(a)

Treatment at 42° C (min)	Live cultures (%)
2	66.66
4	33.33
6	33.33
Control	25.00

Data represent the mean values of six replicates

Table 6.3.   Effect of centrifugation on the response of mature
explants

Explants : Shoot apices   Basal media : MS

Treatment centrifugation at 2000 rpm (min)	Live cultures (%)
10	57.14
20	28.57
30	28.57
Control	25.00

Data represent the mean values of seven replicates

Table 7.   Effect of plant growth substances on the response of
mature explants

Explants : Shoot apices   Basal media :   MS

Treatment	Growing culture (%)
BA 10 mg/l	25.00
GA 1 mg/l	17.85
Control	25.00

Data represent the mean values of six replicates

4.3.2. Treatment with plant growth substances

In addition to stress treatments, effect of plant growth substances on mature explants were studied. The plant growth substances tried were GA 1 mg/l and BA 10 mg/l for 24 hours. The results of the above experiments are summarised in Table 7. BA 10 mg/I for 24 hours yielded better results than GA 1.0 mg/l, the survival rates being 25.00 per cent and 17.85 per cent respectively.

4.3.3. Treatment with antioxidants

To study the effect of antioxidants on mature jack explants three different treatments were tried. The treatments tried were PVP 0.7 % + sucrose 2.0 % for 45 minutes, ascorbic acid 200 mg/l for 20 minutes and cystein HCl 0.1 % for 20 minutes. The results (Table 8) showed that ascorbic acid 200 mg/l treatment for 20 minutes was the best (57.14 per cent survival) followed by PVP 0.7 % + sucrose 2.0 % four 40 minutes (50 per cent survival). Treatment with cystein HCl 0.1 % for 20 minutes was the least effective among the three (25 per cent survival).

4.4. *In vitro* treatments

Three different *in vitro* treatments were tried and the results are presented below.

Table 8.   Effect of treatments reducing phenolics interference on
          the response of mature explants

Explants : Shoot apices   Basal media :   MS

Treatment	Live cultures (%)
PVP 0.7 % + Sucrose 2.0 % for 45 minutes	50.00
Ascorbic acid 200 mg/l for 20 minutes	57.14
Cytein HCl 0.1 % for 20 minutes	25.00
Control	25.00

Data represent the mean values, of seven to ten replicates

Table 9.   Effect of phloroglucinol in the culture establishment
          medium on the response of mature explants

Explants : Shoot apices   Basal media : MS

Treatment (mg/l)	Live cultures (%)
1	20.00
5	50.00
10	66.66
Control	25.00

Data represent the mean values of three to five replicates

4.4.1. Incorporation of phloroglucinol in culture establishment medium

To study the effect of phloroglucinol on mature explants, it was incorporated on to the culture establishment medium at the rate of 1.0, 5.0 and 10.0 mg/l. The results presented in Table 9 shows that phloroglucinol at the rate of 10 mg/l yielded 66.66 per cent growing cultures. When the concentrations were reduced to 5.0 mg/l and 1.0 mg/l, the percentage of growing cultures was reduced to 50.00 and 20.00 respectively.

4.4.2. Incorporation of cobalt chloride in the culture medium

Cobalt chloride at the rate of 13, 130, 650 mg/l were incorporated on to the culture medium. As is evident from Table 10 cobalt chloride was not found to be effective in improving the response of mature explants. Cobalt chloride 130 and 650 mg/l were found to be lethal.

4.4.3. Incorporation of silver nitrate in the culture establishment medium

To study the effect of silver nitrate which is an ethylene inhibitor, on the *in vitro* response of mature explants, it was incorporated in the culture establishment

Table 10.   Effect of cobalt chloride in the culture establishment
medium on the response of mature explants

Explants : Shoot apices   Basal media : MS

Cobalt chloride (mg/l)	Live cultures (%)
13	16.66
130	0
650	0
Control	16.66

Data represent the mean values of four to five replicates

Table 11.   Effect of silver nitrate in the culture establishment
medium   on the   response   of mature explants

Explants : Shoot apices   Basal media : MS

Concentration of silver nitratee (mg/l)	Live cultures (%)
1	36.3
5	46.6
10	60.00
Control	25.00

Data represent the mean values of 11 to 15 replicates

medium at the rate of 1.0, 5.0 and 10.0 mg/l. The results are presented in Table 11. Silver nitrate at the highest concentration (10.0 mg/l) gave 60 per cent live cultures. Percentage of live cultures decreased with decreasing concentration of silver nitrate.

4.5. Effect of season on the response of mature explants

To study the effect of season on the *in vitro* response of mature explants, monthly data on percentage microbial contamination and live cultures for the period from March, 1991 to June, 1992 were recorded (Table 12). Microbial contamination was the lowest in March-April. But during rainy season (June-September), contamination was high.

Table 12.   Effect of season on the *in vitro* response of mature
            explants

Month	Live cultures (%)
1991	
March	70.20
April	70.00
May	20.80
June	23.07
July	9.09
August	52.30
September	30.00
October	33.33
November	15.78
December	12.90
1992	
January	10.70
February	41.66
March	44.44
April	50.00
May	34.46
June	18.75

Data represent the mean values of ten replicates

DISCUSSION

DISCUSSION

Maturity barrier is a major hurdle in the *in vitro* propagation of mature jack trees. In some mature trees species, adult trees produce tissues like epicormic shoots which have juvenile characteristics. *In vitro* establishment could be enhanced when such juvenile tissues are used. But when such juvenile materials are not available, mature tissues have to be rejuvenated by different pretreatments. In the case of jack such juvenile materials are not available. So mature tissues have to be rejuvenated by different pretreatments. Rajmohan (1986) observed distinct difference in the *in vitro* response of jack explants. As the ontogenetic age increases, a significant reduction in the response in the culture establishment, shoot proliferation and rooting was noticed.

The different pretreatments proposed include serial grafting on to juvenile rootstocks (Cauvin, 1981 and Franclet, 1983), treatment of mother plants with plant growth substances (Bouriquet *et al.* 1984) and serial rooting of cuttings (Goublay de Nantois, 1980). Some of these treatments have been partially successful in some tree crops.

The objective of the present investigation was to improve the *in vitro* propagation efficiency of explants from

mature phase jack trees by different pretreatments. The salient findings are discussed below.

The experiment was started in March 1990. In order to study the effect of season on culture establishment in jack, explants were collected at weekly intervals beginning in March, 1990 and cultured. Seasonal effect on culture establishment was observed in apple and chrysanthemum by Monsion and Dunez (1971) and Kodytek (1985). The data on culture establishment, during the different months were tabulated (Table. 12). Culture establishment was the highest during march-April .and the lowest in July, which coincides with the rainy season. Monsion and Dunez (1971) also observed similar results. They found that the optimum season for collecting explants for apple is summer or spring. Similar results were found in Chrysanthemum morifolium, in which explants taken during March-April gave the highest percentage of success (Kodytek, 1985). The present result established the influence of season on culture establishment in jack.

Microbial contamination has for long been a major problem in the culture establishment of explants. Different treatments have been suggested to overcome them. Ramesh (1990) observed that treatment with $HgCl_2$ (0.1 per cent) is the most effective in reducing microbial contamination in seedling shoot apices of jack. Hence, $HgCl_2$ (0.1 per cent)

was used in the present investigation. As is evident, from Table 1, a longer duration of treatment lead to browning of cultures and finally resulted in their death. Culture establishment was the highest (63.60 per cent) when the duration of treatment was 13 minutes. As the duration of treatment was increased to 20 minutes, the culture establishment dropped considerably (12.5 per cent).

Rejuvenation treatments

In order to overcome the problem of maturity barrier in tree crops, various methods have been suggested. These methods include stock plant treatments like ringing and etiolation (Ballester *et al.*, 1989), grafting onto juvenile rootstocks (Franclet, 1983), application of plant growth substances (David *et al.*, 1978) and serial rooting of cuttings (Goublay de Nantois, 1980). Explant treatments like cold shock, heat treatment and centrifugation were suggested by Bonga (1982) to rejuvenate somatic cells. Treatments with plant growth substance (Rogler and Hackett, 1975) and antioxidants (Reynolds and Murashige, 1979) were the other explant treatment tried. *In vitro* treatments like incorporation of phloroglucinol (Jones, 1986) in the culture establishment medium has also found to be a successful rejuvenating method. The salient results of the present investigation with respect to the above treatments are discussed below.

5.1. Stock plant treatments

5.1.1. Ringing, etiolation and grafting onto juvenile rootstocks

Ringing, etiolation and grafting onto juvenile rootstocks were tried as stock plant treatments to enhance rejuvenation. While, grafting onto juvenile rootstock gave the maximum percentage of live cultures (Table 4), it was the lowest in the case of etiolation. The high rate of contamination in the case of etiolation may be because the branches of mature jack trees are exposed in the field for a long time. Hence these plant tissues harbour various microorganisms, many of which grow into the cultures and kill them. Chen and Evans (1990) also got similar results. Ballester *et al.* (1989) reported the importance of etiolation as a pretreatment for *in vitro* establishment and multiplication of mature chestnut. They observed that although there is no significant difference between etiolation and unetiolation treatments in the first and second subcultures, the explants of etiolated origin exhibited a clear advantage over those of unetiolated origin in the number of new segments per explant in the third and fourth subcultures. No firm conclusions like this could be deduced from the present experiment because of the high rate of microbial contamination.

5.1.2. Application of plant growth substances

The effectiveness of BA in rejuvenation was suggested by David *et al*. (1978) in Pinus pinaster; Abo-El-Nil (1982) in conifers; and Bouriquet *et al*. (1984) in woody species. BA and NAA each at three different levels and a control were tried in the present investigation. Among the treatments tried, BA at a lower concentration of 10 mg/l and NAA a higher concentration of 40 mg/l produced high rate of rejuvenation of mature jack cultures (Table 5). David *et al*. (1978), Abo-El-Nil (1982) and Bouriquet *et al*. (1984) also reported that stock plant application of BA plus GA_3 at higher concentration became inhibitory to shoot formation. However, BA at the rate of 10 mg/l gave a higher rate of rejuvenation than NAA 40 mg/l. This may be related to the difference in endogenous levels of auxins and cytokinins.

5.1.3. Use of explants from rooted cuttings

The importance of using explants from rooted cuttings for rejuvenation in micropropagation was suggested by Vermeer (1991) in Quercus robus. Since cuttings of jack did not root as per the procedure of Chattarjee and Mukherjee (1980) further trials could not be done.

5.2. Explants treatments

5.2.1. Stress treatments

Among the stress treatments, cold shock, heat treatments and centrifugation were tried. Initially, cold shock was given for 5, 10 and 20 minutes. It was observed that as the duration of the treatment was increased, the percentage of live cultures decreased. Hence the experiment was repeated with cold shock treatment for 5, 7 and 9 minutes. Cold shock for five minutes was found to be the best among the stress treatments (Table 6.1) which gave 88.88 per cent live cultures on the average. Norreel (1976) observed that cold treatment stimulated rejuvenation following the breaking of physiological relations between the mature phase of the plant.

The explants were first subjected to heat treatment at 55°C for 15, 30 and 60 seconds. None of the cultures survived these treatments. So the experiment was repeated with heat treatment at 42°C for 2, 4 and 6 minutes. Heat treatment at 42°C gave the highest percentage of live cultures (Table 6.2) Fabijanski et al. (1991) had similar observations where initial incubation of anther cultures of Brassica sp. induced the formation of heat shock protiens, which enhanced embryo formation.

In addition to cold and heat treatments, centrifugation was also tried at 2000 rpm for 10, 20 and 30 minutes (Table 6.3). Centrifugation for 10 minutes gave the highest percentage of live cultures. Norreel (1976) also observed that in Datura innoxia, cold treatment followed by centrifugation was androgenic stimulating factor. These treatments combined could also be tried to enhance rejuvenation in jack cultures.

5.3.2. Treatment with plant growth substances

The effect of BA and GA in inducing juvenile characteristics was tried on mature jack cultures by treating the explants with GA at 1 mg/l and another set of explants with BA at 10 mg/l (Table 7). But the treatments were not beneficial.

5.3.3. Treatment to reduce phenolics problems

Phenolics interference was another major problem which hindered the establishment of explants from mature jack trees in vitro. The addition of antioxidants prevent the formation of quinonic compounds thereby controlling browning of explants (Chen and Evans, 1990). In this study, treatments with ascorbic acid, PVP and cystein HCl were tried. Treatment with ascorbic acid 200 mg/l for 45 minutes

was found to be the best preculture treatment for reducing browning of explants (Table 8). Reynolds and Murashige (1979) found that ascorbic acid is far superior to PVP in reducing browning of explants in mature palm tissue cultures. But the result of the present study did not show a significant difference between the effect of ascorbic acid and PVP in reducing browning of cultures.

5.4. *In vitro* treatments

Barleriola-Lucas and Mullins (1984) found that incorporation of phloroglucinol to the culture establishment media is effective in overcoming the difficulty in establishing initial explants of various woody species. Phloroglucinol at three different levels were tried in the present investigation. Phloroglucinol at the rate of 10 mg/l gave the best result (Table 9) followed by 5 mg/l.

The results of treatment with cobalt chloride are given in Table 10. Cobalt chloride was found to be ineffective in improving the response of explants as the treatment did not show any increase in percentage of live cultures over the control treatment. Among the three different concentrations of silver nitrate tried 10 mg/l gave the highest percentage of live cultures. Silver nitrate acts as an ethylene inhibitor which reduces the ethylene content which in turn enhances the survival of explants (Biddington

and Robinson, 1991). Thus among the *in vitro* treatments
tried, phloroglucinol at the rate of 10 mg/l was found to be
the most effective followed by silver nitrate at the rate of
10 mg/l.

The cultures after rejuvenation treatments could
not be subjected to further shoot multiplication and rooting
trials due to microbial contamination and phenolics
interference. However multiple shoots could be induced and
plantlets produced in a limited number of cases. The results
presented in Table 2 and 3 evince that micropropagation from
mature trees of jack could be successfully done.

SUMMARY

SUMMARY

The objective of the present study was to standardise pretreatments to enhance the *in vitro* response of explants from mature jack trees. The experiment was conducted at the College of Agriculture, Vellayani from March 1990 to July 1992. The pretreatments tried involved stock plant treatments, explants treatments and *in vitro* treatments. The stock plant treatments tried were ringing, etiolation, grafting on to juvenile rootstocks, application of plant growth substance, and rooting of cuttings. Stress treatments, treatment with plant growth substance and treatment with agents reducing phenolics oxidation were the different explant treatments. Cold shock, heat treatment and centrifugation were the stress treatments. BA and GA each at different concentrations were used as the plant growth substances. The agents for reducing phenolics oxidation tried were PVP, ascorbic acid and cystein HCl. The *in vitro* treatments included incorporation of phloroglucinol, silver nitrate and cobalt chloride each at different concentrations into the culture. establishment medium. The effect of season on the response of mature explants were also probed.

Surface sterilisation with $HgCl_2$ for 13 minutes was found to be the most effective in reducing microbial

contamination during culture establishment. Among the stock plant treatments tried, grafting on to juvenile rootstock was found to be the most effective for improving the *in vitro* response of explants from mature jack trees. Among the explant treatments, stress treatments were found to be the most effective. Cold shock treatment for 5 minutes at $-20^{O}C$ was found to be the most effective for rejuvenation of mature explants. Heat treatment at $42^{O}C$ for 2 minutes was found to effective followed by centrifugation at 2000 rpm for 10 minutes. Among the *in vitro* treatments incorporation of phloroglucinol at the rate of 10 mg/l was found to be the most effective. The results also revealed the significant influence of season on the response of mature explants. The culture establishment was the highest during March-April which corresponds to summer season. The culture establishment was the lowest during June-August, the rainy season.

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ABSTRACT

The objective of the present study was to improve the propagation efficiency of mature phase jack trees by various pretreatments. The treatments tried involved stock plant treatments, explant treatments and *in vitro* treatments. Surface sterilization with $HgCl_2$ for 13 minutes was found to be the most effective in reducing microbial contamination during culture establishment. Among the stock plant treatments tried, grafting on to juvenile rootstock was found to be the most effective in improving the *in vitro* response of explants from mature jack trees. Stress treatments were found to be the most effective among the explant treatments. Cold shock for 5 minutes at $-20^{\circ}C$ was found to produce the best results followed by heat treatment at $42^{\circ}C$ for 2 minutes. Among the *in vitro* treatments, incorporation of phloroglucinol at the rate of 10 mg/l was found to be the most effective. The results also showed significant influence of season on the response of mature explants, the highest response being observed during March-April.