EX VITRO ESTABLISHMENT OF JACK (*Artocarpus heterophyllus* Lam.) PLANTLETS

BY

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Thesis

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DECLARATION

I hereby declare that this thesis entitled "Ex vitro establishment of jack (Artocarpus heterophyllus Lam.) plantlets" 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 "Ex vitro establishment of jack (Artocarpus heterophyllus Lam.) plantlets" is a record of research work done independently by Shri. B. RAMESH 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

INTRODUCTION

Success of micropropagation depends on the field establishment of *in vitro* derived plantlets. Within the *in vitro* system, the plantlets are heterotrophic and get very favourable conditions for their growth. They are characterised by morphological, anatomical and physiological peculiarities. During *ex vitro* establishment, the plantlets have to switch over to autotrophic nutrition, involving normal photosynthetic activity and water relations. Normally, the plantlets will not be able to withstand the sudden shock of the environmental changes and are easily susceptable to wilting and dessication, resulting in high rate of mortality.

The high humidity within the culture vessels hinders the proper synthesis of cuticle and epicuticular wax on the epidermis of the newly emerging leaves (Brainerd and Fuchigami,1981; Sutter,1985). Consequently, when the plantlets are planted out, they undergo desiccation and drying. Improper functioning of stomata is another problem encountered. The stomata, (which normally close during stress conditions, day/night light changes and abscissic acid treatment) remain open in the *in vitro* grown plantlets (Brainerd and Fuchigami, 1982; Wardle *et al*. 1983).

Water loss due to stomatal malfunctioning can be compensated to some extent if the plantlets have proper root system, with normal vascular root-shoot connection. However, in majority of tissue cultured plantlets, vascular root-shoot connection is not normal. The route of *in vitro* propagation is significant in this respect (Fabbri and Bartocini, 1985). The plantlets developed via somatic embryogenesis normally have proper root-shoot connection. In the other routes, callus is produced at the shoot base which is detrimental for the proper root-shoot connection (Thorpe, 1984). Gradual acclimatization (hardening) is necessary for *ex vitro* establishment in such cases.

Ex vitro establishment of tissue culture derived plantlets has not been studied enough. Serious field mortality is often encountered, while planting out. The problem is severe in the case of woody species. Progress in this aspect will have much practical/commercial significance.

Micro propagation of jack (*Artocarpus heterophyllus* lam.) was seriously handicapped by the high rate of field mortality during planting out (Rajmohan,1985; Rajmohan and Mohanakumaran,1983). The present study addressed this

problem and encompassed the various aspects associated with it. A successful protocol for the ex vitro establishment of tissue culture derived jack plantlets was sought to be developed.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Considerable progress has been made in the *in vitro* propagaton of crop plants. However, the *ex vitro* establishment of plantlets, especially those of woody species, remains critical. Detailed studies on the field establishment of *in vitro* grown plantlets have been limited. *Ex vitro* establishment of plantlets gained importance, consequent on the commercialisation of micropropagation.

This review encompasses the research on the various aspects of *ex vitro* establishment of plantlets, namely morphological, histological and physiological characteristics of *in vitro* propagated plantlets, changes in these characteristics during *ex vitro* establishment, vitrification of cultured shoots and the factors influencing *ex vitro* establishment and its regulation.

A. Morphological physiological and histological characteristics of *in vitro* propagated plantlets

1. Morphological and physiological characteristics

Epicuticular wax has found to be either reduced or absent in the *in vitro* formed leaves, leading to higher rate of water loss (Grout, 1975; Sutter and Langhans, 1979; 1982). The density of wax deposition was found to be

increasing during acclimatization (Wardle et al., 1983). Sutter (1985) observed, in some herbaceous crops, large quantities of wax deposition on the in vitro grown leaves, which implied that the lack of epicuticular wax alone could not explain the water loss. He found no correlation between the amount of epicuticular wax present at the time of planting out and the percentage survival of the plantlets during ex vitro establishment.

Dhawan and Bhojwani (1987) observed that epicuticular wax deposition in the leaves of Leucaena leucocephala was more on the abaxial surface than on the adaxial surface. Sutter (1988) reported that in apple, the relative wax content decreased after ex vitro establishment, which led to an increased conductance through the adaxial surface than through the abaxial surface.

The chemical composition of the epicuticular wax of in vitro grown cabbage plants was studied by Sutter (1984). A higher percentage of polar compounds, like fatty acids, primary alcohols, aldehydes and esters were found. The alkanes and secondary alcohols were low. Dhawan and Bhojwani (1987) observed that during the shoot multiplication stage, leaves had only amorphous wax. Structural wax appeared during in vitro rooting and increased in quantity during ex vitro establishment.

Donnelly et al. (1987) in Silvan blackberry and Donnelly and Skelton (1989) in rose examined the hydathode anatomy. They found that the apex and marginal serration of the leaves of in vitro and green house grown plants had terminal hydathode regions, composed of scattered, primarily adaxial, group of sunken water pores. Water pores and stomata of the in vitro leaves were open with large apertures while those of the green house grown plants were closed with small apertures.

Wetzstein and Sommer (1983) studied the surface morphology of in vitro, acclimatized and field grown leaves. They observed that the in vitro leaves had superficial, circular stomata and irregular epidermal cells. In the acclimatized leaves, the stomata were ellipsoid and depressed; but the epidermal cells were irregular. In the field grown leaves, the stomata were ellipsoid and depressed; but the epidermal cells were well defined and isodiametric in shape. Stomatal density was more in the in vitro leaves than in the acclimatized and the field grown leaves.

Mary et al. (1986) observed that the reduced stature of micro propagated shoots were due to reduced cell division. The cell size as well as the area occupied by vascular

tissues in midrib and petioles were found to be reduced in the *in vitro* grown leaves. Reuther (1986) observed that the roots formed *in vitro* had no root hairs.

2. Histological Characteristics

In vitro propagated plantlets have certain histological characteristics which make their *ex vitro* establishment difficult. In cauliflower, the palisade mesophyll and palisade cells were found to be limited in the *in vitro* grown leaves (Grout and Aston, 1978). In the case of plum plantlets, the palisade cell depth and mesophyll air space were significantly less in the *in vitro* grown leaves than in the field grown leaves (Brainerd *et al.*, 1981).

Wetzstein and Sommer (1982) in *Liquidambar styraciflua* and Fabbri *et al.* (1986) in strawberry, observed that the leaves of micropropagated plantlets lacked well-differentiated palisade parenchyma and spongy parenchyma, and were with large intercellular space. Cells of the *in vitro* leaves had large vacuoles, more cytoplasmic content and flattened chloroplast with irregularly arranged internal membrane system. Donnelly and Vidaver (1984b) and Donnelly *et al.* (1985) reported that the *in vitro* plantlets of red raspberry had thin walled cells without collenchyma and with few phloem fibres. They also found that the ratio of

palisade cells to epidermal cells in the *in vitro* plantlets was less than the field grown plants.

Grout and Aston (1978) observed that the transition zone between shoot and root was abnormal in micropropagated cauliflower shoots. According to Sutter (1981), a continous vascular connetion between the shoot and root was critical for efficient water flow and for reducing the mortality during stress conditions. In *in vitro* grown plantlets, when much callus was produced at the shoot base, roots often originated from the callus and were not strongly connected to the shoots. The vascular connection between roots and shoot was found to be proper when the callus production was the minimum at the shoot base (Cheng and Voqui, 1977; Arnold and Eriksson, 1984; Patel *et al.*, 1988).

Debergh and Maene (1981) reported that the roots formed *in vitro* became non-functional after the plantlets were transplanted to potting mix or soil. Donnelly *et al.* (1985) observed that the roots of *in vitro* cultured red raspberry plantlets had little periderm and those of the transplants had intermediate amount, in comparision to the multilayered periderm of field grown plants.

Fabbri and Bartocini (1985) reported that the method of rooting influenced the anatomy of the roots. Shoots of the

walnut root stock "Paradox" developed *in vitro*, were rooted *ex vitro* under mist, rooted *in vitro* and hardened under mist or kept in culture vessels after rooting. The roots formed under mist showed normal differentiation with no anatomical abnormalities. The roots formed *in vitro* and hardened under mist, had comparatively retarded differentiation of the xylem and the phloem. The roots formed and maintained in the culture vessels were of two types, one having normal thickness with few irregularly arranged xylem and phloem, and the other having thick roots with little differentiation of central cylinder and with large intercellular space in the spongy cortex. The plantlets with roots formed under mist survived better after transplanting than the others.

B. Changes in morphological, histological and physiological characteristics during *ex vitro* establishment of plantlets

1. Changes in leaf structure

Donnelly *et al*. (1985) reported that the changes in the structure of persistent leaves of tissue cultured red raspberry were minimal and only slight secondary wall deposition occurred. Collenchyma formation did not take place even two weeks after transplantation.

Fabbri et al. (1986) observed that the persistent leaves of tissue cultured strawberry enlarged due to increased cell size rather than due to increased cell number. It was associated with increased epicuticular wax deposits on both abaxial and adaxial surfaces. The leaves formed during acclimatization were intermediate in morphology. Dhawan and Bhojwani (1987) found that during the rooting phase, size of the palisade cells decreased and the spongy parenchyma lost the characteristic shape and became elongated.

2. Changes in stomatal mechanism

Brainerd and Fuchigami (1981) and Brainerd et al. (1981) observed that stomata of cultured plantlets regained their normal responsiveness to water stress only after several days of exposure to low relative humidity. Mac9 apple root-stock cultured in vitro and subjected to 30 to 40 per cent relative humidity for six days, when planted out, lost water at high rate for three days due to improper stomatal mechanism. Only by the fourth day, the stomata started functioning normally. They also reported that excised leaves of the plantlets lost 50 per cent of the total leaf water content, three folds faster than the excised, green house grown leaves.

Marin et al. (1988) reported that the non-functional state of the stomata in the persistent leaves of cherry were reversible to certain extent. The shape of the stomata in persistent leaves changed from round to normal elliptical during acclimatization.

Sutter (1988) studied stomatal and cuticular water loss during acclimatization in apple, cherry and sweet gum plantlets. In the acclimatized plantlets, stomatal conductance of persistent leaves decreased to about half of that in the in vitro leaves, while cuticular conductance remained the same. He concluded that increased stomatal closure reduced the conductance.

3. Changes in photosynthetic ability and pigment content

In the case of cauliflower (Grout and Aston, 1978) and strawberry (Grout and Millam; 1988), the leaves formed in vitro failed to develop photosynthetic activity ex vitro and degenerated. The leaves formed ex vitro were photosynthetically capable. The plantlets depended on the stored food material for their growth and development during the early period of ex vitro establishment.

Mary et al. (1986) reported that the photosynthetic ability of the leaves of micropropagated plants was only one-third that of the leaves of the greenhouse grown plants.

The photosynthetic ability depended on the age of the shoots. Sub-cultured shoots, after two to four weeks, were photosynthetically more efficient than those maintained without sub-culturing.

Donnelly and Vidaver (1984a) found that in red raspberry, the pigment contents (chlorophyll a, chlorophyll b and carotenoids, on fresh weight basis) were significantly higher in the newly produced leaves of the transplants than in the leaves of in vitro cultured plantlets. On dry weight basis, chlorophyll a and total chlorophyll were greater in the new leaves of the transplants than in the persistent leaves; chlorophyll b and the carotenoids content did not differ.

C. Vitrification

Vitrification of shoots is frequent in tissue culture. Vitrified shoots or plantlets cannot survive transfer to greenhouse conditions, as they loose too much water and are very susceptible to infection by pathogens.

In vitrified plants, the leaf blades and petioles become humid and the very young leaves in the centre of the rosette become turgid. These turgid leaves elongate suddenly and reach lengths five to six fold, greater than those of

the normal leaves. Finally, the original leaves become almost translucent and necrotic.

The reasons for vitrification are several. Aitken et al. (1981) reported that the free water and the high humidity in the culture vessels lead to vitrification. Hakkart and Versluijs (1983) found that the type of "vessel closure" played an important role in vitrification. Debergh (1983) and Arnold and Eriksson (1984) reported that the culture medium influenced the production of vitrified shoots. They observed that re-vitrification of shoots occurred at higher agar concentrations that reduced the availability of cytokinins and water.

Pasqualetto et al. (1986, 1988) studied the effect of gelling agents and cations on shoot vitrification. Increased concentration of either agar or gelrite resulted in a decreased percentage of vitrification. Certain combinations of gelling agents produced no vitrification. Among the cations, K^+ at lower levels produced higher percentage of vitrified shoots. It also affected the tissue appearence, reduced the shoot number and elongation of shoots, and altered the shoot metabolism.

Meiraziv et al. (1987) observed that reduced calcium levels and increased ammonium nitrate levels in the culture

medium induced vitrification. Similarly, higher water content due to reduced agar concentration induced vitrification.

Kevers et al. (1984) and Kevers and Gaspar (1985) analysed the physiological and biochemical events leading to vitrification of plants cultured in vitro. They found that vitrification resulted from a burst of ethylene, controlled by peroxidase - IAA oxidase system. Higher ethylene concentration in the culture vessels affected various biochemical processes, hindered the lignification and reduced the cellulose content. Deficiency of lignin and cellulose allowed more water uptake, due to reduced wall pressure, and resulted in vitrification.

D. Factors influencing ex vitro establishment of plantlets and their regulation

1. Chemical factors

a. Plant growth substances

There are several reports on the effect of plant growth substances on the in vitro shoot production and rooting, and the ex vitro establishment of plantlets. Lower levels of auxins and cytokinins produced elongated shoots ideal for rooting and acclimatization (Lineberger, 1983; Nair

et al. 1984; Dunstan et al. 1985; Amin and Jaiswal, 1987; Battle and Aldrufeu, 1987).

Leshem (1983) observed no change in shoot quality as influenced by different levels of NAA in the culture medium. Podwyszynska and Hempel (1988) studied the effect of BA and IAA on in vitro rooting and acclimatization of rose hybrids. Rooting and acclimatization were better when lower levels of BA were used in the rooting media and when IAA was omitted from the rooting media.

The type of auxin used for rooting has been found to influence root morphology and plant survival. Williams and Taji (1989) reported that when NAA and NOA were used, the roots produced were thin. IBA produced thicker roots which reduced the establishment of the plantlets during transplanting.

Ranjit and Kester (1988) reported that GA at lower concentrations improved the rooting of tissue cultured cherry root stocks. However, rooting did not occur in the absence or at higher concentration of GA.

b. Other chemical factors

Hainwright and Scrace (1989) studied the effect of sucrose concentration and the type of carbohydrate on

in vitro plant growth. Two to four per cent sucrose concentration gave the maximum shoot height, fresh weight and dry weight of plantlets and registered 97.5 per cent *ex vitro* establishment. Among the different carbohydrates tested, sucrose, glucose and maltose were on par, while sorbitol was the least effective.

Desjardins and Tiessen (1985) observed that very low sucrose concentrations in the medium reduced the rooting percentage. At higher sucrose concentration the rooting percentage and subsequent shoot growth were better. At higher sucrose concentraion, the time required for rooting was found to be reduced.

Leshem (1983), Marin and Gella (1987) and Short *et al.* (1987) reported that higher concentrations of agar in rooting medium increased the *ex vitro* establishment of plantlets; but reduced the rooting. Williams and Taji (1989) reported that higher concentrations of gelrite increased the field establishment of plantlets.

Meiraziv *et al.* (1987) reported that lower levels of calcium and higher levels of ammonium nitrate (culture medium) induced vitrification and reduced *ex vitro* establishment of plantlets. Short *et al.* (1987) observed that addition of polyethylene glycol (PEG) in rooting medium helped *ex vitro*

establishment of plantlets. PEG reduced the humidity within the culture vessels, which in turn caused wax deposition on the leaves. The plantlets thus formed could be directly transplanted to soil with out humidity protection.

Nutrient / fertilizer application, when carefully regulated was found to be useful in promoting the growth of plantlets under ex vitro conditions (Poole and Conover, 1983). Rahman (1988) reported that nutrient application at the time of planting out was inhibitory to the growth of jack plantlets. This was especially significant in the case of nitrogen nutrition. Ammoniacal nitrogen was found to be more inhhibitory than nitrate nitrogen. Slight increase in growth was observed when the nutrients were applied 20 days after planting out. Promotive effect of nitrogen on growth was observed only in treatments given 25 days after planting. When the nutrients were applied 20 days after planting the growth was markedly improved. Interestingly, the growth of these plantlets were on par with the growth of those which received no nutrient application but were irrigated with tap water.

2. **Physical factors**

The physical factors which influence the ex vitro establishment of plantlets are found to be humidity, light,

temperature, gaseous levels in the growth chamber and potting media (Wardle et al., 1983; Borkowska 1984; Desjardins et al. 1987).

a. Humidity

Humidity has been observed to be an important factor in plantlet establishment. Since the plantlets lack effective stomatal closure mechanism, water loss will be high at lower relative humidity, leading to increased field mortality (Donnelly et al., 1987).

Chrysanthemum plantlets, cultured under low relative humidity, exhibited high rate of mortality (Wardle et al., 1983). The survived plantlets had only a few small roots. Stomatal developments was also seen affected. Short et al. (1987) reported that cauliflower and chrysanthemum plantlets cultured at 80 per cent relative humidity had increased wax deposition on their leaves. When they were transferred, water loss from the leaves was reduced and better ex vitro establishment resulted.

In apple, when the plantlets were exposed to low relative humidity, during the initial acclimatization period, the stomatal closure mechanism developed earlier (Brainerd and Fuchigami, 1981). The excised leaves of

plantlets lossed more water when acclimatized at high relative humidity. Mathur et al. (1988) reported that when a relative humidity of 60 to 70 per cent was maintained in the growth chamber,100 per cent establishment of Valeriana wallichi resulted.

Poole and Conover (1983) found that in order to provide humidity, intermittent misting of the plantlets was better than growing them under tents. The increased growth, observed under misting, might be due to the increased availabity of light. However, Sutter and Hutzell (1984) reported that the use of humidity tent was advantageous. Application of antitranspirants was found to be phytotoxic. Treatments with silicone formulations reduced transpiration; but plant growth was found to be affected. Selvapandiyan et al. (1988) observed that smearing the leaves of the plantlets with glycerol, paraffin wax or grease in ether (50% v/v), at the time of transplanting, reduced the rapid water loss from the leaves of tobacco and solanum plantlets. By this method , 100 per cent survival of the plantlets could be obtained.

b. Temperature

The effect of temperature on plantlet establishment has been worked out in many crops. The optimum temperature

range depended on the crop. Tropical crops required a temperature of 30 ± 2 °C. For the subtropical crops, 27 ± 2 °C and for the temperate crops, 25 °C or above were found lethal (Hughes, 1981; Appelgren and Heide, 1972). Borkowska (1984) reported that chilling tissue cultured cherry plantlets improved their growth.

c. Light

Dunstan and Turner (1984) suggested that in order to minimise shock to the plantlets during acclimatization, light intensity should be kept low at first and then increased gradually. Desjardins et al. (1987) reported that supplementary lighting during ex vitro establishment increased the shoot growth and the dry weight of tissue cultured strawberry plantlets. Lee et al. (1988) studied the effect of light intensity on the surface morphology of in vitro developed leaves of sweet gum. High irradiance was found associated with more compact and larger mesophyll cells. The stomatal density was more in the leaves grown under high light intensity than in others.

Read and Economou (1982) reported that quality of light influenced the rooting of micro cuttings raised in vitro. in azaleas, the rooting of micro cuttings was promoted when the shoots were cultured under far-red light

for two weeks prior to planting them in appropriate rooting medium.

d. Gaseous levels in the growth chamber

Lakso et al. (1986) studied the effect of carbon di oxide enrichment on the growth of in vitro propagated grapes during ex vitro establishment. In carbon di oxide enriched environment, dry weight of the plants increased twice in about two weeks and four times after three weeks. Improvement of root growth as well as doubling of leaf area and root:shoot ratio, resulted. Desjardins et al. (1987) studied the effect of carbon di oxide enriched environment during ex vitro establishment of strawberry. It had no effect during the early period of establishment. The net assimilation rate was found significantly increased after 20 to 30 days. The dry weight of roots and shoots also increased. Reuther (1986) found that in vitro plants did not respond to carbondioxide concentration during their initial post transplanting period.

e. Potting media

The medium used for potting the tissue cultured plantlets is an important factor, determining the establishment percentage. Damiano (1979) reported that

either pure peat or a mixture of 1:1 sand and peat was suitable as the potting medium for the ex vitro establishment of strawberry plantlets. The pH of the medium had to be regulated between 5.6 and 7.0.

Kyte and Briggs (1979) observed that a porous potting mixture of sterile peat, perlite and composted bark in 1:1:1 ratio, was the best for rooting tissue cultured rhododendrons. The depth of soil was important, as the survival rate was found to be better in 10 cm pots than in shallow trays. In the case of guava plantlets, when a mixture of sterile soil, sand and compost in 3:3:1 ratio was used for planting, only ten per cent of the plantlets survived (Nair et al., 1983)

Anderson (1984) found vermiculite to be suitable for rooting nephrolepis plantlets. Pena and Biutrago (1984) reported that sterilised slag medium was ideal for planting coffee plantlets. The survival rate of asparagus plantlets in sterile vermiculite - sand mixture (2:1 ratio), vermiculite - sand - peat mixture (2:1:2) ratio and garden loam was 60.0, 57.1 and 78.9 per cent, respectively (Li, 1985). The lower survival rate observed in the two vermiculite mixture was due to greater water accumulation and incidence of diseases.

Reuther (1986) reported that sterile granular rock wool was a better potting medium for asparagus, gerbera, pelargonium and saintpaulia plantlets. In pelargonium, 95 per cent survival of plantlets resulted when perlite medium was used as potting medium (Aldrufeu, 1987). Drew (1988) reported that 90 per cent of papaya plantlets survived when a mixture of sterile peat, perlite and polystrene beads (1:1:1: ratio v/v) was used as the potting medium.

3. Biological factors

a. Vesicular arbuscular mycorrhiza (VAM)

Growth of *in vitro* cultured strawberry plantlets (Kiernan *et al.*, 1984) and *Pistacia integerrima* (Schubert and Martinelli, 1988) increased when inoculated with certain specific mycorrhizal fungi. Rooting and establishment of callus generated garlic plantlets were increased when *Glomus mosseae* was inoculated in the potting medium during transplanting (Fogher *et al.*, 1986).

Ectomycorrhizal fungi increased shoot height, plant dry weight and NPK content of popular (Heslin and Douglas, 1986) and eucalyptus (Malajczuk and Hartney, 1986).

The increased growth of mycorrhiza treatd -plants was due to enhanced mineral uptake (St. John, 1980). The mycelia of mycorrhiza explored large volumes of soil and were to utilize relatively immobile phosphate. Another reason for better growth was the increased production of phytohormones (Allen et al., 1980). Mycorrhizal plants were photosynthetically more efficient than the non-mycorrhizal plants (Sivaprasad and Rai, 1984).

The increased plantlet establishment, consequent on mycorrhizal treatment of the medium, was due to decreased transplant injury (Menge et al., 1978) water uptake and transport (Safir et al., 1971), and low infection of the plantlets by soil-borne pathogens (Schenck, 1981). Root hairs were found to be absent in the tissue cultured plantlets (Reuther, 1986). In the case of mycorrhizal association the mycellium played the role of root hairs and helped in compensating the water loss.

The increased resistance of mycorrhiza treated plantlets to soil-borne pathogens, might be due to higher production of phenolic compounds including phytoalexins within the plant system (Hussey and Rancodori, 1982; Lakshmanan et al., 1987).

E. Recent techniques for increasing the ex vitro establishment of plantlets

Dhawan and Bhojwani (1987) established a new method whereby survival of Leucaena plantlets could be increased to 85 per cent. In this method, the plantlets were transferred to 250 ml Erlenmeyer flasks containing 180 ml quartz sand and irrigated with quarter strength MS nutrient medium. The mouths of the flasks were covered by sterilised aluminium foil. The foil was removed after 10 days and increased light intensity was provided. After 25 days, the plantlets were transferred to polythene bags and kept under natural shade.

Fari et al. (1987) used thin PVC foil covering (TPFC) for tissue culture conta iners. The TPFC facilitated water loss from the cultures. The water loss amounted to 15 to 20 per cent after four weeks and 50 to 60 per cent after six weeks. Under such conditions, the shoots of onion developed strong root system from the fourth week onwards. The leaves were with high dry matter content and profuse wax deposition. Such plants did not need any acclimatization before transplanting.

Goussard and Wiid (1989) formulated a revised approach for the acclimatization of plantlets of grapes cultured

in vitro. In their procedure, the plantlets were removed and rinsed in distilled water and their shoot tips and basal leaves were removed. The plantlets were then transferred to short tubes with the root system immersed in distilled water. After 24 hours the water was replaced with a standard nutrient solution which was changed every 24 to 48 hours. After the emergence of well developed leaves above the top of the tubes, they were transplanted to standard soil media. Survival percentage of 95 to 100 per cent could be obtained by this method.

MATERIALS AND METHODS

MATERIALS AND METHODS

Investigations on the techniques for *ex vitro* establishment of jack plantlets were carried out at the Plant Tissue Culture Laboratory, Department of Horticulture, College of Agriculture, Vellayani from 1988 to 1990.

The materials and methods used for the *in vitro* production of jack plantlets, *in vitro* and *ex vitro* treatments to increase the field establishment of the plantlets as well as morphological, histological and physiological investigations on the characteristics of *in vitro* grown and *ex vitro* established plantlets have been also described in the following pages.

A. *In vitro* production of jack plantlets

The protocol developed by Rajmohan (1985) was adopted with necessary modification for the *in vitro* production of jack plantlets. Shoot apices (1-2 cm) from one-year old seedlings of jack were excised and used as explants for the *in vitro* multiplication. The explants were washed in running tap water for 10 to 15 minutes and then surface sterilised with 0.2 per cent mercuric chloride solution for 12 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 distil water. The leaf

sheaths were removed and the explants were inoculated on establishment medium. The whole process was carried out in a laminar air flow chamber.

The composition of the media used for the various stages of *in vitro* production of jack plantlets is given in Table 1.

The PH of the media was adjusted to 5.7. Erlenmeyer flasks (Borosil) of 100 and 150 ml capacity were used. Sterilisation of the media was done at 15 psi for 15-20 minutes.

All aseptic manipulations were carried out in a laminar air flow chamber. The cultures were incubated at $26 \pm 2^{\circ}$ C with a 16 hour photo period and 40 $\mu E\ m^{-2}\ S^{-1}$ light intensity supplied by cool white fluorescent tubes. The cultures for *in vitro* rooting were incubated in darkness.

The multiple shoots formed were subcultured at 30 day interval. Part of them were transferred to the medium for producing elongated shoots. The remaining were subjected to repeated multiplication process for maintaining a stock of multiple shoots (plate 1). The elongated shoots were then transferred to the rooting medium. The rooting process comprised two stages. First, the shoots were transferred in

Table 1

Composition of media used for *in vitro* production of jack plantlets

No	Stage of *in vitro* propagation	Composition of medium
1	Culture establishment	MS medium supplemented with BA 5.0 mg/l, NAA 0.2 mg/l, ADS 20.0 mg/l, calcium pantothenate 0.1 mg/l, sucrose 30.0 g/l, agar 6.0 g/l.
2	Shoot multiplication	Medium 1 + insoluble PVP 500 mg/l
3	Production of elongated shoots	Medium 2 with BA concentration reduced to 2 mg/l
4	*In vitro* rooting	
	(a) Stage 1	MS medium (with half strength inorganic salts) supplemented with NAA 2.0 mg/l, IBA 2.0 mg/l, calcium pantothenate 0.1 mg/l, sucrose 30.0 g/l and agar 6.0 g/l.
	(b) Stage 2	Medium 4(a) without NAA and IBA

PLATE 1 Multiple shoots formed via enhanced release of axillary buds from jack shoot apex cultured for five weeks on MS proliferation medium

PLATE 2 Three week old jack plantlets


1


2

PLATE 3 Jack plantlet transferred to plastic pot containing sterile sand

PLATE 4 Humidity maintenance device during acclimatization of jack plantlets; mist tunnel

an auxin containing medium and incubated in darkness for seven days. They were then transferred to a medium without the auxins and incubated in darkness for seven to ten days. Roots were seen initiated by this period.

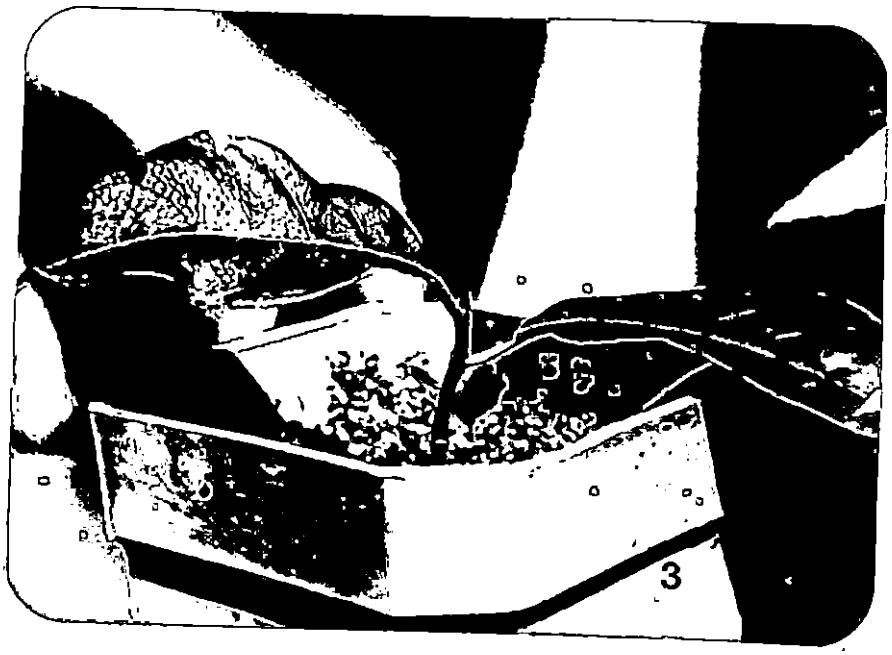
The rooted plantlets were then grown under high light intensity (50 $\mu E\ m^{-2}\ S^{-1}$) for 20 days (plate 2). By this period, the roots were seen well developed. The rooted plantlets were then carefully removed from the agar medium using forceps. The roots were washed with sterile tap water until they were free of agar. The plantlets were then planted in plastic pots of size (5.0 x 5.0 x 7.5 cm having small holes for drainage) filled with sterile sand (plate 3). The plantlets were then kept in diffused sunlight (25 $\mu E\ m^{-2}\ S^{-1}$) at a temparature of 28 $\pm$ 2° C inside a mist chamber to provide high relative humidity for one week. The relative humidity was then gradually lowered to ambient level.

B. Factors influencing plantlet production and their ex vitro establishment

1. At the in vitro stage

a. Composition of the rooting media

The influence of the concentration of inorganic nutrients (in general), nitrogen, calcium, sucrose, agar and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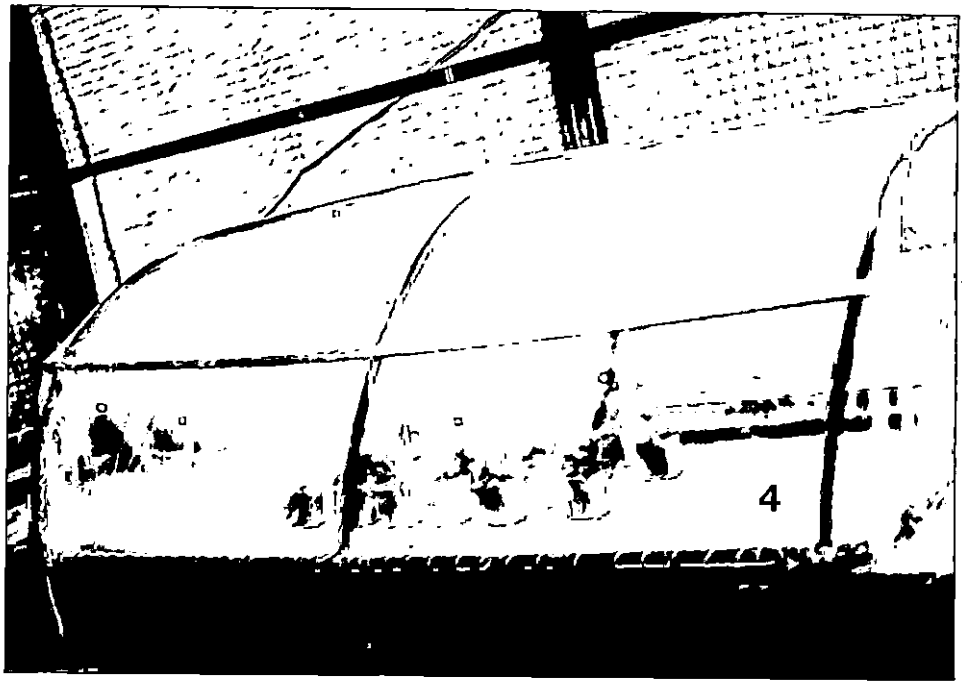
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activated charcoal was studied. The details of the treatments are given in Table 2. Observations on the number of days taken for root initiation, number of shoots rooted, number of roots per shoot and intensity of root branching (based on visual rating; ranging from 1 to 3; 1 = poor, 2 = moderate and 3 = good) were recorded 20 days after root initiation. After transplanting the survival and establishment of the plantlets were recorded.

b. size of shootlets

The influence of the size of shoots on in vitro rooting was studied. The treatments involved shoots of 1.0 cm (with single leaf), 2.0 cm (with two leaves) and 3.0 cm (with three to four leaves) length.

Observations on the days taken for root initiation; the number of shoots rooted, the number of roots per shoot and intensity of root branching were recorded, 20 days after root initiation.

c. Volume of vessels used for rooting

Erlenmeyer flasks of 100 and 150 ml capacity and test tubes of 20 x 150 mm and 25 x 150 mm size were used for

Table 2

Composition of media used for induction of rooting in jack shoots *in vitro*

No	Basal medium	Supplemented with	Treatment	Levels
1	MS with organic nutrients at full concentration	NAA 2.0 mg/l, IBA 2.0 mg/l calcium pantothenate 0.1 mg/l sucrose 30.0 g/l & agar 6.0 g/l	Inorganic nutrients	1/4, 1/2, 1 and 2 times than that in MS
2	MS with half strength inorganic nutrients without ammonium nitrate and organic nutrients at full concentration	Same as in 1	Nitrogen source: ammonium nitrate	0, 1/2, 1 and 2 times than that in MS
3	MS with half strength inorganic nutrients but without calcium chloride and organic nutrients at full concentration.	Same as in 1	Calcium source: calcium chloride	0, 1/2, 1 and 1.5 times than that in MS
4	MS with half strength inorganic nutrients and organic nutrients at full concentration	As in 1 without sucrose	Carbon source: sucrose	0, 10.0, 20.0, 30.0 and 40.0 g/l
5	Same as in 4	As in 1 without agar	Agar	4.0, 5.0, 6.0, 7.0 8.0 and 9.0 g/l
6	Same as in 4	Same as in 1	Activated charcoal	1.0, 5.0, 10.0 and 20.0 g/l

rooting. The days taken for root initiation, the number of shoots rooted, the number of roots per shoot and the intensity of root branching were recorded, 20 days after root initiation.

d. Intensity of light

The influence of light intensity, 20 days prior to planting out, on the ex vitro establishment was studied. The plantlets, just after visible root initiation, were subjected to different light intensities (darkness, 20 $\mu E\ m^{-2}\ S^{-1}$, 35 $\mu E\ m^{-2}\ S^{-1}$ and 50 $\mu E\ m^{-2}\ S^{-1}$). Observation on the number of plantlets established ex vitro was recorded, 30 days after transplanting and the percentage establishment worked out.

2. At the ex vitro Stage

a. Potting media

The following potting media were tried to identify the best for the establishment of the jack plantlets.

Vermiculite
Peat
Soilrite
Coir dust
Saw dust
Sand
Sand + Soil (1:1 v/v)
Sand + Soil + Vermiculite (1:1:1 v/v)
Sand + Soil + Dry powdered cowdung (1:1:1 v/v)
Sand + Soil + Coir dust (1:1:1 v/v)

The potting media were sterilised by autoclaving them at 15 psi for 20 minutes and used. The media were filled in plastic pots of 5.0 x 5.0 x 7.5 cm size. The number of plantlets that established, 30 days after planting out, was recorded and the percentage establishment worked out.

b. Nature and size of pots

Different types and sizes of potting vessels were tested to find out the one that would support the maximum survival of the plantlets. Plastic pots (5.0 x 5.0 x 7.5 cm), mud pots (5.0 x 5.0 x 7.5 cm) and polythene covers (150 gauge and 7.5 x 10 cm) were used. Observation on the number of plants that survived was recorded and the percentage survival worked out.

c. Age of plantlets

The influence of the age of the plantlets (days after visible root initiation) on their survival was studied. Plantlets of 0, 6, 12, 18, 24 and 30 days age were used for planting out. Observation on the number of plantlets that survived was taken and the percentage survival worked out.

d. Devices for regulating relative humidity

Different devices were used for regulating relative humidity around the plantlets for a period of 15 to 20 days after planting out.

i. Mist chamber

A tunnel like structure (150 cm X 50 cm) made by PVC pipes and covered with polythene sheets of 350 gauge was constructed to provide high humidity around the plantlets (plate 4). About 200 plantlets could be accommodated in this Chamber. Diffused sun light was provided by using synthetic shade nets. The pots were placed on a platform and rested on small individual pieces of thermocol to facilitate drainage. The tunnel like structure could be partially lifted to facilitate the regulation of relative humidity. The light intensity within the chamber, during mid day was 25 $\mu E\ m^{-2}\ S^{-1}$. The temperature range was 22 $\pm$ 2°C to 27$\pm$2°C. Regulation of relative humidity from 95 per cent to ambient was possible in the chamber. High humidity was provided by intermittent misting, using the terminal of a misting unit (installed in the net-house). The humidity could be lowered to the desired levels (upto the ambient relative humidity) by the partial lifting of the chamber.

ii. Covering the potted plantlets with glass beakers

The potted plantlets were kept on a platform in the net house under diffused sunlight and covered with glass beakers to maintain high humidity. With a hand sprayer water was sprayed three times daily. For lowering the humidity, the

beakers were partially lifted, with supports, during the later period of acclimatization.

iii. Covering the potted plantlets with polythene bags

The potted plantlets were kept on a platform in the net house under diffused sunlight and covered by polythene bags which were secured with rubber bands, around the pots (plate 5a). Only one initial misting was given.

iv. Placing the potted plantlets in a plastic basin and covering with polythene sheet

The potted plantlets were kept in a plastic basin (60 cm in diameter and 20 cm high) and covered with a polythene sheet to maintain high humidity (plate 6). Water was sprayed using a hand sprayer twice daily. The basin was kept on a platform in the net house under diffused sunlight. For lowering the humidity, during the later period of acclimatization, the polythene sheet was partially uncovered.

v. Covering the potted plantlets kept in petri dishes containing small quantity of water, with glass beakers

The potted plantlets were kept in the net house under diffused sunlight, in petri dishes (15 cm diameter)

PLATE 5 Humidity maintenance device during acclimatization of jack plantlets

a. Individual pot covered with perforated polythene bag

b. Individual pot kept in petri dish containing water and covered with glass beaker

PLATE 6 Humidity maintenance device during acclimatization of jack plantlets; pots kept in plastic basin and covered with polythene sheet


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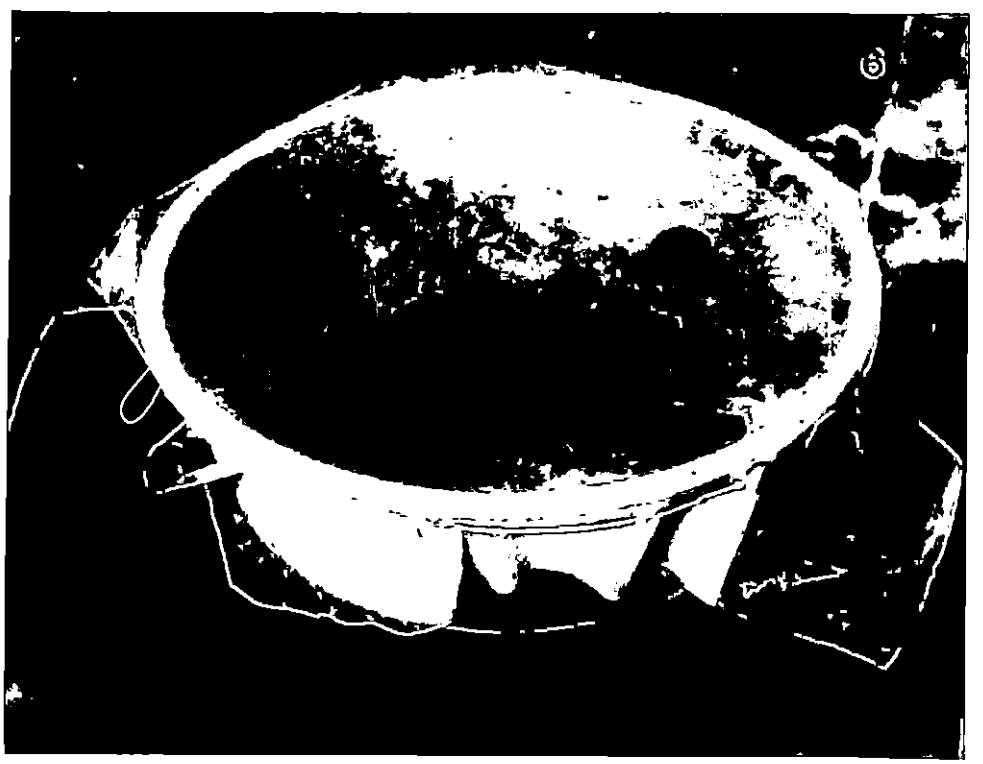
b
a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containing small quantity of water and covered with glass beakers to maintain high humidity around the plantlets (plate 5b). For lowering the humidity, during the later period of acclimatization, the beakers were partially lifted by supports.

As control, the potted plantlets were kept in the net house under diffused sunlight. Intermittent sparying with hand sprayer was given to maintain a thin film of water on the leaves. Observations on the survival of the plantlets were recorded in all the above cases, 30 days after transplanting.

e. Effect of antitranspirants on plantlet establishment

Different antitranspirants were applied on the leaf surface to reduce water loss. Glycerol, silica grease, paraffin wax and starch solution were used as antitranspirants

Solutions of the antitranspirants were prepared by dissolving 100 ml glycerol, 50 g silica grease and 50 g paraffin wax in 100 ml diethyl ether. The solutions were applied on the treatment plantlets by a thin camel brush. Saturated starch solution was applied by spraying with hand sprayer. The potted plantlets were then kept in the mist

chamber as well as in the open. Observations on the survival of the plantlets were recorded.

f. Role of vesicular arbuscular mycorrhiza (VAM) on plantlet establishment

Investigations were carried out to study the influence of vesicular arbuscular mycorrhiza (VAM) fungi on the ex vitro survival and growth of the in vitro produced plantlets. Two species, namely Glomus fasiculatum and Glomus etunicatum were used for the experiment. The in vitro plantlets were planted on sterile sand inoculated with a mixture of 100 chlamydospores of the mycorrhizal fungi [extracted according to Gerdemann and Nicolson (1963) from the rhizosphere soil of guinea grass, infected with the respective VAM fungus and grown for 90 days] and 19 infected root bits (0.5 cm long) of guinea grass. As the control, the plantlets were transplanted to sterilised fine sand. On the 30th day of planting out, the mycorrhizal colonisation percentage (Phillips and Haymann, 1970) and spore count in the rhizosphere soil (Gerdemann and Nicolson, 1963) were recorded.

Observations on survival of the plantlet, plant heiht, leaf number, total leaf area, fresh plant weight, dry plant weight, total uptake of N, P, K, Ca, Mg, Fe, Mn and Zn per

plant and mycorrhizal colonisation in the roots were recorded. The plant growth was observed for 12 weeks.

g. Mineral salt solution for irrigation of plantlets

The influence of mineral salt solutions and inorganic fertilizers on the growth of the plantlets was studied. The effect of time of application of these was also assessed. The following treatments were applied at 10 and 20 days after planting out.

Strength of inorganic salt solution	pH
1/10 MS inorganic salt solution	5.7
1/4 MS inorganic salt solution	5.7
Soluble fertilizer solution (NPK 10:52:10 g/l);	5.7
Control (tap water)	4.4

The plantlets were watered with 5 ml of the solutions/tapwater. Observations were recorded on the survival of plantlets, number of leaves per plant, plant height and fresh plant weight, after 60 days of planting out.

C. Morphological and histological studies

1. Morphological studies

a. Surface morphological studies using scanning electron microscope

Using scanning electron microscope (SEM) [Model : Hitachi S 530] the surface structures of the leaves of the plantlets produced *in vitro* was compared with that of the acclimatized plantlets (new leaves and persistent leaves) and field grown mature plants. The procedure adopted is outlined below.

Fixation → dehydration → critical point drying → gold coating → examining in the SEM.

Samples of the leaf tissues were collected at random from the *in vitro* produced plantlets, acclimatized plantlets (new leaves and persistent leaves) and field grown mature plants. The leaf tissues were treated with a fixative for 24 hrs. The fixative was a mixture of glutaraldehyde solution (2.5%) and phosphate buffer (pH 7.2) in 1:9 ratio.

i. Preparation of glutaraldehyde solution

2.5 ml of glutaraldehyde was added to 100 ml, to get 2.5 percent glutaraldehyde solution.

ii. Preparation of phosphate buffer (pH 7.2)

Two hundred ml of the phosphate buffer (pH 7.2) was prepared by mixing 19 ml of Solution A (0.2 M monobasic sodium phosphate), 81 ml of Solution B (0.2 M dibasic sodium phosphate) and 100 ml distilled water.

Solution A was prepared by dissolving 3.12 g monobasic sodium phosphate ($NaH_2PO_4 : 2 H_2O$) in 100 ml distilled water, Solution B by dissolving 7.16 g dibasic sodium phosphate ($Na_2HPO_4 : 12 H_2O$) in 100 ml distilled water.

After the fixation, the plant tissues were washed in phosphate buffer (pH 7.2) five to six times, to remove the adhering traces of the fixative. The tissues were then dehydrated at room temperature, using the following acetone series.

Strength of acetone (%)	Duration
50	25 mts
70	20 mts
90	1 hr
90	over night
95	25 mts
95	25 mts
100	30 mts
100	30 mts
100	30 mts

The dehydrated plant tissues were then critical point dried in a Model HCP-2 Critical Point Dryer. In order to

facilitate critical point drying, the dehydration solvent, acetone was substituted with amyl acetate. After critical point drying, the tissues were mounted on aluminium stubs with silver epoxy paste, coated with gold and examined.

Observations on stomatal shape, epicuticular wax deposition and structure of the epidermal hairs were made.

b. Stomatal count

A comparative study was made of the stomatal count of the leaves of *in vitro* produced plantlets, acclimatized plantlets (new leaves and persistent leaves) and field grown one-year old seedling plants. Leaf imprints were prepared for the purpose using the clear gum "Quick fix". "Quick fix" was uniformly applied on the surface of the leaf segments and after five minutes, the dry gum membranes were carefully peeled off and mounted on a microscope slide with a drop of water. The stomata were observed and counted, using a 40x objective and a 10x eye piece. The field of the microscope was measured using a stage micrometer and the stomatal count per unit area, was made.

2. Histological studies of leaves

Leaf samples were collected at random from the *in vitro* produced plantlets, acclimatized plantlets (new leaves

and persistent leaves) and field grown one-year old seedlings. Fully mature leaves were selected (fourth leaf from the apical bud was selected in all the leaves, except in persistent leaves). Hand sectioning of the leaves was done with a sharp razor blade for obtaining cross sections containing midrib and without midrib. The sections were stained in 0.1% saffranin for 10 to 12 minutes, observed under biological microscope (Nikon Optiphot), for their cellular tissue arrangements and then photographed.

D. Physiological Studies

1. Water loss through leaves

A comparative study was made of the water loss per unit area at regular intervals from the leaves of *in vitro* grown plantlets, acclimatized plantlets (new leaves and persistent leaves) and field grown seedlings. The excised leaves (with petioles) from the treatment plants were kept immersed in distilled water for three hours at 25° C under diffused light ($20\ \mu E\ m^{-2}\ S^{-1}$) provided by cool white fluorescent tubes. They were then taken out and the petioles excised. After gently wiping the leaves with dry blotting paper, they were placed in shallow aluminium cups with abaxial surface facing up. Water loss from each leaf was estimated by recording the weight at regular intervals of 15 minutes for a total

period of 105 minutes on an electronic digital balance ("Sartorius" make with an accuracy of ± 0.1 mg). Each replication consisted of a single leaf. Throughout the experiment, a temperature of 29 ± 1° C and a relative humidity of 53 percent prevailed. After the experiment, the area of the individual leaves was measured in a leaf area (LI-COR 3100) meter. The relative water loss per unit area of leaves was calculated for all the four types of leaves.

E. Chlorophyll content of leaves

The comparison was made of the chlorophyll content of the leaves of *in vitro* grown plantlets, acclimatized plantlets (new leaves and persistent leaves) and field grown one-year old seedlings at random. Fully mature leaf samples were taken at random (usually fourth leaf from apical buds in all the leaves, except in persistent leaves where the upper most persistent leaf was taken) and chlorophyll a, chlorophyll b and total chlorophyll estimated.

Leaf samples (0.5 g each) were taken without their mid rib portions and ground in glass mortar, using glass pestle with 80 percent acetone. The ground leaf samples were then filtered through Whatman No. 42 paper, until the entire chlorophyll content was filtered off. The filtered solution

was then made up to 100 ml and read at two wave lengths (663 nm and 645 nm) in a Spectrophotometer (Bausch and Lomb Spectronic 2000). Total chlorophyll content, chlorophyll a and chlorophyll b of the different leaves were estimated as below.

$$\text{Total chlorophyll content} = (8.05\ A_{663} + 20.29\ A_{645})\ 0.2$$

$$\text{Chlorophyll a} = (12.72\ A_{663} - 2.58\ A_{645})\ 0.2$$

$$\text{Chlorophyll b} = (22.87\ A_{645} - 4.67\ A_{663})\ 0.2$$

RESULTS

RESULTS

Investigations were carried out which aimed at improving the *ex vitro* establishment of tissue culture derived jack plantlets. The results of these studies are presented in the following pages.

A. Factors influencing plantlet production and *ex vitro* establishment

1. At the *in vitro* stage

a. Rooting medium

i. Strength of inorganic nutrients

The influence of the concentration of the inorganic nutrients in the rooting medium on the *in vitro* rooting was studied first. Half strength MS basal medium supported 93.3 per cent rooting, 6.57 roots per shoot, good intensity of root branching (2.57) and field survival of 53.33 percent plantlets (Table 3). Increasing or decreasing the concentration of inorganic nutrients caused a reduction in the percentage of rooting (83.33 for full and double strength and 66.67 for quarter strength). Full strength of the nutrients recorded 5.4 roots per shoot, good intensity of root branching (2.6) and 40.0 per cent *ex vitro* establishment of the plantlets. The corresponding values

Table 3

Effect of concentration of inorganic nutrients on *in vitro* rooting of shoots and *ex vitro* establishment of plantlets

Treatment No.	Treatment	Per cent shoots rooted(a)	Number of days for rooting	Intensity of root branching	Number of roots per shoot	Per cent plantlets survived
T1	1/4 MS	66.66	22.00	1.75	3.75	50.00
T2	1/2 MS	93.33 b	16.00	2.57	6.57	53.33
T3	MS	83.33	18.00	2.60	5.40	40.00
T4	2MS	83.33	24.00	1.80	3.40	00.00

Note: a Average of 6 observations b Average of 15 observations

were found to be 3.75, 1.75 and 50.0 percent for the quarter strength medium and 3.40, 1.80 and 0.00 percent for the double strength medium.

ii. Strength of nitrogen

The influence of inorganic nitrogen and calcium on in vitro rooting was specifically studied. Half concentration of ammonium nitrate in the rooting medium (MS) supported 100 percent rooting, with 5.7 roots per shoot and good intensity of root branching (2.7) in 17.3 days and 66.6 per cent in vitro establishment of the plantlets (Table 4). The percentage of rooting was 66.6 at the full concentration of ammonium nitrate. Rooting media without ammonium nitrate as well as those containing double concentration, exhibited only 16.6 per cent rooting. The field survival of the plantlets was 50.0 per cent when full strength of ammonium nitrate was used. The control and the highest (double) strength of the nutrient did not support the field survival of the plantlets.

iii. Strength of calcium

Half strength of the calcium salts in the MS medium supported 83.3 per cent rooting of shoots, 5.2 roots per shoot and good intensity of root branching (3) in 20.0 days (Table 5) and 66.7 per cent field survival of the

Table 4

Effect of concentration of nitrogen (ammonium nitrate) on *in vitro* rooting of shoots and *ex vitro* Establishment of plantlets

Treatment No.	Treatment	Per cent shoots rooted a	Number of days for rooting	Number of roots per shoot	Intensity of root branching	Per cent Plantlets survived
T1	1/2 MS-NH_4NO_3	16.6	27.0	1.0	1.0	00.00
T2	1/2 MS+1/2 NH_4NO_3	100.0	17.3	5.7	2.7	66.66
T3	1/2 MS+NH_4NO_3	66.6	20.0	5.3	2.5	50.00
T4	1/2 MS+2NH_4NO_3	16.6	22.0	4.0	2.0	00.00

Note: a Average of 6 observations

Table 5

Effect of concentration of calcium on *in vitro* rooting and *ex vitro* establishment of plantlets

Treatment No.	Treatment	Per cent shoots rooted [a]	Number of days for rooting	Number of roots per shoot	Intensity of root branching	Per cent plantlets survived
T1	1/2 MS - $CaCl_2$	100.00	11.3	1.83	1.16	40.00
T2	1/2 MS+1/2 $CaCl_2$	83.33	20.0	5.20	3.00	66.66
T3	1/2 MS + 1 $CaCl_2$	50.00	16.0	6.00	3.00	50.00
T4	1/2 MS+1.5 $CaCl_2$	66.66	20.5	5.00	2.75	50.00

Note: a Average of 6 observations

plantlets. Though the medium without calcium chloride recorded 100 percent rooting in 17.3 days, it recorded the lowest field survival (40.0 per cent) of the plantlets. Use of the full and 1.5 strength of the salt recorded inferior response.

iv. Strength of sucrose

The percentage of in vitro rooting of the shoots remained constant (83.3) when the sucrose concentration in the culture medium was changed from 20.0 to 40.0 g/l (Table 6). The number of days taken for root initiation was, however, 13.4 for 20.0 g/l, 16.0 for 30.0 g/l and 18.8 for 40.0 g/l. Sucrose at 30.0 g/l recorded the maximum number of roots (5.80) per shoot, the highest intensity of root branching (2.8) and 50.0 percent field survival of the plantlets. Sucrose registered 50.0 percent ex vitro establishment at 40.0 g/l. Sucrose at 20.0 g/l caused a reduction in the field survival (33.3 percent). Sucrose, at 10.0 g/l was inferior with respect to the in vitro rooting and ex vitro establishment. Media devoid of sucrose did not support the field survival of the plantlets.

v. Strength of agar

Standardisation of the optimum concentration of the agar in the rooting medium for maximum ex vitro

Table 6

Effect of concentration of sucrose on *in vitro* rooting of shoots and *ex vitro* establishment

Treatment No.	Treatment	Per cent shoots rooted(a)	Number of days for rooting	Number of roots per shoot	Intensity of root branching	Percent plantlets survived
T1	0	16.6	36.0	1.0	1.0	00.0
T2	10	50.0	18.3	1.6	1.0	33.3
T3	20	83.3	13.4	2.8	1.6	33.3
T4	30	83.3	16.0	5.8	2.8	50.0
T5	40	83.3	20.8	4.2	2.4	50.0

Note: a Average of 6 observations

establishment was attempted. While the survival percentage was 66.6 for 4.0, 5.0 and 6.0 g/l agar, the values were 33.3 and 16.6 for 7.0 and 8.0 g/l. The maximum rooting (100.0 percent) was obtained when 5.0 g/l agar was used. In this case, 6.50 roots (with good intensity of root branching (3.0)) were formed in 26.0 days. Agar at 6.0 g/l supported 83.3 percent rooting in 17.6 days with 5.2 roots per shoot and moderate intensity of root branching (2.4) and 66.6 percent survival of plantlets in the field (Table 7). The number of days for root initiation was found reduced (8.75 days) when 4.0 g/l agar was used. However, the percentage of rooting was less (66.6). The percent field survival of plantlets in this case was also 66.6.

vi. Strength of activated charcoal

The role of activated charcoal in the rooting medium and its influence on *in vitro* rooting and *ex vitro* establishment was studied. Activated charcoal at 1.0 and 10.0 g/l effected 66.6 per cent rooting (Table 8). At 5.0 and 20.0 g/l, 50.0 per cent rooting was observed. The minimum days for root initiation (19.00) was observed in the case of 20.0 g/l, followed by 1.0 g/l (20.25 days), 5.0 g/l and 10.0 g/l registered 58.00 and 35.25 days respectively, for rooting. The number of roots per shoot and the rooting intensity, however, showed a decreasing pattern with

Table 7

Effect of concentration of agar on *in vitro* rooting of shoots and *ex vitro* establishment of plantlets

Treat-ment No.	Treatment	Per cent shoots rooted(a)	Number of days for rooting	Number of roots per shoot	Intensity of root branching	Percent plantlets survived
T1	4	66.6	14.75	5.75	3.0	66.6
T2	5	100.0	26.00	6.50	3.0	66.6
T3	6	83.3	17.60	5.20	2.4	66.6
T4	7	50.0	20.00	4.66	2.0	33.3
T5	8	83.3	15.20	3.80	1.6	16.6

Note: a average of 6 observations

Table 8

Effect of activated charcoal on *in vitro* rooting of shoots and *ex vitro* establishment of plantlets

Treatment No.	Treatment	Per cent shoots rooted(a)	Number of days for rooting	Number of roots per shoot	Intensity of root branching	Percent plantlets survived
T1	1	66.6	20.25	3.5	1.75	66.6
T2	5	50.0	58.00	3.0	1.66	50.0
T3	10	66.6	35.25	2.5	1.75	16.6
T4	20	50.0	19.00	2.3	1.66	50.0

Note: a Average of 6 observations

increasing concentration of activated charcoal. The maxium percent survival of plantlets ex vitro was observed for 1.0 g/l (66.6 per cent) while the least was in the case of 10.0 g/l (16.7). The other two levels tried supported 50.0 percent survival.

b. Size of shootlets

In vitro rooting in relation to size of shootlets was studied. The 3.0 cm long shoots (with three to four leaves) recorded 100.0 per cent rooting, 5.5 roots per shoot and a good intensity of root branching (2.75) (Table 9). The corresponding values were 75.0, 4.0 and 1.83 for 2.0 cm long shoots (with two leaves) and 37.5, 2.66 and 1.33 for 1.0 cm (with one leaf) long shoots.

c. Culture vessels

The effect of type and volume of culture vessels on in vitro rooting percentage, the number of roots per shoot and the intensity of root branching was investigated. Cent per cent rooting, 6.17 roots per shoot and good intensity of root branching (2.83) were observed when 150 ml Erlenmeyer flasks were used (Table 10). These results were on par with those of 100 ml flasks. The corresponding values were 50.0, 3.67 and 1.35, for test tubes of 25 mm x

Table 9

Effect of Size of Shoots on in vitro rooting

Treatment No.	Treatment	No. of Shoots rooted(a)	Per cent shoots rooted	Days for rooting	Number of roots per shoot	Intensity of root branching
T1	1 cm shoot with one leaf	3	37.5	13.66	2.66	1.33
T2	2 cm shoot with two leaves	6	75.0	13.66	4.00	1.83
T3	3 cm shoot with three leaves	8	100.0	13.00	5.50	2.73

Note : a average of 8 observations

Table 10

Influence of size of culture vessels on in vitro rooting

Treatment No.	Treatment	Per cent shoots rooted	Number of days for rooting	Number of roots per shoot	Intensity of root branching
T1	Test tube (25mm x 150mm)	33.3	15.5	2.50	1.00
T2	Test Tube (25mm x 200mm)	50.0	14.6	3.67	1.33
T3	Conical flask (100 ml)	100.0	13.2	6.17	2.67
T4	Conical flask (150 ml)	100.0	14.3	6.17	2.83

200 mm size and 33.3, 2.50 and 1.00 for test tubes of 25 mm x 150 mm size.

d. Light intensity

The light intensity in which the plantlets were exposed during the 21 days "prior to planting out" phase influenced the ex vitro establishment (Table 11). The maximum per cent survival of plantlets (60.0) was observed when they were grown under high light intensity of 50.0 $\mu E\ m^{-2}\ S^{-1}$. The plantlets not exposed to light before planting out did not survive. Medium light intensity (35 $\mu E\ m^{-2}\ S^{-1}$) supported 50.0 per cent survival while low light intensity (20 $\mu Em^{-2}\ S^{-1}$) could effect only 33.3 per cent survival.

2. At the ex vitro stage

a. Potting media

Various potting media were studied for their effect on ex vitro establishment. Sand supported 53.3 per cent survival of the plantlets ex vitro (Table 12). Vermiculite, peat as well as sand:soil (1:1) mixture recorded 40.0 per cent survival. Soilrite, coirdust and sawdust did not support the survival of the plantlets. The remaining treatments tried could effect only 20.0 per cent survival.

Table 11

Influence of light intensity prior to planting out on *ex vitro* establishment of plantlets

Treatment No.	Treatment	No. of plantlets survived(a)	Per cent plantlets survived
T1	Dark	0	0
T2	Low	2	33.3
T3	Medium	3	50.0
T4	High	9[b]	60.0

Note: a average of 6 observations
b average of 15 observations

Table 12

Influence of potting media on ex vitro establishment of plantlets

Treatment No.	Treatment	Number of Plantlets established	Percentage of Plantlets established (a)
T1	Vermiculite	2	40.0
T2	Soilrite	0	00.0
T3	Peat	2	40.0
T4	Coir dust	0	00.0
T5	Saw dust	0	00.0
T6	Sand	8	53.3 (b)
T7	Sand:Soil (1:1)	2	40.0
T8	Sand:Soil:Cowdung (1:1:1)	1	20.0
T9	Sand:Soil:Coirdust (1:1:1)	1	20.0
T10	Sand:Soil:Vermiculite (1:1:1)	1	20.0

Note: a Average of 6 observations b Average of 15 observations

b. Nature and size of pots

The nature and size of containers influence the survival of the plantlets ex vitro. Plastic pots (5.0 x 5.0 x 7.5 cm size) Supported 50.0 per cent survival (Table 13). Among the containers tried, mudpots recorded the lowest percentage of survival (12.5).

c. Age of plantlets

The influence of the age of the plantlets at planting out on the ex vitro establishment was studied. Plantlets of 18 days and above age recorded 60.0 percent survival ex vitro (Table 14). When the plantlets were used just after root initiation, no survival resulted.

d. Devices for regulating humidity

Different devices were used for maintaining high humidity around the plantlets during the initial period of ex vitro establishment. Covering the individual potted plantlets with polythene cover was found to support 80.0 per cent survival (Table 15). Keeping the potted plantlets in mist chamber resulted in 60.0 per cent survival. The other treatments were either not satisfactory or did not support the survival. All the potted plantlets kept in the open (as control) perished.

Table 13

Effect of type of containers used for planting out on *ex vitro* establishment of plantlets

Treatment No.	Treatment	Number of Plantlets established (a)	Per cent Plantlets established
T1	Mud pots	1	12.5
T2	Plastic pots	4	50.0
T3	Poly bags	2	25.0

Note: a Average of 8 observations

Table 14

Effect of age of plantlets used for planting out on *ex vitro* establishment of plantlets

Treatment No.	Treatment	Number of Plantlets established (a)	Per cent plantlets established
T1	Just after root initiation	0	00.0
T2	6 days	1	20.0
T3	12 days	2	40.0
T4	18 days	3	60.0
T5	24 days	3	60.0
T6	30 days	3	60.0

Note: a Average of 5 observations

Table 15

Effect of humidity maintenance devices on *ex vitro* establishment of plantlets

Treatment No.	Treatment	Number of plantlets established (a)	Per cent plantlets established
T1	Mist tunnel	3	60.0
T2	Covered with glass beaker	1	20.0
T3	Covered with plastic cover	4	80.0
T4	Pots placed on a basin and covered with plastic paper	2	20.0
T5	Pots placed on a thin film of water and covered with glass beaker	0	00.0
T6	Control (kept open)	0	00.0

Note: a Average of 5 observations

e. Antitranspirants

Different antitranspirants were tried to reduce water loss from the plantlets and to study their influence on the survival of plantlets ex vitro. Starch solution application supported 40.0 per cent survival of the plantlets ex vitro (Table 16). The plantlets did not survive when applied with the remaining treatments.

f. Vesicular arbuscular mycorrhiza

The jack plantlets, incubated with the VA mycorrhiza Glomus etunicatum and G. fasciculatum at the time of planting out, were observed to be capable of harbouring them in the roots (plate 9;10). The mycorrhizal colonisation by G. etunicatum and G. fasciculatum was 21.4 and 18.8 per cent with a spore count of 7.0 and 9.0 per 10 g soil, respectively, on the 30th day of inoculation. The mycorrhizal plantlets registered 80.0 to 100.0 per cent survival (Table 17). The period required for acclimatization was reduced by one week. The treated plantlets got acclimatized in two weeks.

In the treated plants, plant height was increased (Table 17). The per cent increase in plant height over the control was 59.0 and 46.0, respectively, for the plants

Table 16

Effect of anti transpirants on ex vitro establishment of plantlets

Treat-ment No.	Treatment	Number of plantlets established	Per cent plantlets established
T1	Paraffin wax	0	0.00
T2	Silica grease	0	00.0
T3	Glycerol	0	00.0
T4	Starch	2	40.0

Table 17

Effect of vesicular arbuscular mycorrhizae on the survival, height, fresh weight and dry weight of plantlets, 80 days after planting out

VAM species	Survival of plantlets %	Plant height(cm)	Fresh weight of plants(g)	Dry weight of plants(g)
G.etunicatum	100(a)	9.00(b)	2.57(b)	0.44(b)
G.fasciculatum	80	8.25	2.48	0.39
Control	55	5.67	1.82	0.29

Note a. average of 20 observations b. average of 10 observations

PLATE 7 Jack plantlets established in garden pots containing potting mixture, 45 days after transplanting

PLATE 8 Effect of mycorrhizal treatment on plantlet growth, 80 days after transplanting

a. control plant

b. Plantlet treated with VA mycorrhiza (Glomus etunicatum)

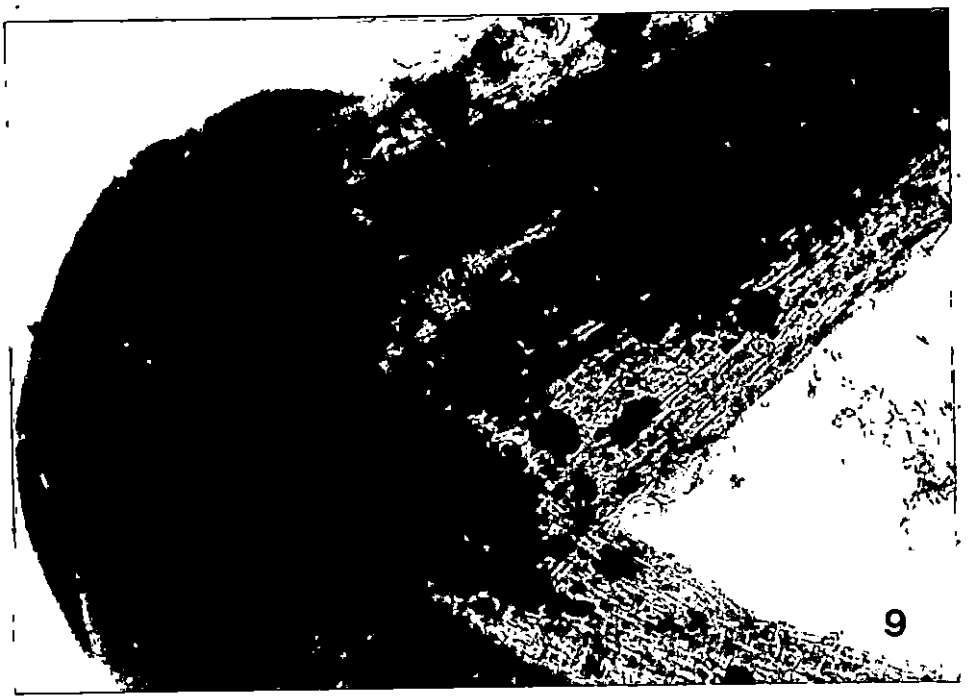

7


a
8

PLATE 9 Root of jack plantlet harbouring VA mycorrhiza

PLATE 10 Root of jack harbouring VA mycorrhiza

a. vesicle
b. hypha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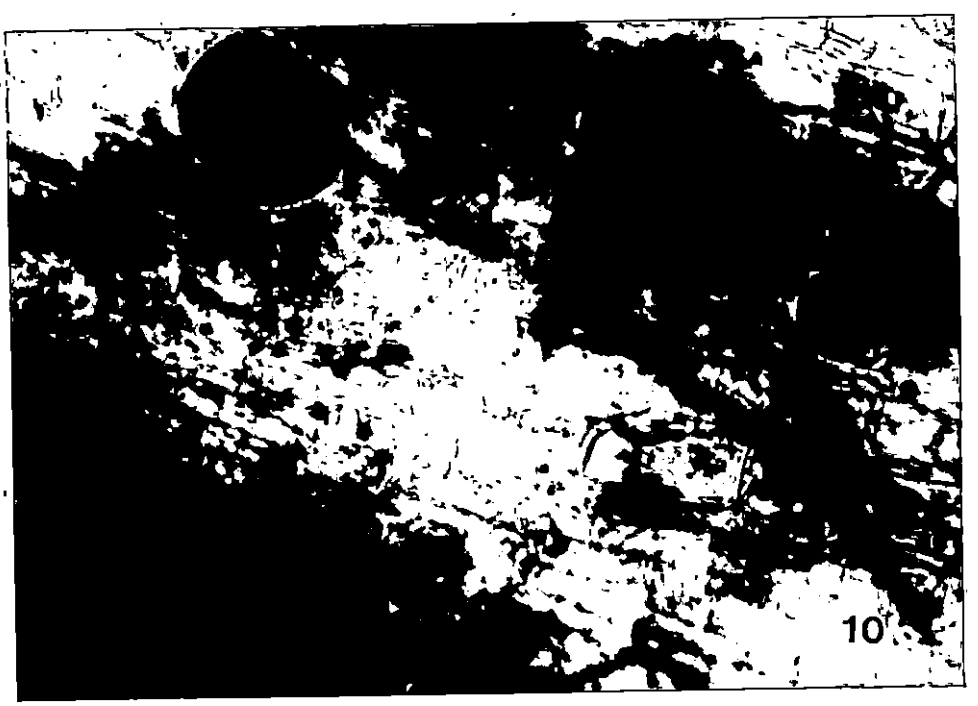
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treated with G. etunicatum (plate 8) and G. fasciculatum. The increase in fresh and dry weights in plants infected with G. etunicatum were 41.0 per cent and 46.0 per cent, respectively, while for G.fasciculatum treated plants the corresponding values were 36.0 and 33.0 per cent.

Increase in the number of leaves and total leaf area per plant was observed in the infected plants. The leaf area was increased by 98.0 percent, compared to control plants, when the plants were treated with G. etunicatum (Fig. I). The value was 49.0 percent for G. fasciculatum.

Plant analysis showed increased total major and minor nutrients status per plant, except iron, in the plants treated with G. etunicatum and G. fasciculatum (Table. 18). The respective percentages of increased status were 45.3 and 7.8 for nitrogen, 37.5 and 50.0 for phosphrous, 67.8 and 63.2 for potassium, 45.7 and 37.0 for calcium, 53.9 and 46.2 for magnesium, 51.7 and 82.1 for manganese and 69.6 and 95.1 for zinc (Fig II ; III). Increase in the case of zinc was the most pronounced. A decrease in the total content per plant was observed in the case of iron, the values being 10.1 and 20.0 percent, respectively, for the plants treated with G. etunicatum and G. fasciculatum.

Table 18

Effect of vesicular arbuscular mycorrhizae on major and minor nutrient elements status 12 weeks after the ex vitro establishment of plantlets

VAM Species (a)	N (mg)	P (mg)	K (mg)	Ca (mg)	Mg (mg)	Fe (µg)	Mn (µg)	Zn (µg)
G.etunicatum	9.3	1.1	14.6	6.7	2.0	338.0	53.4	55.8
G.fasciculatum	6.9	1.2	14.2	6.3	1.9	301.0	64.1	64.2
Control	6.4	0.8	8.7	4.6	1.3	376.0	35.2	32.9

Note: a Average of 3 observations

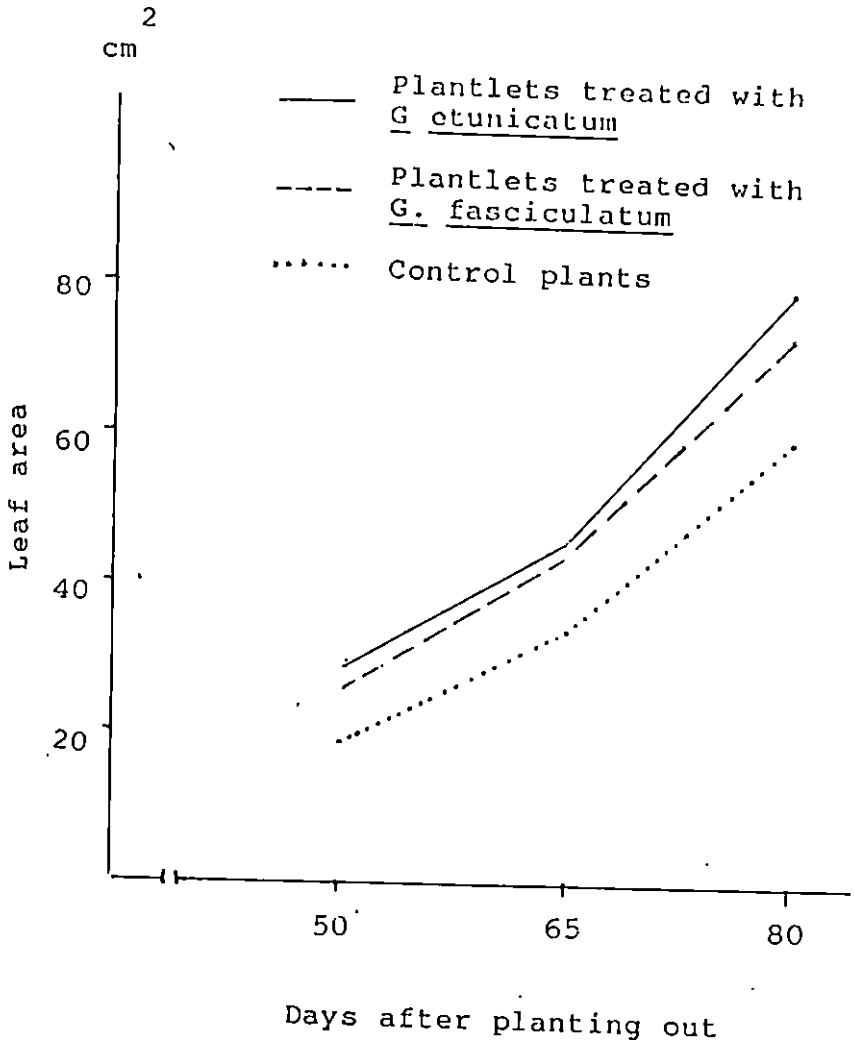


Fig - 1 Leaf area of plantlets as influenced by VA mycorrhizal treatment

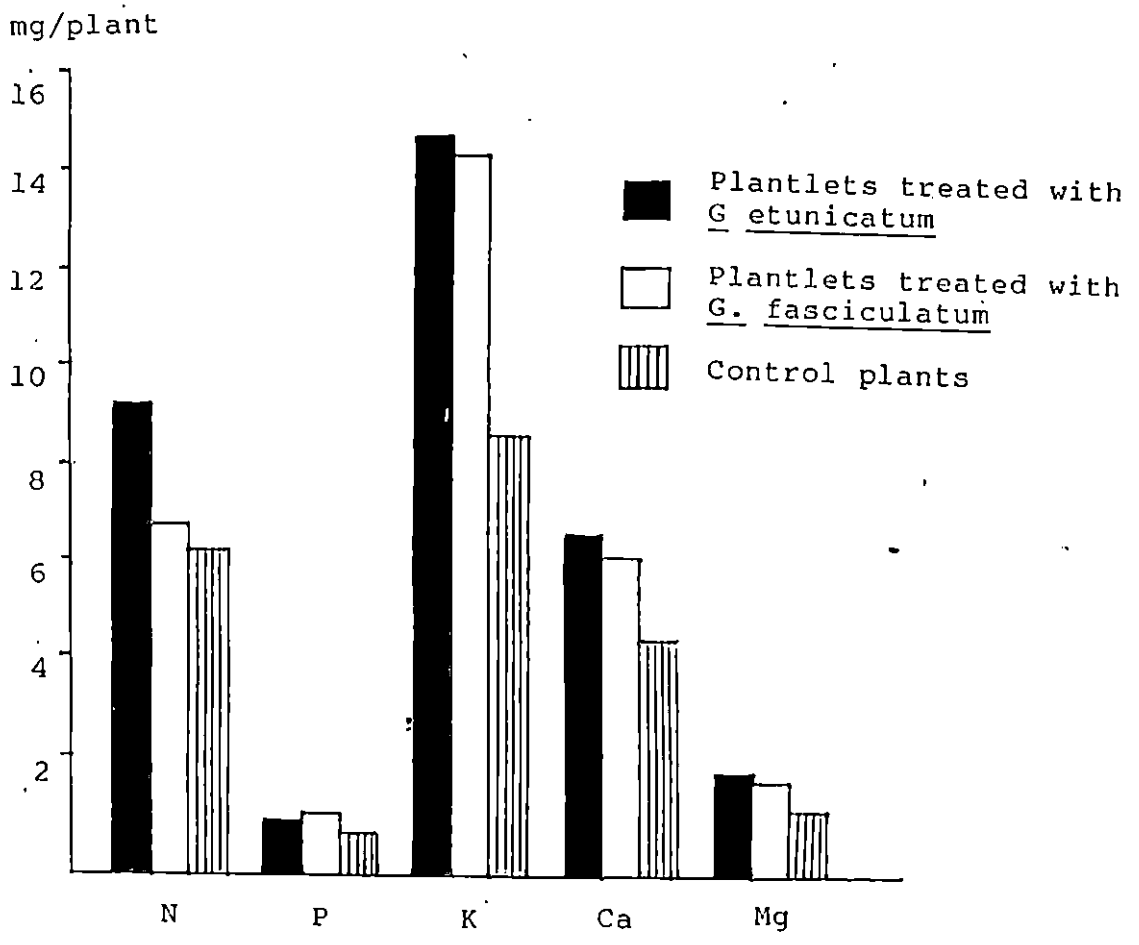


Fig - 2 Content of major nutrients in plantlets (12 weeks after planting out) as influenced by VA mycorrhizal treatment

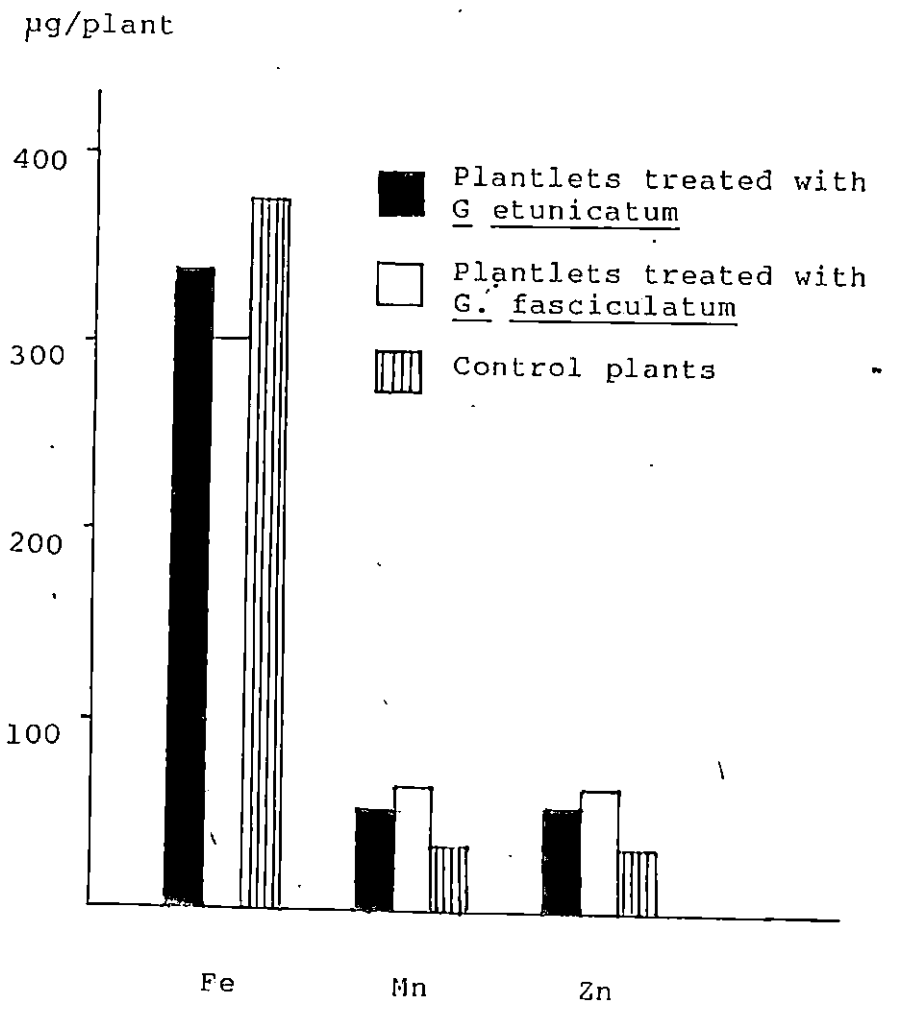


Fig - 3 Content of minor nutrients in plantlets (12 weeks after planting out) as influenced by VA mycorrhizal treatment

g. Nutrient starter solutions

The influence of nutrient starter solutions on ex vitro establishment of jack plantlets was studied. Irrigating them with sterile tap water was sufficient to obtain 80.0 per cent survival (Table 19). Inorganic salts of the MS medium, at one tenth concentration, applied 20 days after planting, registered an establishment percentage of 60.0. However, the same solution, applied 10 days after planting, supported only 40.0 per cent survival. Quarter strength MS inorganic salts and 10:52:10 NPK fertilizer solution, applied 20 days after planting, recorded the same percentage of survival (40.0). None of the plantlets survived when the 10:52:10 NPK fertilizer solution was applied, 10 days after planting.

There was no difference among the growth parameters like number of leaves per plant and plant height, due to the various treatments. However the fresh weight per plant was observed to be higher for the plantlets irrigated with tap water (1.22 g/plant, 60 days after planting out). For the remaining treatments, the values remained on par.

B. Morphological and histological characteristics of plantlets

Table 19

Effect of nutrient starter solutions on *ex vitro* establishment and growth of plantlets

Treatment No.	Treatment		Number of plantlets established (a)	Per cent plantlets established:	No.of leaves after 60 days	Plant height after 60 days (cm)	Fresh weight of plants after 60 days (g)
T1	1/10 MS	10 DAP	2	40.6	4.50	4.75	0.709
T2	1/10 MS	20 DAP	3	60.0	5.00	5.67	0.779
T3	1/4 MS	10 DAP	1	20.0	4.00	4.50	0.722
T4	1/4 MS	20 DAP	2	40.0	4.50	4.50	0.671
T5	10:52:10 NPK	10 DAP	0	00.0	0.00	0.00	0.000
T6	10:52:10 NPK	20 DAP	2	40.0	5.50	5.25	0.676
T7	Tap water		4	80.0	6.25	6.13	1.219

Note: a Average of 5 observations

1. Morphological characteristics

a. Leaf surface morphology

Scanning electron microscopy was used to reveal the surface morphology of jack leaves. In all the four types of leaves studied (in vitro grown leaves, persistent leaves, new leaves produced ex vitro and leaves of field grown plants), trichomes (epidermal hairs) were present. They were found to be unicellular. The trichomes of persistent leaves were found to be collapsed, while in in vitro grown leaves, they were found to be erect (plate 11 ; 12).

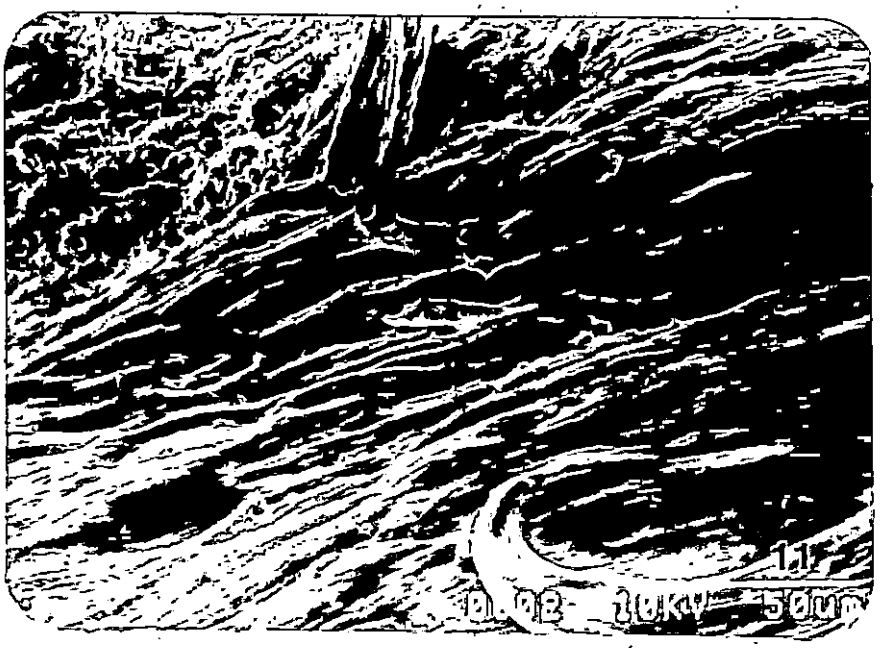
In leaves of field grown plants cystolite (calcium carbonate deposition) was observed (plate 13).

The stomatal shape and guard cells aperture varied among the leaves. The in vitro grown leaves and persistent leaves were found to have circular stomata with large stomatal aperture (plate 14 ; 15). The new leaves produced ex vitro had more or less eliptical shaped stomata with smaller aperture (plate 16). The leaves of control plants were found to have elongated eliptical stomata with very minute stomatal aperture (plate 17).

In in vitro leaves the epicuticular wax deposition was less and irregular (plate 18). In the new leaves

PLATE 11 Adaxial surface of persistent leaf of *ex vitro* established plantlet showing collapsed trichomes (SEM photograph)

PLATE 12 Adaxial surface of leaf of *ex vitro* established plantlet showing erect trichomes (SEM photograph)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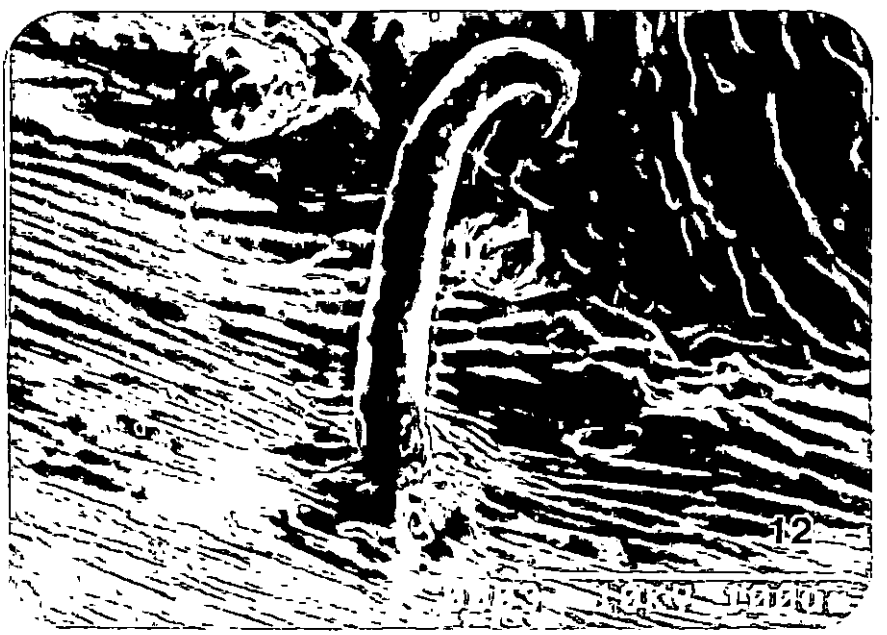
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PLATE 13 Adaxial surface of field grown leaf of control plant showing calcium carbonate deposition (SEM photograph)

PLATE 14 Abaxial surface of *in vitro* grown leaf showing fully opened circular stomata with large stomatal aperture (SEM photograph)


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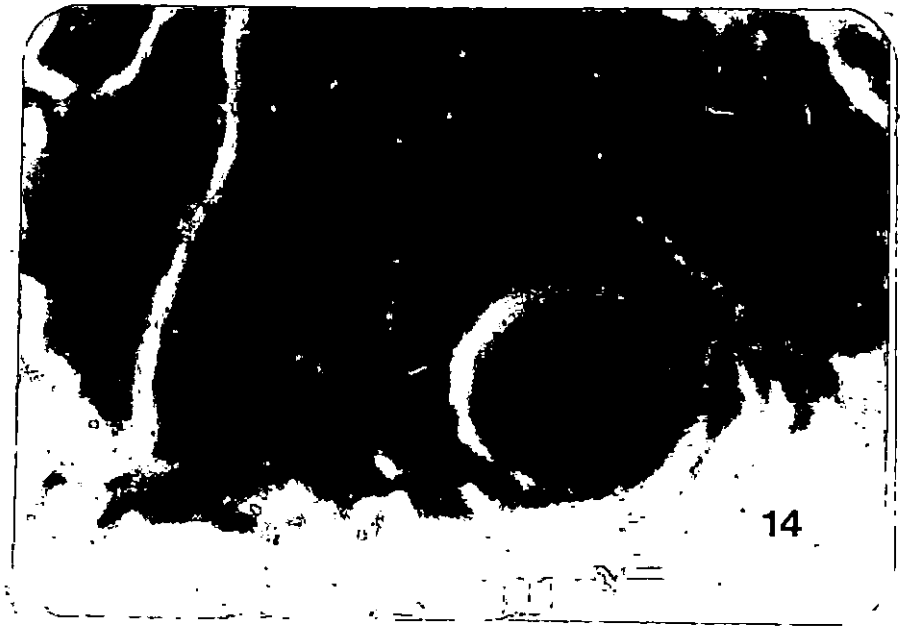
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PLATE 15 Abaxial surface of persistent leaf of *ex vitro* established plantlets showing fully opened circular stomata with large stomatal aperture (SEM photograph)

PLATE 16 Abaxial surface of new leaf formed *ex vitro* showing elliptical stomata with small aperture (SEM photograph)


16
0005 10KV .10um

PLATE 17 Abaxial surface of field grown control leaf showing elongated elliptical stomata with very minute stomatal aperture (SEM photograph)

PLATE 18 Adaxial surface of *in vitro* grown leaf of jack plantlet showing subnormal wax deposition (SEM photograph)


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00000
1.0KV
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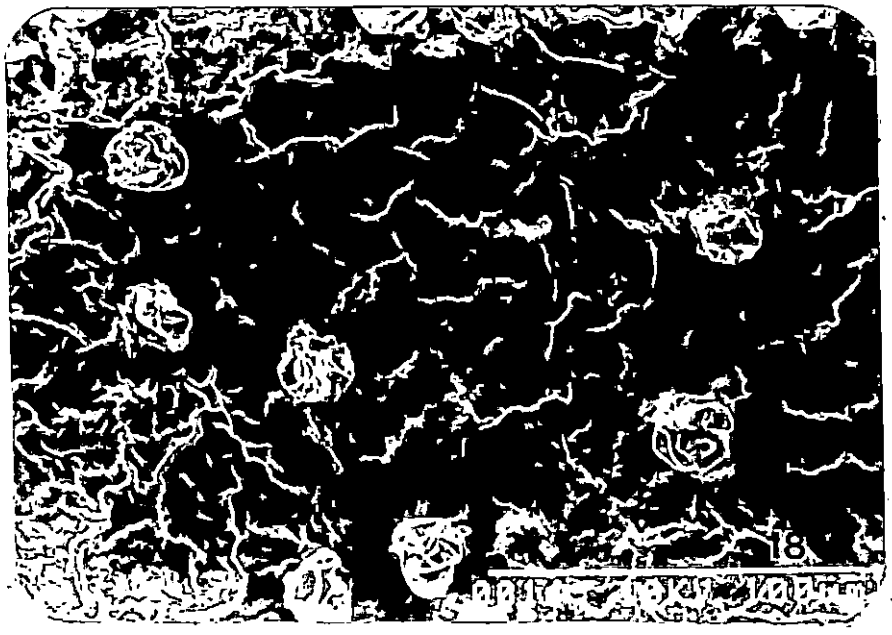
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produced ex vitro, the amount of wax deposition was more but irregularly depicted (plate 19). In field grown leaves there was intense wax deposition in a regular manner (plate 20).

b. Stomatal count

The mean counts of stomata (per 0.5024 mm^2) in the leaves of in vitro plants and persistent leaves were 49.5 and 54.1 respectively (Table 20; appendix - I). The values were lower than those of the new leaves formed ex vitro (66.3) and the leaves of green house grown seedlings (69.7).

2. Histological characteristics

A comparative histological study was conducted among in vitro leaves, acclimatized leaves (persistent and new leaves) and the leaves of field grown plants. The results of the experiment are discussed below.

a. Epicuticular wax

The epicuticular wax was less in in vitro leaves compared to acclimatized and field grown leaves (plate 18 ; 19 ; 20)

b. Epidermis

No marked difference was observed in the epidermis of different leaves. The upper and lower epidermis were found

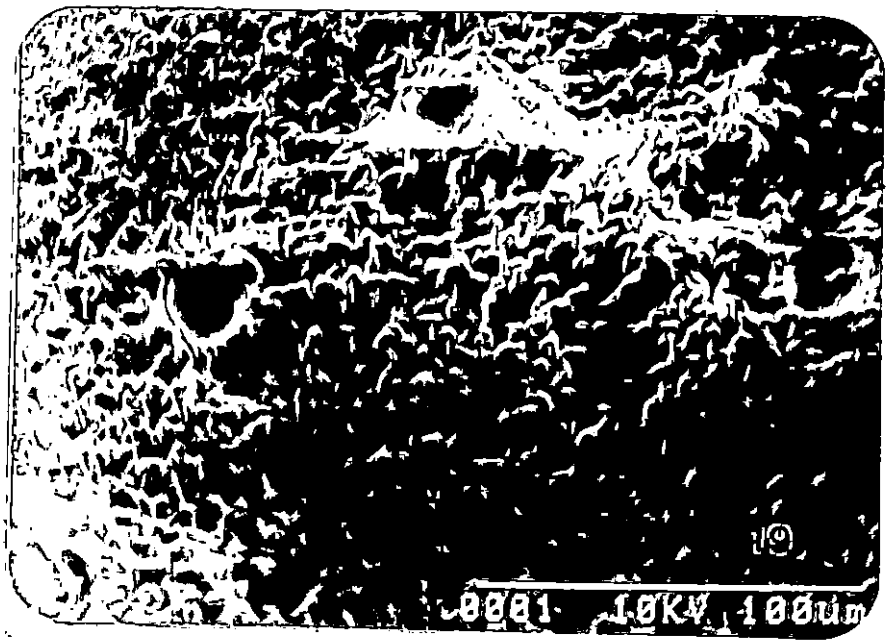
Table 20

Stomatal count of various leaf types of plantlets

Treatment No.	Treatment	Number of Stomata
T1	Control	69.7
T2	New leaves	66.3
T3	Persistent	54.1
T4	*In vitro*	49.5
	CD Value	6.21

PLATE 19 Adaxial surface of new leaf formed ex vitro showing irregular deposition (SEM photograph)

PLATE 20 Adaxial surface of field grown leaf of control plant showing normal wax deposition (SEM photograph)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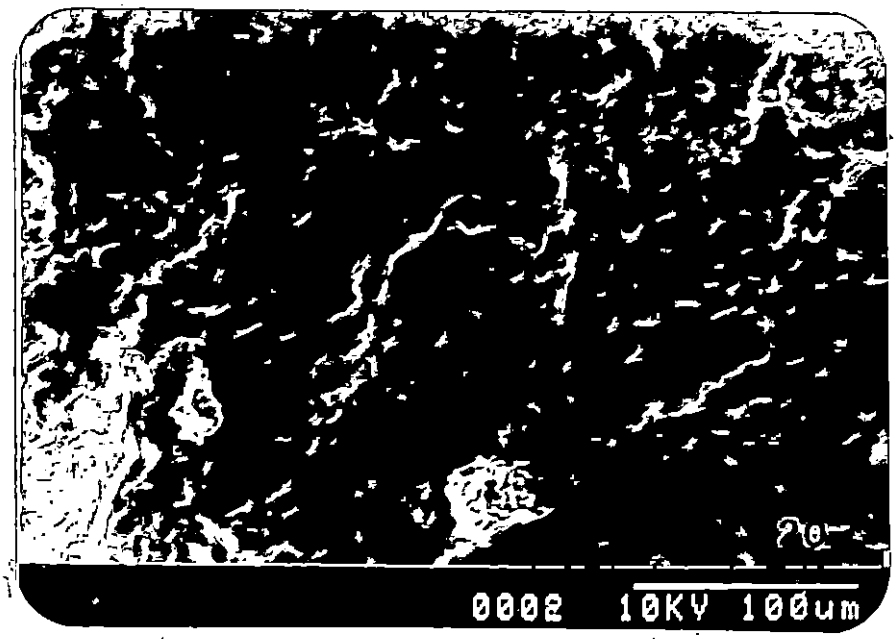
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to be of a single layer of closely packed, slightly elongated, rectangular cells.

c. Palisade parenchyma

In all the types of leaves, only a single layer of palisade cells was observed. The palisade cells of in vitro leaves and persistent leaves were loosely arranged (plate 21) and with inter cellular space. But, in new leaves formed ex vitro and in the leaves of field grown seedlings (plate 22), the palisade cells were more elongated, compactly arranged and without any intercellular space.

d. Spongy parenchyma

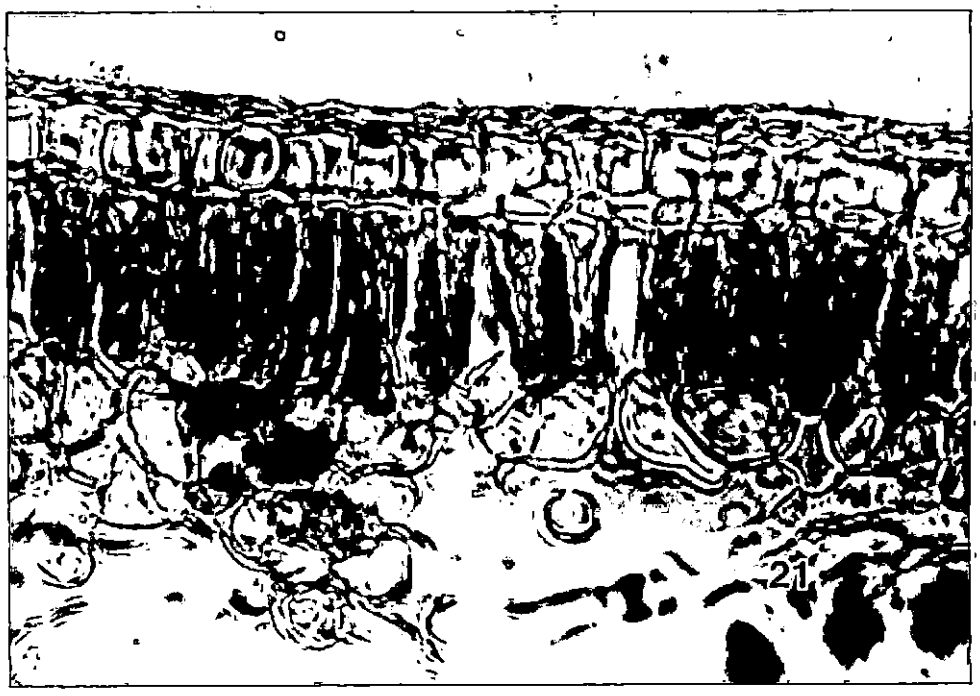
The spongy parenchyma was found to be less differentiated in in vitro and persistent leaves than in the leaves produced ex vitro and the seedling leaves (plate 21 ; 22).

e. Vascular bundles

There was a marked difference in the differentiation of vascular bundles among in vitro leaves, persistent leaves, new leaves produced ex vitro and field grown leaves. In in vitro leaves the number of xylem and phloem vessels were markedly reduced and the sclerenchymatous bundle sheath

PLATE 21 C.S. of *in vitro* grown leaf showing details of palisade and spongy parenchyma

PLATE 22 C.S of field grown control leaf showing details of palisade and spongy parenchyma



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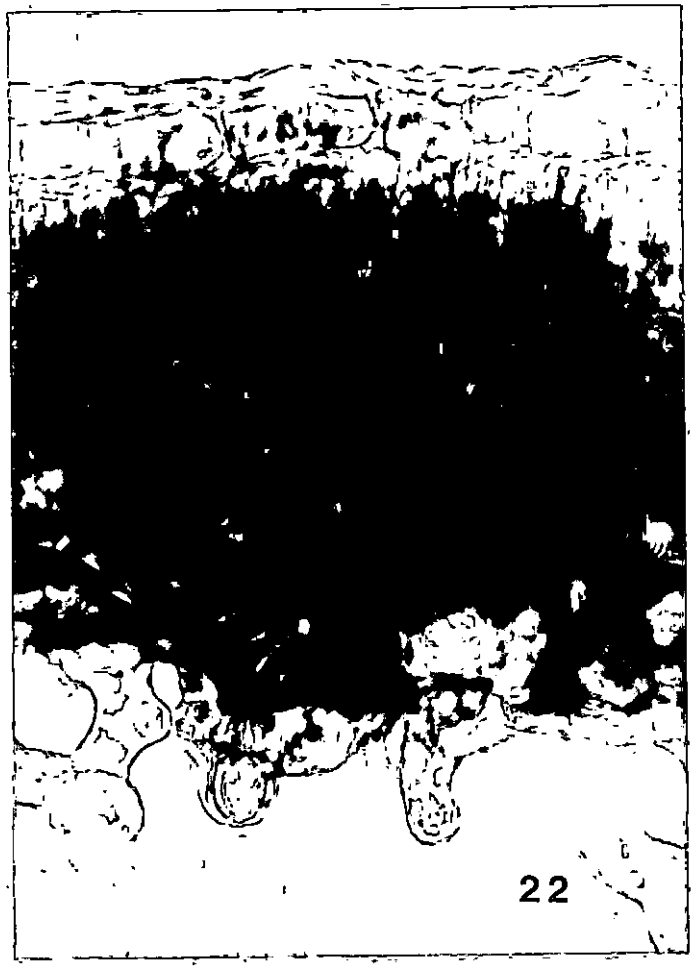

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surrounding the vascular bundles were absent (plate 23). In persistent leaves, leaves formed ex vitro and leaves of field grown plants (plate 24), the vascular bundles were well developed, with regularly arranged xylem and phloem vessels. The vessels of in vitro leaves had annular or spiral thickening (primitive form), while new leaves and leaves of seedlings were pitted (advanced form).

f. Mechanical tissues

Mechanical tissues like collenchyma and sclerenchyma which act as supporting frame work of leaves are generally under developed in in vitro leaves. The collenchymatous cells were not differentiated in in vitro leaves while in all other types of leaves they were in two to three layers on the dorsal side and five to six layers on ventral side, surrounding the vascular bundles. While sclerenchymatous cells were absent in in vitro leaves, they were well developed, surrounding the vascular bundles as a broken ring, in all other types of leaves.

C. Physiological characteristics of plantlets

1. Water loss through leaves

The study revealed that the water loss (per unit area in 105 minutes) from the in vitro leaves (16.0 mg/cm^2) was

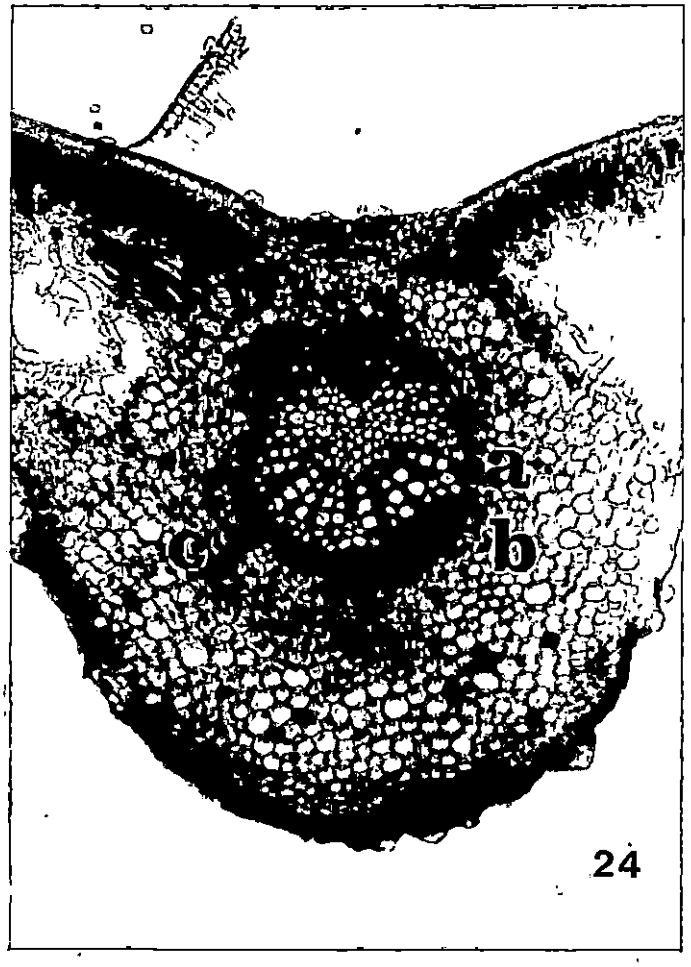
PLATE 23 C.S of *in vitro* grown leaf mid rib of jack plantlet showing details of vascular bundles.

a. xylem vessels
b. phloem vessels

PLATE 24 C.S of field grown control leaf mid rib showing details of vascular bundles

a. xylem
b. phloem
c. bundle sheath

23



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significantly higher than that from the persistent leaves (7.0 mg/cm^2) and new leaves formed ex vitro (2.9 mg/cm^2) (Table 21; appendix-II). The values recorded by the new leaves and the control plants were on par.

Significant difference was observed among the leaf water contents recorded at 15 minute intervals in the in vitro leaves (Table 22 appendix - III). In the case of persistent leaves the values were significantly different upto the fourth interval and were on par subsequently (Fig. IV). In the new leaves the water loss was more or less gradual at the different intervals. The control plants exhibited an initial sudden decrease, followed by a comparatively gradual decrease in water content.

2. Chlorophyll content of leaves

The total chorophyll, chlorophyll a and chlorophyll b contents of the leaves of the green house grown seedlings (3.68, 2.94 and 0.74 mg/g respectively) and of the new leaves produced ex vitro (3.59, 2.65 and 0.93 mg/g respectively) were on par and were significantly higher than those of the in vitro leaves (2.57, 1.89 and 0.67 mg/g respectively) and persistent leaves (1.94, 1.75 and 0.21 mg/g respectively) [Table 23 appendix -IV]. (Fig. V) Values of the latter two were on par.

Table 21

Water loss from various leaf types of plantlets

Treatment No.	Treatment	Mean (mg) water loss at the end of 105 mts/ cm^2
T1	In vitro	16.0
T2	Persistent	7.0
T3	New	3.0
T4	Control	4.5
	CD Value	1.8

Table 22

Water loss at regular invervals from various leaf types of plantlets

Treatment	15	30	45	60	75	90	105
In vitro	3.8	2.6	2.1	2.5	1.5	1.8	1.6
Persistent	2.0	1.3	1.1	0.7	0.6	0.7	0.6
New	0.7	0.6	0.2	0.5	0.3	0.3	0.4
Control	1.1	0.7	0.7	0.6	0.5	0.5	0.3

Table 23

Chlorophyll content of various leaf types of plantlets

Treatment No.	Treatment	Total chlorophyll	Chlorophyll a	Chlorophyll b
T1	In vitro	2.57	1.89	0.67
T2	Persistent	1.94	1.74	0.21
T3	New	3.59	2.65	0.93
T4	Control	3.68	2.94	0.74
	CD Values	0.64	0.46	0.20

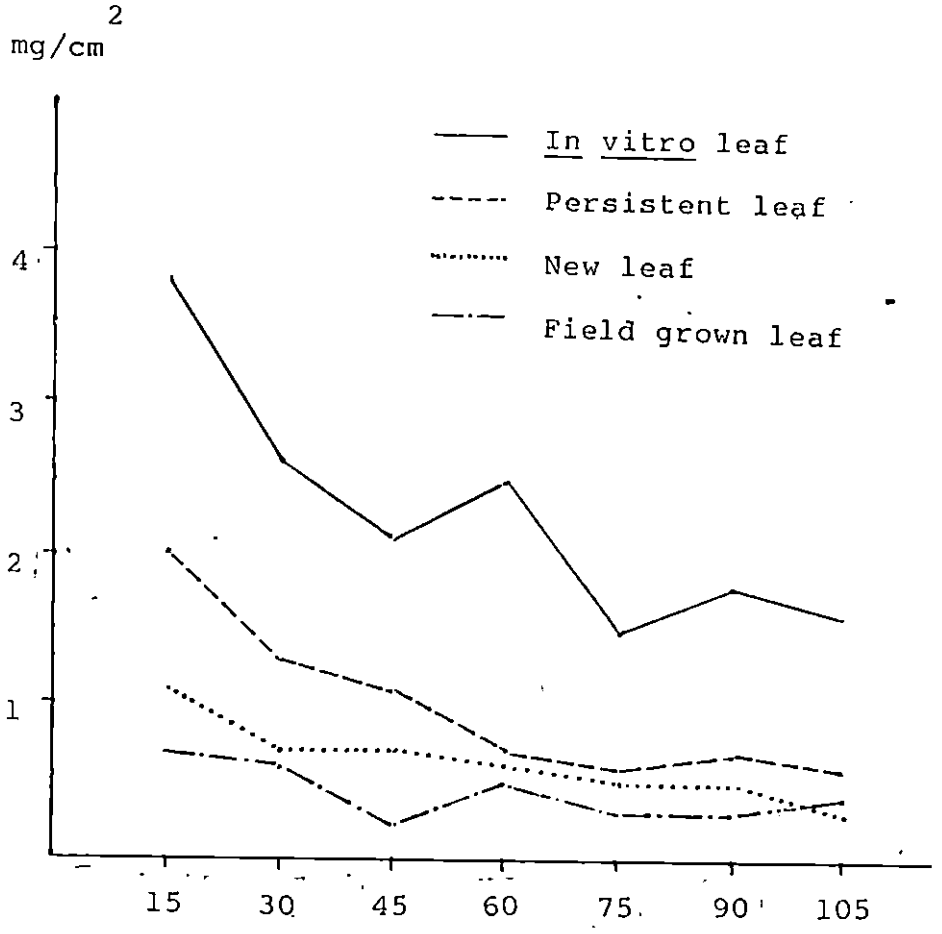


Fig - 4 Water loss at regular intervals from the leaves of plantlets

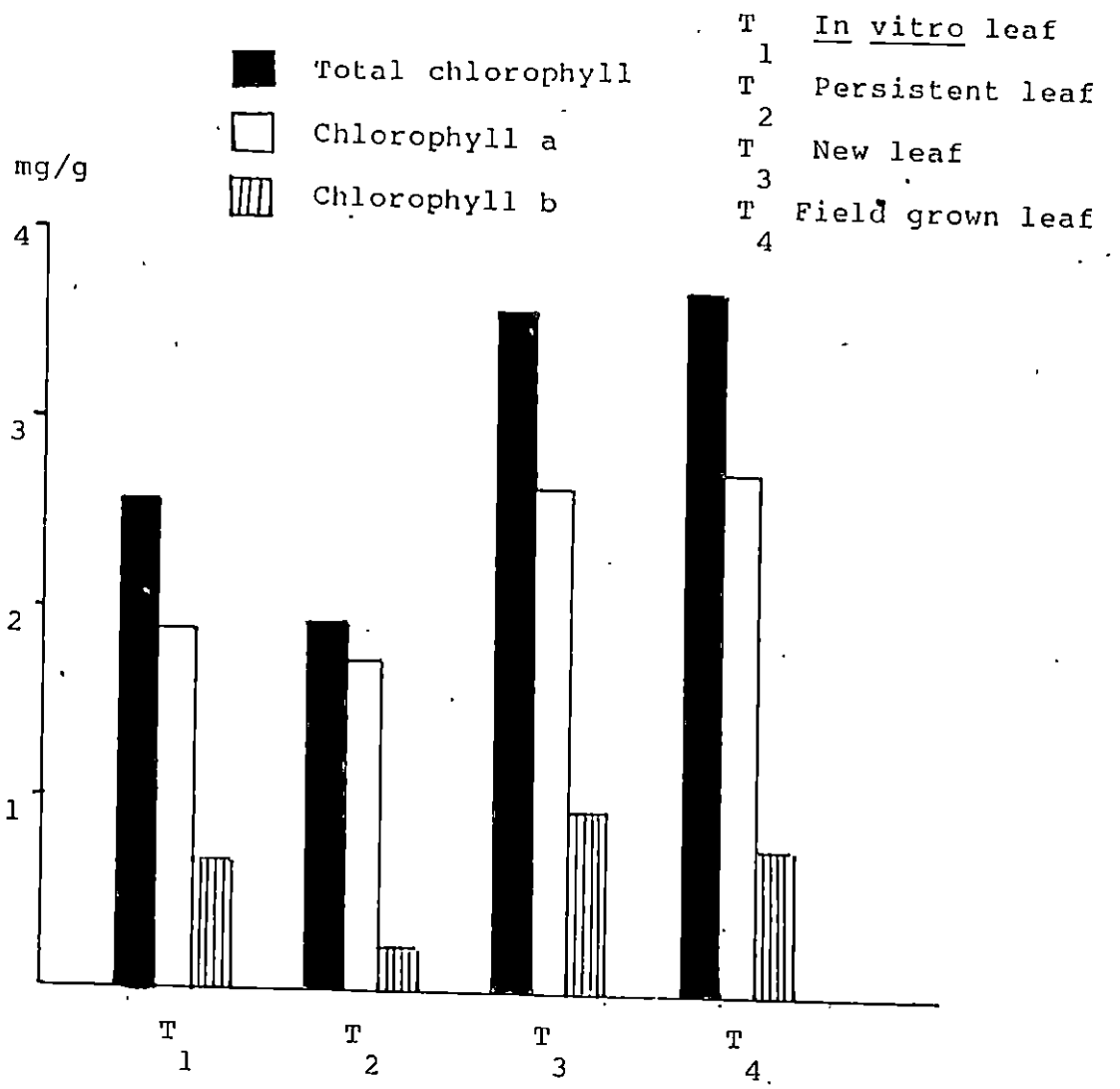


Fig - 5 Leaf chlorophyll content of plantlets as influenced by VA mycorrhizal treatment

DISCUSSION

DISCUSSION

Ex vitro establishment of tissue culture derived plantlets remains critical. Serious field mortality is often encountered, while planting out. The problem is severe in the case of woody species. Micropropagation of jack (Artocarpus heterophyllus Lam.) is also seriously handicapped by the high rate of field mortality during planting out. The present study addressed this problem and encompassed the various aspects associated with it. A protocol for improving the ex vitro establishment of jack plantlets could be evolved. The outcome of the investigations are discussed in the following pages.

In vitro rooting of jack shoot cultures and ex vitro establishment of the plantlets were seen influenced by the concentration of inorganic nutrients in the rooting medium. Half strength of nutrients in the MS medium was found to be the optimum for obtaining maximum rooting (93.3 per cent) number of roots per shoot (6.57), good intensity of root branching (2.57) and ex vitro establishment (53.3%). The same strength was identified as the best for the in vitro rooting of jack shoot cultures by Rajmohan (1985). Higher and lower concentration of the inorganic nutrients reduced the responses. Subnormal strength of inorganic nutrients in MS medium has been found to be favourable for the in vitro

rooting in many instances (Kartha et al., 1974, 1981; Skirvin and Chu, 1979).

The levels of nitrogen (ammonium nitrate) and calcium (calcium chloride) in the rooting medium were observed to have influence on the in vitro rooting of jack shoot cultures and the field survival of the plantlets. Reducing their levels to half strength in the MS medium was beneficial resulting in 100 percent rooting with nitrogen, 83.3 percent with calcium and 66.6 percent ex vitro establishment. Increasing or decreasing their strength resulted in reduced responses. Hyndman et al. (1982) and Mc Comb and Bennet (1982) have also shown the beneficial effects of reducing the nitrogen and calcium contents on in vitro rooting. However, McCown and Mc Cown (1987) observed an increased rooting response in Ulmus spp. when the calcium content in the medium was increased.

It is generally held that lowering the sucrose level in the culture medium is advantageous for the ex vitro establishment as it helps the plantlets to switch over from heterotrophic to autotrophic growth (Conner and Thomas, 1982). This has been found true in several plants like rose (Langford and Wainwright, 1987), cauliflower and chrysanthemum (Short et al., 1987). In contrast, the

present study revealed that the sucrose level should at least be maintained at the normal level of MS medium (30g/l) for the successful establishment (50.0%) of plantlets ex vitro. Wainwright and Scrace (1989) have also concluded like this. The high concentration of sucrose in the medium might have increased the amount of carbohydrate stored in the leaves and increased the utilizable energy available to the plantlets during acclimatization. Sucrose at 30.0g/l maximised the number of roots (5.80) per shoot and good intensity of root branching (2.8) which in turn might have helped the ex vitro establishment of plantlets.

Increased agar concentration in the rooting medium helps the ex vitro establishment in many crops (Leshem, 1983; Marin and Gella, 1987; Short et al., 1987). However, in the case of jack, the in vitro rooting of shoots and ex vitro establishment of plantlets were affected by the increased levels of agar. Increasing the agar concentration above 6.0 g/l inhibited root production and intensity of root branching. Agar is not a totally inert material and contains impurities that can influence the in vitro rooting (Debergh, 1983; Hu and Wang, 1983). The low ex vitro establishment, associated with higher concentration of agar in the rooting medium restricted diffusion of macro

molecules (Romberger and Tabor, 1971) and reduced water availability (Stoltz, 1971) during in vitro hardening.

Improved rooting response has been observed in many instances when activated charcoal was included in the medium, as it absorbed the toxic substances and cytokinins, inhibitory to rooting (Ziv, 1979; Takayama and Misawa,1980). In contrast, in the case of jack, activated charcoal delayed the rooting process and reduced the intensity of root branching. The absorption by activated charcoal is indiscriminate and in the present instance it might have reduced the auxin content, below the required level, for rooting. George and Sherrington (1984) has reported reduced rooting responses with higher concentration of activated charcoal in the medium. The ex vitro survival of the plantlets, however, was supported by the inclusion of 0.1 per cent activated charcoal in the rooting medium.

In vitro rooting of jack shoot cultures was favoured when comparatively larger sized shoots were used. Shoots of >3.0cm length, with three to four leaves, recorded 100.00 per cent rooting, 5.50 roots per shoot and good intensity of root branching (2.75), compared to smaller shoots. Higher food reserves and increased auxin production might have caused the responses.

In vitro rooting of the cultured shoots was found to be influenced by the type and volume of the culture vessels used. Erlenmeyer flasks of 100ml and 150ml capacity were superior to test tubes in this respect. Strong influence of the culture vessels on various *in vitro* responses has been observed earlier (Mc Cown and Sellmer, 1987). However, the mechanism of this influence is not well understood. It may probably be due to the difference in concentration levels of gases, like oxygen and carbondioxide , ethylene and other volatiles within the containers (George and Sherrington,1984).

A significant positive effect of light intensity during the "prior to planting out" stage on the survival of the plantlets ex vitro, was evident in the study. Sixty per cent survival resulted when high light intensity (50 $\mu E\ m^{-2}\ S^{-1}$) was used, while the plantlets not exposed to light did not survive. Murashige (1974) and Hussey (1978) reported that subjecting the plantlets to high light intensity before transplanting helps in better establishment. High light intensity has been shown to increase leaf thickness, mesophyll differentiation, cell division, chlorophyll content and photosynthesis (Lee *et al.*, 1988). Enhanced photosynthesis under the influence of high light intensity helps in building up a high food reserve to be utilised

during transplanting (Murashige, 1978). This might have caused the increased establishment.

Sand was identified as the best suited potting medium, out of the ten tried for supporting the ex vitro establishment of plantlets. This comparatively cheaper material recorded a higher percentage of plantlet survival (53.3) than vermiculite, peat and sand:soil mixture (40.0% each). Sand was an ideal potting medium for maintaining an optimum moisture level and sufficient aeration to the root zone of the plantlets. The moisture level was kept at an ideal 13.5 g/100g of soil. A higher or lower water content in sand itself was proved to be detrimental. Rajmohan (1985), however, observed that vermiculite was superior to sand in supporting the survival of jack plantlets. This contradiction may be due to the difference in the type of device used for humidity regulation around the plantlets.The mist chamber (with intermittent misting)used in the present instance, as against the microscope covers used by Rajmohan to maintain humidity, caused excess moisture accumulation in vermiculite and resulted in decay of roots due to poor aeration.

Plastic pot (5.0x5.0x7.5 cm size) was found to be the best suited container for planting out jack plantlets . It

supported 50 percent survival of the plantlets and was superior to mud pots. Better establishment in plastic pots might be due to its ability to maintain the optimum moisture level of the potting medium, by preventing water loss through the walls.

The maximum field survival (60.0%) resulted when plantlets of 18 to 24 days age were used for planting out. Poor results were obtained when plantlets just after root initiation were used. The active shoot growth that sets in after root initiation and the higher food reserves might have contributed to the higher rate of survival of the aged plantlets. Rajmohan (1985), however, observed that the maximum ex vitro establishment of jack plantlets resulted when one-week old plantlets were used. This contradiction may be, in part, due to the difference in potting medium and humidity maintenance devices used.

Satisfactory percentage of plantlet survival resulted when they were either covered individually with polythene covers (80.0%) or kept in the mist chamber (60.0%). Considering the convenience in handling and maintenance, keeping the plantlets in mist chamber is recommended. The use of the mist chamber (humidity tent), where the vapour pressure deficits of the plantlets, on planting out, is

compensated, remains the method of choice for ensuring survival and maximum growth of the plantlets ex vitro (Sutter and Hutzell, 1984).

As a simple method to avoid rapid water loss from the plantlets, during the ex vitro stage, Selvapandiyan et al. (1988) advocated smearing of the leaves (both surfaces) with glycerol, paraffin wax or grease in ether at the time of planting out. They could obtain 100.0 percent survival of cultured plantlets, due to the mechanical plugging of the stomata,thereby avoiding water loss. However jack plantlets, when given the same treatments did not survive, in the present instance. A low percentage of survival (40.0) was recorded by starch solution application. The treatments might have inhibited the respiratory process and caused poor establishment. Sutter and Hutzell (1984) suggested reduced photosynthesis and phytotoxicity as two possible reasons for the poor results with antitranspirants.

The jack plantlets, incubated with the VAM Glomus etunicatum and G. fasciculatum, at the time of planting out, were observed to have increased ex vitro establishment and growth. The beneficial effects of VA mycorrhizae, during this period, like increased nutrients status, enhanced phytohormone activity and photosynthetic efficiency and

increased stress tolerance might have helped the plantlets for better survival and early establishment. The mycorrhizal mycelia can explore large volumes of soil (St.John, 1980) and help the plants for increased nutrient uptake. This is confirmed by the finding of the present study. However, in the case of cations, the increased absorbtion was preferential, resulting in a relative decrease of iron uptake compared to the other cations like Ca^{2+}, Zn^{2+} and Mn^{2+}. The plants maintain a balance of total anions and cations in the absorption process. The uptake of iron might have been diminished by the influence of the other interacting ions. The increased nutrient uptake and their possible utilisation might have contributed to the enhanced plant growth with respect to the plant height, number of leaves produced, total leaf area, fresh as well as dry weight of plants. Higher photosynthetic efficiency (Sivaprasad and Rai, 1984) and phytohormone production (Allen et al., 1980) have been suggested as the beneficial effects of mycorrhiza in plants. It is likely that the increased uptake of zinc, which is required for the synthesis of IAA within the plant, observed in the present instance, might have caused increased phytohormone activity. A lower percentage of infection by soil borne fungal and other pathogens was noticed in the case of mycorrhizae

treated plantlets. Increased production of phenolic compounds, including phytoalexins has been reported to be responsible in similar cases (Lakshmanan et al., 1987).

Addition of inorganic nutrients to the potting medium is essential for the normal growth of the potted plantlets (Brown and Sommer, 1982; Amerson et al., 1985). However, a negative influence of the nutrient solutions on the survival and growth of jack plantlets was apparent. Irrigation with sterile tap water was sufficient to obtain 80.0 percent survival and initial growth of the plantlets. Little is known of the relationship between applied nutrients and the growth of the plantlets ex vitro. However, the growth can be related to the internal nutrient concentration of the plantlets. It is likely that jack plantlets had adequate nutrient reserves, from the culture media, for supporting their survival and growth under ex vitro conditions in the absence of added nutrients (Rahman, 1988).

The stomatal frequency in new leaves produced ex vitro (66.3) and leaves of field grown seedlings (69.7) were significantly higher than that of in vitro leaves (49.5) and persistent leaves (54.1). Similar results were reported by Brainered et al. (1981) and Dhawan and Bhojwani (1987).

The total water loss from *in vitro* leaves was significantly higher than persistent leaves, new leaves produced *ex vitro* and the leaves of field grown seedlings. The increased water loss from *in vitro* leaves might be due to low epicuticular wax deposition (Grout, 1975; Sutter and Langhani, 1979, 1982) and nonfunctional state of stomata (Brainerd and Fuchigami, 1981 and Marin *et al.*, 1988).

In the *in vitro* leaves excised and exposed to ambient relative humidity, decrease in the water content at fifteen minute interval was significantly different upto the end of the experiment. But in persistent leaves, one hour after the start of experiment, the water loss was on par. In new leaves and field grown leaves the water loss was on par after 15 to 30 minutes of the start of the experiment. The difference in water loss might be due to difference in stomatal functioning among the leaves. The results indicate that in *in vitro* leaves the stomata might have remained open throughout the experiment, while in persistent, field grown and *ex vitro* formed leaves, the stomata might have closed one hour, 30 minutes and 15 minutes, respectively, from the start of the experiment. Similar results were observed by Marin *et al.*, (1988). They found that in detached new leaves and green house grown leaves, the stomatal closure was instantaneous and in *in vitro* leaves it took 20 minutes for

80 percent of stomata to close. However, the significant water loss in in vitro leaves between periods of 15 minutes in the present instance indicates that the stomata would not have functioned.

Scanning electron microscopy has revealed that the epicuticular wax deposition was less on in vitro leaves. The stomata of in vitro leaves were found to be circular with wide stomatal aperture which might have enhanced water loss from tissue cultured plantlets. Similar results were reported by Brainerd et al. (1981) in plum and Donnelly and Vidaver (1984b) in red raspberry .

The histological studies revealed that the palisade parenchyma and spongy parenchyma were underdeveloped in in vitro leaves, which in turn would have enhanced the injury to the tissues of in vitro leaves. In plum the length of the palisade cells was significantly less in in vitro leaves than transferred plantlets (Brainerd et al., 1981). The differentiation of vascular bundles was observed to be improper in in vitro leaves, which might have led to reduced water uptake and enhanced mortality during acclimatization. Similar results were reported by Smith et al. (1986).

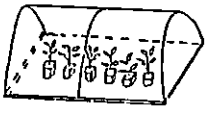
Analysis of chlorophyll content revealed that total chlorophyll, chlorophyll a and Chlorophyll b was

significantly higher in leaves formed ex vitro than in persistent leaves and in vitro grown leaves. The least chlorophyll content being in persistent leaves. Similar results were reported by Donnelly and Vidaver (1984) in red raspberry. However the chlorophyll content in persistent leaves of red raspberry was higher than in in vitro grown leaves.

The various factors influencing the field establishment of jack plantlets, at the in vitro and ex vitro stages could be documented in the present study. An effective protocol for the successful ex vitro establishment of the plantlets could be evolved.(Chart - I) The beneficial influence of VAM in this respect can further be exploited by trying different methods, frequency and stages of inoculation. Inoculating the mycorrhizae at the in vitro rooting stage seems possible and may result in reducing the duration for colonisation in the roots of the plantlets.

Chart - I.

Protocol for the successful ex vitro establishment of the plantlets

factors influencing		stages and duration
MS medium supplemented with BA 5.0 mg/l, NAA 0.2 mg/l, ADS 20.0 mg/l, Calcium pantothenate 0.1 mg/l, sucrose 30.0 g/l and agar 6.0 g/l		Shoot apex cultured in establishment medium
		15 days
MS medium supplemented with BA 5.0 mg/l, NAA 0.2 mg/l, ADS 20.0 mg/l, Calcium pantothenate 0.1 mg/l, sucrose 30.0 g/l, agar 6.0 g/l and insoluble PVP 500 mg/l		Enhanced release of axillary buds
		40 days
MS medium supplemented with BA 2.0 mg/l, NAA 0.2 mg/l, ADS 20.0 mg/l, Calcium pantothenate 0.1 mg/l, sucrose 30.0 g/l, agar 6.0 g/l and insoluble PVP 500 mg/l		Single shoot elongation
		30 days
MS medium (with half strength inorganic salts) supplemented with NAA 2.0 mg/l, IBA 2.0 mg/l, Calcium pantothenate 0.1 mg/l, sucrose 30.0 g/l, and agar 6.0 g/l in 100/150 ml Erlenmeyer flasks with 3.0 cm long shoots inoculated and kept in darkness		In vitro rooting of shoots (stage I)
		7 days
MS medium (with half strength inorganic salts) supplemented with Calcium pantothenate 0.1 mg/l, sucrose 30.0 g/l, and agar 6.0 g/l in 100/150 ml Erlenmeyer flasks and shoots transferred from stage I and kept in darkness		In vitro rooting of shoots (stage II)
		10 days
Plantlets exposed to Light intensity of 50 uE m-2 S-1 prior to planting out		In vitro hardening of plantlets
		18 days
Plantlets of 18 days planted in plastic pot (5.0 X 5.0 X 7.5 cm size) with VAM inoculated sand as potting medium. Irrigation with sterilised tap water maintaining a moisture content of 13.4 g/100 g soil		Transfer to pots
		0 day
Hardening plantlets in mist chamber with an initial relative humidity of 90 per cent and gradually reducing the relative humidity to ambient level. Irrigation with sterilised tap water maintaining a moisture content of 13.4g/100 g soil		Acclimatization
		15 days
		To garden pots containing potting mixture.

SUMMARY

SUMMARY

Ex vitro establishment of jack plantlets was examined in detail, based on the analysis of the influencing factors and the morphological, histological, and physiological characteristics of the plantlets. The study was conducted during 1988-90, at the Plant Tissue Culture Laboratory of the Department of Horticulture, college of Agriculture, Vellayani.

The protocol developed by Rajmohan (1985) was adopted with necessary modification, for the *in vitro* production of jack plantlets.

The salient findings of the study are summarised below.

1. Half strength of MS inorganic salts in the rooting medium was the ideal, supporting 93.3 per cent rooting, 6.57 roots per shoot, good intensity of root branching (2.57) and 53.3 per cent field survival of plantlets.

2. Half concentration of ammonium nitrate in the rooting medium (MS) supported 100.0 per cent rooting (with 5.7 roots per shoot and good intensity of root branching (2.7) in 17.3 days) and 66.6 per cent *ex vitro* establishment of plantlets.

3. Half strength of the calcium salts in the MS rooting medium was the optimum, supporting 83.3 per cent rooting of shoots (with 5.21 roots and good intensity of root branching (3.0) in 20.0 days) and 66.7% field survival of plantlets.

4. Sucrose at 30.0 g/l recorded the maximum number of roots (5.80) per shoot, the highest intensity of root branching (2.8) and 50.0 per cent field survival of plantlets.

5. The use of 6.0 g/l agar was found to be advantageous as it supported 83.3 per cent rooting in 17.6 days (with 5.2 roots per shoot and good intensity of root branching (2.4)) and 66.6 per cent survival of plantlets in the field.

6. The use of activated charcoal was not advantageous for rooting though it supported 66.6 per cent survival of the plantlets.

7. 3.0 cm long shoots (with three to four leaves) were ideal for rooting and recorded 100 per cent rooting, 5.5 roots per shoot and good rooting intensity (2.75).

8. Erlenmeyer flasks of 150.0 and 100.0ml capacity were identified as the superior culture vessels suporting 100.0 per cent rooting, 6.17 roots per shoot and good rooting intensity (2.83).

9. A light of 50 $\mu E\ m^{-2}\ S^{-1}$ for 20 days was found required during the prior to planting out stage for successful *in vitro* establishment of plantlets (60.0%)

10. Sand was identified as the best potting medium out of the ten tried, supporting 53.3 per cent survival of plantlets *ex vitro*.

11. Plastic pots (5.0 x 5.0 x 7.5 cm size) were found to be superior to the other containers tried and supported 50.0 per cent survival of the plantlets.

12. Plantlets of 18 days and above old recorded 60.0 per cent survival *ex vitro*

13. Mist chamber was found to be convenient and successful as a humidity maintenance device for the hardening of the plantlets.

14. Use of the antitranspirants was not advantageous for the establishment of the plantlets.

15. Inoculation of the potting medium with the Vesicular arbuscular mycorrhizae, Glomus etunicatum and Glomus fasciculatum favoured 100.0 and 80.0 per cent ex vitro establishment of plantlets, respectively. The period required for acclimatization was reduced by one week. In the treated plantlets the the plant height, fresh weight, dry weight, number of leaves and total leaf area were significantly increased. Plant analysis showed increased status of total major and minor nutrients, except iron in treated plantlets.

16. Nutrient starter solutions applied during acclimatization did not favour the ex vitro establishment of plantlets. Irrigation with sterilised tap water was ideal.

17. The leaves of the in vitro raised plantlets had improper deposit of epicuticular wax and undeveloped palisade parenchyma, spongy parenchyma, mechanical tissues and vascular bundles, when compared with persistent leaves, new leaves formed ex vitro and field grown seedling leaves.

18. The stomata of in vitro leaves were round and the stomatal apertures were comparatively large. The stomata did not close when exposed to stress conditions,

unlike in new leaves formed ex vitro and leaves of field grown plants.

19. The excised in vitro leaves recorded high rate of water loss, compared to new leaves formed ex vitro and leaves of field grown plants.

20. The mean number of stomata per unit area of leaf was higher in green house grown seedlings compared to the in vitro leaves, persistent leaves and the new leaves formed ex vitro.

21. The total chlorophyll, chlorophyll a. and chlorophyll b contents were significantly less in the in vitro lealves, compared to new leaves formed ex vitro and leaves of field grown plants.

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APPENDICES

APPENDIX I

Analysis of variance table for the stomatal count of different types of leaves.

Source	df	SS	MS	F
Treatment	3	1389.7	463.3	21.56**
Error	16	343.9	21.5	
Total	19	1733.7		

** significant at 1% level

APPENDIX II

Analysis of variance table for the water loss through whole leaves

Source	df	SS	MS	F
Treatment	3	0.5106	0.1700	88.14**
Error	16	0.0310	0.0019	
Total	19			

** significant at 1% level

APPENDIX III

Analysis of variance table for water loss at regular intervals

Source	df	SS	MS	F
Treatment	27	0.1001	0.0037	24.14**
A	3	0.0729	0.0243	158.27**
B	6	0.0188	0.0031	20.46**
AB	18	0.0083	0.0004	3.012*
Error	112	0.0172	0.00015	
Total	166			

** significant at 1% level * significant at 5% level

APPENDIX IV

Analysis of variance table for total chlorophyll, chlorophyll a and chlorophyll b

Source	df	SS	MS	F
Treatment	3	10.51	3.50	15.22**
Error	16	3.61	0.23	
Total	19			
Treatment	3	5.07	1.64	14.26**
Error	16	1.84	0.115	
Total	19			
Treatment	3	1.43	0.4800	21.33**
Error	16	0.36	0.0225	
Total	19			

** significant at 1% level

LIST OF ABBREVIATIONS

ADS	-	Adenine sulphate
BA	-	Benzyl adenine
GA	-	Gibberellic acid
IAA	-	Indole acetic acid
IBA	-	Indole butyric acid
NAA	-	Naphthalene acetic acid
NOA	-	Naphthoxy acetic acid
MS	-	Murashige and Skoog
AC	-	Activated charcoal

EX VITRO ESTABLISHMENT OF JACK (*Artocarpus heterophyllus* Lam.) PLANTLETS

BY

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Abstract of the Thesis

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ABSTRACT

The problem of poor ex vitro establishment of jack plantlets was addressed in a study conducted during 1988- 90 at the Plant Tissue Culture Laboratory of the Department of Horticulture, College of Agriculture, Vellayani. A reliable protocol could be standardised based on the analysis of the factors influencing plantlet production and their ex vitro establishment and characterisation of the morphological, histological and physiological peculiarities of the in vitro raised plantlets.

A rooting medium (MS) containing half strength inorganic nutrients (particularly inorganic nitrogen and calcium salt), sucrose 30.0 g/l and agar 6.0 g/l was identified as ideal for the in vitro rooting and ex vitro establishment of jack plantlets. Activated charcoal was not useful for the purpose. Shoots of 3.0 cm length, with three to four leaves, recorded 100.0 per cent rooting, 5.5 roots per shoot and good root branching (2.75). Erlenmeyer flasks of 100 or 150ml capacity were the superior culture vessels for in vitro rooting. A light intensity of 50 $\mu E\ m^{-2}\ S^{-2}$ for 21 days was required during the prior to planting out stage for successfull ex vitro establishment. Plantlets of 18 days and above old recorded the maximum survival ex vitro. Sand was identified as the best potting medium. However the

moisture level of sand has to be maintained at an optimum of 13.4g/100g of soil. Plastic pot (5.0 X 5.0 X 7.5 cm size with small holes) was found to be superior to the other containers tried. Mist Chamber was found to be convenient and successful as a humidity maintenance device for the hardening of the plantlets. Use of antitranspirants was not advantageous for the establishment of the plantlets. Inoculation of the potting medium with the vesicular arbuscular mycorrhiza Glomus fasciculatum and G etunicatum favoured 80.0 to 100.0 per cent ex vitro establishment of the plantlets; the plant growth was significantly increased in such cases. Nutrient starter solutions cannot be recommended during the initial period of acclimatization as they reduced the survival of the plantlets

Leaves of the in vitro raised plantlets had improper deposit of epicuticular wax and underdeveloped palisade parenchyma, spongy parenchyma, mechanical tissues and vascular bundles. The stomatal aperture was comparatively large. The stomata did not close when exposed to stress conditions. The morphological and histological peculiarities caused high rate of water loss (16.0mg/cm^2 in 105 minutes) from the plantlets when planted out and hence necessitated an initial humidity acclimatization. The mean number of

stomata per unit area, total chlorophyll, chlorophyll a and chlorophyll b contents were less in the *in vitro* grown leaves. The morphological, histological and physiological characteristics of the plantlets were normalised during the *ex vitro* establishment, especially as the new leaves were produced.