STANDARDISATION OF *in vitro* TECHNIQUES FOR ROOTING, HARDENING AND MICROGRAFTING IN COCOA (*Theobroma cacao* L.)

By
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THESIS

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DECLARATION

I hereby declare that the thesis entitled "**Standardisation of *in vitro* techniques for rooting, hardening and micrografting in cocoa (*Theobroma cacao* L.)**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship, associateship or other similar title, of any other University or Society.

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Certified that the thesis entitled "**Standardisation of *in vitro* techniques for rooting, hardening and micrografting in cocoa (*Theobroma cacao* L.)**" is a record of the research work done independently by **Mrs.M.R.Bindu**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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We, the undersigned members of the Advisory Committee of **Mrs.M.R.Bindu**, a candidate for the degree of **Doctor of Philosophy** with major in **Plant Breeding and Genetics**, agree that the thesis entitled "**Standardisation of *in vitro* techniques for rooting, hardening and micrografting in cocoa (*Theobroma cacao* L.)**" may be submitted by Mrs.M.R.Bindu, in partial fulfilment of the requirement for the degree.

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ABBREVIATIONS

ABA	-	Abscissic acid
AC	-	Activated charcoal
$AdSO_4$	-	Adenine sulphate
$AgNO_3$	-	Silver nitrate
BA	-	Benzyl adenine
BAP	-	Benzyl aminopurine
CCC	-	Cycocel
CW	-	Coconut water
GA	-	Gibberellic acid
$HgCl_2$	-	Mercuric chloride
IAA	-	Indole-3-Acetic acid
IBA	-	Indole-3-Butyric acid
2ip	-	2-Isopentinyl Adenine
KIN	-	Kinetin
μM	-	Micromolar
mg l^{-1}	-	milligram per litre
MS	-	Murashige and Skoog
N	-	Normal
PG	-	Phloroglucinol
SH	-	Schenk & Hildebrandt
wks	-	weeks
WPM	-	Woody Plant Medium

Introduction

INTRODUCTION

Tissue culture techniques are becoming increasingly popular as alternative means of plant vegetative propagation. The most significant advantage offered by micropropagation over the conventional methods is that in a relatively short time and space a large number of plants can be produced from a single individual (Bhojwani and Razdan, 1993). Tissue culture plants are reported to grow faster and mature earlier than their seed propagated progenies (Vasil and Vasil, 1980). In tree species, micropropagation offers the scope for large scale multiplication of recalcitrant species within a limited time and reduce the chances of genetic variability in propagules by directly developing shoots from nodal segments (Mittal *et al.*, 1989).

Cocoa (*Theobroma cacao* L.) is one of the most important beverage crops in the world and belongs to the family Sterculiaceae. Cocoa is grown for the beans used in the manufacture of drinking cocoa and chocolate which are very important in the international trade. Cocoa was introduced to India in 1798 and commercial cultivation was started in the 1960's. In India it is grown in an area of 15,000 ha (approximate) with a production of 8,000 t (1991-93) and Kerala stands first in area and production followed by Karnataka and Tamil Nadu. But the estimated internal requirement of cocoa is far higher than the production potential of the cocoa plantation of the country. This shows that there is a need to increase cocoa production in India in order to prevent foreign exchange drain in future.

One of the major reasons for low productivity, and returns from the existing cocoa plantation in the country is that most of these plantation have been

established from seedling progenies which are genetically poor and highly erratic in bearing. The extent of variation is so high that about 75 per cent of the yield is received from about 25 per cent of the plants. This is due to the heterogeneous nature of the plant resulted due to uncontrolled pollination coupled with the self incompatibility system operating. Therefore the recommended practice to achieve yield improvement in this crop is to select elite plants and propagate them vegetatively in order to preserve the unique genetic complexes and heterogeneity that confer superior pod and tree characteristics.

In cocoa, the conventional methods of clonal propagation namely budding and rooting of cuttings are relatively expensive and offer only limited number of propagules. Micropropagation through tissue culture techniques offers the potential for producing large number of uniform plants from selected genotypes in a comparatively shorter time and traits such as faster growth rate, superior yield, quality or disease resistance could potentially be delivered to the farmers 10-25 years earlier than by conventional breeding systems. Micropropagation also preserves the unique genetic complexes and heterogeneity of elite plants.

There are successful reports on the production of shoots from nodal segments under *in vitro* condition both from India and abroad with a remarkable amount of repeatability. But the rooting was always inconsistent and mortality during the hardening stage was very high. In several tree species, rooting remains one of the most critical steps of micropropagation technique. So an attempt was made to standardise the techniques of rooting and hardening of *in vitro* cocoa shoots (Stage III and IV of micropropagation).

Micrografting is a novel grafting technique in which a shoot apex taken from a mother plant is grafted to a young nursery grown plant or to a seedling grown under asceptic condition. The success of this technique suggests that it could also be adopted for the micropropagation of difficult to root tree crops. In cocoa, grafting of *in vitro* derived shoots to *in vitro* or *ex vitro* seedlings was useful to surmount the rooting problem. In the present investigation attempts were also made to standardise micrografting as a method of propagation of cocoa.

Review of Literature

2. REVIEW OF LITERATURE

Tissue culture has been used as a method of micropropagation for the last three decades. The culture of plant tissues has been started as early as 1893, when Rechinger described the formation of callus on isolated fragments of stems and roots. Haberlandt (1902), the father of plant tissue culture was the first to culture plant tissues under *in vitro* condition on a nutrient medium. However his efforts were unsuccessful because he selected a very simple medium which lacked growth regulators. The early development of tissue culture techniques was due to the relentless efforts of many pioneering investigators including White (1934), Gautheret (1939), Nobecourt (1939), Miller *et al.* (1956), Steward *et al.* (1958), Bergman (1960) and Vasil and Hildebrandt (1965, 1967).

The best commercial application of tissue culture techniques has been in the production of true to type plants at a very rapid rate compared to conventional methods (Levy, 1981). Tissue culture plants are reported to grow faster and mature earlier than their seed propagated progenies (Vasil and Vasil, 1980). Multiplication of plants through tissue culture can occur through enhanced formation of axillary shoots, production of adventitious shoots either directly from the explant or through the intermediate stage of callus and also by somatic cell embryogenesis. Shoots obtained by the first two methods were kept for rooting later (Murashige, 1974).

In the first route, meristems like shoot tips or axillary buds are cultured which assume genetic uniformity of the progeny to a great extent (Rao and Lee, 1986). This method is accepted commercially in various crop species for rapid clonal multiplication. The second route, callus mediated organogenesis, is not

recommended for clonal propagation. It may be ideal for variant line selection. Somatic embryogenesis, the third route, is limited to a few species, but results in the most rapid mode of plant production (Evans *et al.* 1981). All these culture systems are achieved in different media constituted by a judicious combination of chemicals, hormones and other growth regulators. In the present investigation, the first route ie. enhanced release of axillary bud has been carried out to get sufficient number of shoots for rooting and micrografting.

2.1 Enhanced release of axillary bud

Axillary buds present in the axils of each leaf has the potentiality to develop into a shoot. In nature, these buds remain dormant for various periods depending on the growth pattern of the plant. Application of cytokinin to the axillary buds can overcome the apical dominance effect and temporally stimulate the lateral bud to grow in the presence of a terminal bud (Sachs and Thimman, 1964).

The conventional method of vegetative propagation by stem cuttings utilizes the ability of axillary buds to take over the function of the main shoot in the absence of a terminal bud. The number of cuttings that can be taken from a selected plant in a year is extremely limited, because in nature vegetative growth is periodic and a minimal size of the cutting (10-12 mm) is necessary in order to establish a plant from it. The enhanced axillary branching method is becoming increasingly popular for clonal propagation of crop plants because the cells of the shoot apex are uniformly diploid and are least susceptible to genotypic changes. It guarantees that the characteristics of the source plant are conserved (Bhojwani and Razdan, 1993).

The advantages offered by enhanced axillary bud release method in tree micropropagation include large scale multiplication of recalcitrant species within a short time and reducing the chances of genetic variability in propagules by directly developing shoots without callus phase (Mittal *et al.*, 1989).

There have been reported attempts to initiate and manipulate tissue culture of *Theobroma cacao* L. since the early work of Archibald (1954). These studies emphasised the recalcitrant nature of this species and the considerable clonal variability in the growth responses *in vitro*.

2.2 Factors influencing success of *in vitro* propagation

The different factors influencing *in vitro* propagation of woody plants are reviewed below.

2.2.1 Size, age and position of explant

As a rule, larger the size of explant, more rapid the growth and greater the rates of survival (Hussey, 1983). If the explant size is small, the cut surface to volume ratio is high and there will be difficulty in the survival of the explant. The type of the explant varies with each plant species and the most suitable one should be determined for each species (Skirvin, 1980). Norton and Norton (1986) studied the effect of explant length (2.5 to 20.0 mm), axillary bud number (0 to 6), presence or absence of apex and explant derivation (top, middle or base of canopy) in the case of *Prunus* and *Spirea*.

Legrand and Mississo (1986) studied the effect of the explant size in the *in vitro* culture of Amelondao cocoa and suggested the existence of minimum explant size below which the buds are incapable of bursting.

The capacity to vegetatively propagate a tree is associated with its juvenility. Generally the more juvenile the specimen, the easier it is to propagate vegetatively. Often some parts of the trees may be mature or senescent while other portions still display juvenile characteristics (Bonga, 1982).

Within any plant, the tissues differ in their degree of determination and thus their ability to undergo morphogenesis. The youngest and less differentiated tissues are found in plant meristems and the culture of this tissue has been successful in a wide range of species (Hughes, 1981). Even the meristematic apices, the centres of growth and organisation in plants undergo changes when the plant matures. Therefore, the tissues derived from these apices behave differently in young and old parts (Bonga, 1980; Hackett, 1980). If true to type vegetative propagation by *in vitro* culture is recalcitrant, it is better to develop methods for vegetative propagation from highly juvenile material.

Rahman and Blake (1988) observed in jack that nodal explant gave more proliferation than shoot tips though there was no significant difference between the two types of explants.

2.2.2 Surface sterilization

The objective of surface sterilization is to remove all the microorganisms present on the explant with minimum damage to the plant part. Explant for surface

sterilization are cut to a size larger than that of the final explant and after sterilization they are trimmed to smaller size and transferred to the medium (Hussey, 1979).

Fungus and bacterial contamination in plant tissue culture are very common. To check these problems fungicides and antibiotics are used either as surface sterilant or medium additive. However, most of the systemic fungicides and some antibiotics inhibit the growth of the plant cultures. Davey *et al.* (1980) suggested that the application of antibiotics like streptomycin, ampicillin or nystatin may be done when the material is infected with known bacterial or fungal contaminants.

The most commonly used surface sterilant is an aqueous solution of sodium hypochlorite. A dilution of 10 per cent (v/v) is normally effective for the purpose, particularly when it is mixed with a surfactant like teepol or similar liquid detergent. Sodium hypochlorite is toxic to plant cells which necessitates the washing of treated tissue twice or thrice with sterile distilled water (Hu and Wang, 1983). Both concentration and time of treatment can be increased or decreased according to the need. Concentration ranging from 1.0 per cent (Minocha, 1980) to 10.0 per cent (Kuo and Tsay, 1977) has been used.

Orchard *et al.* (1979) reported that the excised terminal buds of Amazon cocoa seedling was surface sterilized in 0.1 per cent mercuric chloride for 2 minutes.

Mallika *et al.* (1990) suggested that systemic fungal infection of field explants of cocoa could be substantially controlled by prior fungicidal treatment of mother plants. Freshly prepared chlorine water was found to be an effective sterilant for the explant.

2.2.3 Presence of systemic contaminants

Contamination can be caused by bacteria, fungi or viruses present on the surface of bark, glandular hairs at the nodes and internal tissues (Mathias and Anderson, 1987). Microorganisms present on the outer surface can be eliminated by surface sterilization treatments Those existing within the internal tissues cannot be removed. They cause latent contamination, which is a serious problem associated with woody plant tissue culture (George and Sherrington, 1984).

Wilson and Power (1989) isolated ten systemic microorganisms (bacteria and yeast) from stem sections of *ex vitro* grown rubber plants. To avoid latent infection, plant part may be cultured free of endogeneous microorganisms. Meristem culture was reported in this respect (Eliott, 1972; Galzy, 1972). Growing stock plants under controlled conditions and regularly spraying the plant with systemic and contact fungicides can reduce or avoid the contamination problem to a certain extent (Mallika *et al.*, 1992).

Several workers have reported the use of various fungicides in cultures for reducing fungal contamination (Brown *et al.*, 1982; Shields *et al.*, 1984). Dodds and Roberts (1985) suggested avoiding the use of antibiotics for sterilization because they or their degradation products may be metabolised by plant tissues with unpredictable results.

2.2.4 Seasonal effect on explant establishment

Season of collecting the explant was found to influence the success of plant tissue culture especially for mature trees. It was reported that spring (March-

April) was the best season to initiate tissue culture from mature trees of *Corylus avellana* (Messeguer and Mele, 1987). Explants collected in the months of April-June gave a good response in the micropropagation of *Commiphora wightii*. Sprouting was reduced for the explants collected during July-August and was poor for those collected during September-October (Barve and Mehta, 1993). Contamination rate was found to be higher for the cultures initiated during July-August and September-October. Successful plantlet production in chestnut was obtained with shoots taken during Mid-May (Chauvin and Salesses, 1988). For litchi (*Litchi chinensis*) the test material taken after ten continuous rainy days had a contamination rate of cent per cent and that taken after fifteen continuous sunny days had a low contamination rate of twenty per cent (Yu, 1991).

2.2.5 Culture medium

2.2.5.1 Basal medium

A wide variety of plant tissue and cell culture media have been reported. The earliest and widely used basic media were White (1943) and Heller (1953). Since 1960, most researchers have been using MS (Murashige and Skoog, 1962), B_5 (Gamborg *et al.*, 1968) or SH (Schenk and Hildebrandt, 1972) media. The most popular media used after 1980 were DCR (Gupta and Durzan, 1985) and WPM (Lloyd and McCown, 1980) especially for woody species. The MS salt composition is used very widely, particularly if the desired objective is plant regeneration. The B_5 medium has been used for cell and protoplast culture (Gamborg *et al.*, 1981). The SH medium is similar to B_5, but with slightly higher levels of mineral salts.

The basal media used for the micropropagation of *Acacia aurifolia* was B_5 (Mittal *et al.*, 1989), for tea (Agarwal *et al.*, 1992) and *Passiflora* species

(Drew, 1991) was MS, for *Podocarpus macrophyllus* was White's (Daimon and Mii, 1991) for *Camellia reticulata* cv. Captain Rawes was WPM (San-Jose *et al.*, 1991). The basal media use for cocoa, were liquid LS medium (Orchard *et al.*, 1979), modified MS medium (Passey and Jones, 1983), modified Heller medium (Legrand *et al.*, 1984), half MS (Adu-Ampomah *et al.*, 1987; Mallika *et al.*, 1990, 1992) and MS medium (Adu-Ampomah *et al.*, 1992).

2.2.5.2 Growth regulators

The most important factor in successful tissue culture is the addition of growth regulators (Krikorian, 1982). No universal ratio of auxin and cytokinin has so far been developed for root and shoot induction. For axillary shoot proliferation, cytokinin has been utilized to overcome the apical dominance of shoot and to enhance the branching of lateral buds from leaf axils (Murashige, 1974). The commonly used cytokinins are BAP (Benzyl amino purine), 2ip (2-Iso pentenyl-adenine) and kinetin (furfuryl aminopurine).

Passey and Jones (1983) reported that shoot proliferation in cocoa occured during the first six weeks on media containing BAP, Zeatin or zeatin riboside. Legrand and Mississo (1986) reported a spectacular elongation of buds of cocoa in zeatin containing media. Betrand (1987) reported that the dormant stage of the axillary bud of cocoa could be broken by massive application of cytokinin (BAP) and the buds could be elongated by increasing the exogeneous auxin contents and by applying gibberellic acid (GA). Mallika *et al.* (1990) reported that the bud sprout and leaf expansion from preexisting meristems could be induced in woody plant medium containing kinetin/2iP 1 mg l^{-1} and IAA 0.02 mg l^{-1}.

Some other growth regulators like gibberellins and abscissic acid have sometimes shown dramatic effects in overcoming bud dormancy and achieving organogenesis (Borkowska and Habdas, 1982; Yostuga *et al.*, 1984).

2.2.5.3 Carbon and energy sources

Sucrose is the main carbon energy source for most of the plant tissue culture. Glucose and fructose may be substituted in some cases, but most other sugars are reported to be very poor (George and Sherrington, 1984). Marino *et al.* (1991) reported that shoot proliferation was increased with sorbitol as the carbon source than with sucrose in the case of apricot.

2.2.5.4 Vitamins

The most common vitamins used in plant tissue culture are pyridoxine, nicotinic acid, biotin, riboflavin, folic acid and thiamine. Among these, thiamine is very essential and is usually added in plant tissue culture media at levels of 0.1 mg l^{-1}.

2.2.5.5 Phenolic compounds

Adding phenols to culture media caused an enhancement of callus growth, improved rooting of shoots and a greater rate of shoot proliferation in certain shoot tip cultures (George and Sherrington, 1984). Phloroglucinol added to culture media containing growth substances enhance growth and the rate of axillary shoot production from *in vitro* cultures of *Prunus insitia* (Jones and Hopgood, 1979), *Cinchona ledgeriana* (Hunter, 1979) and *Theobroma cacao* L. (Mallika *et al.*, 1990).

2.2.5.6 Other organic compounds

Other organic compounds which are generally added in plant tissue culture media include myo-inositol, adenine sulphate, activated charcoal, coconut water etc.

The discovery of myo-inositol in coconut water (CW) by Pollard *et al.* (1961) led to the inclusion of inositol in the plant tissue culture media.

Adenine sulphate when added to the medium can enhance growth and shoot formation (Skoog and Tsui, 1948).

2.2.6 Culture conditions

The culture conditions play an important role in the success of tissue culture. The physical form of the medium, light, temperature, relative humidity etc. play an important role in *in vitro* growth and differentiation.

Light requirement for differentiation involve a combination of several components namely intensity, quality and duration. An optimum combination of these is necessary for certain photomorphogenic events. According to Murashige (1977), the optimum day light period is 16 hours for a wide range of plants. Optimum explant establishment and growth of strawberry was at 4000 lux light intensity while maximum propagule growth and development occurs at 6000 lux (Hunter *et al.*, 1983).

Tropical trees require higher culture temperature for culture growth. Hunter *et al.* (1983) reported that the maximum growth for the different

developmental stages of strawberry occured at 28°C. Yeoman (1986) reported that the usual environmental temperature of the species concerned should be taken into account. However most tissue cultures are grown successfully at temperatures around 25 + 2°C.

Relative humidity is rarely a problem except in arid climates, where rapid drying occurs. Hu and Wang (1983) reported that air humidity is infrequently controlled and when it is controlled, 70 per cent has been found to be the most frequent setting.

2.2.7 Genotype

The genotype of the plant chosen for propagation also influence the response in culture. Differences among genotypes in the response of plants to *in vitro* conditions have often been observed (Reed, 1990; Dulieu, 1991; Kristiansen, 1992). Brandt (1994) observed that within clones shoot size was negatively correlated with the number of shoots and positively correlated with the number of roots. Among clones, the number of shoots was not correlated with the number of roots.

2.2.8 Root Induction

Efficient vegetative propagation requires the development of a reliable rooting protocol that work well across a variety of genotypes. The ability of plant tissues to form adventitious roots depends on the interaction of many different endogenous and exogeneous factors. The role of auxins in root formation was well established.

2.2.8.1 Rooting of shoots *in vitro*

The shoots produced *in vitro* can be rooted under *in vitro* conditions and the rooted plantlets can be transferred to soil or the propagules can be treated like microcuttings and rooted *ex vitro* in a non sterile, highly humid, low light environment. Hu and Wang (1983) observed that three phases are involved in rhizogenesis viz. induction, initiation and elongation.

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

2.2.8.2 Factors affecting rooting *in vitro*

2.2.8.2.1 Culture medium

2.2.8.2.1.1 Basal medium

A low salt medium is found satisfactory for rooting of shoots in a large number of plant species. Shoot multiplication was induced in full strength MS medium, while the salt concentration was reduced to half (Garland and Stoltz, 1981; Zimmerman and Broome, 1981) or one third or a quarter (Skirvin and Chu, 1979) for rooting. Relatively high concentration of calcium and nitrogen is essential for rooting (Tripathi, 1971). Calcium prevents leakage of auxin protectors from the tissue into the nutrient medium (Stonier, 1971). Increased calcium content has no stimulatory effect on the rooting of cherry, plum and quince (Nemeth, 1978).

According to Reinert *et al.* (1977), raising the phosphate concentration of the medium can suppress or weaken the root promoting effect of auxin.

Kar and Sen (1985) reported that the roots of *Asparagus racemosus* were initiated when shoots were inoculated in half strength MS basal medium supplemented with 0.5 mg l^{-1} IBA. Rooting of *Eucalyptus grandis* could be achieved on half strength MS salts or White's medium supplemented with low concentration of auxins like IAA, IBA and NAA (Sita and Rani, 1985). Daimon and Mii (1991) reported that the *in vitro* shoots can be rooted on a modified White's medium containing no growth regulators. Rathore *et al.* (1991) reported that the rooting of *in vitro* shoots of desert teak (*Tecomella undulata*) could be done by culturing on half strength MS liquid medium containing IBA 2.5 mg l^{-1} for 48 hours and then transferring to hormone free half strength MS medium. For rooting of cocoa shoots half MS was found to be good (Mallika *et al.*, 1990, 1992; Adu-Ampomah *et al.*, 1992).

2.2.8.2.1.2 Growth regulators

In the micropropagation practice, usually natural auxin IAA and synthetic auxins NAA and IBA are used for rooting. The determination of shoot/root formation is generally dependant on the cytokinin/auxin ratio in the medium (Skoog and Miller, 1957). However the critical balance of growth regulators must be in the tissue itself at the organ forming loci. Lo *et al.* (1980) reported that a high content of cytokinin was deleterious to the initiation and elongation of roots of both monocotyledonous and dicotyledonous plants. But in many plant species, it was shown that optimal root formation occurred in the presence of auxins and cytokinins (Dulits *et al.*, 1975). The best rooting in sour cherries was obtained with BA and

IBA (Ponchia and Roselli, 1980) and in *Dalbergia sisso* with 0.5 mgl^{-1} IAA and 1 mg l^{-1} kinetin (Datta *et al.*, 1983).

Some species of plants require only the transfer of shoots to a hormone free medium for inducing rooting as in *Podocarpus macrophyllus* (Daimon and Mii, 1991). Most of the woody plants require the transfer of shoots to a rooting induction medium (Drew *et al.*, 1993), chronic auxin treatment for a short time (Jha and Sen, 1992) or prolonged exposure in an auxin containing medium (Kar and Sen, 1985).

Pence *et al.* (1979) reported that roots from callus were obtained when leaf, immature ovules and mature cotyledons of cocoa were cultured in a media supplemented with NAA. Passey and Jones (1983) reported that *in vitro* shoots could be rooted in a medium with IBA, NAA and phloroglucinol. Mallika *et al.* (1992) reported that the rooting of the proliferated shoots of seedling cocoa could be achieved by a pulse treatment of IBA (1000 mg l^{-1}) in ethanol. The concentration of IBA was to be enhanced to 5000 mg l^{-1} for rhizogenesis in shoots derived from field explant.

2.2.8.2.1.3 Vitamins and aminoacids

Successful rooting in majority of the tree crops shows that the vitamin formula of MS medium is suitable. Kamada and Haroda (1979) found that amino acids could either stimulate or inhibit direct root formation from internode explants of Torenia, depending on other ingredients presenting in the medium. When IBA and Vitamin D were used together, root formation was improved in some cases (Buchala and Schmid, 1979).

Drew *et al.* (1993) reported that the shoot and root growth of papaya were improved when shoots were transferred after two days from MS medium containing 10 μm IBA to hormone free medium containing 10 μm Riboflavin.

2.2.8.2.1.4 Carbon source

Sucrose is mainly used as the energy source and osmotic agent at a concentration of 20-30 g l^{-1}. Organ initiation is a high energy requiring process and the inhibition of rooting below optimum can be explained by starvations of the heterotrophic shoots and inhibition above optimum may be of osmotic nature.

The mutant cherry clone F 12/1 was successfully rooted even at 50 mg l^{-1} sucrose (Ancora *et al.*, 1982). Elongation of sourcherry roots on hormone free medium depended on the presence of sucrose (20 mg l^{-1}) and the omission of it completely suppressed rooting (Snir, 1983). San-Jose *et al.*, (1991) reported that the best carbon source for the *in vitro* rooting of *Camellia reticulata* was glucose at a concentration of 3 per cent.

2.2.8.2.1.5 Agar

The concentration of agar in rooting experiments varies from zero (liquid medium) to 0.9 per cent, the usual being 0.6-0.8 per cent. Lowering of agar content makes the availability of nutrients and hormones better, but raises the problem of increased water evaporation of the medium. Tabachnik and Kester (1977) found 0.7-0.8 per cent agar to be critical for the rooting of almond. Root primordia formation was successful in apple in agitated, but not in stationary liquid medium (Sriskandarajah and Mullins, 1981). For almond, liquid medium with no auxin and sterile vermiculite as a support was used for rooting (Rugini and Verma, 1982).

2.2.8.2.1.6 Activated charcoal

Activated charcoal (AC) is considered to exert its influence by adsorbing the toxic components released by the inoculum (Fridborg *et al.*, 1978). In the rooting of *in vitro* shoots, activated charcoal exerts a positive influence in stimulating the rooting (Patel and Thorpe, 1984). Rooting rate enhancement in activated charcoal enriched media, has been reported for different species such as raspberry (Welander, 1985), *Sequoiadendron giganteum* (Monteuuis and Bon, 1986). Dumas and Monteuuis (1995) reported that there is a positive influence of the activated charcoal on the rootability of the microshoots resulting in a significant increment in the rooting rates, number of roots, root length and the root score in *Pinus pinaster*. Flynn *et al.* (1990) reported that the activated charcoal at a concentration of 0.15 g l^{-1} was ideal. Mallika *et al.* (1992) reported that 1.0 per cent activated charcoal was best for rooting in cocoa.

2.2.8.2.1.7 Phenolic compounds

Stimulation or inhibition of root initiation by phenolic compounds is due to their interaction with auxins (Nemeth, 1986). The presence of phloroglucinol in media at Stage II can precondition shoots to root at Stage III while they are still multiplying in response to high cytokinin levels (James and Thurbon, 1981). Phloroglucinol at a concentration of 162 mg l^{-1} increased the frequency of rooting and root number in cherry F 12/1 and plum Pixy cultures (Jones and Hopgood, 1979), in M9 and other apple root stocks (James and Thurbon, 1979; Jones and Hatfield, 1976) and in Prunus (Jones and Hopgood, 1979). James and Thurbon (1981) observed that phloroglucinol at 162 mg l^{-1} had no effect per se on the shoot multiplication rate.

But shoot cultures grown in the presence of the compound gave higher rooting percentage than its absence. Effect of phloroglucinol on the rooting of cocoa was reported by Passey and Jones (1983) and Mallika *et al.* (1990).

2.2.8.2.2 Culture conditions

The various culture conditions influencing rooting *in vitro* include light, temperature etc.

Although on intact plants growing in soil, root initiation and growth occurs in darkness, light may influence these processes *in vitro* (George and Sherrington, 1984). But earlier Boxus and Quorin (1974) reported that light was inhibitory for rooting. Tabachnik and Kester (1977) obtained rooting of almond both in light and dark conditions. Keeping the shoots of *Prunus* in the dark during the first five days and then transferring them to illumination increased rooting percentages depending on the quality of light (Standardi *et al.*, 1978). Nemeth (1979) observed higher percentage of rooted plants on BA containing medium, under 2000 lux than 800 lux light intensity. Jordan *et al.* (1982) obtained 80 per cent rooting of *Prunus avium*, although under low light intensity cherry shoots were successfully rooted (Sauer, 1983; Snir, 1983). Best root initiation, development in strawberry was observed at 7000 lux (Hunter *et al.*, 1983). Rathore *et al.* (1991) reported that an initial dark period of 5-7 days favoured root induction in desert teak (*Tecomella undulata*). Good root initiation and root and shoot growth in papaya were obtained when shoots were incubated for 2 days in darkness on a medium containing 10 mM IBA and 3.1 μM Riboflavin before transfer to light (Drew *et al.*, 1993).

Root formation is generally favoured by relatively high temperature and even plants from cool climates have optima much greater than normal soil temperatures. *In vitro* shoots of apple rooted best at 28°C day/22°C night, and if culture temperatures were reduced to 23°/17° or 18°/12°, there were progressive reduction in the number of roots formed (Lane, 1978).

2.2.8.2.3 Genetic background

Rooting ability of the *in vitro* shoots was found to depend largely on the genotype of the explant. It was reported that herbaceous plants can be rooted easily than woody plants. Juncker and Favre (1989) reported that the lack of repeatability in culture establishment, subculture and rooting in the micropropagation of oak is due to between clone difference. But the difference in rooting ability appeared primarily in the clones derived from adult plant material. Such differences were not recorded in juvenile material.

2.2.8.2.4 Physiological influence

Age of the mother tree is important from the point of view of juvenility. Boxus and Quorin (1974) mentioned that the age of *Prunus* propagule influenced root formation. Pierik and Steegman (1975) observed that the ability of Rhododendron stem segments to produce roots decreased with increasing age of the stem. Rooting was higher in juvenile than in adult A 2 apple rootstocks (Welander and Huntrieser, 1981). Vieitez *et al.* (1985) reported that the rooting rate of juvenile *Quercus robus* L. was 83 per cent while that of mature material was 63 per cent by briefly dipping the basal ends of shoots regenerated *in vitro* in concentrated solutions

of IBA. Dumas and Monteuuis (1995) reported that the rooting rates, number of roots, root length and the root score could be affected by the stage of maturity of the explant source in *Pinus* pinaster.

Jones *et al.* (1977) observed that the rooting of M 26 apple was only at the third to fourth month of culture. Increasing number of subcultures have a favourable influence in the rooting of Jonathan and Delicious apple (Sriskandarajah *et al.*, 1982). The length of the final subculture interval (ie. either three, four or five weeks) used immediately prior to the rooting treatment was found to be critical for determining the subsequent rooting responses of *in vitro* shoots of tree paeony and a five weeks subculture regime gave the best rooting performance (Harris and Montall, 1991). The subculture conditions preceding the rooting stage exert influence on root initiation. Quoirin *et al.* (1974) reported that shoots elongated in hormone free medium or with 0.1 mg l^{-1}, GA_3 rooted more easily than small shoots. Rugini and Verma (1982) found a similar response in shoots elongated in low cytokinin media. But Harris and Montall (1991) reported that there was an inverse correlation between rooting capacity and final shoot weight.

2.2.3 Hardening and planting out

Acclimatization or hardening is one of the most important phases of micropropagated plants. The success in acclimatization depends upon not only post-transfer conditions but also the pre-transfer culture condition (Ziv, 1986). Vitrification conditions adversely affect the survival of plantlets *in vivo*.

Light, temperature and relative humidity are the three major factors to be controlled during acclimatisation. Hu and Wang (1983) suggested a period of

humidity acclimatization for the newly transferred plantlets. Three methods of controlling relative humidity are using polythene tent, misting and fogging.

Barnes (1979) maintained high humidity for the newly transplanted watermelon plants under intermittent mist and found the explant survival rate to be poor. Subsequently, the plantlets could be successfully established in the greenhouse by covering them with clean plastic cups to maintain high humidity. The cups were partially lifted for short durations during the second week and later kept removed for 5-6 hours daily. The cups were completely removed subsequently. Broome and Zimmerman (1978) obtained 60 per cent survival rate in black berry by growing the plantlets under inverted glass jars for one to three weeks.

In polythene tent, as the aerial weaning environment is closed, it is possible to take advantage of carbon dioxide enrichment during hardening (Lakso *et al.*, 1986). Rajmohan (1985) reported the use of plastic microscope covers for maintaining 90-100 per cent relative humidity and obtained 55-60 per cent survival of *in vitro* produced jack plantlets.

Sutter *et al.* (1985) reported that the survival of the plantlets depended upon the vigorous growth and newly produced leaves at the time of planting out. Navatel (1982) observed that the success of transplanting and survival of plants greatly depends on the quality of roots. Auxin concentration dependant callus formation may reduce the survival rate of the plant. Lane (1978) reported that the IBA induced roots of apple plants were of better quality than those on NAA medium.

Excessive waterloss was observed from the leaves of apple plantlets immediately after transplanting (Zimmerman and Broome, 1980). High rate of waterloss may be due to the high volume of mesophyll intercellular space (Brainerd *et al.*, 1981), slowness of stomatal response to waterloss (Brainerd and Fuchigami, 1981), reduced quantities of epicuticular wax and reduced layers of pallisade cells. Langford and Wainwright (1987) observed that the leaves grown *in vitro* are incapable of significant photosynthesis because of impaired stomatal mechanism and high water loss.

Improper development of vascular connections between the shoots and the roots might also cause poor establishment of the plantlets (Grout and Asten, 1977; Langford and Wainwright, 1987).

Wainwright (1988) observed that the environment in a tissue culture container is that of very high humidity, low light and usually a constant temperature. As a result leaves on shoots or plantlets of this environment are very poorly adapted to resist the low relative humidity, higher light levels and more variable temperature found *in vivo*.

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

Ramesh *et al.* (1993) found that sand supported 53.3 per cent survival of *in vitro* produced jack plantlets. Vermiculite, peat and sand + soil mixture were found to record 40 per cent survival.

Mallika *et al.* (1992) used a mixture of "Soil rite" and soil for transferring rooted plantets of cocoa. The plantlets were hardened by covering with polythene bags having small holes and by exposing them to ambient conditions after four weeks.

Nutrition of the micropropagules during rooting and hardening has been shown to be species dependant and Scott (1987) has shown that inclusion of fertilizer during hardening can be detrimental of Kalmia, improve plant quality of Rhododendron and is essential for quality Magnolia. Wong (1986) recommended addition of 3 g of nutricate (14:14:14 NPK) to each pot one week after transplanting to get healthier *in vitro* banana plantlets for planting in the main field. Mathur *et al.* (1988) initially irrigated *in vitro* derived java Citronella plantlets with Hoagland and Arnon (1950) nutrient solution for one week. Rahman (1988) reported that a low level or absence of nutrients in the planting substrate for at least 20 days.in the *ex vitro* condition resulted in significantly more growth and survival of plantlets. Nutrient feeding after 20 days improved the further growth and survival. Nitrogen, particularly in the ammonium form was found to be the most inhibitory nutrient during the early stages of establishment. Kesavachandran (1991) obtained cent per cent establishment of Vetiver plantlets with the application of half strength MS nutrient as well as NPK fertilizer solution (10:5:10 g l^{-1}) at weekly intervals. Better vigour of the plantlets was obtained by application of the latter solution. Ramesh *et al.* (1993) found that application of half strength MS basal medium was found to be the best for maximum survival of jack plantlets.

2.2.4 Rooting of shoots *ex vitro*

The cost of production of *in vitro* plantlets could be reduced by changing the rooting stage from an *in vitro* step to an *ex vitro* one. The major cost of producing *in vitro* plants lies in the rooting and hardening stages (Rajeevan and Pandey, 1986). For rooting under *ex vitro* conditions, the shoots for rooting should be handled as microcuttings without using aseptic conditions. By adopting such a technique the sterile tissue culture phase would end with the proliferation of shoots.

Then the rooting could be considered as conventional propagation. Rooting under *ex vitro* condition also facilitates the combining of the rooting stage with acclimatisation which is an essential part of the micropropagation procedure (George and Sherrington, 1984).

2.2.4.1 Methods of *ex vitro* rooting

A. Two step process

The shoots are induced to root and then transferred to rooting media. Micropropagated shoots of *Pinus radiata* were pretreated for rooting by inserting them into water agar medium containing auxins for 5 days. Then they were moved to a potting mixture of peat and pumice for rooting under humidity (Aitken-Christie and Thorpe, 1984).

Maene and Debegh (1983) described a simpler technique of adding a layer of liquid medium over the agar surface in proliferating cultures and *in vitro* proliferated shoots of *Magnolia soulangeana* were successfully rooted *ex vitro* by this method. They further reported that addition of water, auxin solution, sucrose

solution or a combination of auxin and sucrose helped in forming roots in several herbaceous plants. Pulido *et al.* (1994) reported that the rooting of *Pinus canariensis* can be done by pulsing the shoots for 4 h in a 100 μm indole-3-butyric acid aqueous solution and planted in peat : vermiculite : perlite (1:1:1).

B. One step process

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

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

Wochok and Sluis (1980) reported that the wax currant (*Ribes inebrians*) micro shoots can be rooted *ex vitro*. The excised shoots were dipped in Rootone F and planted in trays of sterile medium (peat : perlite : sand 1:1:1) moistened with half strength MS salts. After planting, the trays were covered with clean plastic wrap and placed under the same growing condition as the original cultures. Roots developed in one or two weeks and were gradually hardened by removing the plastic cover for a few hours each day for four weeks. The individual shoots were transplanted in the same medium and kept in a tented mist bench equipped with bottom heat for about two weeks followed by transfer to ordinary green house conditions.

Yeoman (1986) has advocated *ex vitro* rooting approach which may provide a simple, highly efficient and more economic methodology. *In vitro* produced shoots were transferred to pots containing a mixture of peat, vermiculite and sand in a ratio of 4:2:1. The shoots were maintained in a high humidity environment and watered daily. During the first two weeks, a water solution containing 15 mg l^{-1} NAA was administered four times at equally spaced intervals to promote rooting. Twenty per cent shoots rooted after eight weeks.

2.3 Micrografting

Micrografting is a relatively new grafting technique and consists of grafting an apex taken from a mother plant on to (a) a young green house or nursery grwon plant in accordance with accepted grafting technique (*in vivo* micrografting or *ex vitro* micrografting) (b) a decapitated young plant grown from a seedling under aseptic conditions or microcuttings obtained from *in vitro* vegetative multiplication (*in vitro* micrografting) (Jonard, 1986). This technique is usually used to transfer the meristem tips of virus infected plants to virus free seedlings (Novarro *et al.*, 1975). The success of this technique suggests that it could be adapted for the micropropagation of fruit trees to graft known varieties on to dwarfing root stock (George and Sherrington, 1984), to rejuvenate the mature shoot materials (Francelet, 1979; Hackett, 1985) and to study the histological nature of graft unions (Gebhardt and Goldbach, 1988).

In vitro grafting has been described for peach (Alskieff, 1977), apple (Lundergan *et al.*, 1978), plum (Negueroles and Jones, 1979) etc.

Micrografting is effected by inserting small shoot apices in to inverted T shaped incisions immediately below the cut surface of a decapitated rootstock. Alternatively the apices are placed directly on to the cambium layer of the cut surface. The survival of micrografted citrus apices depends on their size. Although very small apices have to be used for virus clones (making the technique difficult and unreliable), larger apices could be used if micrografting were to be carried out for propagation purposes (George and Sherrington, 1984).

Yidana *et al.* (1987) reported that cocoa is recalcitrant to micropropagation with only occasional spontaneous rooting. They suggested that the buds produced under *in vitro* condition can successfully be grafted on to aseptically germinated seedlings. This is of greater utility in the gene banks and also an *in vitro* propagation method for recalcitrant material.

Novardo (1990) reported shoot tip grafting *in vitro* of citrus as a means of obtaining virus free plants. Citrus plants produced by shoot tip grafting maintained the same ontogenic age as the infected shoot tip source plant and in many cases showed an increase in vigour presumably due to elimination of the pathogen. The application of shoot tip grafting to some conifers may produce rejuvenation or at least a reinvigoration of mature trees.

Deogratius *et al.* (1991) studied the growth parameters on apricot shoot tip grafting *in vitro*. Actively growing shoot tips (0.5-1.0 cm) were excised from (a) dormant bud collected from November to February (b) vegetative flushes from plant growing in the field (c) *in vitro* derived vegetative shoots. The effect of rootstocks, cutlivar, temperature, composition of the medium and growth regulator treatments on the success of grafting on rootstock seedlings growing *in vitro* were studied. The

best source of shoot tip was from *in vitro* derived plants. The best time for grafting was March-May and particularly October. Etiolation of the rootstock increased grafting success. GA_3 appeared to stimulate shoot tip growth of grafted plants. The best results were obtained with grafting of 0.5-1.0 mm shoot tips in Nemaguard seedlings which were then cultured at 25°C with a 16 h photoperiod in a medium comprising of MS salts, modified White's vitamins and 3 per cent sucrose.

Aguilar *et al.* (1992) attempted micrografting of somatic embryos of cocoa to *in vitro* derived seedling rootstock and found that complete plant regeneration needed about 10 months. Best results were obtained using simple culture medium, three weeks old rootstocks and somatic embryos without cotyledons.

Starrantino (1992) described a micrografting technique to obtain citrus clones free from virus and virus like diseases. It includes shoot tip grafting of very small shoot apices (0.1-0.3 mm) on to the apical or lateral part of decapitated epicotyl seedlings rootstock cultured *in vitro*. The apical portion of the rootstock in lateral micrografting is decapitated after germination of the grafted apex. The germination percentage was about 40 per cent for apical grafting and 63 per cent for lateral grafting. The percentage success rate was increased by dipping the apex and seedling in a solution of 6 BAP at 0.5 ppm.

A successful micrografting technique was developed for *Pistacia vera* (Abousalim and Montell, 1992). High levels of graft union were achieved when shoots from stage II cultures of four year old *P. vera* cv. Mateur were grafted on to *in vitro* raised seedling roostocks. Light and Flourescence microscopy investigation revealed that vascular continuity was established across grafts by three weeks.

Joseph (1994) reported that the plantlets from somatic embryogenesis of cocoa can be successfully micrografted.

2.4 Anatomical studies

Loewe (1990) studied the histo-anatomy of rooting in walnuts (*Juglans regia* L.) *in vitro*. It was useful to study the origin and development of roots and to find out whether the vascular connection is continuous between shoots and developing roots or there is any intervening callus which prevented the development of vascular connections.

Material and Methods

3. MATERIAL AND METHODS

The present study, 'Standardisation of *in vitro* techniques for rooting hardening and micrografting in cocoa (*Theobroma cacao* L.)' was carried out at the Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara. The *in vitro* studies were conducted in the Plant Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture during 1993-96. The materials and methods used for the present study are detailed in the following sections.

3.1 Explant

Explants were collected from elite 8-10 year old cocoa trees grown in the farm of Cadbury - KAU Co-operative Cocoa Research Project at the College of Horticulture, Vellanikkara. The different genotypes used in the study were GIV 4.1, GVI 50, GVI 67 and S 44.1. Budded plants of these clones maintained in the glass house also served as explant source, especially during the rainy season.

3.1.1 Collection of explant

Single node segments and shoot tips from fan shoots of mother plants either from the field or the glasshouse previously protected with fungicides were used as explants. The mother plants were regularly sprayed with the systemic fungicide Bavistin (0.2%) and the contact fungicide Dithane M-45 (0.3%) in an interval of three days.

3.1.2 Preparation of the explant

Nonwoody stem segments 6-8 cm long were collected from the fan branches of mature trees at the I_2 stage (Greathouse *et al.* 1971) of flushing. The leaves were trimmed and the shoots were immersed in 0.1 per cent Bavistin for 30 minutes. These shoots were taken out and washed thoroughly in tap water containing a few drops of the surfactant teepol. The water was allowed to drain off and the shoots were air dried for about 30 minutes on a blotting paper. The dried shoots were thoroughly swabbed with cotton dipped in 70 per cent ethyl alcohol and moved to the Laminar air flow cabinet. Shoot tips, single node cuttings of 0.5-4.0 cm long retaining the petiole along with a part of the lamina and cuttings without petiole were used.

3.1.3 Surface sterilization

Surface sterilization was carried out under perfect aseptic condition. The Laminar air flow cabinet used was radiated with ultra violet rays. The working table and sides of the Laminar air flow were thoroughly wiped with absolute ethyl alcohol. Sterlized forceps, petridishes, surgical blades and blotting paper were used. The explants were surface sterilized. The different sterilants used are listed in Table 1. In all cases, the explants were kept submerged in the sterilant for the required period shaking at frequent intervals. The solution was drained off and the explants were rinsed three to five times with sterile distilled water to remove all traces of the sterilant from the surface. The explants were then transferred to the culture medium.

Table 1. Sterilants used, concentration and duration of treatment for *in vitro* culture of nodal explants

Pretreatment	Surface sterilant	Concentration (%)	Duration (Min)
Nil	Mercuric chloride	0.05	2 4 6
Nil	Mercuric chloride	0.1	2 4 6
Nil	Chlorine water		2 4 6
Bavistin 0.1%	Chlorine water		2 4

3.2 Culture media

The basal media used in the present study were full strength and half strength MS medium (Murashige and Skoog, 1962), Woody Plant Medium (WPM) (Lloyd and McCown, 1980), White's medium (White, 1954) and SH medium (Schenk and Hildebrandt, 1972). The composition of the different media used are listed in Table 2. The basal media were supplemented with different cytokinins, auxins and other growth regulators.

3.2.1 Preparation of culture media

One litre each of the stock solutions of macro and micro elements including iron and vitamins were prepared by dissolving adequate quantities of each element. The chemicals used for preparing various media were of analytical grade from British Drug House (BDH), SISCO Research Laboratories (SRL), Qualigens, Merck or Sigma. Growth regulators and aminoacids were prepared and stored under refrigerated conditions. Standard procedures (Gamborg and Shyluk, 1981) were followed for preparation of media.

Specific quantities of stock solutions of chemicals were pipetted out. Sucrose, inositol, phloroglucinol and charcoal were added. Glass distilled water was poured to get the required volume of the medium. pH of the solution was adjusted to 5.8 using 1 N NaOH/HCl. To prepare semisolid medium, agar was added and melted. The medium was then poured into culture vessels which were washed thoroughly, rinsed with distilled water and dried. These tubes were plugged with non absorbent cotton and sterilized at 15 psi and 121 °C for 15-20 minutes (Monaco *et al.*, 1971). In the case of liquid medium, strips of Whatman No.1 filter paper

Table 2. Chemical composition of different culture media used for shoot growth and rooting

Constituents	Amount mg l^{-1}			
	Murashige and Skoog MS (1962)	Woody Plant Medium WPM (1980)	Schenk and Hildebrandt SH (1972)	White W (1954)
1	2	3	4	5
Inorganic				
Ammonium nitrate	1650.000	400.000		
Ammonium sulphate	-	-		
Ammonium dihydrogen phosphate	-	-	300.00	-
Borid acid	6.200	6.200	5.000	1.500
Calcium chloride-2 hydrate	446.000	96.000	200.000	-
Calcium nitrate-4 hydrate	-	556.000	-	300.000
Cobalt chloride-6 hydrate	0.025	-	0.100	-
Copper sulphate-5 hydrate	0.025	0.250	0.200	-
Ferric sulphate	-	-	-	2.500
Ferrous sulphate-7 hydrate	27.800	27.800	15.000	-
Manganese sulphate-4 hydrate	22.300	22.300	-	-
Magnesium sulphate-7 hydrate	370.000	370.000	400.000	720.000
Na_2-EDTA-2 hydrate	37.300	37.000	20.000	-

Contd.

Table 2. Continued

1	2	3	4	5
Potassium iodide	0.830	-	1.000	0.750
Potassium nitrate	1900.000	-	2500.000	80.000
Potassium sulphate	-	990.000		
Potassium dihydrogen phosphate	170.000	170.000	-	-
Potassium chloride	-	-	-	65.000
Sodium dihydrogen phosphate	-			
Sodium molybdate-2 hydrate	0.250	0.250	0.100	
Zinc sulphate-7 hydrate	8.600	8.600	1.000	3.000
Organic				
Inositol	100.00	100.000	1000.000	-
Nicotinic acid	0.500	0.500	5.000	
Thiamine HCl	0.100	1.000	5.000	
Pyridoxin HCl	0.500	0.500	0.500	
Glycine	2.000	2.000	-	-
Others				
Sucrose w/v	3.0%	5.0%	3.0%	3.0%
Agar w/v	0.8%	0.8%	0.8%	0.8%

sheets were used to support the explants. The filter paper sheets were cut into long strips and were made in the form of M shaped bridge. These bridges were inserted into the tubes and the medium was poured into these tubes. After sterilization the culture tubes were kept in an air conditioned culture room.

3.3 Inoculation and culturing of explants

The transfer of explants to culture tubes was done in a Klenzoids Laminar air flow chamber under aseptic conditions. For inoculating the explants to the culture medium, the cotton plug of the culture vessel was removed, the vessel neck was flamed and the sterile explants were quickly transferred into the medium using the sterile forceps. The neck of the culture vessel was again flamed and the cotton plug was replaced.

The culture vessels were then kept in the culture rooms where they were incubated. Artificial illumination was provided using white fluorescent lamps for 12-14 hours per day for shoot production. For rooting, cultures were mostly incubated in the dark.

3.4 Season of explant collection on *in vitro* survival of explant

An experiment was conducted to standardise the best season for explant collection and inoculation in which culture establishment was more and contamination rate was the minimum. For this purpose explants were collected and inoculated year round from January to December. Observations on the percentage of cultures survived were recorded after three weeks.

3.5 Shoot induction

The standardised medium for shoot initiation and growth (Mallika *et al.*, 1992) with modified levels of growth supplements were used in the present investigation (Table 3). When the shoots were about three weeks old, subculturing was done in different media for shoot elongation (Table 4).

3.6 Root induction

Rooting was attempted through different methods in the *in vitro* derived shoots. Both *in vitro* and *ex vitro* rooting were tried. *In vitro* rooting included auxin treatment either by incorporation in the media or by pulse treatment. Auxin synergists like phloroglucinol was also tried for rooting.

3.6.1 *In vitro* rooting

Shoots of 2-5 cm long were excised from the shoot proliferating culture. These shoots were then used for root induction.

3.6.1.1 Culture medium

The basal media used for *in vitro* rooting were full strength/half strength/quarter strength MS (Murashige and Skoog, 1962), WPM (Lloyd and Mc Cown, 1980), White's medium (White, 1954) and SH medium (Schenk and Hildebrandt, 1972). Both liquid and solid media with varying levels of agar, sucrose, activated charcoal and the phenolic compound, phloroglucinol were tried at different concentrations (Table 5). Effect of Vitamin B_2 (Riboflavin) on rooting was also tested.

Table 3. Growth regulators for axillary bud release and shoot elongation

Basal medium	Combination of growth regulators ($mg\ l^{-1}$)
WPM + 200 $mg\ l^{-1}$ phoroglucinol	Nil
	2iP 2
	2iP 5
	2iP 5 + Adenine sulphate 0.5
	2iP 5 + $AdSO_4$ 1
	2iP 5 + $AdSO_4$ 1 + $AgNO_3$ 1
	2iP 5 + $AdSO_4$ 1 + $AgNO_3$ 5
	2iP 5 + $AdSO_4$ 1 + $AgNO_3$ 5 + CCC 0.75

Table 4. Subculturing media for shoot elongation and proliferation

Basal media	Growth regulators	Concentration ($mg\ l^{-1}$)	Growth supplements	Concentration
MS	Nil	Nil	Activated charcoal	Nil 0.1 0.25 0.5
WPM	Nil	Nil	,,	Nil 0.1 0.25 0.5
SH	Nil	Nil	,,	Nil 0.1 0.25 0.5
WPM	$AdSO_4$	0.5 1	,,	0.5 0.5
WPM	Cycocel	0.5 0.75	,,	0.5 0.5
WPM	Silver nitrate	1.0 5	,,	0.5 0.5
WPM	IBA	0.1 0.5	,,	0.5 0.5
	Streptomycin Sulphate (antibiotic)	50,100, 150,200 250,300	,,	0.5 0.5 0.5
WPM	Nil	Nil	Phloroglucinol + Activated charcoal	0 0.5
WPM	Nil	Nil	Phloroglucinol + Activated charcoal Phloroglucinol + Activated charcoal	100 0.5 200 0.5
WPM	GA	1,2,3, 4,5	Activated charcoal	0.5
WPM	,,	,, + KIN 3	,,	0.5
WPM	ABA	1.0	,,	0.5
WPM	ABA + KIN	1.0 3.0	,,	0.5

Concentration of AC in percentage

3.6.1.2 Growth regulators

3.6.1.2.1 Prolonged auxin treatment

Auxins like IAA, IBA, NAA and 2,4-D were used in varying concentrations by incorporating in the media (Table 5). The shoots (2-5 cm long) excised from the shoot proliferating cultures were kept in this media either for four days or cultured continously for three weeks. The shoots kept for a short time were then transferred to a charcoal added basal media for further root elongation.

3.6.1.2.2 Chronic auxin treatment

For chronic auxin treatment auxins were tried at higher concentrations and different durations (Table 6). The treatment time varied from a few seconds to few hours. For a very short duration pulse treatment, the required quantity of the growth regulator was dissolved in absolute ethyl alcohol. If the treatment was for a longer duration, the required quantity of auxin was first dissolved in 0.1N NaOH, the volume was made up with distilled water and the solution was sterilised in an autoclave. The excised shoots were then transferred to test tubes in which the auxins were added and kept in an orbital type shaker cum incubator for the required time. After the pulse treatment, the shoots were taken out, allowed to dry and then placed in an auxin incorporated or auxin free media.

3.6.1.2.3 Combinations of growth regulators

Different combinations of growth regulators, both auxins and cytokinins tried for root induction are listed in Table 7. The role of phloroglucinol, the phenolic hormone synergist, in root induction was also studied.

Table 5. Media and physical conditions for *in vitro* rooting

Treatment		Concentrations*
Basal media	WPM, SH, MS, White's	Full, half and quarter strength
Carbon source	Sucrose	0, 30, 50 gl^{-1}
Auxin	IAA, IBA, NAA 2,4-D	0.5, 1.0, 2.0, 5.0, 10.0 0.1, 0.5, 1.0
Cytokinin	BA, 2iP, Kinetin, Zeatin	0.1, 0.5, 1.0
Other growth regulators	Ethrel, GA, ABA	0.1, 0.5, 1.0
Vitamin	Riboflavin	2
Activated charcoal		0, 2.5, 5.0, 7.5, 10.0 g^{-1}
Agar		0, 2.0, 4.0, 6.0, 8.0 gl^{-1}
Phenolic compound	Phloroglucinol	100, 200, 400, 1000
Physical conditions	Temperature Light Intensity	$28\pm2^{\circ}$, $24\pm2^{\circ}$ 0, 4000 lux

* mg l^{-1} wherever not specified

Table 6. Pretreatments for *in vitro* rooting

Growth regulator	Concentration mg l^{-1}	Duration	
		Pulse treatment	Long duration treatment (hours)
IBA, NAA, IAA	100, 500, 1000		2, 4, 10, 24, 36, 48
	1000, 2000, 3000, 4000, 5000	3 sec, 1 min	
IBA + NAA	1000 + 1000 3000 + 3000	3 sec, 1 min	

Table 7. Combination of growth regulators for *in vitro* rooting

Basal media : ½ MS + AC 0.5%

Growth regulators	Concentration mg l^{-1}
IBA + NAA	0.5, 1.0, 2.0, 5.0, 10.0 (5 x 5 combinations)
IBA + IAA	1.0, 2.0, 5.0, 10.0 (4 x 4 combinations)
IBA + 2,4-D	1.0, 2.0, 5.0, 10.0 x 0.1, 0.5, 1.0 (4 x 3 combinations)
IBA + BA	1.0, 2.0, 5.0, 10.0 x 0.1, 0.5, 1.0 (4 x 3 combinations)
IBA + 2iP	1.0, 2.0, 5.0, 10.0 x 0.1, 0.5, 1.0 (4 x 3 combinations)
IBA + KIN	1.0, 2.0, 5.0, 10.0 x 0.1, 0.5, 1.0 (4 x 3 combinations)
IBA + Zeatin	1.0, 2.0, 5.0, 10.0 x 0.1, 0.5, 1.0 (4 x 3 combinations)
IBA + Ethrel	1.0, 2.0, 5.0, 10.0 x 0.1, 0.5, 1.0 (4 x 3 combinations)
IBA + GA	1.0, 2.0, 5.0, 10.0 x 0.1, 0.5, 1.0 (4 x 3 combinations)
IBA + ABA	1.0, 2.0, 5.0, 10.0 x 0.1, 0.5, 1.0 (4 x 3 combinations)
IBA + Phloroglucinol	1.0, 2.0 x 100, 200, 400, 1000 (2 x 4 combinations)

3.6.1.3 Physical, physiological and genetic factors

The physical conditions tried for rooting were temperature and light (Table 5). To find out whether physiological maturity has an influence on rooting, shoots from juvenile seedlings, from mature plants after the stage II culture and shoots after the fifth subcultures were used for rooting. Different genotypes like S 44.1, G VI 67 and G VI 4.1 were selected to find out the effect of genotype on rooting.

Observations like number of cultures established, number of cultures rooted, characteristics of roots like root length, presence of laterals, etc. were recorded. Anatomical sections were taken from the point of origin of roots (root-shoot transition zone) to study whether there was establishment of vascular connections between the roots and shoots. The procedure followed for anatomical studies are described in detail in section 3.12. The rooted plants were planted out and subjected to different hardening procedures as described in section 3.8.

3.6.2 *Ex vitro* rooting

A trial was attempted to get rooting under *ex vitro* condition by providing high humidity (90 per cent RH) and cool temperature (24 + 2°C). The rooting was tried by two methods.

3.6.2.1 One step method

In this method, the shoots excised from the shoot proliferating cultures were directly planted in sterilized potting mixture (Table 8) with and without

pretreatment (Table 6). The shoots were then subjected to different hardening procedures as described under section 3.8.

3.6.2.2 Two step method

The excised shoots were first cultured in a root induction medium for 4 days. The shoots were then planted out in different potting mixtures for *ex vitro* rooting.

The number of days taken for plant establishment, number of plants established, number of plants rooted etc. were recorded.

3.7 Planting out

In vitro produced plantlets were planted out from the test tube following a series of hardening processes. After proper root development under *in vitro* condition, the plants were selected. The cotton plug was removed and distilled water was poured into the tube. This was kept for half an hour for softening the agar. The plants were then taken out carefully and washed gently under running tap water. The medium adhering to the roots was removed completely by using a camel hair brush. The roots were then dipped in 0.2 per cent Bavistin for five minutes and the plantlets were transferred to small pots containing potting mixture. The pots were immediately protected under high humidity.

3.7.1 Standardisation of potting mixture

Different potting mixtures were used to select the best one for maximum survival and establishment of the plantlets (Table 8). The potting mixtures were used after autoclaving for 45-60 minutes.

Then it was filled in small pots or in polythene bags with a hole for drainage.

3.8 Hardening procedures

The temperature and humidity during hardening was controlled by different methods (Table 8).

3.8.1 Covering the potted plants with polybags

The potted plants were covered with polybags of size 20 x 15 cm and the plants were kept in a hardening unit. After two days holes were punched on these bags for better aeration.

3.8.2 Covering with microscope cover

Two or three potted plants were covered together by a polyprophylene microscope cover of 60 cm height and 45 cm diameter at the base. The hardening procedure was carried out in the Laboratory. The covers were removed and the pots were transferred to the hardening unit when the plants were showing signs of establishment.

3.8.3 Covering with polybags under mist

The plants in the pots were covered with polybags of size 20 x 15 cm. Holes were punched on the cover and the pots were then kept under mist in a mist chamber.

3.8.4 Keeping the plants in brick tunnel

The plants were planted out in small polythene bags having a size of 10 x 5 cm. Small holes were punched at the basal half and filled with gravel. The top portion of the bag was filled with sand. The plants were then kept in a tunnel made of bricks which was tightly covered with polythene sheets. The sheet was positioned by using bricks. The whole structure was placed in a hardening unit.

About 15-20 days after planting out, the plants showed initial signs of establishment in the pot. The plants were exposed for increasing periods during the day for hardening. A convenient schedule used was up to 10 am during the first week, up to 11 am during the second week and up to 12 noon during the third week. From the fourth week onwards, they were kept exposed throughout the day under shade. After 50-60 days the individual plants were transferred to larger polythene bags or pots containing a homogeneous mixture of equal proportion of sand, soil and farm yard manure. At this stage the plants were similar to any other container plant.

The percentage of survival and hardening time under different treatments were recorded. Growth rate of established plants indicated by increase in height and number of leaves at definite intervals were also observed.

3.9 Nutrient supply

Different treatments like drenching with ½ MS and ½ WPM salt solution without organics at weekly intervals were carried out (Table 8).

Table 8. Treatments for hardening and planting out

Potting mixture	Soil rite, Sand, Vermiculite, Potting mixture (Sand:Soil:FYM 1:1:1) Sand + Potting mixture (1:1) Soil rite + Potting mixture (1:1) Vermiculite + Sand (1:1)
Nutrient supply	Drenching with ½ MS/½ WPM without organics
Hardening procedure	Covering with polybag, covering with microscope cover, covering and keeping under mist, keeping in brick tunnels

3.10 Field planting

The plantlets were ready for field planting after four months of growth in the polybag.

3.11 Micrografting

Cocoa shoots were found to be recalcitrant to rooting. So an attempt was made to standardise the technique of micrografting in cocoa to help to save the time and resources in micropropagation. Two types of micrografting were tried - *in vitro* and *ex vitro* micrografting.

3.11.1 *In vitro* micrografting

3.11.1.1 Rootstock material

The rootstock used was axenic seedlings of cocoa raised in test tubes of size 200 x 25 mm.

3.11.1.1.1 Raising of axenic seedlings

Tree ripe pods were collected and surface sterilized by immersing in 0.1 per cent mercuric chloride for one hour. The seeds were extracted and the mucilage along with the seed coat was peeled off. Surface sterilization of the seeds were done in the Laminar air flow unit by treating them in mercuric chloride (0.1%) for three minutes.

3.11.1.1.2 Culture media and conditions for raising seedlings

In order to standardise an ideal medium which would favour quicker and

better germination, the cocoa seeds were cultured in different media and conditions (Table 9).

3.11.1.2 Scion material

Scion material proposed to be micrografted included the following:

1. Shoots regenerated from nodal segments of field grown trees
2. Plantlets/shoots recovered from the germinating somatic embryos
3. Haploids recovered from anther culture

The procedure followed for the production of *in vitro* shoots from field explants is described in detail in section 3.5.

3.11.1.2.1 Regeneration of plantlets through somatic embryogenesis

Developing cocoa pods (90-100 days old) were collected form 8-10 year old trees growing in the field. Pods were surface sterilized with 0.1 per cent $HgCl_2$ for one hour and the zygotic embryos were excised aseptically. The embryonic axis was removed and the cotyledons were cultured. The medium used was MS + NAA 1.8 mg l^{-1} + thiamine 1 mg l^{-1} + CW 15 per cent + sucrose 4 per cent (Joseph, 1994). The embryoids produced were then germinated in ½ MS liquid medium with 5 per cent sucrose.

3.11.1.2.2 Production of haploids through anther culture

Young flower buds were collected from profusely flowering plants in the field. Size of the flower buds was standardised by correlating it with the binucleate stage of the pollen grains. The buds were surface sterilized in 0.1 per cent $HgCl_2$

Table 9. Culture media for raising seedlings of *Theobroma cacao* L.

Basal media	Physical condition	Sucrose percentage	Temperature	Light intensity
Full MS	Solid/liquid	0, 3, 5	24±2°C, 28±2°C	0, 4000 lux
Half MS	Solid/liquid	0, 3, 5	24±2°C, 28±2°C	0, 4000 lux

Table 10. Culture media for anther culture in *Theobroma cacao* L.

1	MS + CW 15 + 0.1 BAP + 1 NAA + 126 PG
2	MS + CW 15 + 0.25 BAP + 1 NAA + 126 PG
3	MS + CW 15 + 0.5 BAP + 1 NAA + 126 PG
4	MS + CW 15 + 0.75 BAP + 1 NAA + 126 PG
5	MS + CW 15 + 1 BAP + 1 NAA + 126 PG
6	MS + Ascorbic acid 100 + 2iP 4
7	MS + Ascorbic acid 100 + 2,4-D 4
8	MS + Ascorbic acid 100 + IAA 4
9	MS + Ascorbic acid 1000 + Kinetin 4

for 5 minutes and washed thrice in sterile distilled water. The anthers were dissected out aseptically under a dissection microscope and were cultured in different media (Table 10).

3.11.1.3 Methods of micrografting

The different methods of micrografting tried were side grafting, cleft grafting and epicotyl grafting. In side grafting, the stock plant should have a smooth and straight section in the stem. The diameter of the scion should be slightly smaller than that of the stock. A slanting cut was made on the stock at the desired position. The base of the scion was also prepared by making a long slanting cut at one side and a small cut on the other side so that it would be like a wedge. The scion was then inserted into the stock and was fixed in position.

In cleft grafting the top portion of the seedling was cut and removed. A vertical split for a distance of 1-1.5 cm was made down the centre of the stock. The basal end of the scion was cut into a gently sloping wedge of about 0.5-1 cm long. The scion was gently inserted into the stock and was fixed in position.

In epicotyl grafting, the age of the rootstock should be one week old and the leaves should not started unfurling. The stem portion above the cotyledon (epicotyl region) was beheaded. Seedling with a straight stem at the area of grafting was carefully selected. The grafting technique was as that of wedge grafting.

Grafting was done with surgical blades (No.22 and 24) fitted on a long blade handle and forceps of different size. The graft union was tightly held in position until proper joining took place. Copper wire, cotton thread etc. were used for tying.

3.11.1.4 Age of rootstock

To find out the optimum physiological age of the rootstock for micro-grafting, seedlings of two, three, four and five weeks were selected and the different methods of grafting were tried with scions of 2 cm having at least two leaves **(Table 11).**

3.11.1.5 Position of grafting

To find out the right position of grafting, a slanting cut was made on the rootstock. The different positions of grafting tried were above the cotyledons, 2 cm and 4 cm below the cotyledons **(Table 11).**

3.11.1.6 Size of scion

Scions of different size - 0.5 cm, 1.0 cm, 1.5 cm, 2.0 cm and more than 2.0 cm - were used for micrografting. Plantlets regenerated from embryoids were also used. The position of grafting was 4 cm below the cotyledons **(Table 11).**

The micrografted seedlings were then replaced into the liquid medium and allowed to grow in the same culture tube. The culture was frequently replenished with fresh medium until the graft was planted out.

3.11.1.7 Planting out of micrografts

After 2-3 weeks, the grafted seedlings were planted out in a sterilized potting mixture and covered with polythene covers of size 20 x 15 cm or with microscope covers to maintain the humidity. A slanting cut was made on the seedlings above the graft joint and the seedling tip was snapped back until the shoot was properly hardened. The top of the rootstock was completely removed in about two months and the grafts were transferred to larger pots.

Table 11. Micrografting trials conducted in *Theobroma cacao* L.

Method of micrografting	Side grafting, cleft grafting, epicotyl grafting
Age of rootstock	2 wks, 3 wks, 4 wks, 5 wks
Position of grafting	Above the cotyledon or 2 cm and 4 cm below the cotyledon
Size of scion	0.5 cm, 1.0 cm, 1.5 cm, 2.0 cm or more

3.11.2 *Ex vitro* micrografting

The *ex vitro* micrografting was carried out by using nursery grown cocoa seedlings. Grafting was tried both on one month old seedlings and on two months old seedlings with a second tier of leaves. The method involved was cleft grafting. The seedlings were decapitated and the stem tip was split open. The scion with a wedge shaped base was inserted in the split of the rootstock and fixed in position using polythene film. Scions of different sizes viz. bud initials, shoots 1-1.5 cm long with one tender, expanded leaf and shoots with one or more hardened leaves were used. The grafted seedling was immediately covered with polybags and fastened in position using rubber bands. The grafts were gradually exposed to ambient conditions from the second week onwards and by fifth week the plants were completely hardened. At this stage they were transferred to larger pots. Usual phytosanitary measures were taken to avoid infections. Observations like the percentage of survival of the grafts, number of days taken for graft union etc. were taken.

3.12 Anatomical studies

Anatomical sections of collar region of rooted plantlets and graft union of micrografts were taken. It was then stained with saffranin and examined under a light microscope. Photomicrographs were taken.

3.13 Statistical analysis

Statisticall analysis of the data was carried out in Completely Randomised Design following Panse and Sukatme (1985). Transformation of the data was carried out wherever necessary.

Results

4. RESULTS

The results of various experiments conducted to standardise the optimum culture conditions for the rooting, hardening and micrografting of *Theobroma cacao* L. are presented in the sections to follow.

4.1 Shoot induction

The shoots required for rooting, hardening and micrografting were produced under *in vitro* condition as follows.

4.1.1 Explant

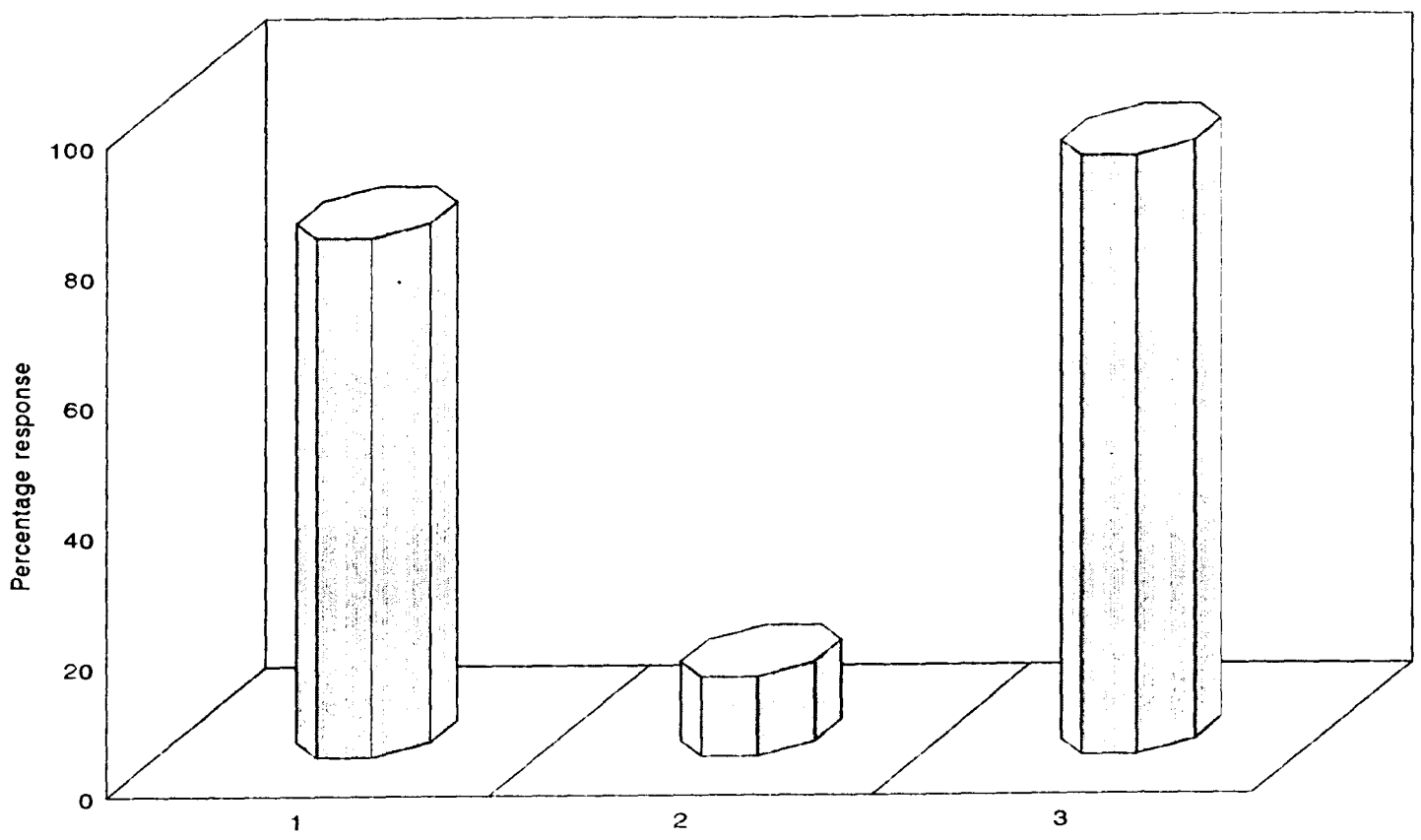
Size, age and position on the mother plant influenced the response of explant under *in vitro* conditions. Data pertaining to the effect of various sized explants of *Theobroma cacao* L. for initiating bud release are furnished in Table 12. The nodal segments with a subtending leaf bit of about 1 cm was necessary for culture establishment (Plate Ia). Bud burst was less in the shoots inoculated without the leaf bit and they showed callusing at the base. But the nodal segment (4.0 cm long) with the leaf bit gave 84.44 per cent bud burst. The nodal segments of 3.0 cm and 4.0 cm size, gave 80 per cent and 84.44 per cent response respectively. Chi-square analysis showed no significant difference between these two treatments. If the size was more than 3.0 cm, the chances of contamination was more. Hence the ideal size of explant was fixed as 3.0 cm.

When the age of the explants were compared, the seedling explants responded in a better way than the adult material. Juvenile shoots of 3.0 cm long

Table 12. Response of explants on initiating shoot bud release

Explant	Explants showing response %					
	Without petiole	Length of explant with petiole and leaf bit in cm				
		0.5	1.0	2.0	3.0	4.0
Nodal segments from field explant	21.11	5.55	28.88	56.66	80.00	84.44
Shoot tips from field explant		0.00	1.11	6.66	12.22	16.66
Nodal segments from juvenile material	22.22	6.66	45.55	75.55	92.22	96.66

* All the treatments replicated three times with 30 tubes
** Culture period 3 weeks
*** Culture medium WPM + 2ip 2 mg l^{-1}



1 = Nodal segments from field; 2 = Shoot tips from field; 3 = Nodal segments from juvenile material

Fig.1. Effect of explant types on shoot bud release

showed 92.22 per cent response while shoots of 3.0 cm long from the field explants showed only 80.0 per cent response (Fig.1).

Regarding the position of nodal segments taken from fan branches, explants from the middle of the shoots responded in a better way than the tip or base. Bud burst was poor in shoot tips cultured under identical conditions.

4.1.2 Surface sterilization of explant

The field grown material is known to be heavily infested with many fungi and bacteria. So special care was taken for the surface sterilization of explants. The results are presented in Table 13. Statistical analyais (Chi-square) showed that the treatments differed significantly. Bavistin (0.1%) pretreatment for 30 minutes followed by sterilisation with freshly prepared chlorine water was the best for cocoa nodal segments. This treatment for 4 minutes gave 94.66 per cent of living contamination free cultures. Surface sterilization using freshly prepared chlorine water for four minutes gave 88.00 per cent contamination free living cultures and 12.00 per cent infected cultures. Drying was not observed in cultures treated with chlorine water. But this treatment for a shorter period (2 minutes) recorded only 34.66 per cent contamination free living cultures. Prolonged treatment in Chlorine water led to high rate of death of cultures and bleaching of leaves and shoots. Mercuric chloride was toxic to cocoa shoots at all the levels tried. The treatment produced a high percentage of dead cultures.

4.1.3 Control of systemic contaminants

The subculturing medium for shoot elongation was WPM supplemented with 0.5 per cent activated charcoal and during subculturing bacterial contamination

Table 13. Effect of various sterilizing agents on the explant survival

Sterilizing agent	Duration	Explant %		
		Infected	Living	Dead
Chlorine water	2	65.33	34.66	Nil
	4	12.00	88.00	Nil
	6	6.66	46.68	46.66
Mercuric chloride 0.05%	2	44.00	48.00	6.00
	4	37.33	41.33	21.33
	6	20.00	45.33	34.66
Mercuric chloride 0.1%	2	21.33	42.66	36.00
	4	14.66	37.33	49.33
	6	8.00	6.66	85.33
Bavistin (0.1%) Pretreatment + chlorine water	2	17.33	78.66	4.00
	4	4.00	94.66	1.33

* All the treatments replicated three times with 30 tubes
** Culture period 3 weeks
*** Culture medium WPM

was regularly observed. The antibiotic Streptomycin sulphate was tried at different concentrations ranging from 50 to 300 mg l^{-1} to overcome this. Streptomycin sulphate (SMS) at 200 mg^{-1} was the optimum concentration controlling the infection. A lower concentration could not control bacterial infection and a higher concentration markedly retarded growth of the cultures.

4.1.4 Seasonal influence on explant contamination and culture establishment

Table 14 presents the results of seasonal influence on contamination rate and culture establishment of nodal segments. Poor culture establishment was due to the high rate of microbial infection. Prophylatic spraying of the mother trees with the systemic fungicide Bavistin (0.2%) and contact fungicide (Dithane M-45 (0.3%) greatly reduced the contamination of the explants. The standardised surface sterilization treatment viz., pretreatment in Bavistin (0.1%) for 30 minutes followed by chlorine water treatment for 4 minutes had been tried at monthly intervals throughout the year. The basal medium was WPM supplemented with 2iP (2 mg l^{-1}). Observations were taken after a period of 3 weeks in culture. The percentage of cultures established varied significantly over different months. Maximum culture establishment was during the month of March (82.22%) followed by April (81.11%). Minimum culture establishment was in June (22.22%) followed by July (23.33%). In general, culture establishment was more from January to May.

4.1.5 Bud break and shoot elongation

4.1.5.1 Culture medium

The results of the experiment on the effect of the different basal media for bud break are furnished in Table 15. The percentage establishment and the

Table 14. Seasonal influence on contamination rate and culture establishment

Month	Per cent cultures established	Per cent cultures contaminated
January	73.33	17.77
February	67.77	28.88
March	82.22	14.44
April	81.11	13.33
May	71.11	20.00
June	22.22	68.88
July	23.33	65.55
August	42.22	52.22
September	54.44	36.66
October	46.66	50.00
November	66.66	30.00
December	57.77	38.88

* All the treatments replicated three times with 30 tubes
** Culture period 3 weeks
*** Culture medium - WPM + 2ip 5

growth were recorded by visual scoring. The basal media used were full strength and half strength of MS, WPM and SH and they had varying effects on culture establishment. Good response indicated by bud break, shoot elongation and leaf production was obtained in full strength WPM supplemented with $AdSO_4$ 1 + CCC 0.75 + 2iP 5 + $AgNO_3$ 5 + PG 200 mg l^{-1} (Plate Ib). In the high salt MS media the performance was only average on visual rating and the culture estabishment was 85 per cent. In full strength SH and half strength of MS, WPM and SH, the culture establishment was low, the bud break was delayed and subsequent growth was poor.

4.1.5.2 Effect of growth regulator and other media supplements

The media used to study the effect of growth regulators and media supplements were the modifications of the medium proposed by Mallika *et al.* (1992). This experiment was conducted to select the most ideal medium for maximum shoot elongation which as essential for rooting and micrografting. The results of the experiment are recorded in Table 16.

The role of different media on the number of days taken for bud break was compared. Bud break was absent in WPM supplemented with phloroglucinol alone (T_1). Days taken for bud break was minimum in the treatment T_8 (WPM + 2iP 5 + CCC 0.75 + $AdSO_4$ 1 + $AgNo_3$ 5 + PG 200 mg l^{-1}) and was maximum in T_2 (WPM + 2iP 1 + PG 200 mg l^{-1}). Statistical analysis showed significant variation among the treatments. The treatments T_3 and T_4 were on par. The treatments T_5, T_6 and T_7 were on par and were superior to the first set.

When the effect of media on number of axillary shoots produced were compared, the treatments differed significantly. The number of axillary shoots

Table 15. Effect of basal media on culture establishment

Basal media	Percentage culture establishment	Growth response
MS	85.00	+++
½MS	38.33	++
WPM	90.00	++++
½WPM	41.66	++
SH	65.00	++
½SH	33.33	+

* All treatments replicated three times with 30 tubes
** Culture period 3 weeks
*** Media supplements 2iP 5 + CCC 0.75 + $AdSO_4$ 1 + $AgNo_3$ 5 + PG 200 mg l^{-1}

\+ No bud break
++ Delayed bud break
+++ Bud break with small sprouts
++++ Bud break, shoot elongation and leaf expansion

produced was maximum in T_6 and T_8 (2.66) (Plate Id and IIa). But this was statistically on par with T_5 and T_7 (2.33). T_2 and T_4 produced significantly lower number of shoots per axillary bud (1.0) (Plate Ic).

Study of the effect of different media on height of the longest shoot produced revealed that the treatments differed significantly. The treatment T_8 was significantly superior to T_2, T_3 and T_4 but was on par with T_5, T_6 and T_7. The shoot length was maximum when cultured in T_8 (2.73 cm) and minimum in T_2 (0.4 cm).

Number of leaves produced per plant also followed the same trend as that of shoot length. Maximum number of leaves (4.3) were produced in T_8 which was significantly superior to all other treatments. It was minimum in T_2 (0.33) and was on par with T_3 (0.66). Profuse callusing was seen in T_2, T_3, T_4 and T_5. Callusing was minimum in T_6 and was completely absent in T_7 and T_8.

The best medium for bud initiation and shoot elongation was T_8 ie., WPM supplemented with 5 mg l^{-1} 2iP, 1 mg l^{-1} Adenine sulphate, 5 mg l^{-1} silver nitrate, 0.75 mg l^{-1} cycocel and 200 mg l^{-1} phloroglucinol.

WPM supplemented with 0.5 per cent activated charcoal, 3 per cent sucrose and 200 mg l^{-1} streptomycin sulphate was the best subculturing medium for shoot elongation (Plate IIb and c). When IBA (0.1 and 0.5 mg l^{-1}) was added to the subculturing medium, shoot growth was retarded. Effect of gibberellic acid (GA_3) on shoot elongation was tried singly (1, 2, 3, 4, 5 mg l^{-1}) and in combination with kinetin (3 mg l^{-1}). GA had no favourable effect on shoot elongation. ABA (1 mg l^{-1}) along with kinetin (3 mg l^{-1}). This medium favoured growth of dormant buds and unfurling of leaves, but shoot elongation was suppressed.

Tabel 16. Effect of media on bud initiation and shoot elongation

Culture media ($mg\ l^{-1}$)	Days for bud break	No. of axillary shoot	Height of longest shoot (cm)	No. of leaves	Remarks
T_1 WPM + 200 PG	Nil	Nil	Nil	Nil	No growth
T_2 WPM + 2iP 1 + 200 PG	16.66	1.00	0.40	0.33	Callus at the base
T_3 WPM + 2iP 5 + 200 PG	12.33	1.66	0.90	0.66	"
T_4 WPM + 2iP 5 + $AdSO_4$ 0.5 + 200 PG	11.66	1.00	1.16	1.33	"
T_5 WPM + 2iP 5 + $AdSO_4$ 1 + 200 PG	9.00	2.33	2.60	3.00	"
T_6 WPM + 2iP 5 + $AdSO_4$ + 200 PG + $AgNo_3$ 1	9.33	2.66	2.66	4.00	Slight callus at the base
T_7 WPM + 2ip 5 + $AdSO_4$ + PG 200 + $AgNo_3$ 5	9.00	2.33	2.70	4.00	No callus
T_8 WPM + 2ip 5 + $AdSO_4$ 1 + $AgNo_3$ 5 + CCC 0.75 + PG 200	7.33	2.66	2.73	4.30	"
SE	0.78	0.2999	0.1083	0.4147	

* All treatments replicated three times with 30 tubes
** Culture period - 3 weeks
*** Genotype S 44.1

4.1.5.3 Carbon and energy source

The data presented in Table 17 indicate the effect of different levels of sucrose on enhanced release of axillary buds. The basal media, MS and WPM were used in the present experiment.

The treatments differed significantly on shoot elongation. Maximum shoot elongation was in WPM supplemented with sucrose 5 per cent (T_6 - 95.0%). Response was poor in medium with lower level of sucrose T_1 (3.0%). The treatments T_1 and T_4 (WPM + sucrose 1%) and T_2 (MS + 3% sucrose), T_3 (MS + 5% sucrose) and T_5 (WPM + 3% sucrose) were on par.

The treatments differed significantly on multiple shoot production. It was absent at lower concentration of sucrose (1%). T_6 (WPM + sucrose 5%) recorded the maximum number of cultures with multiple shoots.

Number of shoots produced per explant was maximum in WPM with 3 per cent and 5 per cent sucrose (four shoots) and minimum in MS with 1 per cent sucrose (single shoot). Maximum number of leaves per shoot was in T_6 (4.33) and minimum was in T_1 (1.66).

Statistical analysis showed that the treatments differed significantly on the length of longest shoot produced. T_6 was significantly better than all other treatments and produced shoots with maximum length. Shoots with minimum length was produced in T_1.

Table 17. Effect of sucrose on shoot induction of *Theobroma cacao* L.

Treatment	Explant showing shoot elongation (%)	Explants with multiple shoots (%)	No. of shoots/ explant	Number of leaves/ shoot	Longest shoots (cm)
T_1 MS + sucrose 1	30.0	0.0	1.00	1.66	0.66
T_2 MS + sucrose 3	78.0	18.0	2.00	2.33	1.93
T_3 MS + sucrose 5	87.0	43.0	2.33	3.00	1.10
T_4 WPM + sucrose 1	33.0	1.3	2.00	2.00	1.10
T_5 WPM + sucrose 3	71.0	24.0	2.33	3.33	2.00
T_6 WPM + sucrose 5	95.0	56.0	2.66	4.33	2.96
SE		0.1522	0.4172	0.3897	0.1652

* All treatments replicated three times with 30 tubes
** Culture period 3 weeks
*** Media supplements 2iP 5 + $AdSO_4$ 1 + CCC 0.75 + $AgNo_3$ 5 + PG 200 mg l^{-1}

The best medium for bud break and shoot elongation was WPM supplemented with 5 per cent sucrose along with other supplements like 2iP, $AdSO_4$, Phloroglucinol (PG), cycocel (CCC) and silver nitrate ($AgNO_3$).

4.1.5.4 Effect of culture conditions

The results of the experiment to study the effect of different culture conditions like temperature, light intensity and physical condition of the media are recorded in Table 18. Culture establishment was better in solid media than in liquid media. High light intensity (4000 lux) and high temperature (28 + 2°C) had a positive influence on plant growth.

Highest percentage of cultures showing bud burst (85.0%) was obtained in solid medium kept at maximum light intensity (4000 lux) and at a temperature of 28 ± 2°C and the lowest (3.33%) was in solid medium under dark incubation and low temperature (24 ± 2°C). The number of days taken for shoot bud release was minimum under high temperature and light condition. After bud burst, the shoot elongation and leaf expansion was not observed under dark incumbation. The shoot length was maximum in liquid cultures kept at high temperature and light intensity. Number of leaves produced was maximum (3.66) in solid cultures at 28 ± 2°C and high light intensity. The results showed that the best culture condition for shoot production of cocoa was culturing in solid medium and incubation at high light intensity (4000 lux) and high temperature (28 ± 2°C).

4.1.5.5 Effect of genotype

The data pertaining to the effect of different genotypes viz., S 44.1,

Table 18. Effect of physical conditions on explant establishment

	Light	Temperature	Explants showing bud release (%)	No. of days taken for bud release	Length of shoot	No. of leaves
Solid (0.7% Agar)	Dark 0 lux	24 + 2°C	3.33	21.33	-	-
		28 + 2°C	8.33	20.33	-	-
	Light 4000 lux	24 + 2°C	35.00	16.00	15.0	1.0
		28 + 2°C	85.0	7.33	30.0	3.6
Liquid (filter paper bridges)	Dark	24 + 2°C	8.33	21.33	-	-
		28 + 2°C	10.00	20.00	-	-
	Light	24 + 2°C	28.33	15.0	18.0	1.0
		28 + 2°C	75.0	8.0	32.0	3.0

* All treatments replicated 3 times with 20 tubes
** Culture period 1 month
*** Culture medium WPM + 2iP 5 + CCC 0.75 + $AdSO_4$ 1 + $AgNo_3$ 5 + PG 200 mg l^{-1}
**** Genotype used S 44.1

Table 19. Effect of genotypes in establishment of cultures

Genotype	No. of days taken for shoot bud release	Length of longest shoot (cm)	No. of leaves/ shoot
S 44.1	7.33	2.00	3.66
G VI 50	14.00	1.76	3.00
G IV 4.1	18.66	0.56	1.33

* All treatments replicated 3 times with 20 tubes
** Culture period 3 weeks
*** Culture media WPM + 2iP 5 + CCC 0.75 + $AdSO_4$ 1 + $AgNo_3$ 5 + PG 200 mg l^{-1}

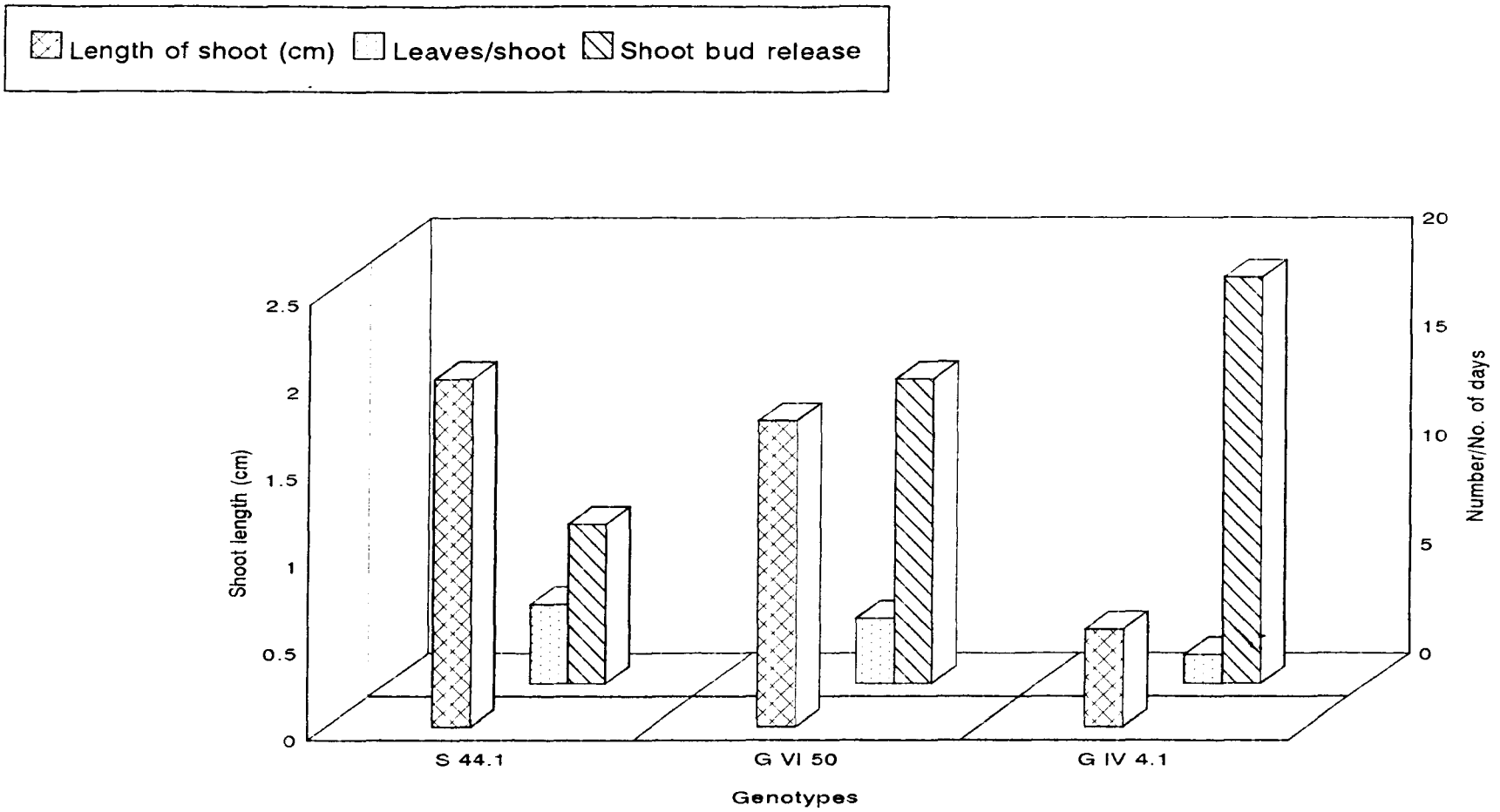


Fig.2. Effect of genotypes in culture establishment

G VI 50 and G IV 4.1 on culture establishment are presented in Table 19 (Fig. 2). Number of days taken for shoot bud release differed significantly among the genotypes. But there was no significant difference in length of longest shoot or number of leaves per shoot. Genotype S 44.1 responded better in culture compared to G VI 50 and G IV 4.1. Bud burst was quicker in this genotype producing longest shoots with maximum number of expanded leaves. Days taken for bud burst was minimum (7.3) in S 44.1 compared to G IV 4.1 (18.6) and a shoot length of about 2 cm was achieved in three weeks compared to that of only 0.56 cm in G IV 4.1. There was about three fold increase in the number of leaves/shoot in S 44.1 compared to G IV 4.1.

4.2 Root Induction

The shoots produced under *in vitro* conditions were rooted by *in vitro* as well as *ex vitro* methods.

4.2.1 *In vitro* rooting of shoots

4.2.1.1 Effect of basal medium

All the basal media were provided with sucrose 3 per cent and AC 0.5 per cent. Since no rooting was achieved in the basal media, a pulse treatment of auxin was given. The excised shoots were given a quick dip in IBA 5000 mg l^{-1} for 3 seconds. In general, response of cocoa to rooting was poor (Table 20). Maximum rooting was in half strength MS medium (23.33%) and in quarter strength MS medium, 16.66 per cent of the cultures rooted. Three other media in which rooting occurred were half strength WPM, quarter strength WPM (6.66%) and half strength SH (6.66%). The more common root culture media, White's

Table 20. Effect of basal media on *in vitro* rooting

Basal media	Rooting (%)	No. of days taken for rooting
MS	0	
½ MS	23.33	32.14
WPM	0	
½ WPM	13.33	35.25
White's	0	
½ White's	0	
SH	0	
½ SH	6.66	30.00
1/4 MS	16.66	37.20
1/4 WPM	6.66	38.50
1/4 SH	0	
1/4 White's	0	

* All treatments replicated 3 times with 10 tubes
** Media supplements - Sucrose 3 per cent + Activated charcoal 0.5 per cent
*** Pretreatment - Pulse treatment in IBA 5000 mg l^{-1} for 3 seconds
**** Culture period - 3 weeks

medium was not suitable for inducing rooting in cocoa. The best medium identified for rooting was ½ MS supplemented with 3 per cent sucrose and 0.5 per cent activated charcoal. A pulse treatment with a higher concentration of auxin was a prerequisite for rooting.

4.2.1.2 Effect of growth regulators

The different growth regulators tried for rooting in cocoa are presented in Table 21. Rooting was observed in treatments highlighted. Details of rooting response are recorded in Table 22. Observations were recorded for a period of 6 weeks.

The percentage of cultures which remained green was maximum when the pretreated shoots (IBA 5000 mg l^{-1} for 3 seconds) were cultured in the ½ MS medium. Maximum rooting was recorded in IBA (0.5 mg l^{-1}) incorporated medium and in auxin free medium after a pretreatment with IBA 5000 mg l^{-1} for 3 seconds (33.33%). The latter treatment recorded minimum days for rooting (30 days) and maximum roots (3 roots). The average length of roots in this medium was 4.5 cm (Plate IVa). When Riboflavin was added at a concentration of 2.0 mg l^{-1} to the rooting medium (½ MS + 0.5% AC), no positive response was observed.

The best rooting treatment for cocoa was identified as pretreating in IBA 5000 mg l^{-1} for 3 seconds followed by culturing in ½ MS media with 0.5 per cent AC.

4.2.1.3 Effect of carbon source

Table 23 gives the information on the effect of different levels of carbon

Table 21. Combinations of basal media and growth regulators tried for *in vitro* rooting

MS + 0.5 AC	+ 0.5 NAA/IAA/IBA
,,	+ 1.0 NAA/IAA/IBA
,,	+ 2.0 NAA/IAA/IBA
,,	+ 5.0 NAA/IAA/IBA*
,,	+ 10.0 NAA/IAA/IBA
,,	+ 20.0 NAA/IAA/IBA
½MS + 0.5 AC	+ 0.5 NAA/IAA/IBA
,,	+ 1.0 NAA/IAA/IBA
,,	+ 2.0 NAA/IAA/IBA
,,	+ 5.0 NAA/IAA/IBA*
,,	+ 10.0 NAA/IAA/IBA*
,,	+ 20.0 NAA*/IAA/IBA
1/4MS + 0.05 AC	+ 0.5 NAA/IAA/IBA
,,	+ 1.0 NAA/IAA/IBA
,,	+ 2.0 NAA/IAA/IBA
,,	+ 5.0 NAA/IAA/IBA
,,	+ 10.0 NAA/IAA/IBA
,,	+ 20.0 NAA/IAA/IBA
WPM + 0.5 AC	+ 0.5 NAA/IAA/IBA
,,	+ 1.0 NAA/IAA/IBA
,,	+ 2.0 NAA/IAA/IBA
,,	+ 5.0 NAA/IAA/IBA
,,	+ 10.0 NAA/IAA/IBA
,,	+ 20.0 NAA/IAA/IBA
½WPM + 0.5 AC	+ 0.5 NAA/IAA/IBA
,,	+ 1.0 NAA/IAA/IBA
,,	+ 2.0 NAA/IAA/IBA
,,	+ 5.0 NAA/IAA/IBA
,,	+ 10.0 NAA/IAA/IBA
,,	+ 20.0 NAA/IAA/IBA
1/4WPM+0.5 AC	+ 0.5 NAA/IAA/IBA
,,	+ 1.0 NAA/IAA/IBA
,,	+ 2.0 NAA/IAA/IBA
,,	+ 5.0 NAA/IAA/IBA
,,	+ 10.0 NAA/IAA/IBA
,,	+ 20.0 NAA/IAA/IBA
SH + 0.5 AC	+ 0.5 NAA/IAA/IBA
,,	+ 1.0 NAA/IAA/IBA
,,	+ 2.0 NAA/IAA/IBA
,,	+ 5.0 NAA/IAA/IBA
,,	+ 10.0 NAA/IAA/IBA
,,	+ 20.0 NAA/IAA/IBA
½SH + 0.5 AC	+ 0.5 NAA/IAA/IBA
,,	+ 1.0 NAA/IAA/IBA
,,	+ 2.0 NAA/IAA/IBA
,,	+ 4.0 NAA/IAA/IBA
,,	+ 10.0 NAA/IAA/IBA
,,	+ 20.0 NAA/IAA/IBA
1/4SH + 0.5 AC	+ 0.5 NAA/IAA/IBA
,,	+ 1.0 NAA/IAA/IBA
,,	+ 2.0 NAA/IAA/IBA
,,	+ 5.0 NAA/IAA/IBA
,,	+ 10.0 NAA/IAA/IBA
,,	+ 20.0 NAA/IAA/IBA
White's + 0.5 AC	+ 0.5 NAA/IAA/IBA
,,	+ 1.0 NAA/IAA/IBA
,,	+ 2.0 NAA/IAA/IBA
,,	+ 5.0 NAA/IAA/IBA
,,	+ 10.0 NAA/IAA/IBA
,,	+ 20.0 NAA/IAA/IBA
½ White & 1/4 White	
½MS + 0.5 AC	+ 0.1 2,4-D
,,	+ 0.5 2,4-D
,,	+ 1.0 2,4-D
,,	+ 0.1 BA
,,	+ 0.5 BA
,,	+ 1.0 BA
,,	+ 0.1 2iP
,,	+ 0.5 2iP
,,	+ 1.0 2iP

Contd.

Table 21. Continued

½MS + 0.5 AC + 0.1 Kin	½MS + 0.5 AC + IBA 10 + NAA 1.0
,, + 0.5 Kin	,, + NAA 2.0
,, + 1.0 Kin	,, + NAA 5.0
	,, + NAA 10.0
,, + 0.1 Zeatin	,, + NAA 20.0
,, + 0.5 Zeatin	
,, + 1.0 Zeatin	½MS + 0.5 AC + IBA 10 + IAA 5.0
	,, + IAA 10.0
,, + 0.1 Ethrel	+ IAA 20.0
,, + 0.5 Ethrel	½MS + 0.5 AC + IBA 1.0 + 2,4-D 0.1
,, + 1.0 Ethrel	,, + 2,4-D 0.5
	,, + 2,4-D 1.0
,, + 0.1 GA	
,, + 0.5 GA	½MS + 0.5 AC + IBA 2.0 + 2,4-D 0.1
,, + 1.0 GA	,, + 2,4-D 0.5
	,, + 2,4-D 1.0
,, + 0.1 ABA	
,, + 0.5 ABA	½MS + 0.5 AC + IBA 5.0 + 2,4-D 0.1
,, + 1.0 ABA	,, + 2,4-D 0.5
	,, + 2,4-D 1.0
½MS + 0.5 AC + IBA 0.5 + NAA 1.0	½MS + 0.5 AC + IBA 10.0 + 2,4-D 0.1
,, + NAA 2.0	,, + 2,4-D 0.5
,, + NAA 5.0	,, + 2,4-D 1.0
,, + NAA 10.0	
,, + NAA 20.0	½MS + 0.5 AC IBA 1.0 + BA 0.1
	,, + BA 0.5
½MS + 0.5 AC + IBA 1.0 + NAA 1.0	,, + BA 1.0
,, + NAA 2.0	
,, + NAA 5.0	½MS + 0.5 AC + IBA 2.0 + BA + 0.1
,, + NAA 10.0	,, + BA 0.5
,, + NAA 20.0	,, + BA 1.0
½MS + 0.5 AC + IBA 2.0 + NAA 1.0	½MS + 0.5 AC + IBA 5.0 + BA 0.1
,, + NAA 2.0	,, + BA 0.5
,, + NAA 5.0	,, + BA 1.0
,, + NAA 10.0	
,, + NAA 20.0	½MS + 0.5 AC + IBA 10 + BA 0.1
	,, + BA 0.5
½MS + 0.5 AC + IBA 5.0 + NAA 1.0	,, + BA 1.0
,, + NAA 2.0	
,, + NAA 5.0	½MS + AC 0.5 + IBA 1.0 + 2iP 0.1
,, + NAA 10.0 *	,, + 2iP 0.5
,, + NAA 20.0	,, + 2iP 1.0

Contd.

Table 21. Continued

½MS + AC 5.0 + IBA 2.0 + 2iP 0.1	½MS + AC 0.5 + IBA 1.0 + Kin 0.1
,, + 2iP 0.5	,, + Kin 0.5
,, + 2iP 1.0	,, + Kin 1.0
½MS + AC 0.5 + IBA 5.0 + 2iP 0.1	½MS + AC 0.5 + IBA 2.0 + Kin 0.1
,, + 2iP 0.5	,, + Kin 0.5
,, + 2iP 1.0	,, + Kin 1.0
½MS + AC 0.5 + IBA 10 + 2iP 0.1	½MS + AC 0.5 + IBA 5.0 + Kin 0.1
,, + 2iP 0.5	,, + Kin 0.5
,, + 2iP 1.0	,, + Kin 1.0
½MS+AC 0.5 + IBA 1.0 + Zeatin 0.1	½MS + AC 0.05 + IBA 10 + Kin 0.1
,, + Zeatin 0.5	,, + Kin 0.5
,, + Zeatin 1.0	,, + Kin 1.0
½MS+AC 0.5 + IBA 2.0 + Zeatin 0.1	½MS + AC 0.5 + IBA 1.0 + GA 0.1
,, + Zeatin 0.5	,, + GA 0.5
,, + Zeatin 1.0	,, + GA 1.0
½MS+AC 0.5 + IBA 5.0 + Zeatin 0.1	½MS + AC 0.5 + IBA 2.0 + GA 0.1
,, + Zeatin 0.5	,, + GA 0.5
,, + Zeatin 1.0	,, + GA 1.0
½MS+AC 0.5 + IBA 10 + Zeatin 0.1	½MS + AC 0.5 + IBA 5.0 + GA 0.1
,, + Zeatin 0.5	,, + GA 0.5
,, + Zeatin 1.0	,, + GA 1.0
½MS+AC 0.5 + IBA 1.0 + Ethrel 0.1	½MS + AC 0.5 + IBA 10 + GA 0.1
,, + Ethrel 0.5	,, + GA 0.5
,, + Ethrel 1.0	,, + GA 1.0
½MS+AC 0.5 + IBA 2.0 + Ethrel 0.1	½MS + AC 0.5 + IBA 1.0 + ABA 0.1
,, + Ethrel 0.5	,, + ABA 0.5
,, + Ethrel 1.0	,, + ABA 1.0
½MS+AC 0.5 + IBA 5.0 + Ethrel 0.1	½MS + AC 0.5 + IBA 2.0 + ABA 0.1
,, + Ethrel 0.5	,, + ABA 0.5
,, + Ethrel 1.0	,, + ABA 1.0
½MS+AC 0.5 + IBA 10 + Ethrel 0.1	½MS + AC 0.5 + IBA 5.0 + ABA 0.1
,, + Ethrel 0.5	,, + ABA 0.5
,, + Ethrel 1.0	,, + ABA 1.0

Table 21. Continued

½MS + AC 0.5 + IBA 10	+ ABA 0.1	
"	+ ABA 0.5	
"	+ ABA 1.0	

* Observed rooting
** All treatments were tried with pretreatment (Table 5) also

Table 22. Effect of different growth regulators on rooting of *Theobroma cacao* L.

Growth regulator	Concentration mg l^{-1}	Cultures showing growth (%)	Cultures rooted (%)	No. of days taken for rooting	No. of roots	Length of roots (cm)
NAA	20.0	26.66	16.66	36.25	2.0	2.0
IBA	10.0	30.00	13.33	32.00	2.0	5.0
NAA + IBA	20.0 + 5.0	16.66	10.00	31.66	3.0	3.5
IBA + IBA	1000.0 (3 s) + 2.0	9.0	3.33	33.00	1.0	5.0
IBA	5000.0 (3 s)	33.33	23.33	32.00	2.0	4.0
IBA + IBA	5000.0 (3 s) + 0.5	26.66	23.33	30.00	3.0	4.5

* Culture period 6 weeks
** Media ½ MS + 0.5 AC + 0.6% Agar
*** Genotype S 44.1
**** All treatments replicated three times with 10 tubes

source ie. sucrose on rooting. The cultures retaining chlorophyll after a period of six weeks was maximum at five per cent level of sucrose. The rooting percentage was equal at three per cent and five per cent concentration of sucrose. The number of roots produced and average length of roots was slightly higher at three per cent level but was not significantly different from five per cent concentration. So the most ideal concentration of sucrose selected for rooting of cocoa shoots was three per cent.

Stray instances of rooting was observed on prolonged culture in the establishment medium (Plate IIIa) and in the shoot elongation medium.

4.2.1.4 Effect of agar

Data given in Table 24 indicates the results of the experiment conducted to standardise the concentration of agar for rooting. Rooting percentage (23.33%), number of roots and root length were maximum (2.42 and 3.08 cm respectively) at 0.6 per cent concentration. Agar at 0.4 per cent and 0.8 per cent concentration recorded 20 per cent rooting. The number of roots and root length at 0.4 per cent of agar were 2.33 and 2.63 cm respectively. At a higher concentration of agar (0.8%) the number of roots was 1.5 and root length was 0.5 cm. These observations showed that the optimum concentration of agar for rooting was 0.6 per cent.

4.2.1.5 Effect of activated charcoal

Table 25 gives the information on the effect of charcoal on rooting of cocoa. Rooting was equal when the charcoal concentration was 0.5, 0.75 and 1.0 per cent (23.33%). But maximum roots were produced at 1.0 per cent concentration of AC (2.4). Root length was maximum (3.2 cm) at this concentration. Rooting was

Table 23. Effect of sucrose on rooting of *Theobroma cacao* L.

Concentration of sucrose (%)	Cultures remaining green (%)	Cultures rooted (%)	No. of shoots	Length of roots (cm)
0	Nil	Nil	Nil	Nil
3	36.66	23.33	2.28	2.17
5	46.66	23.33	2.42	2.48

* Agar - 0.6 per cent

Table 24. Effect of agar on rooting of shoots

Concentration of agar (%)	Cultures remaining green (%)	Cultures rooted (%)	Average No. of roots	Length of longest root (cm)
0.0	-	-	-	-
0.2	-	-	-	-
0.4	23.33	20.00	2.33	2.63
0.6	33.33	23.33	2.42	3.08
0.8	26.66	20.66	1.50	0.5

* Sucrose 3 per cent
** All treatments replicated three times with 10 tubes
*** Culture period 6 weeks
**** Pretreatment IBA 5000 mg l^{-1} for 3 seconds
***** Basal medium ½MS + 0.5 AC

not recorded in medium with 0.10 per cent activated charcoal and in charcoal free medium. At 0.25 per cent concentration, the rooting was 3.33 per cent, the root length was 1.2 cm and average roots produced was one. The results proved that 0.5 to 1.0 per cent of AC may give good rooting.

4.2.1.6 Effect of phloroglucinol

When phloroglucinol was added in the media, rooting was absent. But the cultures rooted when this compound at 200 mg l^{-1} was used in combination with IBA 2 mg l^{-1}. Profuse callusing was observed at the base of the shoot and the roots were originated from the callus (Plate IIIb). The plants dried off within one week on transferring to the external condtiions. Anatomical studies of the collar region revealed the presence of granular callus in this area (Plate IIIc). Vascular continuity between root and shoot was absent because of the intervening callus. These roots were unbranched and without any laterals.

4.2.1.7 Effect of culture conditions

Data on the effect of various culture conditions on rooting are presented in Table 26. Rooting rate was maximum under dark incubation (0 lux) and at a temperature of 28 $\pm$ 2°C (23.33%). Number of days taken for rooting was least (26.33) and the average number and length of roots was maximum (3.1 and 3.6 cm respectively). Incubating at high light intensity (4000 lux) and at high temperature (28 $\pm$ 2°C), a lower rate of rooting (13.33%) was recorded. The number of days taken for rooting was 31.66 and the average number and length of roots were 2.0 and 3.0 cm respectively. Culturing at 24 $\pm$ 2°C under dark condition resulted in 3.33 per cent rooting and needed about 36 days for rooting. On an average 2.6 roots

Table 25. Effect of activated charcoal on rooting of *Theobroma cacao* L.

Concentration of activated charcoal	Cultures remaining green (%)	Cultures rooted (%)	Average number of roots	Length of roots (cm)
0.00	6.66	0	0	0
0.10	6.66	0	0	0
0.25	10.00	3.33	1.0	1.2
0.50	30.00	23.33	2.2	3.0
0.75	33.33	23.33	2.2	3.2
1.00	33.33	23.33	2.4	3.0

* All treatments replicated 3 times with 10 tubes
** Culture period 6 weeks
*** Pretreatment in 5000 mg l^{-1} IBA for 3 seconds
***** Basal medium ½MS + 3% sucrose + 0.6% agar

Table 26. Effect of culture conditions on rooting of *Theobroma cacao* L.

Light intensity	Temperature	Rooting (%)	No. of days for rooting	No. of roots	Length of roots (cm)
0 lux	24+2°C	3.33	36.00	2.6	2.5
	28+2°C	23.33	26.33	3.1	3.6
4000 lux	24+2°C	0.00	-	-	-
	28+2°C	13.33	31.66	2.0	3.0

* All treatments replicated 3 times with 10 tubes
** Culture media ½MS + 3% sucrose + 0.5% AC + 0.6% Agar
*** Pretreatment IBA 5000 mg l^{-1} for 3 seconds
**** Culture period 6 weeks

were produced with a length of 2.5 cm. Rooting was completely absent under nigh light intensity (4000 lux) and low temperature (24 + 2°C).

Before subjecting the rooted plantlets to different hardening treatments, a pretreatment was given by keeping under high light intensity (4000 lux) for one week. This had a favourable effect on plant establishment and survival.

4.2.1.8 Effect of genotype and physiological age

The findings of the experiment on the effect of genotype and physiological age on *in vitro* rooting are presented in Table 27. The genotypes, viz., S 44.1, G VI 67 and G IV 4.1 differed in their ability to produce roots. Rooting percentage was 23.33, 20.00 and 13.33 respectively. The shoots used for rooting were from stage II cultures of field grown mature trees.

The genotype, S 44.1 recorded minimum number of days for rooting (36 days) as well as maximum percentage of rooting (23.33). The genotype G VI 67 also recorded 36 days for rooting and maximum root length (2.8 cm). G IV 4.1 registered maximum days for rooting (38 days), minimum roots (only 1.0) and root length (1.5 cm).

Increasing the number of subculturing had a slight positive influence on rooting of the genotype S 44.1. The rooting percentage was not changed on subculturing for the genotype G VI 67 and G IV 4.1. Average number of roots produced were increased only for the genotype G IV 4.1. Length of roots of the genotypes S 44.1 and G IV 4.1 was increased but for G VI 67 it was not changed.

Table 27. Effect of genotype and physiological age on rooting of *Theobroma cacao* L.

Genotype	Physiological age of explant	Rooting (%)	No. of days taken for rooting	No. of roots	Length of roots
S 44.1	Shoots after stage II culture	23.33	36.2	2.0	2.0
	Shoot after five subcultures	26.66	33.3	2.0	2.2
G VI 67	Shoots after stage II culture	20.00	36.0	3.1	2.8
	Shoots after five subcultures	20.00	36.0	3.1	2.8
G IV 4.1	Shoots after stage II culture	13.33	38.1	1.2	1.5
	Shoots after five subcultures	13.33	38.1	2.0	1.6
Unknown	Shoots from seedlings material	93.33	21.0	5.0	3.5

* All treatments replicated 3 times with 10 tubes
** Culture period 6 weeks
*** Basal medium ½MS + 0.5 AC + 0.6% Agar
**** Pretreatment IBA 5000 mg l^{-1} for 3 seconds
***** Pretreatment IBA 1000 mg l^{-1} for 3 seconds for seedling material

Shoots derived from *in vitro* seedlings rooted very easily and rooting percentage was very high (93.3). Rooting of these shoots could be achieved by an auxin pulse treatment followed by culture in an auxin free medium. The base of the shoots were dipped in 1000 mg l^{-1} IBA dissolved in absolute ethyl alcohol for a few seconds and then transferred to the hormone free ½ MS medium supplemented with 0.5 per cent activated charcoal. In this medium the shoots rooted easily within 21 days. Length of roots as well as number of roots were maximum (3.5 cm and 5.0 cm respectively).

4.2.2 *Ex vitro* rooting

Ex vitro rooting was tried by one step method and two step method. But none of the treatments were useful in inducing rooting. The leaves of the shoot remained green for two weeks after which yellowing was observed. The shoots completely dried off after one month.

4.2.3 Effect of potting mixture on establishment and survival of tissue cultured plantlets

The results of the experiment to know the effect of different potting mixtures on the rooting of cocoa shoots are presented in Table 28. The rooting substrate containing soilrite and potting mixture at equal proportion recorded maximum survival of plantlets after two weeks (80.00%) (Plate IVb). The rate of establishment was also maximum (60.00%). Two months after plant out, increase in height was 2.0 mm per week and increase in mean number of leaves produced was 2.33. When potting mixture (sand : soil : cowdung 1 : 1 : 1) alone was used, plantlet survival and establishment was lower than the above treatment (20% and 6.66% respectively). The growth in this potting medium was very poor. The

Table 28. Effect of potting mixtures on plantlet survival

Potting mixture	Number planted out	No. of plants survived after two weeks	Details of growth		
			Number established	Increase in height/week (mm)	Mean number of leaves produced 2 months after plant out
Soilrite	15	2	-	-	-
Vermiculite	15	-	-	-	-
Sand	15	-	-	-	-
Potting mixture	15	3	1	1.0	1.0
Sand + Soil (1:1)	15	1	-	-	-
Soilrite + potting mixture (1:1)	15	12	9	2.0	2.33
Vermiculite + Sand (1:1)	15	Nil	-	-	-

increase in height was only 1.0 mm per week and the unfurling of leaves was only at the rate of one after two months. In all other media tried, the plantlets dried or decayed in a period of 2 weeks. From the observations, the best potting medium for rooting of shoot was a mixture of soil rite and potting mixture in equal proportions.

4.2.4 Effect of different hardening methods

The results of the experiment conducted for selecting the best hardening method are presented in Table 29. Covering with polybags was found to be better than covering with microscope cover. The survival percentage one month after planting out was 66.66 and 33.33 respectively with ½ MS drenching. Increase in height was 5.0 mm and 2.5 mm per week and increase in leaf number was 2.33 and 1.33 respectively.

4.2.5 Effect of nutrient supply

Table 29 presents the effect of weekly spraying of nutrient starter solution on plant growth. The results showed that this treatment had marked influence in plantlet survival. But there was slight increase in height after one month in sprayed plants and mean number of leaves produced was more in these treatments. Spraying with ½ MS or ½ WPM recorded the same results.

A study on survival and growth of plantlets during acclimatization (Table 30) showed that the survival percentage decreased upto the second month of planting out (Fig.3). It remained the same from the second month onwards. Increase in plant height and leaf number showed that the growth rate was very poor for *in vitro* raised cocoa plantlets.

Table 29. Hardening procedures and nutrient supply on growth of plantlets

Hardening methods	Nutrient supply	Plant characters		
		Growth after one month		
		Survival (%)	Height (mm)	Mean No. of leaves
Covering with poly bags	½ MS drenching	66.66	5.0	2.33
	½ WPM drenching	50.00	3.3	1.33
	Control	50.00	2.3	-
Covering with microscope cover	½ MS drenching	33.33	2.5	1.33
	½ WPM drenching	33.33	2.5	1.33
	Control	16.66	1.0	-
Covering and keeping under mist	½ MS drenching	Nil	-	-
	½ WPM drenching	Nil	-	-
	Control	Nil	-	-
Keeping in moist brick tunnels	½ MS drenching	Nil	-	-
	½ WPM drenching	Nil	-	-
	Control	Nil	-	-

* All treatments replicated three times with 6 pots
** Potting mixture - Soilrite + potting mixture (1:1)

Table 30. Survival and growth of plantlets during acclimatisation

Period after planting out	Survival (%)	Increase in height (mm)	Mean No. of leaves produced
2 weeks	80.00	0.0	Nil
1 month	53.33	2.3	1
2 months	33.33	5.7	3
3 months	33.33	12.0	5
4 months	33.33	32.6	7

* Potting mixture - Soilrite + potting mixture (1:1)
** Hardening method covering with polybags
*** Average of 5 observations

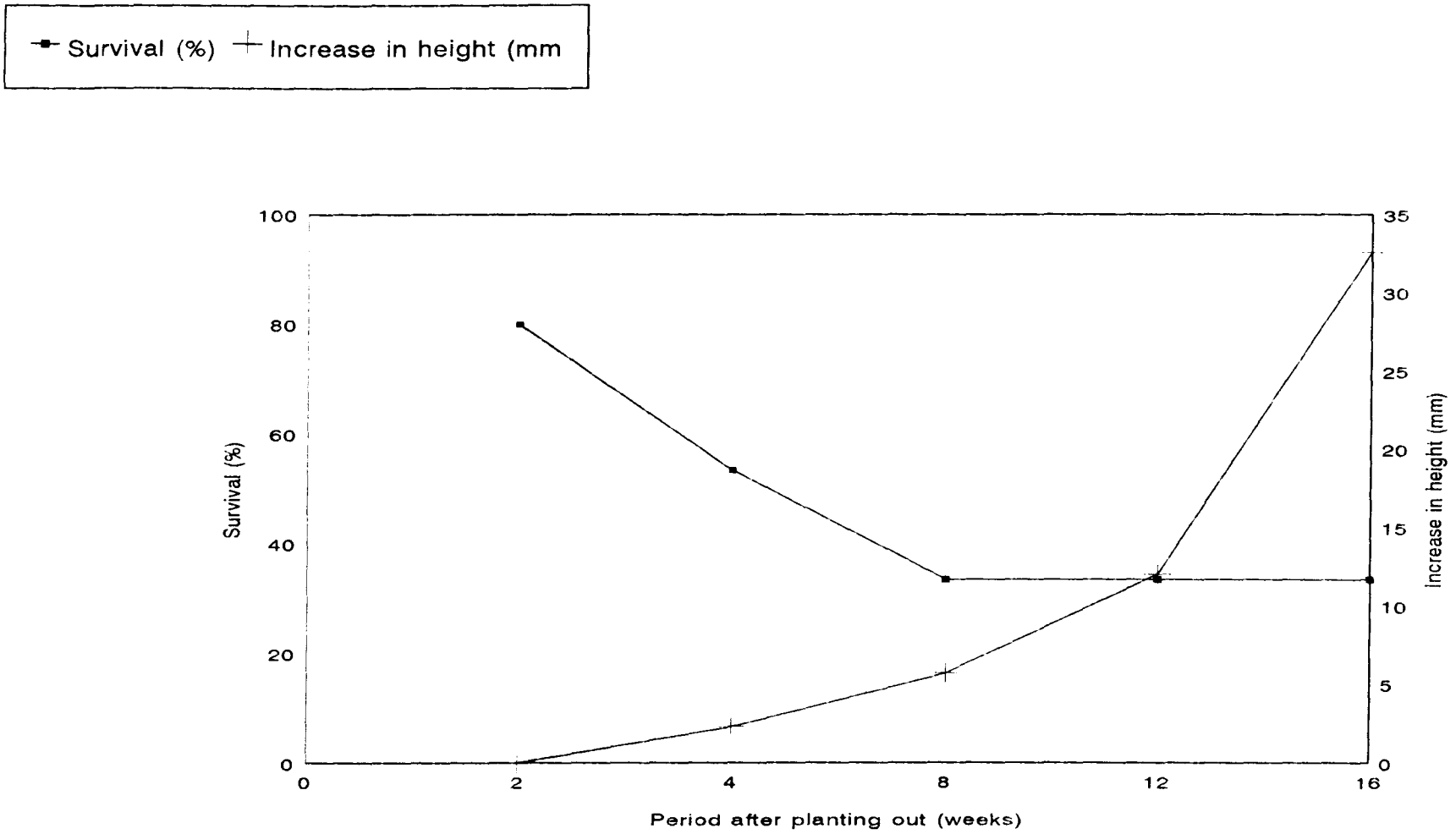


Fig.3. Survival and growth of plantlets during acclimatisation

4.3 Micrografting

The result of the micrografting techniques tried for the micropropagation of cocoa are presented below.

4.3.1 *In vitro* micrografting

In *in vitro* micrografting, the rootstock was axenic seedlings. Shoots regenerated from nodal segments of field grown trees and plantlets/shoots recovered from germinating somatic embryos were used as scion.

4.3.1.1 Rootstock material

Axenic seedlings were raised using seeds of tree ripe pods. Surface sterilized seeds were cultured in different media and observations were recorded (Table 31). All the seeds germinated in Full MS liquid medium with three per cent as well as five per cent sucrose and in half MS liquid medium with five per cent sucrose. Quicker germination and ideal rootstock for micrografting were obtained in liquid medium irrespective of the nutrient composition. When sucrose was added, microbial contamination was high on micrografting. The solid medium was not preferred because it was not easy to replace the grafted seedlings after micrografting in this hard medium.

The results of the effect of different physical conditions on the seed germination and production of ideal rootstock for micrografting are presented in Table 32. Higher seed germination (95%) and highest percentage of graftable seedlings (90%) were produced when the seeds were cultured at high light intensity (4000 lux) and high temperature (28 $\pm$ 2°C). Dark incubation (0 lux) and low

Table 31. Effect of different media for raising axenic seedlings

Basal medium	Physical condition of the medium	Sucrose (%)	Germination (%)	Mean No. of days taken for germination
Full MS	Solid	0	96.66	14.2
		3	98.33	15.1
		5	98.33	16.2
	Liquid	0	93.33	12.5
		3	100.00	10.2
		5	100.00	10.8
Half MS	Solid	0	91.66	15.3
		3	96.66	14.8
		5	98.33	15.6
	Liquid	0	95.00	12.3
		3	98.33	10.8
		5	100.00	11.0

* All treatments replicated 3 times with 20 tubes

temperature (24 ± 2°C) was not ideal and resulted in 62.00 per cent seed germination and lanky seedlings. Percentage of seedlings ideal for micrografting was only 65.00 when they were grown in the dark.

4.3.1.2 Scion material

The effect of different scion materials ie. shoots regenerated from nodal segments and plantlets/shoots germinated from somatic embryos was analysed. The success percentage was highest (80-90%) when shoots regenerated from nodal segments were used as the scion. Plantlets/shoots germinated from somatic embryos recorded a very low percentage success. The different treatments for haploid production from anthers resulted only in callus formation. Micrografting using haploids were not attempted since no haploids were recovered.

4.3.1.3 Method of micrografting

The different micrografting techniques tried were side grafting, wedge grafting and epicotyl grafting (Table 33). The success was maximum with side grafting (80%) and graft union was achieved in about two weeks. In wedge grafting, the percentage success was much less and longer time was required for the healing of the graft union. Epicotyl grafting was not successful in cocoa.

4.3.1.4 Position of micrografting and age of rootstock

Micrografting was attempted at different positions of the seedling stock, viz., above and below the cotyledons at varying distances. Seedlings of different maturity were used as rootstocks. The results are presented in Table 34. Maximum success and survival was recorded by grafting scions at a position of 4 cm below the

Table 32. Effect of culture conditions for raising seedlings

Culture condition		Percentage of germination	Percentage of ideal rootstock produced
Light intensity	Temperature		
Dark (0 lux)	24+2°C	62.00	60.00
	28+2°C	80.00	60.00
Light (4000 lux)	24+2°C	80.00	51.00
	28+2°C	95.00	90.00

* All treatments replicated 3 times with 20 tubes
** Basal media - ½ MS without sucrose

Table 33. Effect of different methods on success of grafting

Method of grafting	Percentage of success of graft union	Days taken for graft union
Side grafting	80.0	13.2
Wedge grafting	10.0	16.0
Epicotyl grafting	Nil	Nil

* All treatments replicated 3 times with 20 tubes
** Rootstock 4-5 weeks old
*** Scion 3 cm long with 2 hardened leaves

cotyledon of four weeks old seedlings (83.33% and 81.66% respectively) (Plate Va). This treatment was the best since minimum days were needed for graft union (8.2 days) and further growth was satisfactory. A slightly lower percentage of success and survival was recorded when the stocks were five weeks old and grafting was 4 cm below the colyledons (80.00%), but scion growth was better. When the position of grafting was 4 cm below the cotyledon of five weeks old rootstock, increase in height and leaf number was maximum. The increase in height two weeks after grafting was 11.0 mm and four weeks after grafting was 18.0 mm. The mean increase in leaf number in the respective periods was 1.2 and 1.66 respectively.

The position of the grafting above the cotyledon was not congenial. Grafting 2 cm below the cotyledons recorded a low per cent of success and survival. The cotyledons would have created some physical obstructions. Seedlings below the age of three weeks were also not ideal due to the presence of more mucilage as well as absence of expanded leaves.

The survival was very poor when two weeks old seedlings were used (11.66%) and moderate when rootstocks were of three weeks old (66.66%). The results showed that seedlings of 4-5 weeks old should be selected for getting maximum success and better growth.

4.3.1.5 Size of scion

Scions of different sizes starting from bud initials to long shoots with expanded leaves were tied for micrografting and the results were presented in Table 35. Survival of the graft was obtained when the scion had at least one hardened leaf. Highest success and survival percentage were (90.00 and 83.33%)

Table 34. Effect of the age of rootstock and position of grafting in cocoa micrografting

Age of rootstock week	Position of grafting	Success (%)	Survival (%)	Days taken for graft union	Growth of scion			
					Increase in height		Increase in no. of leaves	
					2 weeks	1 month	2 weeks	1 month
2	Above cotyledon	Nil	-	-	-	-	-	-
	2 cm below cotyledon	10.0	5.0	16.0	-	1.0	-	-
	4 cm below cotyledon	16.66	11.6	15.2	-	2.0	-	-
3	Above cotyledon	Nil	-	-	-	-	-	-
	2 cm below cotyledon	53.33	46.66	14.2	2.0	8.0	-	1.0
	4 cm below cotyledon	73.33	66.66	13.8	3.0	9.0	-	1.25
4	Above cotyledon	3.33	-	-	-	-	-	-
	2 cm below cotyledon	66.66	63.33	10.2	4.0	10.0	-	1.5
	4 cm below cotyledon	83.33	81.66	8.2	8.0	15.0	0.6	1.6
5	Above cotyledon	6.66	-	-	-	-	-	-
	2 cm below cotyledon	63.33	61.66	9.6	10.0	18.0	1.0	1.3
	4 cm below cotyledon	80.00	78.33	8.5	11.0	18.0	1.2	1.66

* Treatments are replicated 3 times with 20 tubes

** Culture period - 1 month

obtained when scions of more than 2 cm long with at least two hardened leaves were used. In this trial, better scion growth in terms of increase in height and leaf number was recorded. There was a mean increase in height of 9.0 mm and leaf number of 1.2 after two weeks and 15.0 mm and 2.5 after four weeks. When grafting was done with scions having bud initials only or shoots without any hardened leaf, the grafting operation was not successful. The buds remained green for a few days and after that the leaves and shoots dried off.

4.3.1.6 Anatomical studies

The anatomical observations on the graft union was periodically recorded and the results revealed that after 14 days, a callus bridge was formed connecting the stelar cylinder of the rootstock and the scion (Plate VIa, b). By about three weeks time, from the callus bridge, a cambial ring was differentiated which was in line with the cambial ring of the rootstock and the scion (Plate VIc and VIIa). By about 40 days the newly formed cambium was found to produce secondary xylem towards the innerside and secondary phloem towards the outside. The rootstock and scion become so intimate that it forms a single common stelar cylinder. This union enables the rootstock and scion to lose its identity and perform as a single physiological functional step (Plate VIIb, c).

4.3.1.7 Growth of micrografts after planting out

The survival and growth of micrografted plants were studied and the results are furnished in Table 36. The survival of the grafts planted out were only 90.00 per cent. The increase in height was 0.2 cm and mean number of leaves produced was 0.1 by two weeks period. The survival percentage was decreasing up

Table 35. Effect of the size of scion on micrografting

Characteristics of scion	Success (%)	Survival (%)	Scion growth			
			After 2 weeks		After 1 months	
			Height (mm)	Leaf No.	Height (mm)	Leaf No.
0.5 cm long bud without any leaf	10.00	-	-	-	-	-
1.0 cm long with two tender leaves	16.66	-	-	-	-	-
1.0 cm long with one expanded leaf	33.33	26.66	2.0	-	5.0	0.25
1.5 cm long with at least one hardened leaf	83.33	76.66	5.0	0.25	9.0	0.66
2.0 cm long with at least 2 hardened leaves	86.66	80.00	6.0	1.0	12.0	2.16
More than 2.0 cm long with hardened leaves	90.00	83.33	9.0	1.2	15.0	2.5

* All treatments replicated three times with 20 tubes
** Culture period - 1 month
*** Rootstock - 1 month old

Table 36. Survival and growth of micrografted plants during acclimatisation

Period after micrografting	Survival (%)	Increase in height (cm)	Mean number of leaves produced in the scion
2 weeks	90.00	0.2	0.1
3 weeks	83.33	0.5	0.6
4 weeks	75.00	1.2	1.3
5 weeks	71.66	2.0	2.0
6 weeks	71.66	3.0	3.0
2 months	71.66	5.5	4.0

* Average of 20 observations

to the fifth week and after that it became static. The increase in height by about five weeks time was 2.0 cm and the increase in leaf number was 2.0 (Fig.4). At this stage, the polythene cover was removed. After two weeks, the plants were transferred to larger pots containing potting mixture and kept in the hardening unit for another one month.

4.3.2 *Ex vitro* micrografting

Ex vitro micrografting was carried out on nursery raised plants and the results are furnished in Table 37. In this method success was generally less than grafting on *in vitro* seedlings. Stock plants of different ages were used for grafting and older stocks having a few hardened leaves favoured better growth of the scion. In this method scions without a hardened leaf failed to survive. Retaining leaves on the rootstock favoured rapid growth of the scion shoot. Scion shoots having bud initial only and/or with one or two tender leaves did not show any signs of development. Later the graft was lost due to the death of the scion even after proper graft union. In grafts using *in vitro* shoots accompanied by one or two tender expanded leaves, the recovery was poor due to the drying of the leaves and death of shoots. Though some of these shoots exhibited successful graft union with the root-stock, no signs of further growth of the terminal buds was noticed. Encouraging results were obtained when healthy shoots having one or more hardened leaves were used as the scions. Rapid and extensive elongation of the scion was observed in *ex vitro* grafting.

Table 37. Success rate and growth of *ex vitro* micrografts in *Theobroma cacao* L.

Stock plant	Scion shoot	Graft success (%)		Scion growth
		After 2 weeks	After 6 weeks	
4 weeks old decapitated seedling without retaining any leaf	Bud initials only	20.0	0.0	No growth
	One tender expanded leaf	10.0	0.0	Slight elongation
	One or more hardened leaves	30.0	20.0	Moderate growth
8 weeks old decapitated seedlings retaining 3-4 leaves	Bud initials only	30.0	0.0	No growth
	One tender expanded leaf	40.0	0.0	Decay of leaves
	One or more hardened leaves	50.0	35.0	Rapid growth

Grafting method - Cleft grafting
Age of rootstock - 3-4 weeks
20 grafts/treatment

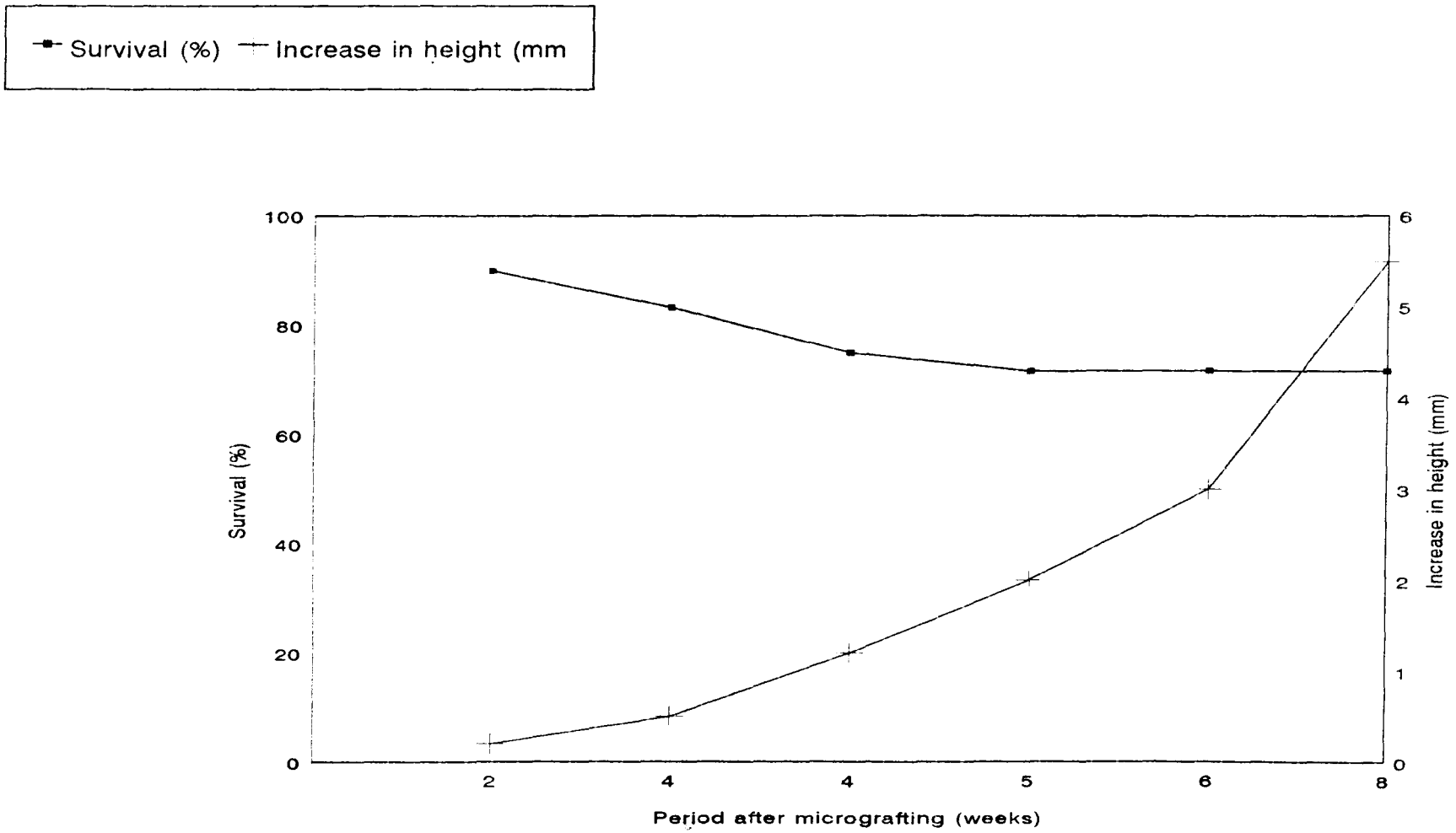


Fig.4. Survival and growth of micrografted plants during acclimatisation

Discussion

5. DISCUSSION

Cocoa (*Theobroma cacao* L.) is an important beverage crop of the world and the various products, particularly those obtained from its seeds are of immense economic value. It is highly heterogeneous because of open pollination and incompatibility. Therefore, the recommended practice to achieve yield improvement in this crop has been to select elite plants and propagate them vegetatively. The two vegetative propagation methods, budding and rooting of cuttings are relatively expensive and offer only limited number of propagules. Tissue culture techniques can be used for the production of true to type plants at a very rapid rate (Levy, 1981). But an efficient vegetative propagation method requires the development of a reliable rooting protocol that works well across a variety of genotypes. According to Duncan (1992), cocoa has been recalcitrant to tissue culture propagation and satisfactory shoot proliferation has not been obtained so far. This is more so when explants from field grown adult trees were used. In the present study attempts were made to standardise the *in vitro* techniques for rooting, hardening and micrografting for rapid multiplication of elite cocoa plants.

5.1 Enhanced release of axillary buds

5.1.1 Explant

The survival percentage and growth rate of shoots depended on the size of the explant. For larger sized explants, higher survival percentage and growth rate were reported (Hussey, 1983). A positive correlation between explant size and release of axillary bud was also observed in the present investigation. The bud break percentage was increased as the size of explant increased. But if the size is more

than 3.0 cm microbial contamination was very high. This high contamination rate may be because of the longer explant size. Legrand and Mississo (1986) and Flynn *et al.* (1990) had reported similar observations in cocoa. Smaller sized explants showed callusing at the base which may be due to the high surface to volume ratio causing difficulty in the survival of the explant. Litz (1986) reported that one of the major problems that has been encountered in cocoa tissue culture was the excessive callusing.

The present trial also showed that a large explant size ie. 1.5-2 cm long nodal segment with a subtending leaf bit was necessary for bud burst as reported by Mallika *et al.* (1990).

Explant derived from seedling material responded in a better way than adult material (92.22 per cent and 80.00 per cent respectively). The capacity of vegetative propagation of a tree is associated with the juvenility. Juvenile material responded to tissue culture more easily than adult material as reported in many plants by Bonga (1982).

The position of the explant on the fan shoot influenced the bud release under *in vitro* condition. Shoot tips recorded a poor response (12.22 per cent) when compared to nodal explants (21.11 per cent). A similar observation was made in jack (Rahman and Blake, 1988) and in mature *Morus nigra* (Yadav *et al.*, 1990). This probably may be due to differences between the physiological status of the buds on different regions of the stem (Vieitez *et al.*, 1985). Shoot tips contain a higher content of auxins which may inhibit bud break and shoot growth but favour callusing.

5.1.2 Surface sterilization

Microbial contamination in cultures of field explants is a serious problem in tree micropropagation. These microbes should be removed without causing any damage to the explant by surface sterilization. General surface sterilization procedures have been outlined by various workers (George and Sherrington, 1984; Dodds and Roberts, 1985), but specific method has to be evolved for each crop and each tissues to be handled. Regular spraying of the mother trees with the systemic fungicide Bavistin (0.2%) and the contact fungicide Dithane M-45 (0.3%) brought down the contamination percentage greatly which was in confirmity with the observations made by Legrand and Mississo (1986) and Mallika *et al.* (1992). The results of the present investigation showed that the best surface sterilization treatment for cocoa shoots was pretreatment in Bavistin (0.1%) for half an hour followed by fresh chlorine water treatment for four minutes. This treatment registered 94.66 per cent living contamination free cultures. Contaminated cultures were only 4.00 per cent and 1.33 per cent were dead cultures. Prolonged treatment in chlorine water led to the bleaching of the tissues. Mercuric chloride treatment resulted in a high percentage of dead cultures. This chemical may be toxic to cocoa explants. Better growth observed in Bavistin (0.1%) pretreated cultures may be due to the cytokinin like effect which can enhance the plant growth (Sundaram and Oblisami, 1979).

5.1.3 Presence of systemic contaminants

On subculturing in the shoot elongation media (WPM + 0.5 per cent AC), bacterial contamination was frequently observed. This may be due to the presence of bacteria in the plant which can make its appearance later. Bacterial

infection was not observed in the establishment medium which contains phloroglucinol. Zimmerman and Broome (1980) in their *in vitro* studies on apple, suggested that this compound can act as a bactericide. This may be the reason for the absence of bacterial contamination in the establishment medium. Zimmerman and Broome (1980) also reported that the phloroglucinol can increase the shoot regeneration in shoot tip cultures carrying concealed bacterial infections.

The bactericide (Streptomycin sulphate) should be used at a concentration of 200 mg l^{-1} for controlling bacterial infection in cocoa subcultures. A higher concentration caused retarded growth of the cultures and a lower concentration was not sufficient for controlling the bacteria. But Dodds and Roberts (1985) suggested that the antibiotic or their degradation products may be metabolised by plant tissues with unpredictable results. However, it was better to avoid antibiotics for sterilization.

5.1.4 Seasonal effect on explant establishment

The results of the present investigation revealed that the culture establishment and the incidence of explant contamination depended on the season of collection. Maximum culture establishment was observed in March (82.22%) and minimum was in June (22.22%). The high contamination rate during June-July may be due to the prevailing rainy days. Yu (1991) reported a similar report of high contamination during rainy days in litchi. Maximum culture establishment obtained during March-April may be due to the presence of hot, sunny days unsuitable for proliferation of microbes.

5.1.5 Bud break and shoot elongation

Buds present in the axils of every leaf have the potential to develop into a shoot. In nature, these buds are normally dormant due to apical dominance. The application of cytokinin to the axillary bud can overcome the apical dominance effect (Murashige, 1974). The multiplication of axillary shoots can be substantially enhanced by providing suitable cytokinin at an appropriate concentration either with or without auxins. The direct effect of cytokinin is believed to be an enhancement of cell division. The effect of cytokinin in tissue culture may vary according to the particular compound used, the type of culture and the variety of plant from which it was derived.

Among the different basal media tried, maximum culture establishment indicated by bud break, shoot elongation and leaf production was obtained in full strength WPM. This confirms the observation made by Mallika *et al.* (1990, 1992). In MS medium also shoot growth was obtained but WPM was preferred because of its lower salt concentration. Passey and Jones (1983) succeeded in getting whole plant from nodal segments of 2-3 year old seedling plants on modified MS medium. Adu-Ampomah *et al.* (1992) reported the use of MS medium for getting complete plants.

The objective of the present study was to get elongated shoots which was a prerequisite for trying rooting and micrografting. With a view of this different combinations of growth regulators were tried based on the media proposed by Mallika *et al.* (1992). The results showed that 2iP (2-Isopentinyl adenine) at a concentration of 5 mg l^{-1} should be used for getting maximum shoot elongation. This is a naturally occuring cytokinin and its superiority over BA (Benzyl adenine)

or Kinetin has been reported in a number of species like Rhododendron (Anderson, 1975), Blue berry (Cohen, 1980), garlic (Bhojwani, 1980) and cocoa (Mallika *et al.*, 1992).

Adenine sulphate at a concentration of 1 mg l^{-1} had a favourable influence on shoot growth in cocoa. The possible growth regulatory effect of adenine and its use in tissue culture were first noted by Bonner and Haagensmit (1939). Adenine and related substances are precursors of endogeneous cytokinin (Nitsch *et al.*, 1967). The favourable effects of adenine at lower levels in inducing axillary shoots may be due to its promotive effects caused by counteracting the inhibitory action of auxins on lateral bud release. There are reports regarding the action of adenine as synergist to cytokinins like kinetin and zeatin (Nitsch *et al.*, 1967). Role of adenine sulphate in enhancing the growth and shoot formation was suggested earlier by Skoog and Tsui (1948). Mahato (1992) reported that addition of adenine sulphate at 6 mg l^{-1} induced multiple shoot formation in *Dalbergia latifolia*.

Addition of the phenolic compound, phloroglucinol at 200 mg l^{-1} was essential for shoot growth in cocoa. The role of phloroglucinol to enhance growth and rate of axillary shoot production has already been reported in *Prunus insitia* (Jones and Hopgood, 1979), *Cinchona ledgeriana* (Hunter, 1979) and *Theobroma cacao* L. (Mallika *et al.*, 1992). Jones (1976) reported the promotive effects of PG in shoot proliferation in apple. But Whiteley and Abbot (1976) recorded a negative result on the stimulation of growth in *Malus* cultures by Phoroglucinol. They explained that its physiological role is unclear and likely to be complex. There are strong indications that its action is not simply one of auxin synergism brought about by an inhibition of IAA oxidase activity (Jones and Hatfield, 1976). Moreover the

promotive effect of phloroglucinol was observed only in presence of ethylene inhibitors and antigibberellins and PG showed some synergistic effect with these media additives (George and Sherrington, 1984). There are suggestions that phloroglucinol in certain plant cultures acts in an indirect way by the suppression of contamination. This also may be a reason for the improved growth exhibited by cocoa shoots in the presence of PG.

Addition of silver nitrate at 5.0 mg l^{-1} reduced callusing at the base of cocoa shoots. Any explant of cocoa placed in a medium without growth regulators resulted in the formation of callus (Mallika *et al.*, 1990). Calusing at the base arrested further growth of shoots. The callusing was followed by drying of leaves leading to gradual death of the shoots. The appearance of callus has already been cited as a factor interfering with culture success in cocoa (Passey and Jones, 1983; Dublin, 1984). Addition of silver nitrate (5 mg l^{-1}) suppressed the callus and improved the growth. The addition of the high doses of cytokinin, ie. 2iP may enhanced the production of ethylene. The inhibitory effect of ethylene on organogenesis is already known (Khalid *et al.*, 1991). Silver nitrate has the ability to inhibit the action of ethylene and thereby help in organogenesis (Mallika *et al.*, 1992).

The growth retardant Chlormequat (CCC), an antigibberellin showed positive response to shoot growth of cocoa in culture. The most effective concentration of this chemical was 0.75 mg l^{-1}. This has the ability to suppress the endogeneous level of growth regulators which may be high in cocoa plants. In the absence of CCC, the sprouts appeared to be feeble and pale. The growth regulating effect of this compound was typical of a gibberellin biosynthesis inhibitor (Davis

and Curry, 1991). Cycocel treated plants have thicker and shorter internodes and have potential to enhance the net photosynthesis. The potential to enhance the net photosynthetic activity is not completely clear but may be related to the increased leaf and mesophyll layer thickness in the treated plants. Leaves of treated plants have been reported to contain increased palisade cell length and spongy parenchyma layer thickness. In addition to directly increasing photosynthesis, CCC may sometimes prolong the period of normal photosynthetic activity for a leaf by delaying senescence (Davis and Curry, 1991). In the present experiment in cocoa bud cultures also, proper expansion and greening of leaves in presence of CCC may be due to the enhanced net photosynthesis. Being a gibberellin biosynthesis inhibitor, it may be acting by suppressing the action of endogeneous gibberellic acid to promote the release of buds from the pre-existing meristem and inhibiting shoot elongation. Gibberellic acid (GA_3) is already reported to be inhibitory in cocoa tissue culture (Blake and Maxwell, 1984). The role of Chlormequat to improve morphogenesis in various plants at a concentration of 0.1 mg l^{-1} had already been reported by Reynolds *et al.* (1980), Blackmon *et al.* (1981), Blackmon and Reynolds (1982) and Mallika *et al.* (1992).

Addition of gibberellic acid alone or in combination with kinetin had no favourable influence on shoot elongation of cocoa. But Mallika *et al.* (1990) reported a positive response when filter sterilized GA_3 1-3 mg l^{-1} was incorporated in the medium (WPM + kinetin + IAA). In the present experiment, gibberellic acid was directly added to the medium before sterilization. GA_3 being a thermolabile compound may be lost on sterilization. Death of explants was not observed in the present study in the presence of gibberellic acid. A similar observation was made by Mallika *et al.* (1990). But this is contradictory to the report of Blake and Maxwell

(1984). Bud dormancy in cocoa could be broken by ABA (1 mg l^{-1}) in combination with kinetin (3 mg l^{-1}). The response of cocoa shoots to this growth inhibitor indicates that the endogeneous level of growth promoters like auxins and gibberellins may be high in the plant.

In the present investigation, sucrose at 5 per cent level was ideal for shoot induction as well as multiple shoot production. Sucrose is a source of carbon energy and act as an osmoticum which is generally needed for growing tissues under *in vitro* condition. Sucrose has invariably been found as the best carbohydrate for many crops. Sucrose is an ideal source carbon energy and osmoticum for supporting the growth of shoot bud cultures of cardamom (Reghunath, 1989). Higher concentration is required for dilute media when compared to the concentrated ones (George and Sherrington, 1984). In the present study, a high level of sucrose was better because of the use of the low salt basal medium, WPM. The sucrose concentration may also be related to the specific carbohydrate metabolism through which water relation and endogeneous phytohormones are regulated. Gamborg *et al.* (1974) has suggested that sucrose concentration affect the efficiency of nitrate and ammonium ion in the medium and the effect of cytokinin on cell division may also be dependant on the sugar availability.

In an attempt to standardise the physical condition of the media, it has been found that solid cultures are better than liquid cultures. In cocoa, liquid cultures are usually contaminated by bacteria.

High light intensity (4000 lux) and high temperature (28 $\pm$ 2°C) were ideal for cocoa tissue culture. The daylength period given was 12 hours. Light requirement for tissue differentiation involves a combination of several factors,

namely intensity, quality and duration. An optimum combination of these factors is necessary for certain photomorphogenic effect (George and Sherrington, 1984). Tropical trees were reported to require a high culture temperature for growth (Hunter *et al.*, 1983). Cocoa being a tropical tree may need a high temperature for growth. Hence the culture room temperature was maintained at 28 $\pm$ 2°C. Yeoman (1986) reported that the usual environmental temperature of the species concerned should be taken in to account while optimising the culture condition.

Three different genotypes were compared for shoot initiation using the most favourable culture conditions identified in the present study. The rate of shoot initiation from the primary explants showed variation between clones. The number of buds initiated per culture as well as the vigour of buds varied with genotype even when cultured under identical conditions. Most favourable and consistent response was obtained in the accession S 44.1 when compared to G VI 50 and G IV 4.1. These observations strongly suggest clonal variation in their response to *in vitro* culture of cocoa.

Variation in genotypic response to culture has been reported earlier in a number of crops. In cocoa, Pence *et al.* (1979) had shown that genotypic differences and seasonal effects played a great role in callus and organ forming cultures. Clone difference has been reported to be significant in oak (*Quercus robur*) micropropagation also (Juncker and Favre, 1989). Vieitez *et al.* (1985) also observed marked differences in the responses in *in vitro* cultures of three clones of adult chestnut and they attributed the reason to the genetic differences. Similar differences among genotypes in various crops in the *in vitro* conditions has already been reported (Reed, 1990; Dulieu, 1991; Kristiansen, 1992).

In cocoa, the shoot apex shows alternating periods of activity and dormancy. This endogeneous control of intermittent bud growth of the shoot apex in the intact plant may be determined by hormonal stimuli derived from other parts of the plant (Orchard *et al.*, 1979). Wood (1979) has suggested that flush growth in cocoa under *in vitro* condition is made possible by using reserves of nutrients already stored in the tissues. A positive response by the addition of the diverse growth regulators as media additives to promote the successful growth of shoots suggests the involvement of complex factors in controlling the flushing under *in vitro* conditions also.

5.1.6 Root induction

Efficient vegetative propagation requires the development of a reliable rooting protocol that works well across a variety of genotypes. A few plants produce roots during the regime used to encourage multiple shoot formation. A separate rooting protocol was then unnecessary and rooted plantlets can be moved directly into external environment for hardening off. In most instances, the presence of cytokinin at stage II inhibits root formation and a separate root inducing medium has to be used at stage III. The shoots produced under *in vitro* condition can be rooted by *in vitro* or *ex vitro* methods (George and Sherrington, 1984).

5.1.6.1 *in vitro* rooting

Rooting rate of cocoa shoots was low. Nair *et al.* (1992) reported that the cocoa cuttings produced only 20-30 per cent rooting under *in vivo* conditions also.

In the present study, various factors for rooting of cocoa shoots were attempted. Among the basal media tried, MS basal medium with half strength inorganic salts and full strength organic salts was the best and recorded 23.33 per cent rooting. This observations confimed the earlier report by Mallika *et al.* (1992). But this medium had some adverse effect in certain species (Gupta *et al.*, 1981) which is not observed here. The concentration of inorganic salts in the basal media was reported to influence the *in vitro* rooting regardless of the growth substances present. Several researchers had shown that *in vitro* rooting can successfully be achieved by reducing salt concentration in the media particularly in high salt media like MS and its derivatives (Skirvin and Chu, 1979; Kar and Sen, 1985; Daimon and Mii, 1991; Rathore *et al.*, 1991). Normal strength of organic growth factors was essential for root initiation. The favourable effect of reduced macroelement concentration in rooting may probably be due to the less ionic concentration. Tripathi (1971) reported that a high concentration of calcium and nitrogen was essential for rooting. MS media contains a high level of these elements. Rooting of cocoa shoots in this media confirmed the observation made by Tripathi (1971). Calcium has the ability to prevent leakage of auxin protectors in to the nutrient medium (Stonier, 1971). Reinert *et al.* (1977) reported that an increase in phosphate concentration of the medium can suppress or weaken the root promoting effect of auxin. In the present study, the absence of rooting in full strength SH medium may be due to the high concentration of phosphate ions (2.6 mM). But in half strength SH medium which had 1.3 mM phosphate ion, 6.66 per cent rooting was observed. Eventhough White's medium contains a very low level of phosphate ions (0.1 mM) rooting was not observed.

In the present investigation, vitamins of the group B are reported to be essential for rooting (Nemeth, 1986). Drew *et al.* (1993) reported that the vitamin B_2 (Riboflavin) had a favourable effect on rooting of papaya. But in the present experiment, riboflavin had no positive effect in the rooting of cocoa when added to ½ MS medium containing 0.5% AC.

The ability of plant tissues to form adventitious roots depends on interaction of many endogeneous and exogeneous factors. The role of auxin in root formation was well established. In the micropropagation practice, usually the natural auxin IAA, synthetic auxins like IBA, NAA, 2,4-D etc. were used for rooting. In the present investigation treatment with higher concentrations of both NAA (20 mg l^{-1}) and IBA (5 and 10 mgl^{-1}) resulted in rooting.

For improving rooting, different combinations of growth regulators were tried. But the result was not satisfactory. The best rooting was obtained when shoots pretreated with auxins were cultured in auxin free ½ MS basal media or in IBA (0.5 mg l^{-1}) incorporated media (23.33%). But in the latter media, the percentage of cultures remaining green was less. So the best rooting treatment selected for cocoa shoots was pretreating in IBA (5000 mg l^{-1}) for 3 seconds followed by culturing in auxin free medium. The number of days taken for roots to appear was minimum (30 days) in this medium. But the results were inconsistent. Mallika *et al.* (1992) reported rooting of cocoa by a pulse treatment n 5000 mg l^{-1} IBA for 2-3 seconds which is in confirmity with the present observation.

Ancora *et al.* (1982) reported that IBA and NAA were the most effective auxins for root induction, the first phase of rhizogenesis while cytokinins inhibited

it. In the present experiment also, IBA and NAA are identified as the most potent auxins for rooting. Higher concentration of cytokinin generally inhibit root formation (Schraudolf and Reinert, 1959; Harris and Hart, 1964) and prevent root growth and the promotive effects of auxins on root initiation (Hamphries, 1960). But there have been reports that the cytokinins promoted root growth (Fries, 1960) or root formation in the absence of auxins. In all cases, only low rates of cytokinins were effective. In the present experiment, addition of cytokinins at lower concentrations (1 mg l^{-1}) had no favourable effect on rooting.

The carbon energy source tried for rooting was sucrose and the standard concentration of three per cent was found to be optimum. Sucrose can act as energy source and osmotic agent. Organ initiation is a high energy requiring process and the inhibition of rooting below optimum can be explained by starvation of the heterotrophic shoots and inhibition above optimum may be of osmotic nature. Sucrose is clearly involved in the differentiation of xylem and phloem elements in cultured cells; but the actual role it plays is not yet known. Sucrose is generally required to be present in addition to IAA, before tracheid elements are differentiated in tissue culture. The number of both sieve and xylem elements formed depend on sucrose concentration. Apart from the very clear morphogenetic effects of optimum sucrose content there are reports that an excess concentration can inhibit rooting (George and Sherrington, 1984). Snir (1983) reported complete supression of rooting in sour cherry by the omission of sucrose in the medium and root elongation occurred at the optimum level (20 mg l^{-1}).

In the present experiment, the optimum concentration of agar for rooting was 0.6 per cent. In most of the rooting trials, the optimum was recorded to vary

from zero to 0.9 per cent. Agar is usually used as a carrier material to solidify the medium supporting root initiation and growth *in vitro*. Physiologically agar is not a completely inert material and is a source of various types of substances which may influence growth in sensitive species. In cocoa, solid media with 0.6 per cent agar gave 23.33 per cent rooting and rooting was absent in liquid medium. It was reported that in solid media, the plant material is held above the medium so that no special means of aerating the culture is required. Moreover shoots and roots grow in a more orderly fashion on a stationary medium. The physical condition also influence the oxygen tension of the medium. In conditions of partial or complete anaerobisis, the activity of enzymes which catalyse reducing reactions may be increased and that of oxidative enzymes may be decreased. A diminution of oxygen availability within plant cells might therefore cause a decrease in IAA oxidase activity (Kessel and Carr, 1972) and/or an increase in the activity of nitrate reductase (Schulp and Stouthamer, 1970).

Kitto and Young (1981) observed an inverse relationship between the rooting ability and the agar concentration in Carrize Citrange cultures. In the present study, a concentration above 0.6 per cent has found to have a similar effect on rooting.

Addition of activated charcoal (AC) in the medium had a remarkable influence in the initiation of roots as well as in the number of roots produced per shoot. Addition of activated charcoal (0.5%) was found to essential for rooting in cocoa. Mallika *et al.* (1992) had already reported the use of AC (1.0%) in the rooting of cocoa shoots. The capacity to adsorb inhibitory compounds and excessive concentration of plant growth hormones and the property to darken the medium

which mimics the soil condition are the factors proposed in favour of the favourable effects of AC in rooting (Proskauer and Berman, 1970; Wang and Huang, 1976; Fridborg *et al.*, 1978). Enhanced rooting in activated charcoal enriched media has been reported for different species such as raspberry (Welander, 1985), *Sequoiadendron giganteum* (Monteuuis and Bon, 1986) and *Pinus pinaster* (Dumas and Monteuuis, 1995).

Rooting was observed when phloroglucinol was added in combination with the auxin, IBA. PG has not any independant influence on rooting. Nemeth (1986) reported that stimulation or inhibition of root initiation by phenolic compounds is due to their interaction with auxins. According to James and Thurbon (1981), the presence of phloroglucinol in media at stage II can precondition shoots to root at stage III while they are still multiplying in response to high cytokinin levels. In the present investigation PG was invariably added in stage I media. Addition of it in stage III media has a favourable effect on rooting which was already been reported (Passey and Jones, 1983; Mallika *et al.*, 1990). But callusing was found at the base of the shoot and the roots were seemed to have been originating from it. On transferring the plantlets to the potting mixture for hardening, they dried off within a week. Anatomical section of the collar region revealed that vascular bundle connections are absent between the root and shoot. There is a layer of corky callus in between. Vascular connection is essential for efficient water flow as otherwise water stress during acclimatisation causes high mortality of tissue cultured plantlets (Mohammed and Vidaver, 1988).

Root formation was generally favoured by relatively high temperature. The best temperature ideal for rooting in cocoa was 28 $\pm$ 2°C (23.33%). The

rooting percentage was reduced when the temperature was 24 + 2°C. Similar favourable effect of high temperature on rooting has already been reported in apple (Lane, 1978) in Myrobalan and plum (Hammerschlag, 1982).

The light intensity of 0 lux (dark incubation) was found to be best for the rooting in cocoa. The plant tissues need to be supplied with carbohydrate before they will produce adventitious roots and it has been suggested that there is an optimum concentration of internal carbon reserves above and below which the formation of adventitious roots is inhibited (Lovell *et al.*, 1972). The necessary supply of carbon energy can be provided both by sugar in the medium and in green tissues, through photosynthesis. If the combined source is too great, rhizogenesis may be inhibited. If photosynthesis was prevented chemically or by keeping cultures in the dark, applied sucrose was less inhibitory (Lovell *et al.*, 1972).

The effect of genotype on rooting showed that it has a profound influence on rooting. The genotype S 44.1 showed better rooting ability than G IV 4.1. Similar clonal difference were reported in oak (Juncker and Favre, 1989), and *Eucalyptus marginata* (McComb and Bennet, 1982).

When the effect of physiological age (juvenility) on rooting was compared, shoots from seedlings tissues rooted easily and that of field grown plant was found to be very low. Identical observations were made in Eucalyptus (de Fossard *et al.*, 1977), apple (Welander and Huntrieser, 1981), *Quercus robus* L. (Vieitez *et al.*, 1985) and *Pinus pinaster* (Dumas and Monteuuiis, 1995).

Increasing the subculturing time and frequency has a small but favourable influence in rooting of the genotype S 44.1. But it has no effect for the

genotype G VI 67 and G IV 4.1. Jones *et al.* (1977) reported the rooting of M 26 apple can be improved by subculturing, which was not observed here. The different subculturing media having different cytokinins or GA_3 at various levels has not any positive effect which was in contrary to the reports of Quoirin *et al.* (1974), Rugini and Verma (1982) and Sriskandarajah *et al.* (1982).

5.1.6.2 *Ex vitro* rooting

Ex vitro rooting was not successful in cocoa. Cocoa is very much difficult to root under *in vitro* and *in vivo* conditions and recorded only 20-30% rooting. The lack of rooting may be due to the physiological maturity attained by mother plants.

Rooting was very poor for *in vitro* raised cocoa shoots from field explants. This may be due to several biochemical and physiological reasons such as the presence of naturally occurring rooting inhibitors. According to Haissig (1974) lack of root initiations in response to applied auxins in cuttings may be due to lack of necessary enzymes to synthesise the root inducing auxins - phenol conjugates, lack of enzyme activators, presence of enzyme inhibitors, lack of substrate phenolics or due to physical separation of enzyme reactants due to cellular compartmentalization. Any one of this may possibly be the reason for lack of rooting of cocoa microshoots under *in vitro* condition. Hartman *et al.* (1993) reported that in difficult to root woody plant species, ease of adventitious root formation declines with age of parent stock. Juvenility in relation to rooting may possibly explained by the increasing production of rooting inhibitors as the plant grows older. Reduced rooting potential may also be due to the lowering of phenolic levels, which can act as auxin synergists in root initiation. This may be the reason for rooting of cocoa shoots in phlorogluci-

nol added medium. Moreover phloroglucinol can reduce the activity of IAA oxidase and can act as an auxin protectant.

5.1.6.3 Hardening and plant out

Bavistin (0.1%) treatment for five minutes before transferring to the soil increased the survival percentage of the plantlets as reported by Mallika *et al.* (1992). The media adhering to the root system should completely be removed, otherwise it will act as a substrate for microbial growth.

The most ideal rooting substrate (potting mixture) for establishing cocoa plantlets was a mixture of soil rite and potting mixture (1:1). Mallika *et al.* (1992) reported that better establishment of cocoa plantlets was obtained in a media containing soil rite and sterile sand (3:1). The type of potting mixture, used for transferring *in vitro* grown plantlets is an important factor determining the establishment percentage. Media which maintain an optimum moisture level and sufficient aeration to the root zone of the plantlets promote better establishment and growth of the transplanted plantlets (Ramesh *et al.*, 1993). Ramesh *et al.* (1993) observed that for transferring jack plantlets, sand was the best potting mixture followed by vermiculite and peat.

Improved rooting of cocoa plantlets in the soil rite - potting mixture media may be due to the ideal rhizosphere provided. Better ventillation and drainage were obtained which created a favourable condition for root growth and vigorous vegetative growth of the plantlets (Wainwright, 1988). This media also helped to maintain optimum moisture status.

In the present study, drenching with ½ MS or ½ WPM salt solution at weekly intervals produced healthier plantlets. The beneficial effect of addition of inorganic nutrients to the pot mixture has been reported by several workers (Brown and Somer, 1982; Rajmohan, 1985; Kesavachandran, 1991). Wong (1986) recommended the addition of 3.0 g of nutricate urea (14:14:14 NPK) to each pot one week after transplantation to get healthier *in vitro* banana plantlets for field planting. Kavangh *et al.* (1987) found in *Prunus serotina* that application of full strength Hoaglands solution at weekly intervals produced the most vigorous plantlets. Dewald *et al.* (1988) reported that for best results with pineapple plantlets, initial application of a fertilizer biweekly followed by ten percentage MS salts solution in the latter stages was found to be the optimum. Application of 5 ml nutrient solution containing ½ MS inorganic salts (pH 5.7) at weekly intervals enhanced the survival and promoted normal growth of the plantlets. In cassava, liquid fertilizer application yielded good results on the growth (Roca, 1984).

The *in vitro* derived plantlets have to be acclimatised to the entirely new environment prevailing *in vivo* as they are poorly adapted to resist the lower relative humidity, higher light intensity and temperature. A precise control of weaning environment alone can ensure maximum survival rates (Grout and Aston, 1977; Langford and Wainwright, 1987; Wainwright, 1988). A period of humidity acclimatisation was considered essential for the newly transferred plantlets to adapt to the outside environment. During this time, the plantlets undergo morphological and physiological adaptation enabling them to develop typical terrestrial plant water control mechanism (Grout and Aston, 1977; Sutter *et al.*, 1985). In the present experiment, it was observed that subjecting the plantlets to high light intensity

(4000 lux) for one week before transplantation helps in better establishment as reported in some other crops by Murashige (1974) and Hussey (1979). Enhanced photosynthesis under the influence of high light intensities before transplantation helps in building up a high food reserve to be utilized during the transformation period from partially heterotrophic to autotrophic growth of the plantlets after transplantation (Murashige, 1977).

The hardening, prior to transplantation is a crucial step deciding the success of micropropagation. The internal anatomy and ultrastructure of shoots propagated *in vitro* is often different to that of greenhouse or field grown plants (Wetzstein *et al.*, 1981; Dunstan and Sutter, 1982). The success in acclimatisation depends on the post-transfer and pre-transfer conditions (Ziv, 1986). Some of the established aberrant features of the *in vitro* raised plants are (a) improper development of leaf which consists of poor or absence of cuticular wax on leaf surface, poor development of palisade layers and pronounced mesophyll air spaces (Grout and Aston, 1977; Leshem, 1983; Donelly and Vidaver, 1984). (b) impaired stomatal mechanism with nonclosing stomata (Brainerd and Fuchigami, 1981; Capellades *et al.*, 1990). The impaired stomatal mecahnism has been attributed to the abnormal orientation of microfibrils as well as to high levels of sodium ions in guard cells, which may interfere with the movement of potassium ions (Wardle *et al.*, 1981). (c) poor photosynthetic ability caused due to the availability of sugar (mostly sucrose) in the medium. The *in vitro* growing plantlets are not truely photoautotrophic but mixo or heterotrophic. Poor organization of grana in the chloroplasts along with the etiolated effect produced by ethylene in the glass vessels, greatly contribute to their decreased photosynthetic ability (Grout and Aston, 1977; Lee *et al.*, 1985). (d) Vitrification of shoots to different extent and associated poor vascular

tissue differentiation both in leaves and stem. (e) Rooting of shoots preceded by intervening callusing and poor association of conducting tissues of root and shoot and lack of root hair in general.

The poor survival of cocoa plantlets transferred to the external environment may be attributed to any one of the above mentioned reasons. Desication was the main cause of mortality on transplantation. In the present study, high relative humidity was maintained during the initial period of planting out by different methods. Covering the plantlets with microscope cover or with polybags was equally effective. But polybags are cheap, readily available and are easy to handle. Humidity in the cover was maintained by intermittent water sprays.

5.1.7 Micrografting

In the present study, micrografting was successful. It is a method which can save much time and resources in the micropropagation of cocoa. Rooting - a crucial and difficult step in the micropropagation of this crop can be surmounted by this technique. Yidana *et al.* (1987) proposed the usefulness of micrografting in cocoa for gene banks and for *in vitro* propagation. Several scientists reviewed the importance of micrografting in the micropropagation of fruit trees for grafting known varieties to dwarfing rootstocks (George and Sherrington, 1984), in the rejuvenation of mature shoot materials (Francelet, 1979; Hackett, 1985) and in the study of histological nature of graft union (Gebhardt and Goldbach, 1988). Aguilar *et al.* (1992) and Joseph (1994) attempted micrografting in cocoa to regenerate plantlets from somatic embryos. In the present study, different types of scions viz. *in vitro* produced shoots and embryoids were tried and the grafting technique was standardised for the shoots. The technique of micrografting has great significance in

a tree crop like cocoa where a viable micropropagation protocol is handicapped by the low level of rooting of *in vitro* shoots and high level of mortality during hardening.

Seedlings raised in liquid ½ MS media devoid of sucrose was used as the rootstock. Cultures were maintained in a culture room at 28 + 2°C and exposed to a light intensity of 4000 lux. Seed culture in liquid medium facilitated the *in vitro* grafting operation. Liquid medium was favourable for the establishment of the grafted cultures also. Moreover, the seedlings can easily be taken out and can be replaced with least damage to the root system in a liquid medium. A medium soldified by agar quickly asphyxiated the roots and the blockage prevented the development of secondary roots (Mosella Chancel, 1979). But providing of a filter paper bridge allowed more oxygen to the rootstock system. Addition of sucrose to the culture media increased the contamination of grafts. This was contradictory to the observation made by Mampouya (1983). He reported that in citrus species the sucrose content upto 85 g l^{-1} improved the success from 40 per cent (30-50 g l^{-1}) to 95 per cent.

Effect of age of rootstock on percentage success of micrografting showed that maximum success was obtained when seedlings of 4-5 weeks old were used. A higher establishment and quicker callusing was observed. Growth rate of the scion measured in terms of increase in height and number of leaves were also good.

The results of different scion material on the success of micrografting was studied. The shoots from nodal segments recorded maximum success and establishment. The embryoids recorded a poor survival rate. Haploids were not used as scions because the regeneration from anther callus was not possible. The presence

of expanded leaves on the shoots may be the reason for the high percentage of survival of shoots.

Among the different methods of grafting attempted, maximum success was recorded in side grafting. Wedge grafting can also be done but with a limited success percentage. Successful *in vivo* side grafting techniques in cocoa had been reported for detecting virus in bud wood (Wood, 1979). Wedge or saddle grafting under nursery condition as a successful method with limited practical application was also reported (Wood, 1979).

Maximum success and survival were recorded when the position of grafting was 4 cm below the cotyledons of four weeks old seedling. Minimum days were needed for graft union and recorded satisfactory growth rate. But better scion growth with maximum increase in height and leaf number was recorded when the position of grafting is 4 cm below the cotyledon of five weeks old seedlings. Grafting above the cotyledon was difficult. In most of the cases, the shoots bend downward just above the cotyledon and a convenient position for grafting was absent. Grafting just below the cotyledon was also difficult because cotyledons created some physical obstructions. Completely removing the cotyledons at this stage was not good for the growth of the rootstock. Presence of at least one expanded mature leaf on the rootstock was essential for grafting success. Seedlings below the age of three weeks were not ideal due to the presence of mucilage and absence of expanded leaves. Seedlings of 4-5 weeks old should be used as the rootstocks and the grafting should be done 4 cm below the cotyledons.

When scions of different sizes were tried for grafting, it was observed that highest successs and survival was recorded when 2 cm long shoot with at least

two hardened leaves were used. Scion growth was better with maximum rate of growth in terms of increase in plant height and leaf number. The effect of scion length on the success of graft 'take' was well established in mango (Ram and Bist, 1982; Ratan, 1985). The superiority of longer scion could perhaps be attributed to high reserve food materials in them compared to shorter scion. In shorter scion exhaustion of food material would take place before union is completed.

The length of the scion or rootstock appears to be concerned with graft union and subsequent growth of the graft. This was contradictory to the observation of Ratan (1985) in mango. He reported that the growth of scion in stone grafting was not influenced by the girth of stock or scion and number of leaves produced.

Histological studies at the graft position showed that the callus bridge connecting the stelar cylinder of the rootstock and the scion was found 14 days after grafting. The mechanism of the healing of graft union depends to a large extent as the activities of the cambium and its derivative tissue (Fahn, 1982). A cambial ring was differentiated from the callus bridge after 21 days. Secondary xylem and secondary phloem were produced from the newly formed cambium by at least 40 days after grafting. The rootstock and scion became so intimate that it forms a single common stelar cylinder and perform as a single functional unit.

When the survival and growth of the micrografts were studied, it was observed that 90.00 per cent of the planted out micrografts were survived. The survival rate was decreasing up to the fifth week after planting out and then it became static. The growth during the early days was slow and later it became faster. The reason may be the transformation from the heterotrophic to photoautotrophic

nature. The increase in height by about five weeks was 2.0 cm and the increase in leaf number was 2.0.

Ex vitro micrografting is a method in which the scion used was *in vitro* raised shoots and the rootstock used was nursery sown seedlings. The success of *ex vitro* micrografting was found to be very low in cocoa when compared to *in vitro* micrografting. The scion should have atleast one hardened leaf and the rootstock should be 2-3 months old. If scion shoots with tender leaves were used, death occurred even after proper graft union. This may be due to the absence of hardened leaves. The tender leaves may get dried easily under the high temperature and low humid environment. It was observed that the growth of successful graft was rapid and good.

Summary

SUMMARY

Investigations on 'Standardisation of *in vitro* techniques for rooting, hardening and micrografting of cocoa (*Theobroma cacao* L.)' were carried out at the Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara utilising the facilities of Plant Tissue Culture Laboratory of the Department of Plantation Crops and Spices. The results of the study are summarised below:

1. Nodal segments of 3.0 cm size taken from the middle of the fan branches were the best explant source for establishing cultures of elite field grown trees. Nodal segments taken from *in vitro* seedlings responded in a better way than that of adult tissues.

2. Pretreatment with the systemic fungicide bavistin (0.1%) for half an hour followed by treating with freshly prepared chlorine water for four minutes was the best method of surface sterilization.

3. Systemic bacterial infection of the cultures could be controlled by incorporating the antibiotic, Streptomycin sulphate at a concentration of 200 mg l^{-1}, in the subculturing media.

4. Poor culture establishment was due to the high rate of microbial infection which varied with weather conditions. Maximum culture establishment and minimum contamination was recorded during the month of March.

5. The best basal medium ideal for the explant establishment was WPM.

6. Among the different combinations of growth regulators tried, WPM supplemented with 2iP 5 + $AdSO_4$ 1 + CCC 0.75 + $AgNO_3$ 5 + PG 200 mg l^{-1} was the best for producing ideal shoots for rooting and micrografting.

7. Maximum shoot elongation was obtained when subcultured in growth regulator free WPM supplemented with 0.5 per cent AC and 3 per cent sucrose.

8. Growth of dormant bud and unfurling of leaves were obtained when subcultured in a media containing ABA 1 mg l^{-1} and kinetin 3 mg l^{-1}.

9. The optimum concentration of sucrose for bud break and shoot elongation was 5 per cent.

10. More number of elongated shoots were produced on culturing in solid media and incubating at high light intensity (4000 lux) and high temperature (28 + 2°C).

11. The cultures showed differential response to genotypes. When the three genotypes S 44.1, G VI 50 and G IV 4.1 were compared, genotype S 44.1 produced maximum shoot elongation and more leaves than G VI 50 and G IV 4.1.

12. Rooting percentage was very low both under *in vitro* and *ex vitro* conditions. Among the different basal media tried for *in vitro* rooting 1/2 MS supplemented with activated charcoal was found to be the best.

13. Among the different combinations of growth regulators, rooting was observed only in a few cases. Among the treatments, maximum rooting was obtained by pretreating with IBA 5000 mg l^{-1} for 3 seconds followed by culturing in 1/2 MS media containing 0.5 per cent AC.

14. Optimum concentration of sucrose for rooting was 3 per cent and that of agar was 0.6 per cent.

15. Stray cases of rooting was observed during prolonged culture in the shoot elongation media.

16. Addition of phloroglucinol along with IBA resulted in callus mediated rooting and the plantlets did not survive on planting out.

17. Among the different physical conditions tried better rooting was recorded under dark incubation at a temperature of 28 + 2°C.

18. Transferring the rooted plantlets from dark incubation to light for one week before planting out had a favourable effect on plantlet establishment.

19. Among the three genotypes, S 44.1, G VI 67 and G IV 4.1, genotype S 44.1 recorded better rooting.

20. Increasing the number of subculture during shoot proliferation had a slight but positive influence only on rooting of S 44.1.

21. Shoots from axenic seedlings rooted readily.

22. *Ex vitro* rooting was not successful in shoots from field explants.

23. Best potting medium for establishing *in vitro* cocoa plantlets was a mixture of soilrite and potting mixture in equal proportion.

24. The best hardening method for cocoa plantlets was covering with polybags for 2-3 weeks followed by periodical exposure to ambient conditions.

25. Spraying with nutrient starter solution 1/2 MS or 1/2 WPM improved the plantlet survival.

26. The plantlet survival decreased up to the second month of planting out and became static thereafter. The growth rate was very poor for *in vitro* raised cocoa plantlets from field explant.

27. *In vitro* and *ex vitro* micrografting were possible in cocoa.

28. Axenic seedlings cultured in half MS liquid medium devoid of sucrose was the ideal rootstock for micrografting. These seedlings were ready for grafting in two weeks when raised under high light intensity (4000 lux) and high temperature (28 + 2°C).

29. *In vitro* shoots from nodal segments recorded a high percentage of success as scion compared to plantlets from somatic embryos.

30. Among the different methods of grafting tried, viz. side, wedge and epicotyl, maximum success was recorded in side grafting.

31. Grafting success was maximum when the scions were grafted 4 cm below the cotyledons of 4-5 weeks old axenic seedlings.

32. Highest percentage of success and survival of the graft was recorded when scions of more than 2 cm long with at least two hardened leaves were used.

33. Anatomical studies revealed that after 14 days, a callus bridge was formed connecting the stelar cylinder of the rootstock and the scion. By about 3 weeks time, a cambial ring was differentiated from the callus bridge which was in line with the cambial ring of the rootstock and the scion. The union became so intimate and act as a single physiological step by about 40 days period.

34. The survival rate of planted out micrografts decreased up to the fifth month and after that it was static. Quicker and better growth was recorded when compared to the *in vitro* plantlets.

35. *Ex vitro* micrografting recorded lower success than *in vitro* micrografting.

36. Older plants with a few hardened leaves was ideal as rootstock. Scions should have at least one hardened leaf.

37. Rapid and extensive scion elongation was observed in *ex vitro* micrografting.

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* Originals not seen

Plates

Plate Ia. Nodal segment with part of the subtending leaf used as explant

b. Bud initiation one week after culturing in WPM + $AdSO_4$ 1 + CCC 0.75 + 2ip 5 + $AgNO_3$ 5 + PG 200 mg l^{-1}

c. Single shoot produced from leaf axil two weeks after culturing in WPM + 2ip 1 + $AdSO_4$ 0.5 + PG 200 mg l^{-1}

d. Multiple shoots produced from leaf axil two weeks after culturing in WPM + 2ip 5 + $AdSO_4$ 1 + CCC 0.75 + $AgNO_3$ 5 + PG 200 mg l^{-1}


a


b

Plate IIa. Multiple shoots produced three weeks after culture in WPM + 2ip 5 + $AdSO_4$ 1 + CCC 0.75 + $AgNO_3$ 5 + PG 200 mg l^{-1}

b. Single shoot elongation in WPM + AC 0.5% + Sucrose 3% + Streptomycin sulphate 200 mg l^{-1}

c. Multiple shoot elongation in WPM + AC 0.5% + Sucrose 3% + Streptomycin sulphate 200 mg l^{-1}


a


b

Plate IIIa. Appearance of root initials on prolonged culture in the establishment medium

b. Rooting in phloroglucinol added medium

c. L.S. of the root-shoot transition zone showing an intervening callus between vascular tissues of root and shoot


a


b

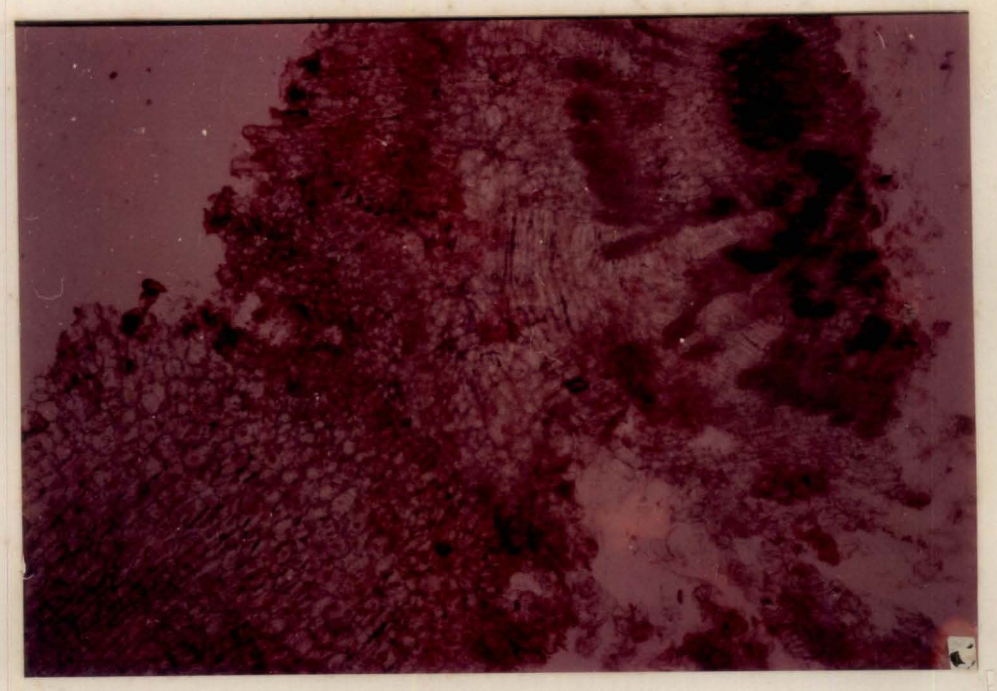


Plate IVa. A rooted plantlet producedby IBA (5000 mg l^{-1}) pulse treatment followed by culture in ½ MS + AC 0.5%

b. A rooted plantlet showing continued growth after hardening in the rooting medium - soilrite + potting mixture (1:1)


a

Plate Va. Micrografted plant one week after grafting - 2 cm long *in vitro* scions is grafted to one month old sterile rootstock by side grafting

b. Micrografted plant covered with a polybag kept for hardening in the hardening unit

c. A micrografted plant two weeks after hardening

d. A six months old micrografted plant in the larger pot ready for field planting


a

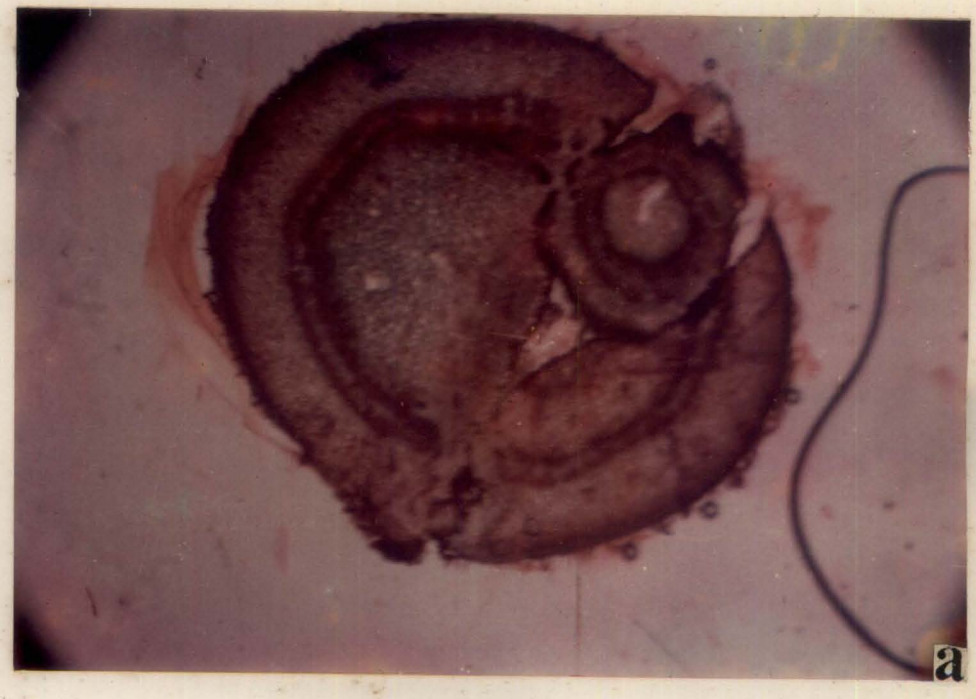

c

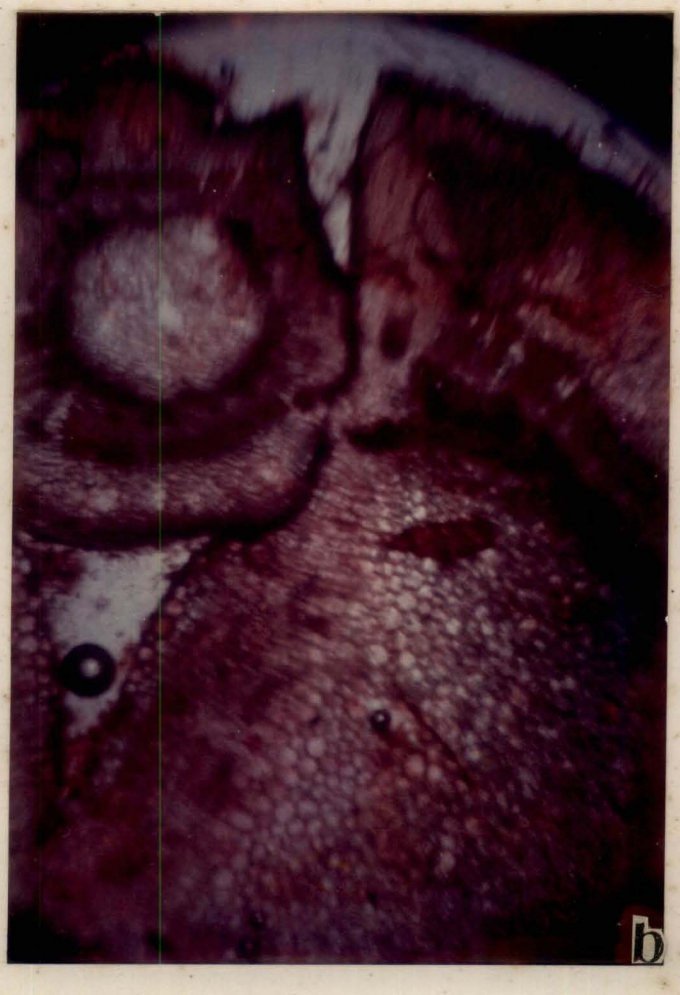

b


d

Plate VIa, b. 14 days old micrograft showing a callus bridge connecting the stelar cylinder of the rootstock and scion

c. 21 days old micrograft showing a cambial ring differentiating from the callus bridge


a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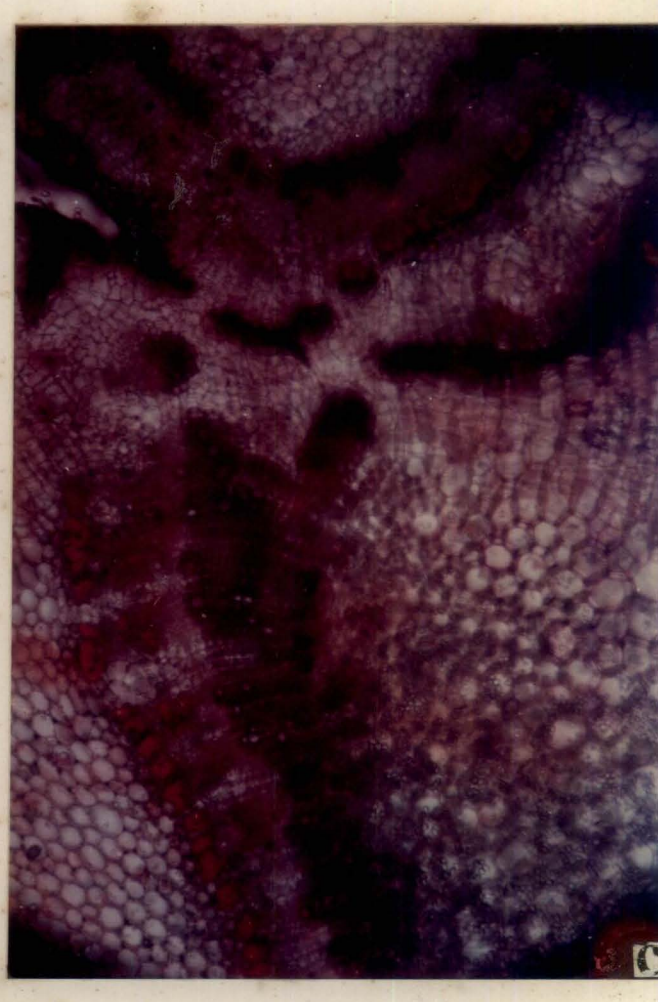
c

Plate VIIa. Better establishment of the graft union three weeks after micrografting

b, c. 90 days old micrograft showing a single common stelar cylinder for rootstock and scion


a


b


c

STANDARDISATION OF *in vitro* TECHNIQUES FOR ROOTING, HARDENING AND MICROGRAFTING IN COCOA (*Theobroma cacao* L.)

By
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ABSTRACT OF THE THESIS

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ABSTRACT

Investigations on 'Standardisation of *in vitro* techniques for rooting, hardening and micrografting in cocoa (*Theobroma cacao* L.)' were carried out at the Department of Plant Breeding and Genetics, College of Horticulture, Vellanikkara utilising the existing facilities of the Plant Tissue Culture Laboratory of the Department of Plantation Crops and Spices during the period from 1993-1996. Studies were conducted to identify the best rooting protocol and also to refine the technique of micrografting.

The nodal segments taken from the field were pretreated with Bavistin (0.1%) for half an hour and then surface sterilized in chlorine water for four minutes. The explants were then cultured in the medium, WPM + 2ip 5 + $AgNO_3$ 5 + CCC 0.75 + $AdSO_4$ + PG 200 mg l^{-1} and incubated at 28 + 2°C under 4000 lux light intensity for shoot bud release. For getting sufficient number of elongated shoots, subculturing was done in WPM supplemented with 0.5% activated charcoal and 200 mg l^{-1} streptomycin sulphate. Observations of three different genotypes revealed that they responded differently to *in vitro* contidition and the genotype S 44.1 exhibited a very good growth.

Rooting was very poor under *in vitro* condition and was completely absent under *ex vitro* condition. Among the different basal media for *in vitro* rooting, ½ MS supplemented with activated charcoal was the best. Maximum rooting was obtained when the shoots were pretreated in IBA 5000 mg l^{-1} for 3 seconds followed by culturing in the basal medium. Optimum concentration of sucrose for rooting was 3 per cent and that of agar was 0.6 per cent.

For rooting, the cultures should be kept at a temperature of 28 + 2°C under dark condition. The genotypes differed in their response to rooting. Among the three genotypes - S 44.1, G VI 67 and G IV 4.1 tested, S 44.1 recorded better rooting.

The rooted shoots should be potted in a medium containing a mixture of soilrite and potting mixture in equal proportion for establishment. The plantlets should be covered with polybags for 2-3 weeks and then exposed to ambient conditions periodically for hardening. Plantlet survival rate decreased up to the second month of planting out and after that it became static.

In vitro micrografting and *ex vito* micrografting were possible in cocoa. The best rootstock for *in vitro* micrografting was axenic seedlings cultured in half MS liquid medium devoid of sucrose. These seedlings were ready for grafting in two weeks when raised under high light intensity (4000 lux) and high temperature (28 + 2°C).

In vitro shoots from nodal segments were found to be a very good scion material for grafting. Among the different grafting techniqes, side grafting was the most ideal one. Success was the highest when scions with two or more hardened leaves were grafted 4 cm below the cotyledons in 4-5 weeks old axenic seedlings. Anatomical studies revealed that the graft union was complete in about a month. Grafted seedlings showed profuse growth after planting out.

Ex vitro micrografting recorded lower percentage of success than *in vitro* micrografting. Older plants with a few hardened leaves were the most ideal root-

stock and the scions should have at least one hardened leaf. Rapid and extensive scion elongation was observed in *ex vitro* micrografting. The most significant achievement of the present investigation was the standardisation of the technique of *in vitro* micrografting by which the rooting problem can be surmounted to a great extent.