IN VITRO STUDIES ON MORPHOGENESIS AND PLANT REGENERATION IN ELITE CLONES OF COCOA (*Theobroma cacao* L.)

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"*In vitro* studies on morphogenesis and plant regeneration in elite clones of 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 or other similar title, of any other university or society.

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ABBREVIATIONS

µM	- Micromolar
2,4-D	- 2,4-dichlorophenoxy acetic acid
2-ip	- 2- Isopentinyl Adenine
BA	- Benzyl Adenine
BAP	- Benzylamino purine
FAA	- Formaldehyde, acetic acid and ethanol
$g\ l^{-1}$	- gram per litre
GA_3	- Gibberellic Acid
$HgCl_2$	- Mercuric chloride
IAA	- Indole-3-Acetic Acid
IBA	- Indole Butyric Acid
LAF	- Laminar Air Flow
$mg\ l^{-1}$	- milligram per litre
min	- minute
MS	- Murashige and Skoog
NAA	- Naphthalene Acetic Acid
ppm	- parts per million
sec	- second
SH	- Schenk and Hildebrandt
TBA	- Tert-butanol
UV	- Ultraviolet
v/v	- volume in volume
w/v	- weight in volume
WPM	- Woody Plant Medium

INTRODUCTION

1. INTRODUCTION

Cocoa (*Theobroma cacao* L.) is a beverage crop of huge economic significance. It is a key raw material for chocolate manufacturing. It is popularly known as 'Food of God' and considered divine from time immemorial. It is known to have originated from Central and Southern America. It belongs to family Malvaceae and the genus Theobroma. Among the twenty two species of this genus, *Theobroma cacao* is the only commercially important one due to the value of its seeds. Seeds are commonly known as beans and are obtained from pods. The three cultivars of cocoa are Criollo, Forastero and Trinitario, which differ in its genetic origin, pod morphology, size, colour and flavour of beans (Wood and Lass, 1987). In addition to its flavour, cocoa is known to have notable health benefits. High levels of flavonoids have been linked to cardiovascular health benefits and reduces cholesterol level.

Cocoa is cultivated on land between 20 degrees north and south of equator. According to ICCO (International Cocoa Organization), the worldwide production of cocoa beans in 2015-16 is around 4 million metric tons and 70 per cent of world production is from African countries like Cote d 'Ivorie, Ghana, Nigeria and Cameroon. The commercial cultivation of cocoa in India was started in 1980s and now it is mainly cultivated in the southern states like Kerala, Karnataka, Andhra Pradesh and Tamil Nadu. It is also considered as an ideal intercrop in coconut and arecanut based cropping systems. The present domestic availability is only about 30 per cent. Considering the market growth in the chocolate segment in India, which is about 15 per cent per annum, cocoa has a great potential to develop in coming years.

Cocoa is a perennial, cross pollinated and diploid plant. It is mainly propagated through seeds and are highly heterozygous due to open pollination. As a result, cocoa plants derived from seeds are highly variable in terms of their agronomic performance. However, the out crossing nature of the crop insist to develop seedlings only from specially designed polyclonal gardens, otherwise the quality of planting material will be

drastically affected. The polyclonal gardens are available only with limited government institutions and the seedlings produced from here cannot meet the present demand. Also, due to the non- availability of land and labour, there is limited scope for further expansion of poly clonal garden.

The vegetative clonal propagation of superior cocoa genotypes has long been recognized as a potential means to increase cocoa production (Wood and Lass, 1987). However, progress in the development of improved methods for vegetative propagation of cocoa has been slow. In addition, there are a number of disadvantages associated with the propagation of cocoa plants via rooting, grafting or budding of plagiotropic cuttings including the intensive labour and associated costs and an undesirable bush like growth pattern which can occur.

In vitro propagation in cocoa can be considered as an alternative method of propagation that facilitate international exchange of planting material, speed up the diffusion of selected clones, keep the plant habit preferred and limit the dissemination of diseases which can be transmitted by conventional methods. It can also address the question of availability of bulk planting material. Thus, clonal propagation of superior genotypes through *in vitro* methods was one of the identified thrust areas of cocoa biotechnology. Under this background, the present study was proposed with an objective to develop a protocol for *in vitro* regeneration among elite cocoa clones and to determine the variation in morphogenesis.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Cocoa is one of the important commercial plantation crops of India. It is self-incompatible (Cope, 1962) and highly cross pollinated. The most common method of propagation is through seeds and the source of seed pod is very important in deciding the quality of seedling. In order to produce seeds of known parentage and more genetic superiority, seed pods are collected from polyclonal seed gardens. These gardens must be isolated from other cocoa plantations at a distance of 200 m in order to prevent undesirable cross pollination (CPCRI, 2007). Minimol *et al.* (2015) highlighted the importance of maintaining polyclonal gardens in cocoa for hybrid seed production by exploiting self-incompatibility. Even then, there are chances of segregation to some extent and the progenies may not be true to type. Moreover, the desired crosses are mainly done through hand pollination which requires properly skilled labour.

Another commonly relied method of propagation in cocoa is budding. The budded plants from fan shoots usually have a diffuse branching system and bushy growth habit. This kind of growth habit often cause difficulties in carrying out cultural operations and harvesting. Thus, appropriate pruning is necessary and this also requires skilled labour.

In cocoa, the clonal propagation through *in vitro* methods are expected to address these limitations of seed propagation and budding. *In vitro* propagation facilitates rapid multiplication of plants within a short period of time and tissue culture plants are true to type. In addition to this, it allows improvement, conservation and distribution of cocoa germplasm (Maximova *et al.*, 2005). Another potential contribution of *in vitro* propagation is the proper screening of plants for various biotic and abiotic stresses under laboratory conditions, which is practically easier than field conditions. Thus, *in vitro* propagation aids in production and testing of plants with resistance to diseases like cocoa swollen shoot virus (Quainoo *et al.*, 2008) and drought tolerance. The review highlights not only the micropropagation in cocoa but also in other woody perennials with special emphasis on enhanced release of axillary buds.

2.1. BOTANY OF COCOA

Theobroma cacao is a small tree of 6-8 m high under Malvaceae family. The tree yields cocoa beans, which is the source of chocolate and cocoa butter. It is tap rooted with few lateral branches. The roots are mainly concentrated at a depth of 15-20 cm below the soil surface (Wahid *et al*., 1989). It grows in tiers and shows dimorphic branching. The single main stem or chupon grows to a height of 1-2 m. Then the terminal bud breaks out and grows into lateral plagiotropic 'fan branches'. Leaves are simple, petiolate with long lamina and wavy margins. Depending on the level of management, cocoa starts flowering after 1-2 years of planting. In Kerala, two peak flowering periods are observed, one in May-June and the other in November-December (Rajamony and Mohanakumaran, 1995). Flowers are cauliferous, produced on thickened portion of woody trunk called cushion. Fruit is botanically a drupe referred as pod. Seeds are called beans and each pod contains about 20-60 beans.

2.2. PROPAGATION IN COCOA

Cocoa is amenable to both sexual as well as asexual methods of propagation. It can be vegetatively propagated by stem cuttings, buds and grafts. But it is conventionally propagated through seeds.

2.2.1. **Seed propagation**

Cocoa seeds become physiologically mature for germination in about 2 weeks before the ripening of the pod starts. Seeds are preferably sown in December - January, so that 4 - 6 month old seedlings become available for planting in May - June. The seeds of cocoa are non-dormant and lose viability within a week. To retain viability, extracted seeds can be stored in moist charcoal and then packing in polythene bags. Cocoa is naturally cross pollinated through insects and hence highly heterozygous. Thus, seed propagation did not give true to type progeny.

2.2.2. **Vegetative propagation**

The common vegetative propagation methods in cocoa are cuttings, budding and grafting.

2.2.2.1. ***Cuttings***

For vegetative propagation through cuttings, fan branches are more preferred though chupons also can be used. Young branches of 10-15 cm long and green at the top and brown below are selected. Each branch can give two to four cuttings and the number of leaves in each cutting should be at least two. Laminar portions of leaves are cut off and stumps of 5 cm are made. The cut portion is then dipped in rooting hormone preparation. The most commonly used preparation in Ghana is a mixed solution containing NAA (5 g) and IBA (5 g) dissolved in 480 ml of 50 per cent alcohol (Nair *et al.*, 1993). For planting, polythene bags are filled with saw dust and then kept in moist chamber with sprinkling of water at frequent intervals. It takes a month for rooting.

2.2.2.2. ***Budding***

Root stocks of 6-12 month old seedlings are mostly preferred for budding. Nair *et al.* (1993) described budding on older rootstock. Bud wood is usually taken from fan branches or chupons. Any of the common methods of budding like T- budding, patch budding, or modified forkert method can be used. After three weeks, the budding tape is cut off and if the union is successful, the stock portion is snapped back. The stock portion is cut back after the bud has grown to a shoot. The plant is ready to transplant after 3-6 months.

In Ghana, T- budding was reported to have highest percentage of success but according to Keshavachandran (1981) modified forket method was found to be better than T, inverted T and patch budding methods.

2.2.2.3. ***Grafting***

Seedlings of four months growth are used as rootstock. Shoots of comparable thickness which has just turned brown with at least two hardened leaves are used as scion. Usually wedge grafting is done in cocoa. For this, the stock stem is cut to convenient height (about 5 cm from ground level) and then a longitudinal slit of about 2 cm is made. In stem portion of scion shoot, slanting cut is given on two sides to a similar length and it is made into a wedge. The scion portion is then inserted into the stock portion and tied round with a budding tape to keep it in position. It takes about three weeks for the graft union to be completed (Nair *et al.*, 1993).

2.2.2.4. ***In vitro propagation***

Since the early work of Archibald (1954), attempts were made to propagate cocoa through *in vitro* techniques. Mallika *et al.* (1992) worked on *in vitro* propagation in *Theobroma cacao* using nodal segments as explants in WPM supplemented with 2-ip and IAA. Bindu (1997) investigated on the standardization of *in vitro* techniques for rooting, hardening and micrografting in cocoa. Kurian and Peter (2007) reported shoot proliferation and elongation in cocoa nodal explants on MS medium containing kinetin (5 mg l^{-1}), NAA (1 mg l^{-1}) and brossinolide (0.1 mg l^{-1}).

2.2.2.4.1. ***In vitro* propagation in other crops**

In *Terminalia arjuna*, the terminal bud and mature nodal explants were cultured to obtain shoots (Thomas *et al.*, 2003). For shoot induction, WPM with BA (2 mg l^{-1}) was found to be best and the plantlets rooted in media containing IAA (1 mg l^{-1}).

Antony (2008) stated that for *in vitro* culture of nodal segments of nutmeg, SH medium was found superior to half strength MS and WPM. When the medium was supplemented with thidiazuron (0.03 mg l^{-1}) and activated charcoal (0.5 %), 50 per cent of cultures showed bud expansion within 9 days. When nodal segments were taken from juvenile as well as coppiced trees, maximum culture establishment was seen on SH

medium supplied with thidiazuron (0.03 mg l^{-1}), calcium hydrolysate (25 mg l^{-1}), sucrose (2 %), glucose (1 %) and activated charcoal (0.5 %).

Adiga *et al.* (1996) regenerated *Artocarpus heterphyllus* using nodal explants and the results shown that MS medium containing BA (2 mg l^{-1}) showed highest number of shoots, adventitious buds per shoot and number of leaves per shoot.

Micropropagation by axillary bud proliferation from nodal segments of *Terminalia bellirica* was studied by Ramesh *et al.* (2005). The *in vitro* produced shoots showed rooting in both Gamborg's medium and WPM, when supplemented with IBA (4.9 μM).

Keshavachandran and Riji (2005) studied *in vitro* propagation of cashew using nodal segments as explants. For shoot initiation, MS medium was supplemented with kinetin (5 mg l^{-1}), NAA (1 mg l^{-1}) and brassinolide (0.1 mg l^{-1}). The cultures were then kept in dark for 7 days followed by light. *In vitro* shoots were rooted when pulse treatment was given in IBA (1000 mg l^{-1}) for 2-3 min followed by transferring to quarter strength MS with IBA (1 mg l^{-1}).

An efficient tissue culture regeneration system was developed for *Elliotia racemosa* by Woo and Wetzstein (2008). Maximum shoot elongation system was obtained in WPM incorporated with 2-ip (25 μM). Moreover, 90 per cent rooting was obtained when pulse treatment was given to *in vitro* shoots in IBA (150 μM) for 5 days.

2.2.2.5. ***General aspects of tissue culture***

Haberlandt (1902) was the first to envision the concept of plant tissue culture by culturing the tissues under *in vitro* condition on a nutrient medium. Schleiden (1838) and Schwann (1839) proposed the cell theory which states that plants are composed of cells in their structures and these cells show totipotency. *In vitro* techniques exploit this to regenerate plants from cells, tissues and organs. Thus, it forms a basis for plant tissue culture. Moreover, the efforts of many pioneer investigators like White (1934), Gautheret (1939), Nobecourt (1939), Miller *et al.* (1956), Steward *et al.* (1958), Bergmann (1960),

Cocking (1966), Carlson *et al.* (1972), Hu and Wang (1983) and Litz (1986) also contributed to the development of plant tissue culture. Skoog and Miller (1957) developed a completely defined plant nutrient medium for tissue culture.

Vasil and Vasil (1980) stated that tissue culture plants grow and mature at a faster rate as compared to conventional seed propagated progenies. Also plant tissue culture has a wide range of applications in agriculture like clonal propagation, axillary shoot multiplication, virus elimination, *in vitro* grafting, embryo rescue, secondary metabolite production, *in vitro* gene banks, production of haploid plants and embryo rescue (Akin-Idowu *et al.*, 2009). Thus, tissue culture is a method of micropropagation for commercial production of true to type plants that grow in relatively short period of time.

Multiplication of plants in tissue culture can be achieved in three different ways *viz.* enhancing axillary bud breaking, production of adventitious buds directly or indirectly via callus and somatic cell embryogenesis. The shoots produced *in vitro* through axillary bud breaking and callus mediated organogenesis were later kept for rooting in different media (Murashige, 1974). Callus mediated organogenesis and somatic embryogenesis are not suggested for clonal propagation of plants since the chance of producing aberrant is more. But in axillary bud or shoot tip culture, genetic fidelity is considerably more (Neelannavar, 2006).

2.2.2.5.1. **Enhanced release of axillary bud**

Axillary buds are those present in the axils of leaves having the potential to develop into a shoot. Generally, these buds remain dormant in plants due to apical dominance. They grows out only if the terminal bud is removed or injured. The phenomenon of apical dominance is controlled by growth regulators. Thus, exogenous application of growth regulator like cytokinin can stimulate the growth of axillary buds even in presence of terminal buds (Sachs and Thimann, 1967). Culturing of axillary buds offer better genetic uniformity in progenies (Lee and Rao, 1988). Few attempts were made for *in vitro* propagation of *Theobroma cacao* through enhanced release of axillary

buds. Traore *et al.* (2003) cultured nodal explants of cocoa on thidiazuron medium and reported that, axillary buds proliferated and developed into shoots. When pulse treatment was given for root induction, the total percentage of rooted micro cuttings increased up to 89 per cent.

2.2.2.6. ***Factors controlling success of in vitro plant regeneration***

2.2.2.6.1. **Explant - age, size and position**

Explants can be taken from different parts of a plant, including portions of shoots, leaves, stems, flowers and roots. The most commonly used explants are meristematic tips of shoots, axillary bud tips and root tip. Usually these tissues shows a high rate of multiplication and can produce growth regulating substances like auxin and cytokinin (Akin-Idowu *et al.*, 2009).

Pierik (1991) reported that nodal meristems are an important explant source for micropropagation and plants obtained by culturing these were comparatively more resistant to genetic variation. In rough lemon (*Citrus jambhiri)*, for callus induction, nodal segments were reported to be better as compared to leaf and root segments (Savita *et al.*, 2010).

In *Vanillla planifolia*, nodal stem segments as explants were found to be easy for clonal propagation (Agarwal *et al.*, 1992). The study conducted by Anirudh and Kanwar (2008) in *Pyrus pyrifolia* showed that the culture establishment frequency was higher for nodal segments (71.95 %) as compared to shoot tips (69.07 %). In *Jatropha curcas*, various explants like shoot tip, stem and axillary buds can be successfully used for micropropagation (Kaewpoo and Te-Chato, 2009).

The physiological state of shoots from which explants are collected influences the establishment and development of cultures. Passey and Jones (1983) recommended axillary buds from actively flushing shoots. Lardet *et al.* (1998) demonstrated that, buds from dormant shoots respond better than those collected during active meristem growth.

Gymnema sylvestre showed maximum shoot induction when 30 day old seedling axillary node explants were used for *in vitro* propagation (Komalavalli and Rao, 2000).

The position of nodes relative to the shoot apex can also affect establishment and the axillary buds distal from the shoot tip are more likely to respond. Sharma *et al.* (2003) stated that the position of bud on the foliage twig of *Crataeva adansonii* effects the rate of shoot multiplication.

Hussey (1983) stated that if larger the size of explant, it shows more growth and survival. In *Mangifera indica*, irrespective of the age of tree, shoot tips of 5 cm long and 0.4-0.6 cm thick from current season's shoots was found to be best for culture establishment and shoot initiation. In *Bixa orellana*, nodal explant of size 0.5 cm produced maximum multiple shoots (D'Souza and Sharon, 2001).

In cocoa, *in vitro* propagated plants can be obtained from a wide range of its tissues like zygotic embryos (Pence, 1989), nodal segments (Mallika *et al.*, 1992), nucellar tissues (Figueira and Janick, 1993) and petals and staminodes (Boutchouang *et al.*, 2016). The shoot explants taken from mature trees are difficult to regenerate due to slow growth, browning, poor rooting and high rate of contamination. Thus, explants are usually taken from grafted plants of 2-3 years old with regular spraying of fungicides.

Bindu (1997) used non-woody stem segments of cocoa with 6-8 cm long from fan branches of mature trees for *in vitro* propagation. According to Kurian and Peter (2007), the ideal explant for micropropagation in cocoa was single node cutting of 2-3 cm long having maximum length of lower internode and a portion of subtending leaf.

2.2.2.6.2. **Season of collection of explant**

Seasonal changes greatly influence not only the culture establishment but also the concentration of plant growth regulators in the cambial zone (Siril and Dhar, 1997). It was reported that in tamarind the explants collected during April - June showed maximum bud break under *in vitro* condition (Mahale *et al.*, 2005). In *Vitex trifolia*, maximum bud break

(90%), along with maximum number of shoots per nodal explant was recorded when explants were collected during mid-September to November (Ahmed and Anis, 2014).

The major constrain in the establishment of *in vitro* cultures in *Bauhinia valii* was browning (Dhar and Upreti, 1999). Usually, the explants collected in April-June (growing phase) showed maximum browning. So, the collection of explants were mainly done during March-April (dormant phase) and this season showed better shoot initiation also.

Hohtola (1988) studied the seasonal effect on explant viability and degree of contamination in *Pinus sylvestris*. It was observed that, callus proliferation was highest in explants collected from December - January and April - July and lowest in February and from September - November. In tamarind, maximum bud break under *in vitro* condition was when explants were collected in April.

In vitro response of apical bud explants from mature trees of *Artocarpus heterophyllus* showed that buds collected from November - January was found to be the best for initiation of cultures from trees grown under field condition (Amin and Jaiswal, 1993).

Season of explant collection influences the culture establishment in *Swietenia macrophylla* (Sankri, 2009). *In vitro* studies showed least contamination when explants were collected during November to April as compared to other months.

2.2.2.6.3. **Genotype**

Coleman and Ernst (1989) observed *in vitro* adventitious shoot regeneration response from three genotypes of *Popolus deltoides* using internodal explants. Genotypic differences were observed in shoot regeneration when cultured on media supplemented with 0.5 mg l^{-1} zeatin. Mallika *et al.* (1997) reported that genotype has no influence on *in vitro* shoot initiation in nutmeg. In *Theobroma cacao*, the genotypic background plays a vital role in all steps of embryogenic process. According to Ajijah *et al.* (2016), the genotype responses in cocoa were highly dependent on the type of explant used.

Bindu (1997) standardized the *in vitro* techniques for root induction, hardening and micrografting in cocoa using the genotypes GIV 4.1, GVI 50, GVI 67 and S 44.1.The study showed that, all genotypes responded differently to *in vitro* propagation and genotype S 44.1 responded better than others.

2.2.2.6.4. **Surface sterilization**

A wide range of microbial contaminants are present on plant surface. So, when explants are inoculated to a nutrient medium, the entire *in vitro* system gets contaminated. Thus, surface sterilization is a crucial step in plant tissue culture and for this different surface sterilizing agents can be used at varying durations of time.

Surface sterilization of pods of Acacia for *in vitro* culture was done by treating with 2 per cent (v/v) solution of detergent Teepol for 10 min and then disinfection using $HgCl_2$ solution for 20 min (Gantait *et al.*, 2018). Sankri (2009) reported that surface sterilization of *Swietenia macrophylla* was done using solution of Bavistin and Indofil M-45 each at 0.2 per cent for 1 hour followed by surface sterilization with 0.1 per cent $HgCl_2$ for 15 min. In almond, surface sterilization of shoot tips was achieved by using 0.1 per cent $HgCl_2$ for 10 min followed by use of 70 per cent alcohol for 10 sec.

Bindu (1997) standardized the pretreatment of nodal segments of cocoa with systemic fungicide Bavistin (0.1%) for 1 hour followed by treatment with freshly prepared chlorine water for 4 min. Silva *et al.* (2008) reported that surface sterilization of immature flower buds of cocoa by immersion in 70 per cent (v/v) Ethyl alcohol for 1 min and 30 sec followed by 20 min in 2.5 per cent (v/v) Sodium hypochlorite solution containing 0.1 per cent (v/v) Tween-20 and then rinsing in sterile water reduces maximum contaminants.

2.2.2.6.5. **Presence of systemic contaminants**

Surface sterilization of explants has its own limitations and it is often inefficient and finally leads to culture contamination (Leifert and Woodward, 1998). This may be either due to the use of inactive disinfectants or the microorganisms being protected inside the plant tissue used as explant. It is easy to eliminate the contamination due to

microorganisms present on the surface but difficult to control those present exogenously. These are usually associated with latent contamination and a major constrain in woody plant tissue culture (George and Sherrington, 1984). Thus, proper sterilization procedure is inevitable.

The use of fungicides in cultures for reducing fungal contamination was suggested by Shields *et al.* (1984). Mallika *et al.* (1992) highlighted that systemic and contact fungicidal spray of mother plants of cocoa could control the culture contamination to a certain limit.

Dodds and Roberts (1985) proposed that, it is preferable to avoid the use of antibiotics for the surface sterilization of explant as it often give unpredictable results in tissue culture.

2.2.2.6.6. **Culture medium**

Culture medium and its composition is one of the most important factor that affects the growth and morphogenesis of plant tissues. Several media formulations have been reported by many researches. The earliest media proposed was the root culture medium by White (1934). Murashige and Skoog (1962) proposed the MS medium, a nutrient blend of amino acid, inorganic salts and vitamins. Since then, it became the widely accepted medium for micropropagation, callus culture and suspension culture. Later many derivatives of MS medium were formulated like B5 medium (Gamborg *et al.*, 1968), SH (Schenk and Hildebrandt, 1972) and Woody Plant medium (Lloyd and McCown, 1980). WPM is a mineral salt medium most widely used for the microculture of woody crops (Lloyd and McCown, 1980). This is because, many woody species could not tolerate the relatively high salt and chloride levels encountered in medium formulations like MS.

Shahinozzaman *et al.* (2012) analyzed the differential effect of basal media on shoot proliferation utilizing MS and WPM as experimental media in *Acacia catechu*. The result showed that maximum explants produced highest number of shoots on MS medium, but explants produced longest shoots in WPM medium.

Tiwari *et al.* (2002) used nodal explants of teak for developing a micro propagation protocol and the maximum average number of shoots were reported in MS media supplemented with BAP (22.2 μM) and IAA (0.57 μM). In *Pyrus pyrifolia*, the highest number of shoots per culture was obtained with WPM followed by MS medium (Thakur and Kanwar, 2008).

In *Morinda citrifolia*, an improved tissue culture protocol was developed by Shekhawat *et al.* (2015). Shoot regeneration from nodal meristem explant was maximum when MS media supplemented with 6-BAP (4 mg l^{-1}).

In cocoa, the basal media, WPM was found to be superior to half strength MS in which cultures could be maintained for a long time without leaf necrosis and abscission. Bindu (1997) stated that the best basal medium ideal for culture establishment in cocoa was WPM.

2.2.2.6.7. **Gelling agent**

Plant tissue culture is commonly done in a semi-solid or solid media. The gel provides a surface support for the tissue to grow in static condition. It should be firm enough to hold the explant in position. Hardness of culture medium is very important and it influences the growth of culture. Too much hardy medium inhibits proper contact between medium and tissue (Debergh, 1983).

Hamidah *et al.* (1997) reported that, for micropropagation of *Anthurium scherzerianum*, the better solidifying agent for culture medium was gelrite as compared to agar. Neelannavar (2006) standardized the best solidifying agent for shoot proliferation and growth in *Vanilla planifolia*. A combination of sago at 50 g l^{-1} and agar 1 g l^{-1} was recognized as best than solo use of agar.

2.2.2.6.8. **Growth regulators**

The role of plant growth regulators in organ formation was reported by Skoog and Miller (1957). Generally, root formation is favoured by high auxin to cytokinin ratio

whereas high cytokinin to auxin ratio promote shoot development. Murashige (1974) stated that, cytokinin enhances the lateral bud expansion from leaf axils. In woody plants, growth regulators like IBA and NAA are mostly preferred for root induction (Pierik, 1988).

Woody species like *Rhododendron* and *Azalea* of Ericaceae family are intolerant of growth regulator like BA and media like MS, and produce stunted shoot growth in *in vitro* cultures. Due to this reason 2-ip or zeatin in WPM are commonly used (McCown, 2000).

Axillary shoot proliferation from nodal explants of three pummelo varieties were studied by Begum *et al.* (2004). Half strength MS medium supplemented with BA was found to have highest shoot induction as compared to kinetin and NAA.

In *Bixa orellana*, from shoot apex and nodal explants, plants were regenerated on B5 medium supplemented with 4.9 μM 2-ip (D'Souza and Sharon, 2001). *In vitro* plant regeneration of *Acacia catechu* was done in WPM supplemented with 13.9 μM kinetin and 2.7 μM NAA (Gantait *et al.*, 2018).

In *Saraca asoca, in vitro* propagation was done using shoot tip, nodal and inter nodal explants in MS medium with different concentration of BAP, kinetin, and 2,4-D (Ramasubbu *et al.*, 2008). The result revealed that shoot organogenesis (82 %) was high when nodal explants were cultured in MS media supplemented with BAP (0.5 mg l^{-1}) and callus were formed more on 2, 4-D.

In cocoa, WPM containing kinetin/2-ip (1 mg l^{-1}) and IAA (0.02 mg l^{-1}) was reported to have successful bud sprout and leaf expansion from pre-existing meristems (Mallika *et al.*, 1992).

2.2.2.6.9. **Carbon source**

Carbohydrates are indispensable for growth and development of tissue culture plants (Gamborg *et al.*, 1976). It is essential for differentiation of xylem and phloem

elements in cultured cells (Aloni, 1980). In apple, maltose was found to be more effective than fructose, glucose or sucrose (Daigny *et al.*, 1996). Fitch and Maureen (2003) reported that 3 per cent sucrose was best for *in vitro* propagation of papaya.

Traore and Guilitinan (2006) reported that cocoa explants (petals and staminodes) showed no growth when cultured on media containing sorbitol and slow growth on media containing maltose. Moreover, the percentage of explants producing one or more embryos on media containing glucose, fructose, and sorbitol ranged from 6 per cent to 99 per cent, 18 per cent to 19 per cent and 3 per cent to 82 per cent respectively based on the genotype.

2.2.2.6.10. **Vitamins**

Vitamins are essential component supplemented in the culture media for better growth of tissues. The most commonly used vitamins in tissue culture media are thiamine, nicotinic acid, pyridoxine and myo-inositol in the ratio 0.1: 0.5: 0.5: 100 mg l^{-1} (Murashige and Skoog, 1962).

Antonopoulou *et al.* (2005) studied the effect of addition of riboflavin to the *in vitro* rooting medium of peach plant. It was reported that rooting percentage increased with the addition of riboflavin from 0-2 mg l^{-1}.

2.2.2.6.11. **Silver nitrate**

Giridhar *et al.* (2005) studied the influence of silver nitrate on shoot multiplication of vanilla under *in vitro* condition. Ozden-Tokatli *et al.* (2005) conducted *in vitro* study using nodal explants from pistachio. When the culture medium was added up to 48 µM of silver nitrate, there was an improved regeneration frequency and shoot growth and a reduced basal callus formation. In tomato, the beneficial effect of silver nitrate on reducing browning was observed during callus induction (Shah *et al.*, 2014).

In cocoa explants, profuse callusing at the expense of differentiation could be suppressed by the addition of silver nitrate (5 ppm) to the medium (Mallika *et al.*, 1992). Kouassi *et al.* (2017) studied the effect of antioxidants in tissue browning of different

genotypes of cocoa. When compared with the control, callus browning was reduced two to three times when various concentrations of the silver nitrate were supplemented to the culture media.

2.2.2.6.12. **Activated charcoal**

Activated charcoal is often used in plant tissue culture for improving cell growth and development (Pan and Staden, 1998). It has a promotary effect on morphogenesis due to its irreversible adsorption of inhibitory compounds in the culture medium and thereby decreasing the toxic metabolites, phenolic exudation and brown exudate accumulation. Moreover, it has some stimulatory and inhibitory activities including the release of substances that promote growth, alteration and darkening of culture media, and adsorption of vitamins, metal ions and plant growth regulators like abscissic acid and gaseous ethylene. Thus plays an important role in micropropagation, somatic embryogenesis, anther culture, synthetic seed production, protoplast culture, root and stem elongation. The major constrain in using activated charcoal in the medium is that, in addition to adsorbing unwanted substances, it may adsorb needed hormones (Ebert *et al.*, 1993), vitamins (Pan and Staden, 1998) or metal ions such as Cu^{2+} and Zn^{2+} (Van Winkle *et al.*, 2003).

Mallika *et al.* (1992) stated that development of natural roots is promoted by activated charcoal in the medium and presence of 1 per cent activated charcoal is good for thinner and healthier roots.

Phenol exudation from the explant is a major constrain in *Ensete ventricosum*. Birmeta and Welander (2004) overcame this problem by adding 0.05-0.1 g l^{-1} activated charcoal together with dark incubation of the cultures without retarding the growth of explants. *Hagenia abyssinica*, a multipurpose tree was micropropagated through terminal and lateral shoots on WPM, supplemented with BA, IBA and 0.1 g l^{-1} activated charcoal. The activated charcoal added to the medium avoided media browning (Feyissa *et al.*, 2005).

2.2.2.6.13. **Culture environment**

The rate of growth and extent of differentiation of tissues are greatly influenced by physical conditions such as pH of the medium, light, temperature and relative humidity.

Srividya *et al.* (1998) reported that, in neem, for the optimum growth of axillary buds, the cultures should be maintained in a temperature of 28 ± 2^0C with a light intensity of 3000 lux. In papaya, most of the *in vitro* cultures were grown successfully under 16 h photoperiod receiving 2000-3000 lux in culture rooms (Suthamathi *et al.*, 2002).

Sivanesan and Jeong (2009) reported that, in *Plumbago zeylanica, the* cultures maintained at 25 ± 2^0C under 16 h photoperiod using cool, white fluorescent light produced successful shoot induction. In *Dioscorea alata,* the cultures were maintained at 25 ± 1^0C under 16 h photoperiod using white fluorescent tubes were shown successful shoot induction (Das *et al.*, 2013).

Bindu (1997) obtained maximum elongated shoots in cocoa when cultured in solid media and incubating at a temperature of 28 ± 2^0C and a high light intensity of 4000 lux.

2.2.2.6.14. **Exudation from explant and methods to overcome this under *in vitro* condition**

Browning of explant along with exudations in cultures is a major obstacle for *in vitro* establishment of many plant species, especially the woody perennials. Phenolic compounds leach out from cut ends of explants, oxidized by the major protease, polyphenol oxidases leads to this lethal browning of explant tissues as well as the culture medium (Bhat and Chandel, 1991). Hassan and Zayed (2018) suggested the addition of anti-oxidants like citric acid (150 mg l^{-1}) or ascorbic acid (100 mg l^{-1}) to the medium before culturing for minimizing the polyphenol interference.

Warrier *et al.* (2005) investigated the phenol interference in Indian rose wood (*Dalbergia latifolia*) under *in vitro* conditions. To overcome this, the explants were

soaked in a solution of ascorbic acid and citric acid at 150 mg l^{-1} for 3 hours. Also, the culture medium was incorporated with 150 mg l^{-1} each of ascorbic and citric acid and 0.7 per cent polyvinyl pyrrolidone. This combination of treatments greatly reduced the problem.

Komalavalli and Rao (2000) found that, in *Gymnama sylvestre,* addition of citric acid (100 mg l^{-1}) to the culture medium prevented phenolic exudation. Moreover, it enhanced the production of healthy shoots and shoot bud differentiation. Dalal *et al.* (2006) reported that in apple, dark treatment given to the initiated shoots for few days reduced the release of phenolic compounds from it. The effect of antioxidant treatment on total polyphenol exudation was studied by North *et al. (*2012) in *Strelitzia reginae.* The total phenol content was reduced to 59 per cent when activated charcoal was added to the medium as compared to other anti-oxidants like ascorbic acid.

In vitro propagation of *Dioscoria alata* using nodal cuttings showed leakage of exudate from the cut ends of explant and then browning of the culture medium. To overcome this problem, sealing of the cut ends of explants using paraffin wax was found to be best with 90 per cent success (Bhat and Chandel, 1991).

The effect of activated charcoal on browning of medium in different *Piper* species was studied by Madhusudhanan and Rahiman (2000). The explants used were nodal and internodal segments, stem, petiole and leaf. Addition of activated charcoal 200 mg l^{-1} to MS medium was proved to be the best with minimum browning. Among the various species, *Piper longum* showed maximum browning of cultures and *Piper nigram* showed minimum.

2.2.2.6.15. **Multiple shoot induction**

Siddique and Anis (2007) evaluated the effect of two cytokinins, BA and thidiazuron as supplements to MS for inducing multiple shoots in *Cassia angustifolia*. The highest rate of shoot multiplication was reported in MS media supplemented with 5 µM thidiazuron and 1 µM IAA at pH 5.8. In *Vitex trifolia*, optimum shoot multiplication

and elongation was achieved when nodal segments were cultured on MS media containing 5.0 µM thidiazuron, 1.0 µM BA and 0.5 µM NAA (Ahmed and Anis, 2014).

Singh *et al.* (1994) studied the multiple shoot formation from shoot tips of mature explants of *Citrus reticulatum* and *Citrus limon* when cultured on MS media fortified with 0.25 mg l^{-1} BAP, 0.5 mg l^{-1} IBA and 0.5 mg l^{-1} NAA. Mendanha *et al.* (1998) reported multiple shoot induction from axillary buds of *Havea brasiliensis* in MS media supplemented with 1 mg l^{-1} kinetin; 1 mg l^{-1} 2, 4-D; sucrose 20 g $l^{-1}$and agar 4 g l^{-1}.

In lime (*Citrus aurantifolia*), nodal explants gave eight multiple shoots per node when media was supplemented with 1.0 mg l^{-1} BAP and 0.5 mg l^{-1} kinetin (Al-Khayri and Al-Bahrany, 2001). Cobo *et al.* (2018) studied the enhanced release of axillary bud under *in vitro* condition in *Vaccinium floribundum*. WPM supplemented with 3 mg l^{-1} 2-ip and 1 mg l^{-1} NAA was found to be ideal for culture establishment. This combination also produced relatively high number of shoots per bud.

2.2.2.6.16. **Rooting of *in vitro* produced shoots**

In vitro produced shoots can be rooted through either *in vitro* or *ex vitro* methods. *In vitro* root induction varies with species, explant and PGR. Usually, due to the presence of meristematic tissue, explants utilizing juvenile plant parts root more effectively and easily than the mature parts (Gantait *et al.*, 2018).

Phulwaria *et al.* (2012) found that *Terminalia bellirica* shoots raised under *in vitro* condition can be rooted best by culturing in half MS media supplemented with 24.6 µM IBA and 100 mg l^{-1} activated charcoal. For reducing labour, cost and time, *ex vitro* rooting was also tried and it was observed that highest percentage of shoots rooted *ex vitro* when treated with 2,460 µM IBA for 5 min.

In guava, adventitious rooting occurs on excised shoots after subculturing on medium containing half strength MS with 1 µM each of IBA and NAA and 1 g l^{-1} activated charcoal (Amin *et al.*, 1988).

In *Saraca asoca,* rooting of microshoots from nodal explant culture was maximum in MS media supplemented with BAP (4 mg l^{-1}) (Ramasubbu *et al.*, 2008). Girijashankar (2011) reported that in *Acacia auriculiformis* the best rooting response by the elongated shoots was on half MS basal, when compared to other treatments imposed.

In *Cassia angustifolia*, the maximum percentage of rooting was observed from isolated shoots when cultured on MS media supplemented with IBA (60 µM) with 1 per cent activated charcoal and then transferring the shoots to half MS liquid medium without IBA and activated charcoal (Siddique and Anis, 2007).

Komalavalli and Rao (2000) studied the root induction in *Gymnema sylvestre* and found that half MS media with IBA (3 mg l^{-1}) is the best with maximum root emergence. *In vitro* rooting in *Morinda citrifolia* on half strength agar gelled MS medium supplemented with 1.0 mg l^{-1} IBA produced 44.30 roots per shoot from the cut ends (Shekhawat *et al.*, 2015).

In *Punica granatum*, 86 per cent rooting of regenerated shoots was induced in a half strength MS media supplemented with IBA (1 mg l^{-1}) (Naik *et al.*, 1999). For further elongation of primary roots, the rooted shoots were later transferred to an auxin free half strength MS media. Sadeghi *et al.* (2015) reported that *Prunus empyrean*, cent per cent *in vitro* rooting was achieved on half strength MS media with 0.5 mg l^{-1} IBA, 1.6 mg l^{-1} thiamine and 150 mg l^{-1} iron sequestrene.

Mansseri-Lamrioui *et al.* (2011) studied the influence of different type and concentration of auxin for root induction in *Prunus avium*. They reported that the highest average number and length of roots were obtained when the medium was incorporated with IBA 1 mg l^{-1}.

In vitro clonal propagation of guava var Banarasi local was studied by Amin and Jaiswal (1988). Blomstedt *et al.* (1991) conducted *in vitro* studies in *Eucalyptus regnans* and found maximum rooting when pulse treatment was given in 20 mg l^{-1} IBA solution followed by culturing in WPM supplemented with activated charcoal. Excised shoots

were rooted on media containing half strength MS salts, 1.5 per cent sucrose, 1 μM each of IBA and NAA and 1 g l^{-1}activated charcoal. Das and Rout (2002) standardized *in vitro* rooting in *Plumbago rosea* and *Plumbage zeylanica* shoots on MS media supplemented with 1.2 μM IBA and 2 per cent (w/v) sucrose. Martin (2002) achieved in developing a successful *in vitro* rooting media for *Holostemma adakodien*. 90 per cent rooting was reported in half MS media fortified with 0.05 mg l^{-1} IBA.

Mallika *et al.* (1992) reported that plantlet regeneration *in vitro* from nodal segments of cocoa showed maximum rooting by a pulse treatment of IBA in ethanol.

2.2.2.6.17. **Hardening and planting out**

The ultimate success of *in vitro* propagation lies in the successful establishment of plant under *ex vitro* condition. The wider use of micropropagation is often restricted by the high rate of plant loss and damage when transferred to potting media. This may be due to the abnormalities of *ex vitro* conditions like variation in light intensity, humidity and moisture (Fila *et al.*, 1998). Relative humidity can be successfully controlled by using polythene tent, fogging and misting. Proper rhizosphere environment is also necessary for better growth and acclimatization of plantlets (Zimmermann and Fordhams, 1985).

In *Bixa orellana*, 80 per cent survival of plantlets was reported in hardening trial when coco peat was used as the substrate. For proper acclimatization, the plants were initially potted in a growth chamber and then moved to a greenhouse (D'Souza and Sharon, 2001). Nath and Buragohain (2005) used a mixture of sterilized sand and soil in the ratio 3:1 to harden the *in vitro* produced plantlets of *Adhatoda vasica*. The plantlets were also covered with polythene bags to maintain humidity for a week before transferring to the main field.

Highest survival percentage was obtained in micropropagation of *Olea europaea* when hardened in a media containing a mixture of vermiculate and perlite in the ratio 3:1 (Peixe *et al.*, 2007). In *Woodfordia fruticosa*, 89 per cent of rooted plantlets were established *ex vitro* in potting media containing sand, soil and manure in the ratio 1:1:1.

The plantlets were maintained under proper microclimatic condition in greenhouse for 8 weeks before field planting (Krishnan and Seeni, 1994).

Neelannavar (2006) found cent percentage survival of *in vitro* plantlets of *Vanilla planifolia* in soil + coco pith (1:1) and sand + soil+ coco pith (1:1:1). The hardened plantlets were also found to be more vigorous. In *Plumbago zeylanica*, the rooted plants were potted in a mixture of sterile soil, sand and vermiculate in the ratio 1:1:1 for successful establishment. The potted plants were also supplied with quarter strength MS basal salt solution devoid of sucrose and myoinositol for four weeks under greenhouse condition (Sivanesan and Jeong, 2009). The micropropagated plants when grown for several months in green house produces functional tap root in acacia (Gantait *et al.*, 2018).

Mallika *et al.* (1992) reported that successful hardening of the rooted cocoa plantlets can be done by transferring to a potting mixture composed of soil rite and soil. Bindu (1997) stated that for better establishment of tissue culture cocoa plants, a mixture of soil rite and potting mixture in the ratio 1:1 was found to be ideal. After planting, the tissue culture plant should be covered using polybags for 2-3 weeks followed by exposure to ambient conditions for better survival.

2.3. Anatomical studies

Adventitious root formation differs in micro cuttings and depends on genotype. Induction of roots is more difficult in woody species than in herbaceous species (Hackett, 1985). During *in vitro* propagation, the root formation can be either direct or indirect. In direct root formation, the roots are developed from plant tissues but in indirect form, the roots are developed following callus formation (Pinker, 2000).

Smith *et al.* (1991) reported that the vascular connections between the new *in vitro* roots and the micro cutting stems of maple, bitch and apple were often found to be discontinuous. This was because the *in vitro* roots were often subtended by callus or it may be emerged from callus. Anatomical studies help to reveal the differences in

involvement of various cell layers and organelles in adventitious root formation (George *et al*., 2008).

De Klerk *et al.* (1995) described the three different phases of rooting in micro shoots of apple *(Malus domestica*) *viz*. dedifferentiation, induction and morphological differentiation. An intermediate callus phase before root initiation is an evidence for the occurrence of these three phases. Prolonged phases of dedifferentiation and re-differentiation is usually associated with indirect rooting via callus.

The histo-anatomy of *in vitro* rooting in walnut was studied by Loewe (1990). This helped to study the origin and development of roots and also to find out whether vascular connections between shoot and root is continuous or there is any intervening callus preventing the development of vascular connections.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present study '*In vitro* morphogenesis and plant regeneration in elite clones of cocoa (*Theobroma cacao* L.)' was carried out at the Tissue Culture Laboratory of Department of Plantation crops and Spices, College of Horticulture, Vellanikkara during 2016-18. The materials and methods used for the present study are detailed in the following sections. The study was undertaken through the conduct of following four experiments.

Experiment 1. Standardization of culture establishment

Experiment 2. Standardization of multiple shoot induction and proliferation

Experiment 3. Standardization of rooting and root growth

Experiment 4. Standardization of hardening

3.1. CULTURE ESTABLISHMENT

3.1.1. **Chemicals and Glass wares**

The major and minor elements used for the study were of analytical grade. Glass wares used were of Borosilicate brand. The cleaning of glass wares were done by soaking them overnight and thorough washing with detergent solution (0.1 % Tween 20) followed by rinsing with potassium dichromate solution in sulphuric acid. They were again washed with tap water till free of acid and finally rinsed with double distilled water. Then the glass wares were kept in a hot air oven for proper drying and then stored in cup broads until used.

3.1.2. **Source of explant**

Explants were collected from budded plants of cocoa varieties *viz*. CCRP 2, CCRP 6, CCRP 8, CCRP 15 and Scavina (Plate 1).

CCRP 2 - Variety evolved through single plant selection from local population. Trees are high yielding with smooth green pods.

CCRP 6 - Variety evolved through single parent selection from exotic collection (IMC 10).

CCRP 8 - Hybrid between CCRP 1 and CCRP 7. High yielding and produces medium sized pods.

CCRP 15- Newly released hybrid from KAU. Parentage – G VI 55 X Criollo.

Scavina - Exotic and a high tissue culture responding genotype.

These plants were maintained in the poly house of Department of Plantation crops and Spices, College of Horticulture, Vellanikkara.

3.1.2.1. *Preparation of budded plants for collection of explants*

Seedlings of 6 month old showing good vegetative growth and comparatively disease free were taken as rootstock and bud wood from fan branches of elite trees of different genotype were used for budding. Precuring of bud wood was done by cutting off the lamina of all the leaves of selected branch about 30 cm from the tip. This practice was done to increase the bud take. Patch budding was done and after three weeks budding tape was cut off. When the bud union was proper, a vertical cut was made half way through the stem above the bud and snapped back. Later, the stock portion was cut back when the bud had grown with two to three hardened leaves. After three months the plant became ready for explant collection.

3.1.2.2. *Collection of explant*

Single node segment from fan shoots of mother plants already protected with fungicides were used as explants. The source plants were regularly sprayed with a systemic fungicide Carbendazim (0.2 %) and a contact fungicide Mancozeb (0.3 %) in an interval of three days to reduce fungal attack.

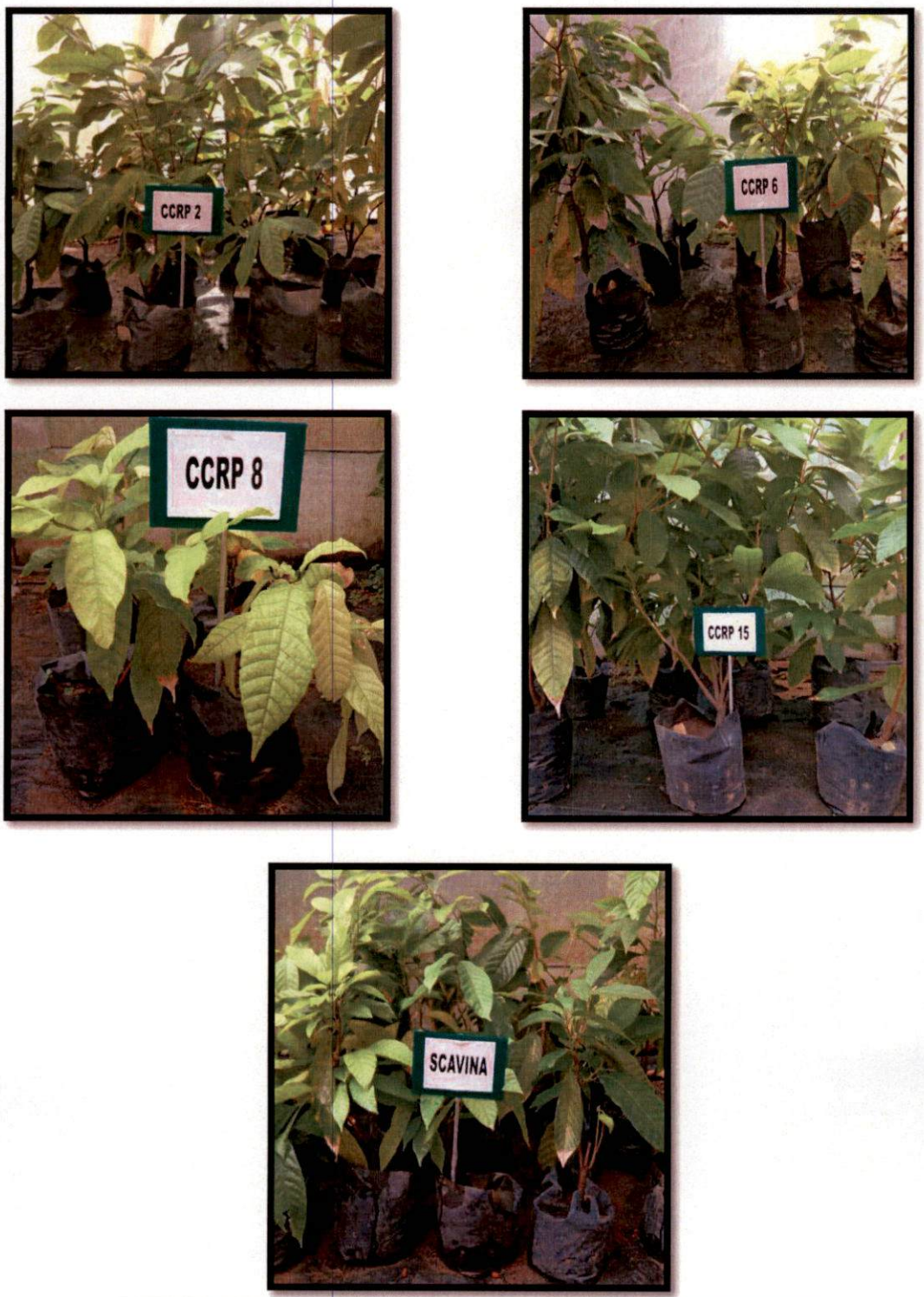


Plate 1. Budded cocoa varieties used as explant source

3.1.2.3. *Preparation of explant*

Stems of approximately 10-15 cm were cut from the budded plants of cocoa maintained in the polyhouse using a secature and brought soon to the lab for avoiding desiccation. Leaves were trimmed by maintaining a part of the petiole and single node cuttings of 5 cm were made. These segments were taken in a net bag .The bag was tied under running tap water for 1 hour till the mucilage exuded from the cut ends got washed off. Then the segments were washed thoroughly in tap water containing 2 per cent solution of Teepol. Each surface sterilization treatments were tried to eliminate fungal and bacterial contaminants. The surface sterilization treatments done outside the laminar air flow chamber are furnished in Table 2. After each surface sterilization treatment, explant was thoroughly rinsed 5 times with sterilized distilled water to remove all traces of chemicals and then the segments were moved to laminar air flow cabinet.

3.1.3. **Culture establishment medium**

3.1.3.1. *Composition of culture establishment medium*

Basal medium used in the present study for culture establishment was WPM (Lloyd and McCown, 1980). The composition of this medium is given in Table 1. The medium was also supplemented with different levels of growth regulators like 2-ip and IAA for studying the effect of these on culture establishment.

Table 1. Composition of various media tried for *in vitro* culture of *Theobroma cacao*

Ingredients (mg/l)	MS	WPM
Inorganic Constituents		
$(NH_4)NO_3$	1650.00	400.000
KNO_3	1900.000	-
K_2SO_4	-	990.000
KH_2PO_4	170.000	170.000
$Ca(NO_3)_2.4H_2O$	-	556.000
$CaCl_2.2H_2O$	446.000	96.000
$MgSO_4.7H_2O$	370.000	370.000
$FeSO_4.7H_2O$	27.800	27.800
Na_2EDTA	37.300	37.000
$MnSO_4.7H_2O$	22.300	22.300
$ZnSO_4.7H_2O$	8.600	8.600
H_3BO_3	6.200	6.200
KI	0.830	-
$Na_2MoO_4.2H_2O$	0.250	0.250
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	-
Organic Constituents		
Inositol	100.00	100.00
Nicotinic acid	0.500	0.500
Pyridoxine HCl	0.500	0.500
Thiamine HCl	0.100	1.000
Glycine	2.000	2.000
Others		
Sucrose w/v	3.0 %	3.0 %
Agar w/v	0.8 %	0.8 %

3.1.3.2. *Preparation of the stock solution*

For easy preparation of the media, stock solutions of major and minor nutrients were prepared according to the standard procedure (Gamborg and Shyluk, 1981). For preparing stock solution, required quantities of chemicals were weighed accurately. Then distilled water was taken in a beaker and chemicals are added one by one and dissolved by constant stirring. Since the iron stock precipitates readily, care should be taken while preparing it. In two separate beakers Na_2 EDTA and $FeSO_4.7H_2O$ were dissolved in approximately 200 ml distilled water each. Then both the beakers were placed on hot plates and brought up to almost boiling. In next step $FeSO_4.7H_2O$ was slowly added to Na_2 EDTA with constant stirring. Then the volume was made up to one liter by adding distilled water. The mixture was then allowed to cool in room temperature and stored under refrigerated condition in amber coloured bottles. All stocks were labelled indicating the stock number and date of preparation. These stock solutions were replaced with fresh one after three months. The stock solutions of vitamins and growth regulators were also prepared fresh after eight and four weeks respectively.

3.1.3.3. *Preparation of culture medium and sterilization*

Specific quantity of stock solutions were pipetted out into a conical flask. Required quantity of sucrose and inositol were added. Plant hormones were also pipetted out to the beaker. The volume was made up to the required level using double distilled water. The pH of the solution was checked using a pH paper and adjusted if required using 1.0 N NaOH and 1.0 N HCl. The solidifying agent agar along with charcoal was also added to the medium. The media was sterilized using an autoclave at 121^0C and 15 psi (1.06 kg cm^{-2}) for 20 min.

Dispensing of media into test tubes was done under perfect aseptic condition in a laminar air flow chamber. The media was UV sterilized along with cotton plugged test tubes and Cefotaxim for 20 min. The required quantity of Cefotaxim was pipetted out and added to the media. About 15 ml of media was quickly poured into each of the test tubes after removing the plug. Neck of the test tubes were again flamed and cotton plug was

replaced. These test tubes were allowed to store in a cool and dry place. After one week, the test tubes were checked for contamination and those contaminated were avoided before inoculation.

3.1.4. **Surface sterilization**

Surface sterilization was carried out under perfect aseptic condition. The laminar air flow cabinet was radiated with UV rays. The working table and sides of the laminar air flow were thoroughly wiped with absolute ethyl alcohol. All metal and glass wares were wrapped in aluminium foil and autoclaved at 1.06 kg cm^{-2} for 30 min at 121°C. Sterilized forceps, petri dishes, and knives were again dipped in alcohol and flamed on a spirit lamp at the time of use. Hands were washed with soap under running tap water followed by wiping with absolute alcohol before inoculation.

The surface sterilization treatments done inside the laminar air flow chamber are furnished in Table 2. When the sterilants were used, it should be kept submerged in the explants for required period by shaking at frequent intervals. Then the segments were also thoroughly washed with sterile distilled water to remove all the traces of sterilants from the surface. From each of the segments, the top, bottom and remaining part of petiole were cut and then air dried for about 30 min on a blotting paper. After proper drying, the explants were transferred to culture medium.

Table 2. Surface sterilization treatments for nodal segments of cocoa

Treatments	Treatment details
T_1	Carbendazim 0.2 % for 30 min (outside LAF), Streptocycline 0.1 % for 5 min (outside LAF), Ethyl Alcohol 70 % for 1 min (inside LAF), $HgCl_2$ 0.1 % for 2 min (inside LAF)
T_2	Carbendazim 0.2 % for 30 min (outside LAF), Streptocycline 0.1 % for 5 min (outside LAF), Ethyl Alcohol 70 % for 1 min (inside LAF), $HgCl_2$ 0.1 % for 3 min (inside LAF)
T_3	Carbendazim 0.2 % for 30 min (outside LAF), Streptocycline 0.1 % for 10 min (outside LAF), Ethyl Alcohol 70 % for 3 min (inside LAF), $HgCl_2$ 0.1 % for 5 min (inside LAF)
T_4	Carbendazim 0.2 % for 30 min (outside LAF), Streptocycline 0.1 % for 10 min (outside LAF), Ethyl Alcohol 70 % for 3 min (inside LAF), $HgCl_2$ 0.1 % for 7 min (inside LAF)
T_5	Mancozeb 0.2 % for 30 min (outside LAF), Streptocycline 0.1 % for 5 min (outside LAF), Ethyl Alcohol 70 % for 1 min (inside LAF), $HgCl_2$ 0.1 % for 2 min (inside LAF)
T_6	Mancozeb 0.2 % for 30 min (outside LAF), Streptocycline 0.1 % for 5 min (outside LAF), Ethyl Alcohol 70 % for 1 min (inside LAF), $HgCl_2$ 0.1 % for 3 min (inside LAF)
T_7	Mancozeb 0.2 % for 30 min (outside LAF), Streptocycline 0.1 % for 10 min (outside LAF), Ethyl Alcohol 70 % for 3 min (inside LAF), $HgCl_2$ 0.1 % for 5 min (inside LAF)
T_8	Mancozeb 0.2 % for 30 min (outside LAF), Streptocycline 0.1 % for 10 min (outside LAF), Ethyl Alcohol 70 % for 3 min (inside LAF), $HgCl_2$ 0.1 % for 7 min (outside LAF)

3.1.5. **Inoculation of the explants**

Inoculation of explants were done in laminar air flow chamber under aseptic condition. It was done by quickly transferring the sterile explants into the culture medium using sterile forceps after removing the cotton plug of the culture tube. After inoculation, the neck of the culture tube was again flamed and the cotton plug was replaced.

3.1.6. **Culturing of explants**

The culture tubes were then incubated in culture rooms at 28±2°C with a relative humidity of 80-90 per cent under white fluorescent lamps provided for artificial illumination.

3.1.7. **Standardization of culture establishment**

The experiment was done in two factorial CRD. The first factor was media, at four different levels (Table 3) and the second factor was variety. Each treatment was done in 5 cocoa varieties like CCRP 2, CCRP 6, CCRP 8, CCRP 15 and Scavina. So there were 20 different treatment combinations. The number of replications were two.

Table 3. Combination of growth regulators for culture establishment

Basal medium: WPM

Treatments	Plant growth regulators (ppm)
CE_1	2-ip 1.00 + IAA 0.02
CE_2	2-ip 2.00 + IAA 0.04
CE_3	2-ip 3.00 + IAA 0.06
CE_4	2-ip 4.00 + IAA 0.08

CE – Culture Establishment

3.2. INDUCTION OF MULTIPLE SHOOTS AND PROLIFERATION

For multiple shoot induction, the surviving cultures were sub cultured at an interval of 2-3 weeks to medium containing silver nitrate. Addition of silver nitrate was suggested to overcome the problem of callus production at the cut ends of explants and also improve shoot regeneration. Each treatment of multiple shoot induction was done in 5 cocoa varieties like CCRP 2, CCRP 6, CCRP 8, CCRP 15 and Scavina.

3.2.1. **Standardization of multiple shoot induction and proliferation**

The experiment was done in two factorial CRD. The first factor was media, at four different levels (Table 4) and the second factor was variety. Each treatment was done in 5 cocoa varieties like CCRP 2, CCRP 6, CCRP 8, CCRP 15 and Scavina. So there were 20 different treatment combinations. The number of replications were 2.

Table 4. Combination of growth regulators for multiple shoot induction

Basal medium: WPM + $AgNO_3$ 5 ppm

Treatments	Plant growth regulators (ppm)
MS_1	2-ip 1.00 + IAA 0.02
MS_2	2-ip 2.00 + IAA 0.04
MS_3	2-ip 3.00+ IAA 0.06
MS4	2-ip 4.00+ IAA 0.08

MS – Multiple Shoot

3.3. INDUCTION OF ROOTING AND ROOT GROWTH

The shoots obtained from the above experiment was subjected to rooting under *in vitro* condition. The basal medium used for *in vitro* rooting was half strength MS medium. The composition of the media is given in Table 2. For rooting, pulse treatment was given

to the shoots with IBA at different concentrations for 2-3 sec. Each treatment was done in 5 cocoa varieties like CCRP 2, CCRP 6, CCRP 8, CCRP 15 and Scavina.

3.3.1. **Standardization of media for rooting**

The experiment was done in two factorial CRD. The first factor was media, at five different levels (Table 5) and the second factor was variety. Each treatment was done in 5 cocoa varieties like CCRP 2, CCRP 6, CCRP 8, CCRP 15 and Scavina. So there were 25 different treatment combinations. The number of replications were 2.

Table 5. Combination of growth regulators used for rooting

Basal medium: ½ MS+ AC 0.2 per cent

Treatments	Rooting hormone (ppm)	Pulse treatment (sec)
R_1	IBA 1000	2-3
R_2	IBA 2000	2-3
R_3	IBA 3000	2-3
R_4	IBA 4000	2-3
R_5	IBA 5000	2-3

R – Rooting

3.4. HARDENING

Ex vitro establishment of plantlets in different potting media and containers were studied (Table 6). Each treatment was replicated two times. For hardening, the culture tubes were taken out of the culture room and the cotton plugs were removed. Then sterile water was added and kept as such for 10 -15 min. The rooted plants were then taken out

carefully using a sterile forceps. By thorough washing the agar adhering to the roots was completely removed.

The plantlets were treated with Mancozeb (0.2 %) for 5 min before planting out. The plantlets were then planted in disposable cups filled with sterilized potting media.

Table 6. Potting media tried for *ex vitro* establishment of tissue culture cocoa plants

Treatments	Potting media
PM_1	Sand: Vermicompost compost (1:1)
PM_2	Vermiculate: Perlite: Vermicompost (1:1:1)

PM – Potting Mixture

3.5. ROTARY MICROTOME SECTIONING

3.5.1. **Procedure**

I. Preparation of material and then, embedding it in paraffin wax

1. The material was treated in FAA at least for 24 hours.
2. The plant material was then subjected to a TBA series of process for one week.

Table 7. TBA series

Contents	50 %	70 %	85 %	95 %	100 %
Water	50 ml	30 ml	15 ml	0	0
95 % Ethyl alcohol	40 ml	50 ml	50 ml	45 ml	0
TBA	10 ml	20 ml	35 ml	55 ml	75 ml
100 % Ethyl alcohol	0	0	0	0	25 ml
Total	100 ml	100 ml	100 ml	100 ml	100 ml

- Monday

  12 pm- The TBA series was started by immersing the plant material in 50 per cent TBA

  4 pm- The material was taken out from 50 per cent TBA and then immersed it in 70 per cent TBA overnight.

- Tuesday

  9 am- The material was then taken out from 70 per cent TBA and then immersed in 85 per cent TBA.

  12 pm- The material was taken out from 85 per cent TBA and then immersed in 95 per cent TBA.

  4 pm- Then the material was taken out from 95% TBA and then immersed in 100 per cent TBA overnight.

- Wednesday

  8 am- The material was taken out from 100 per cent TBA and then immersed in pure TBA.

  12 pm- The material was taken out from pure TBA solution and then immersed in fresh pure TBA and then kept overnight.

- Thursday

  9 am- Make TBA –liquid paraffin mixture in 1:1 ratio and was shaken well to obtain a clear solution. Then material along with small amount of TBA was added to the mixture and then kept overnight in hot air oven. Also some solid paraffin was kept in hot air oven.

- Friday

  9 am- A new container was taken and three-fourth of it was filled with melted paraffin and then waited for 4-5 minutes. Then it was kept in hot air oven at 55^0C – 60^0C without shaking.

11 am- The material was reached the bottom of the bottle otherwise material may not good or suit for further processing. Then, one-fourth of paraffin was decanted paraffin and one-fourth new melted paraffin was added.

1 pm- Half of melted paraffin was decanted half of new melted paraffin was added.

2.30 pm- Three-fourth of paraffin was decanted and three- fourth of new melted paraffin was added.

3.30 pm- Full paraffin was decanted and the material was changed to pure melted paraffin and then kept overnight.

- Saturday

  8.45 am- Full paraffin was decanted and fresh melted paraffin was added.

  9.45 am- Full paraffin was again decanted and then fresh melted paraffin was added.

  10.45 am- Full paraffin was decanted.

  4.30 pm- Block was made in ice water.

II. Serial Section Staining

1. First an adhesive was prepared using equal volume of egg white (25 ml) and glycerol (25 ml) by proper mixing.
2. The adhesive was then brushed in glass slide using a brush.
3. Then the ribbon of wax having the material was kept on adhesive coated slide.
4. Then the slide was kept under the flame of a candle and then, the melted wax was rubbed using a tissue paper
5. The slide was then dipped in xylene for dewaxing for 10-20 sec.
6. Then dipped in alcohol- xylene (1:1) for 20 sec.
7. A single dip was given in absolute alcohol.
8. The slide was dipped in saffranin stain for 2-3 sec.
9. Then the slide was dipped in absolute alcohol to remove the excess stain.
10. A dip was given in clove oil which act as a clearing agent.
11. The slide was then mounted in DPX (Distyrene, Plasticizer, and Xylene).

3.6. OBSERVATIONS

Each trial was carried out with a minimum of 12 tubes and with two replications. Observations were taken for a period of four weeks at seven days interval. The data collected is presented on the basis of number of cultures remained uncontaminated. The main observations recorded are the following.

1. **Percentage of culture establishment**

   This observation was recorded for treatments done in culture establishment media, multiple shoot induction media and rooting media. This was calculated on the basis of number of uncontaminated cultures per treatment.

2. **Percentage of shoot induction**

   Number of cultures showing shoot induction were expressed as percentage of total number of surviving cultures. A culture was recorded with shoot induction when the dormant axillary bud has just emerged.

3. **Period of shoot induction**

   It was the time taken for shoot induction usually expressed as number of days.

4. **Number of multiple shoots per culture**

   It was the number of shoots in each uncontaminated tube while kept in multiple shoot induction media.

5. **Percentage of rooting**

   It was the number of rooted cultures in different hardening media expressed as percentage.

6. **Percentage survival of plantlets in different hardening media**

   Percentage of plants showing survival under *ex vitro* condition in different potting media.

3.7. STATSTICAL ANALYSIS

The data recorded were statistically analyzed wherever necessary using OP stat.

RESULTS

4. RESULTS

The results of various experiments conducted to standardise the *in vitro* propagation of different genotypes of *Theobroma cacao* conducted at the Tissue Culture Laboratory of Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara during 2016-2018 are presented in the following section.

4.1. SURFACE STERILIZATION OF EXPLANT

The nodal segments containing axillary buds used as explants in the present study may be contaminated with different microorganisms in its surface. Therefore, these were subjected to different surface sterilization treatments for preventing culture contamination. Observation on culture contamination was recorded in weekly interval for three weeks. The results showed that, there were variation in the percentage of culture contamination when different treatments were done for establishing aseptic cultures.

The surface sterilization treatments (Table 2) were carried out with nodal segments from grafted plants of cocoa and the results obtained in the study were presented in Table 8. The percentage of cultures surviving in various treatments ranged from 8.33 per cent to 91.67 per cent after one month of inoculation in the culture medium WPM + 2-ip 1 ppm + IAA 0.02 ppm. The culture damage was due to microbial contamination (Plate 3 and 4) and drying of tissues due to high concentration of sterilant.

The most effective surface sterilization treatment was T_7 and the treatment involved washing the nodal segments in Teepol followed by 0.2 per cent Mancozeb for 30 min followed by Streptocycline 0.1 per cent for 10 min outside the laminar air flow chamber. Inside the laminar air flow, Ethyl Alcohol 70 per cent was treated for 3 min followed by $HgCl_2$ 0.1 per cent for 5 min. The survival percentage reported was 91.67 per cent and rest of the cultures were destroyed by microbial contamination (8.33 %).

The least effective treatment was T_1, in which the explants were treated with Carbendazim 0.2 per cent for 30 min followed by Streptocycline 0.1 per cent for 5 min

outside the laminar air flow chamber. Inside the laminar air flow, Ethyl Alcohol 70 per cent was treated for 1 min and then $HgCl_2$ 0.1 per cent for 2 min. In this treatment, contamination was maximum (91.67 %) and but no explant mortality. In T_2 and T_3 survival percentage was increased up to 25 per cent and 41.67 per cent but still a higher contamination rate was observed in the cultures without any explant mortality. In T_4, when the duration of $HgCl_2$ 0.1 per cent was increased up to 7 min, a high rate of explant mortality (75 %) was observed along with contamination rate of (8.33 %).

Treatments using Mancozeb as fungicide showed more rate of survival. In T_5, half of the total number of cultures survived but the other half got contaminated. In T_6, more survival percentage was obtained (66.67 %) but still the contamination rate was higher (33.33 %). T_8 was treatment with cent per cent contamination free cultures but maximum number of explants got dried (83.33 %) in the same treatment (Table 8).

Table 8. Effect of surface sterilisation treatments in the culture establishment of nodal segments of cocoa

Treatment	Survival one month after inoculation (%)	Contamination (%)	Type of contamination	Cultures dried by tissue damage (%)
T_1	8.33	91.67	B, F	NIL
T_2	25.00	75.00	B, F	NIL
T_3	41.67	58.33	F	NIL
T_4	16.67	8.33	F	75.00
T_5	50.00	50.00	B, F	NIL
T_6	66.67	33.33	B, F	NIL
T_7	91.67	8.33	F	NIL
T_8	16.67	NIL	-	83.33

Culture medium – WPM + 2-ip 1 ppm + IAA 0.02 ppm

Number of explants – 12

B – Bacteria, F – Fungus

Plate 2. Fungal contamination

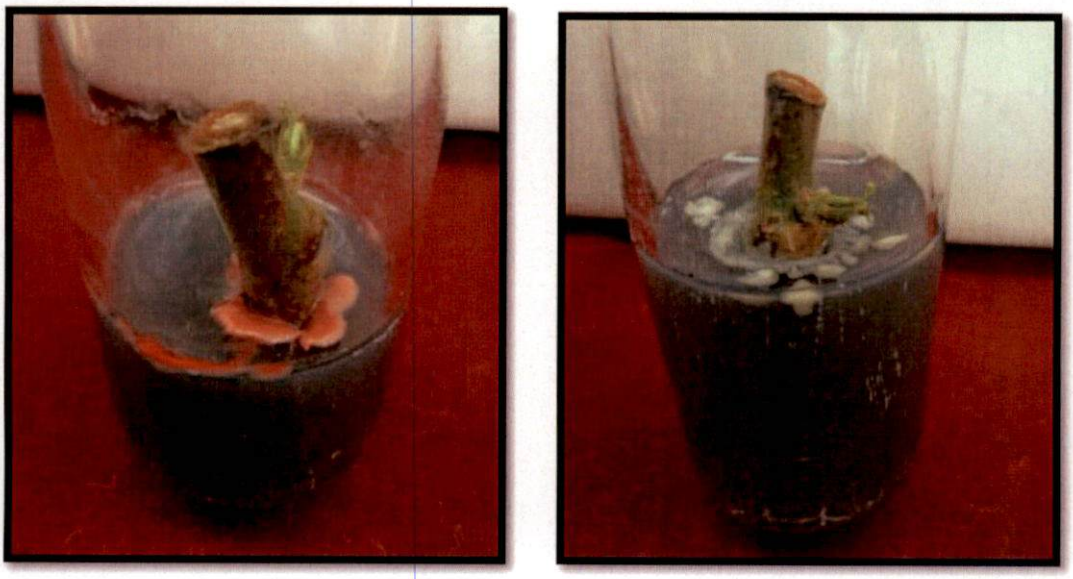


Plate 3. Bacterial contamination

4.2. CULTURE ESTABLISHMENT

4.2.1. **Culture establishment percentage (%)**

Culture establishment percentage did not vary significantly among media. Highest culture establishment percentage was recorded in CE_2 (95 %) which was followed by 93.33 per cent in CE_1, CE_3 and CE_4.

There were no significant variations in culture establishment percentage among the different varieties. The highest culture establishment percentage was recorded in V_5 (97.91 %), followed by V_1 (95.83 %).

There was no significant variation in culture establishment percentage with respect to the different media and varieties. Culture establishment percentage varies from 83.33 per cent to 100 per cent. Cent per cent culture establishment was recorded in CE_1V_4, CE_1V_5, CE_2V_1, CE_2V_2, CE_2V_5, CE_3V_3, CE_4V_1 and CE_4V_5 (Table 9).

Table 9. Effect of culture establishment media, varieties and their interactions on culture establishment percentage

	CCRP 2 (V_1)	**CCRP 6 (V_2)**	**CCRP 8 (V_3)**	**CCRP 15 (V_4)**	**SCAVINA (V_5)**	**Mean (Media)**
CE_1	9.61 (91.67)	9.61 (91.67)	9.13 (83.33)	10.05 (100.00)	10.05 (100.00)	9.69 (93.33)
CE_2	10.05 (100.00)	10.05 (100.00)	9.61 (91.67)	9.18 (83.33)	10.05 (100.00)	9.79 (95.00)
CE_3	9.61 (91.67)	9.61 (91.67)	10.05 (100.00)	9.61 (91.67)	9.61 (91.67)	9.70 (93.33)
CE_4	10.05 (100.00)	9.18 (83.33)	9.61 (91.67)	9.61 (91.67)	10.05 (100.00)	9.70 (93.33)
Mean (Varieties)	9.83 (95.83)	9.61 (91.67)	9.60 (91.67)	9.61 (91.67)	9.94 (97.91)	

$\sqrt{x} + 0.5$ transformed values, original values are given in parenthesis

CD (CE) – N/A CD (V) – N/A CD (CE x V) – N/A

CE_1 – WPM + 2-ip 1 ppm + IAA 0.02 ppm

CE_2 – WPM + 2-ip 2 ppm + IAA 0.04 ppm

CE_3 – WPM + 2-ip 3 ppm + IAA 0.06 ppm

CE_4 – WPM + 2-ip 4 ppm + IAA 0.08 ppm

CCRP 2

CCRP 6

CCRP 8

CCRP 15

SCAVINA

Plate 4. Single shoot produced from leaf axils in different varieties after one week of culturing

4.2.2. **Shoot induction percentage (%)**

Shoot induction percentage did not vary significantly among media. Highest shoot induction percentage was recorded in CE_2 (95 %) which was followed by 93.33 per cent in CE_3 and CE_4.

There were no significant variations in shoot induction percentage among the different varieties. The highest shoot induction percentage was recorded in V_5 (97.92 %), followed by V_1 (93.75 %).

There was no significant variation in shoot induction percentage with respect to the different media and varieties. Shoot induction percentage varies from 83.33 per cent to 100 per cent. Cent per cent shoot induction was recorded in CE_1V_4, CE_1V_5, CE_2V_1, CE_2V_2, CE_2V_5, CE_3V_3, and CE_4V_5 (Table 10).

Table 10. Effect of culture establishment media, varieties and their interactions on percentage of shoot induction

	CCRP 2 (V_1)	**CCRP 6 (V_2)**	**CCRP 8 (V_3)**	**CCRP 15 (V_4)**	**SCAVINA (V_5)**	**Mean (Media)**
CE_1	9.61 (91.67)	9.18 (83.33)	8.70 (83.33)	10.05 (100.00)	10.05 (100.00)	9.52 (91.67)
CE_2	10.05 (100.00)	10.05 (100.00)	9.61 (91.67)	9.18 (83.33)	10.05 (100.00)	9.79 (95.00)
CE_3	9.61 (91.67)	9.61 (91.67)	10.05 (100.00)	9.61 (91.67)	9.61 (91.67)	9.70 (93.33)
CE_4	9.61 (91.67)	9.61 (91.67)	9.61 (91.67)	9.61 (91.67)	10.05 (100.00)	9.70 (93.33)
Mean (Varieties)	9.72 (93.75)	9.61 (91.67)	9.49 (91.67)	9.61 (91.67)	9.94 (97.92)	

$\sqrt{x} + 0.5$ transformed values, original values are given in parenthesis

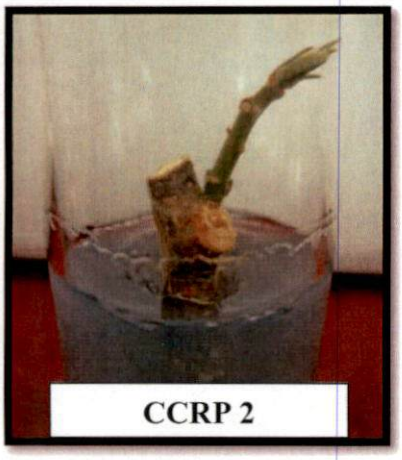
CD (CE) – N/A CD (V) – N/A CD (CE x V) – N/A

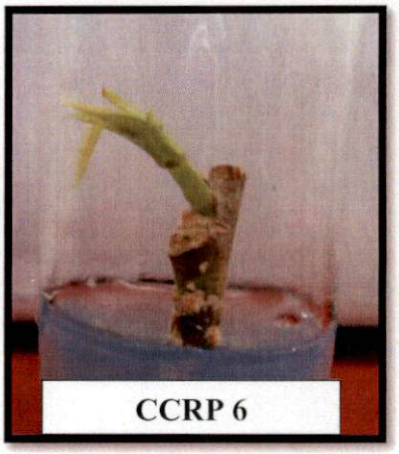
CE_1 - WPM+2-ip 1 ppm + IAA 0.02 ppm

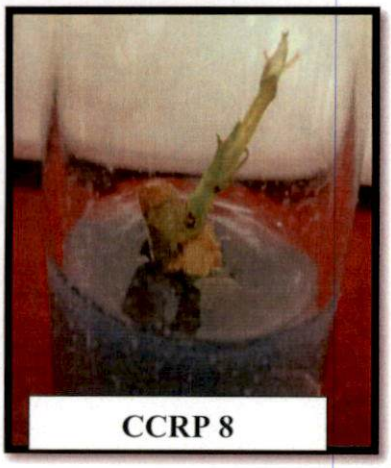
CE_2 - WPM+2-ip 2 ppm + IAA 0.04 ppm

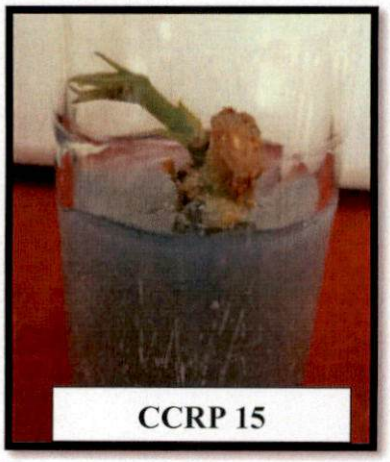
CE_3 - WPM+2-ip 3 ppm + IAA 0.06 ppm

CE_4 - WPM+2-ip 4 ppm + IAA 0.08 ppm









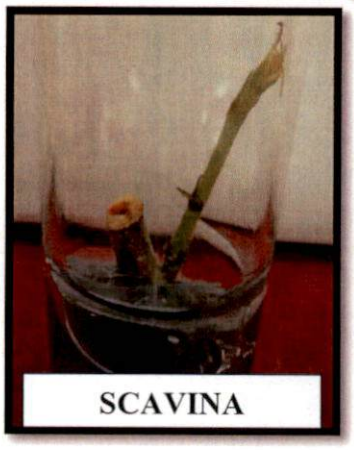
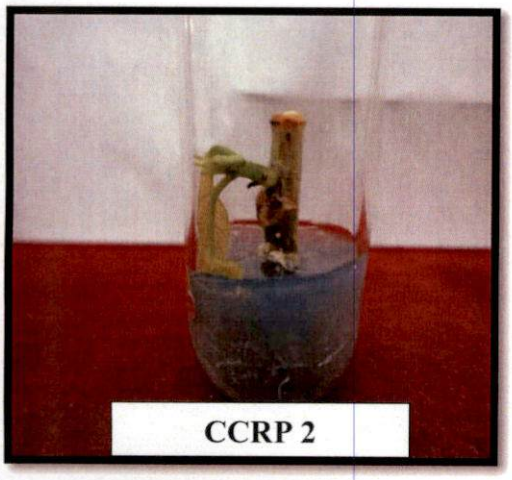
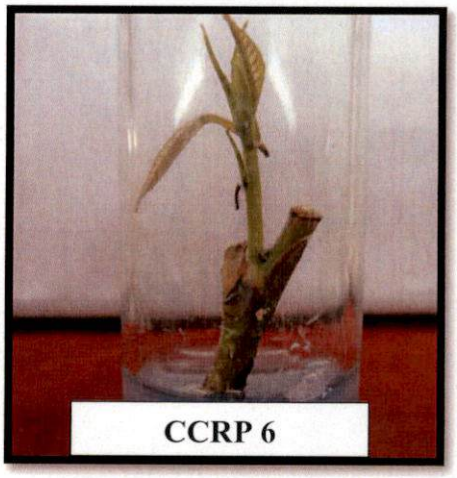


Plate 5. Single shoot produced from leaf axils in different varieties after two weeks of culturing





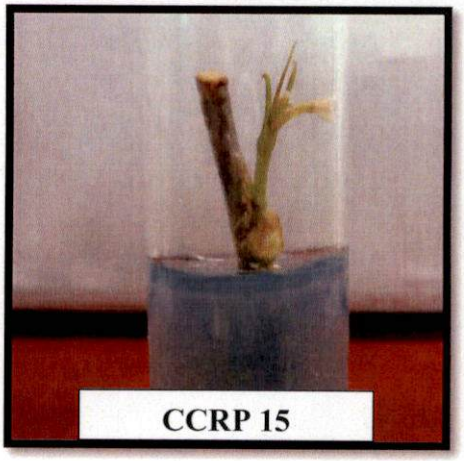


Plate 6. Single shoot produced from leaf axils in different varieties after three weeks of culturing

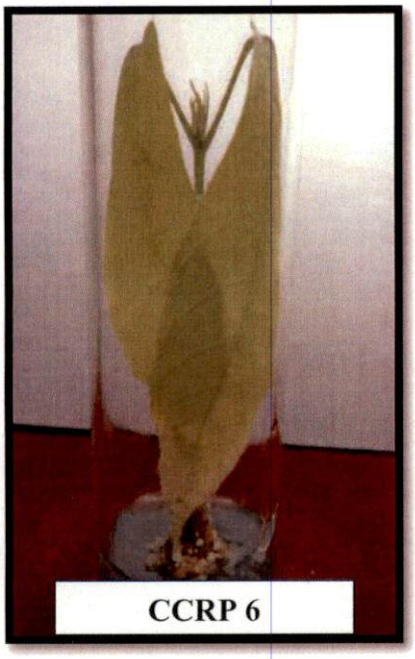


Plate 7. Single shoot produced from leaf axils of different cocoa varieties after four weeks of culturing

Plate 7. Continued: Single shoot produced from leaf axils of different cocoa varieties after four weeks of culturing

4.2.3. **Period of shoot induction (Days)**

Period of shoot induction was significantly influenced by different media. It was recorded lowest in CE_1 (8.41) which was followed by CE_2 (9.65). Highest period of shoot induction was found in CE_3 (10.09). Irrespective of varieties, CE_1 (8.41) proved to be the best for a relatively shorter period and superior.

Period of shoot induction showed significant difference among different varieties. Lowest period of shoot induction was noted in V_1 (7.10) and the requisite period of shoot induction was statistically on par with variety V_5 (8.37). Highest period of shoot induction was found in V_3 (11.07).

Interaction effect of media and varieties varied significantly. In respect to the differential response of variety with different media, the period of shoot induction was lowest in CE_4V_5 (6.33). However, this was statistically on par with CE_1V_1 (7.33), CE_1V_2 (7.25), CE_1V_4 (6.90), CE_2V_1 (7.5), CE_2V_2 (6.95), CE_2V_5 (8.65), CE_3V_1 (7.00), CE_3V_3 (8.75), CE_3V_5 (8.65) and CE_4V_1 (6.58) (Table 11).

Table 11. Effect of culture establishment media, varieties and their interactions on period of shoot induction

	CCRP 2 (V_1)	CCRP 6 (V_2)	CCRP 8 (V_3)	CCRP 15 (V_4)	SCAVINA (V_5)	Mean (Media)
CE_1	7.33	7.25	10.75	6.90	9.83	8.41
CE_2	7.50	6.95	15.47	9.67	8.65	9.65
CE_3	7.00	12.05	8.75	14.00	8.65	10.09
CE_4	6.58	10.65	9.30	16.58	6.33	9.89
Mean (Varieties)	7.10	9.23	11.07	11.79	8.37	

CD (CE) – 1.21 CD (V) – 1.35 CD (CE x V) – 2.72

CE_1 – WPM + 2-ip 1 ppm + IAA 0.02 ppm

CE_2 – WPM + 2-ip 2 ppm + IAA 0.04 ppm

CE_3 – WPM + 2-ip 3 ppm + IAA 0.06 ppm

CE_4 – WPM + 2-ip 4 ppm + IAA 0.08 ppm

4.3. MULTIPLE SHOOT INDUCTION

4.3.1. **Culture establishment percentage (%)**

Culture establishment percentage varied significantly among media. Highest culture establishment percentage was recorded in MS_3 and MS_4 (95 %) which were on par with MS_1 (91.67 %). The lowest culture establishment percentage was in MS_2 (83.33 %).

There were no significant variations in culture establishment percentage among the different varieties. The highest culture establishment percentage was recorded in V_5 (97.91 %), followed by V_3 (93.75 %).

There was no significant variation in culture establishment percentage with respect to the different media and varieties. Culture establishment percentage varies from 75 per cent to 100 per cent. The culture establishment percentage was cent per cent in MS_1V_3, MS_1V_5, MS_2V_5, MS_3V_3, MS_4V_1, MS_3V_4, MS_3V_5, MS_4V_1 and MS_4V_2 (Table 12).

Table 12. Interaction effect of varieties and multiple shoot induction media on culture establishment

	CCRP 2 (V_1)	**CCRP 6 (V_2)**	**CCRP 8 (V_3)**	**CCRP 15 (V_4)**	**SCAVINA (V_5)**	**Mean (Media)**
MS_1	9.61 (91.67)	9.61 (91.67)	10.05 (100.00)	8.70 (75.00)	10.05 (100.00)	9.60 (91.67)
MS_2	8.70 (75.00)	8.70 (75.00)	9.18 (83.33)	9.18 (83.33)	10.05 (100.00)	9.16 (83.33)
MS_3	9.18 (83.33)	9.61 (91.67)	10.05 (100.00)	10.05 (100.00)	10.05 (100.00)	9.79 (95.00)
MS_4	10.05 (100.00)	10.05 (100.00)	9.61 (91.67)	9.61 (91.67)	9.61 (91.67)	9.79 (95.00)
Mean (Varieties)	9.38 (87.50)	9.49 (89.59)	9.72 (93.75)	9.38 (87.50)	9.94 (97.91)	

$\sqrt{x} + 0.5$ transformed values, original values are given in parenthesis

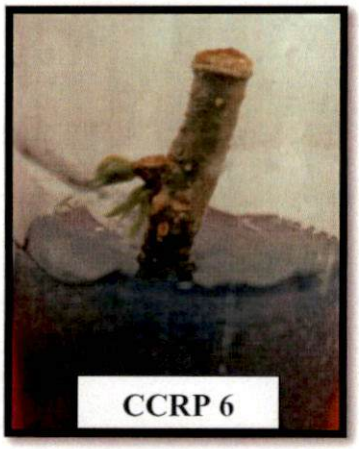
CD (MS) – 0.4 CD (V) – N/A CD (MS x V) – N/A

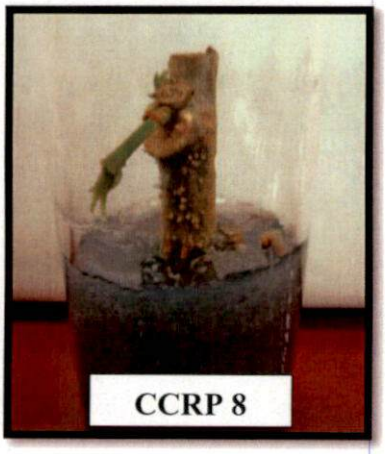
MS_1 – WPM + 2-ip 1 ppm + IAA 0.02 ppm + $AgNO_3$ 5 ppm

MS_2 – WPM + 2-ip 2 ppm + IAA 0.04 ppm + $AgNO_3$ 5 ppm

MS_3 – WPM + 2-ip 3 ppm + IAA 0.06 ppm + $AgNO_3$ 5 ppm

MS_4 – WPM + 2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5 ppm





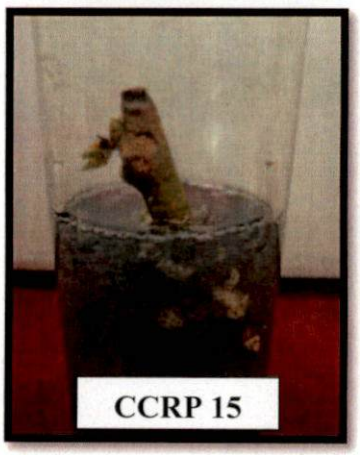


Plate 8. Multiple shoots produced from leaf axils of different cocoa varieties after two weeks of culturing in multiple shoot induction media

4.3.2. **Shoot induction percentage (%)**

Shoot induction percentage vary significantly among media. Highest shoot induction percentage was recorded in MS_4 (91.67 %) which was followed by MS_3 (86.67 %). The lowest shoot induction percentage was reported in MS_1 (75 %) and this was on par with MS_2 (76.67 %).

The shoot induction percentage vary significantly among the different varieties. The highest shoot induction percentage was recorded in V_5 (93.75 %), followed by V_1 (85.42 %). The lowest shoot induction percentage was reported in V_4 (72.92 %).

There was significant variation in shoot induction percentage with respect to the different media and varieties. Shoot induction percentage varies from 66.67 % to 100 %. The shoot induction was recorded as cent percent in MS_1V_5, MS_2V_5, MS_3V_3, and MS_4V_1. This was statistically on par with MS_1V_1 (91.67 %), MS_1V_2 (83.33 %), MS_3V_3 (91.67 %), MS_3V_5 (91.67 %), MS_4V_2 (91.67 %), MS_4V_3 (91.67 %) and MS_4V_4 (91.67 %). The lowest shoot induction percentage was 66.67 % in MS_2V_1, MS_2V_2 and MS_2V_4 (Table 13).

Table 13. Effect of multiple shoot induction media, varieties and their interactions on percentage shoot induction

	CCRP 2 (V_1)	CCRP 6 (V_2)	CCRP 8 (V_3)	CCRP 15 (V_4)	SCAVINA (V_5)	Mean (Media)
MS_1	9.62 (91.67)	9.19 (83.33)	7.14 (50.00)	7.14 (50.00)	10.05 (100.00)	8.63 (75.00)
MS_2	8.23 (66.67)	8.23 (66.67)	9.18 (83.33)	8.23 (66.67)	10.05 (100.00)	8.79 (76.67)
MS_3	9.18 (83.33)	9.18 (83.33)	9.62 (91.67)	9.18 (83.33)	9.62 (91.67)	9.36 (86.67)
MS_4	10.05 (100.00)	9.62 (91.67)	9.62 (91.67)	9.62 (91.67)	9.18 (83.33)	9.68 (91.67)
Mean (Varieties)	9.27 (85.42)	9.05 (81.25)	8.89 (79.17)	8.54 (72.92)	9.74 (93.75)	

$\sqrt{x} + 0.5$ transformed values, original values are given in parenthesis

CD (MS) – 0.31 CD (V) – 0.35 CD (MS x V) – 0.70

MS_1 – WPM + 2-ip 1 ppm + IAA 0.02 ppm + $AgNO_3$ 5 ppm

MS_2 – WPM + 2-ip 2 ppm + IAA 0.04 ppm + $AgNO_3$ 5 ppm

MS_3 – WPM + 2-ip 3 ppm + IAA 0.06 ppm + $AgNO_3$ 5 ppm

MS_4 – WPM + 2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5 ppm

4.3.3. **Period of shoot induction (Days)**

The variation in period of multiple shoot induction was not significant among the different media used. It varied from 19.70 to 21.30. Irrespective of varieties, MS_2 (19.70) proved to be the best for a relatively shorter period. The highest period of shoot induction was reported in MS_4 (21.30).

The period of shoot induction varied significantly among the varieties. The lowest period of shoot induction was recorded in V_5 (17.38) which was statistically on par with V_1 (19.38) and V_3 (18.75). The highest period of shoot induction was in V_4 (24.25), which was statistically on par with V_2 (23.75).

There was significant variation in period of shoot induction among different media and varieties. The lowest period of shoot induction MS_1V_5 (14.50) which was statistically on par with MS_2V_3 (17.50), MS_2V_5 (16.50), MS_3V_3 (16.50), MS_3V_5 (18.50) and MS_4V_1 (15.50). The highest period of shoot induction reported was MS_4V_2 (26.00), which was statistically on par with MS_1V_2 (25.00), MS_1V_3 (21.50), MS_1V_4 (23.50), MS_2V_4 (23.50), MS_3V_2 (23.50), MS_3V_4 (24.50), MS_4V_2 (26.00) and MS_4V_4 (25.50) (Table 14).

Table 14. Effect of multiple shoot induction media, varieties and their interaction on period of shoot induction

	CCRP 2 (V_1)	**CCRP 6 (V_2)**	**CCRP 8 (V_3)**	**CCRP 15 (V_4)**	**SCAVINA (V_5)**	**Mean (Media)**
Ms_1	21.00	25.00	21.50	23.50	14.50	21.10
MS_2	20.50	20.50	17.50	23.50	16.50	19.70
MS_3	20.50	23.50	16.50	24.50	18.50	20.70
MS_4	15.50	26.00	19.50	25.50	20.00	21.30
Mean (Varieties)	19.38	23.75	18.75	24.25	17.38	

CD (MS) – N/A CD (V) – 2.28 CD (MS x V) – 4.55

MS_1 – WPM + 2-ip 1 ppm + IAA 0.02 ppm + $AgNO_3$ 5 ppm

MS_2 – WPM + 2-ip 2 ppm + IAA 0.04 ppm + $AgNO_3$ 5 ppm

MS_3 – WPM + 2-ip 3 ppm + IAA 0.06 ppm + $AgNO_3$ 5 ppm

MS_4 – WPM + 2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5 ppm

4.3.4. **Number of multiple shoots per culture**

The effect of different multiple shoot induction media on number of multiple shoots per culture was significant. The highest number of multiple shoots per culture was in MS_4 (1.76) which was statistically on par with MS_3 (1.74). The lowest number of multiple shoots was in MS_2 (1.14) which was statistically on par MS_1 (1.30).

Number of multiple shoots per culture did not vary significantly among varieties. Highest number of multiple shoots per culture was recorded in V_2 (1.66) and the lowest number was in V_4 (1.38).

Interaction effect of media and varieties on number of multiple shoots differed significantly. The highest number of multiple shoots per culture was in MS_4V_5 (2.25) which was statistically on par with MS_3V_2 (2.08), MS_3V_3 (2.00), MS_3V_4 (2.00), MS_4V_1 (2.00) and MS_4V_2 (1.83). The lowest number of multiple shoots per culture was in MS_2V_3 (1.00) which was on par with MS_1V_1 (1.40), MS_1V_2 (1.40), MS_1V_3 (1.16), MS_1V_4 (1.23), MS_1V_5 (1.33), MS_2V_1 (1.07), MS_2V_2 (1.15), MS_2V_4 (1.15), MS_2V_5 (1.33), MS_3V_1 (1.33), MS_3V_5 (1.33) and MS_4V_4 (1.16) (Table 15).

Table 15. Effect of multiple shoot induction media, varieties and their interactions on number of multiple shoots per culture

	CCRP 2 (V_1)	**CCRP 6 (V_2)**	**CCRP 8 (V_3)**	**CCRP 15 (V_4)**	**SCAVINA (V_5)**	**Mean (Media)**
MS_1	1.40	1.40	1.16	1.23	1.33	1.30
MS_2	1.07	1.15	1.00	1.15	1.33	1.14
MS_3	1.30	2.08	2.00	2.00	1.33	1.74
MS_4	2.00	1.83	1.58	1.16	2.25	1.76
Mean (Varieties)	1.44	1.66	1.43	1.38	1.56	

CD (MS) – 0.22 CD (V) - N/A CD (MS x V) – 0.48

MS_1 – WPM + 2-ip 1 ppm + IAA 0.02 ppm + $AgNO_3$ 5 ppm

MS_2 – WPM + 2-ip 2 ppm + IAA 0.04 ppm + $AgNO_3$ 5 ppm

MS_3 – WPM + 2-ip 3 ppm + IAA 0.06 ppm + $AgNO_3$ 5 ppm

MS_4 – WPM + 2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5 ppm

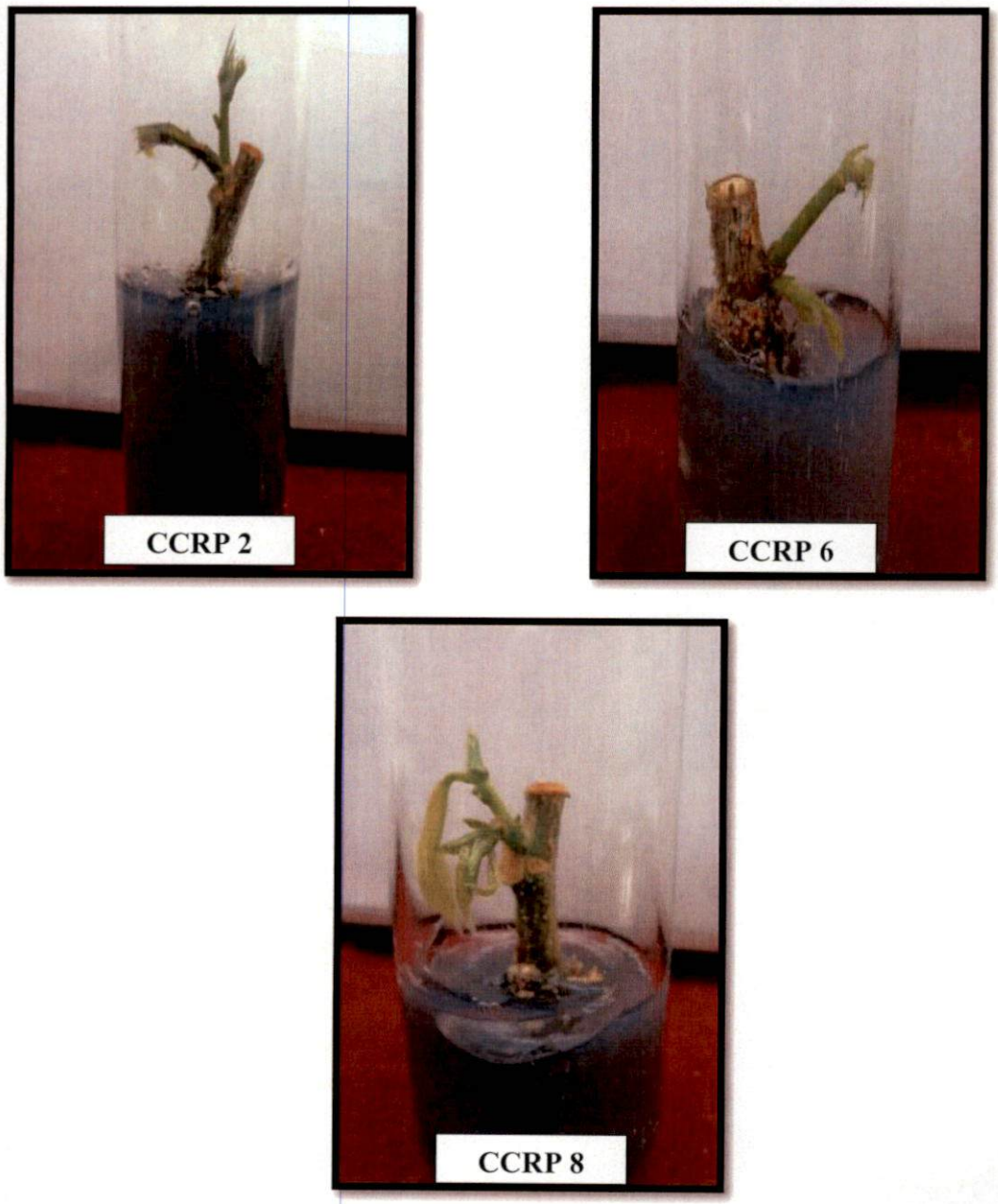


Plate 9. Multiple shoots produced from leaf axils of different cocoa varieties after four weeks of culturing

Plate 9. Continued: Multiple shoots produced from leaf axils of different cocoa varieties after four weeks of culturing

4.4. ROOTING OF *IN VITRO* PRODUCED SHOOTS

Shoot induction percentage and multiple shoot induction percentage were together high in cocoa but the number of shoots with minimum growth for inoculation in culture tubes for rooting was low. Thus, the rooting experiment was tried in limited number of cultures. *In vitro* shoots were kept for rooting in half strength MS medium after pulse treatment with IBA at different concentrations. Some of the cultures showed rooting after 1 month. The details are furnished in Table 16.

Root induction *in vitro* shoots of five different varieties of cocoa viz. CCRP 2, CCRP 6, CCRP 8, CCRP 15 and Scavina was found to be congenial in the media ½ MS + IBA 4000 ppm (pulse treatment for 2-3 sec) and ½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec) were found to be congenial (Table 16). In ½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec), varieties, CCRP 2, CCRP 6, CCRP 8, CCRP 15 and Scavina showed 20 per cent, 60 per cent, 60 per cent, 80 per cent and 70 per cent root induction respectively. However, in two varieties, CCRP 15 and Scavina, had successful root induction in ½ MS + IBA 4000 ppm (pulse treatment for 2-3 sec). Hence in general, it can be concluded that ½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec) was superior to all the media tried for root induction.

Table 16. Effect of different concentration of IBA on root induction in five varieties of cocoa

Varieties	Treatments	Number of shoots inoculated	Number of plants rooted	Percentage of root induction (%)
CCRP 2	½ MS + IBA 1000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 2000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 3000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 4000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec)	10	2	20.00
CCRP 6	½ MS + IBA 1000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 2000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 3000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 4000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec)	10	6	60.00
CCRP 8	½ MS + IBA 1000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 2000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 3000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 4000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec)	10	6	60.00
CCRP 15	½ MS + IBA 1000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 2000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 3000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 4000 ppm (pulse treatment for 2-3 sec)	10	1	10.00
	½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec)	10	8	80.00
Scavina	½ MS + IBA 1000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 2000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 3000 ppm (pulse treatment for 2-3 sec)	10	Nil	Nil
	½ MS + IBA 4000 ppm (pulse treatment for 2-3 sec)	10	2	20.00
	½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec)	10	7	70.00

Root initiation in *in vitro* produced cocoa shoot after 30 days of culturing in ½ MS media after pulse treatment with IBA 5000 ppm

Fully developed roots in *in vitro* shoots after 50 days of culturing in ½ MS media after pulse treatment with IBA 5000 ppm

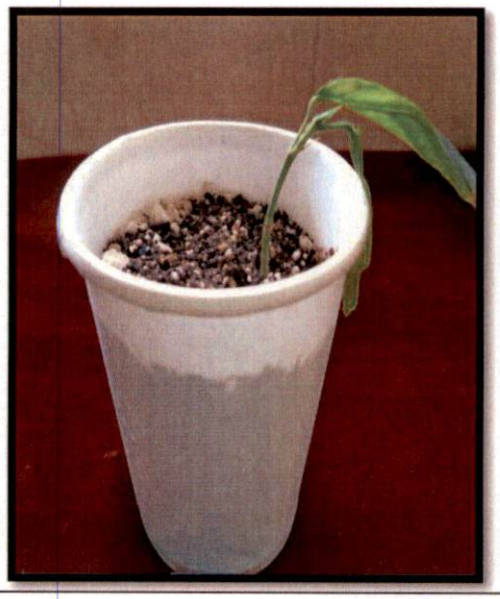
Plate 10. Rooting of *in vitro* shoots of cocoa in half MS media after pulse treatment with IBA

4.5. HARDENING

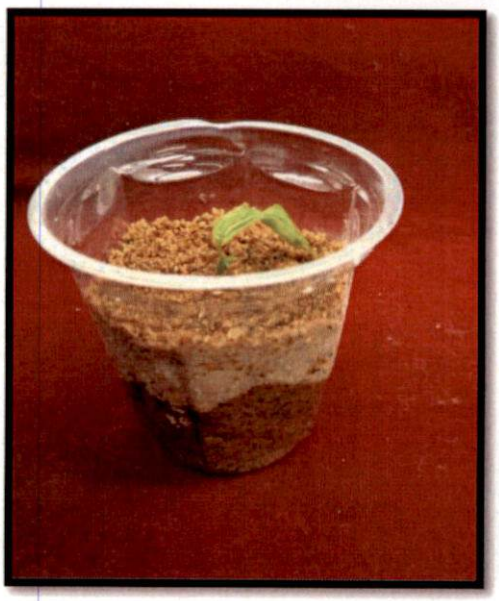
The results of the effect of different potting mixtures on establishment and survival of tissue culture plants of cocoa were studied and presented in Table 17. For hardening of tissue culture plants of cocoa, two different potting media were used. The potting media were Vermiculite: Perlite: Vermicompost in equal proportion (PM_1) and Sand: Vermicompost in equal proportion (PM_2). The tissue culture cocoa plants were initially planted in disposable cups made of paper but due to high moisture content retained in these cups, fungal attack of the plants were prominent. Hence, these cups were later replaced with plastic disposable cups which were found to be better for controlling the contamination. Among the potting media tried, PM_2 recorded the maximum survival of plantlets for one week. However, later the plants showed yellowing and finally the shoots were completely dried off. When the potting mixture PM_1 was used, the plantlets survived for comparatively lower period than the above treatment.

Table 17. Effect of potting media on plant survival

Treatments	**Potting media**	**Number of plants kept for hardening**	**Percentage of survival**
PM_1	Sand: Vermicompost (1:1)	10	Nil
PM_2	Vermiculite: Perlite: Vermicompost (1:1:1)	15	Nil



Hardening in potting mixture – Vermiculite: Perlite: Vermicompost (1:1:1)



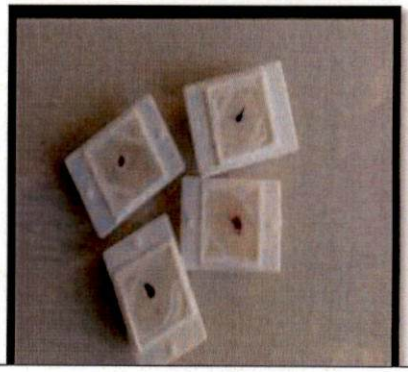
Hardening in potting mixture – Sand: Vermicompost (1:1)

Plate 11. Hardening of tissue culture cocoa plants

4.6. ANATOMICAL STUDIES

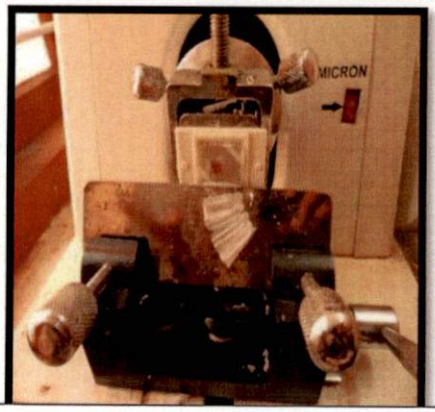
The anatomical observation on root-shoot transition zone using rotary microtome sectioning showed the presence of intervening callus between the vascular tissues of root and shoot (Plate 13). This is a usual problem encountered in tissue culture of cocoa. The intervening callus inhibits proper vascular connection and thus, the plants kept for hardening were difficult to get established.

Fixation of material in FAA for 24 hours



Material embedded in wax block

Ultra-thin sectioning using rotary microtome

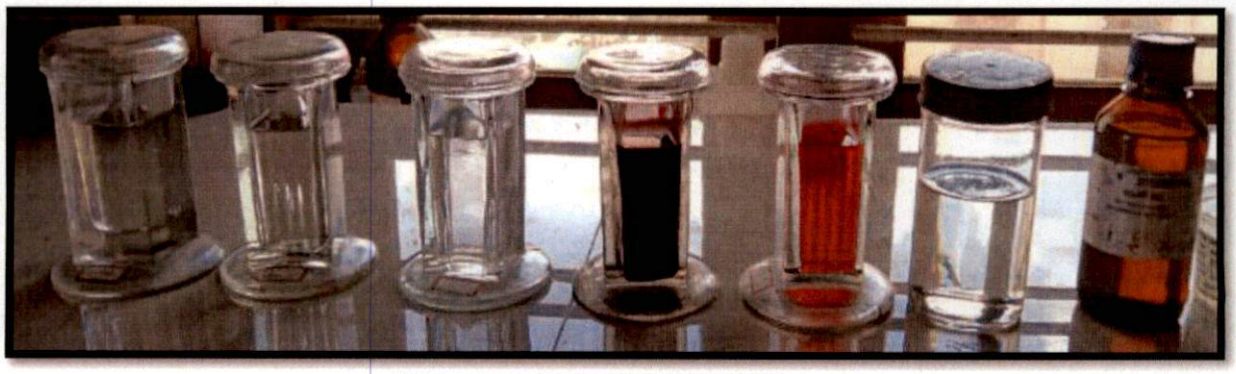


Thin ribbon of wax containing plant material

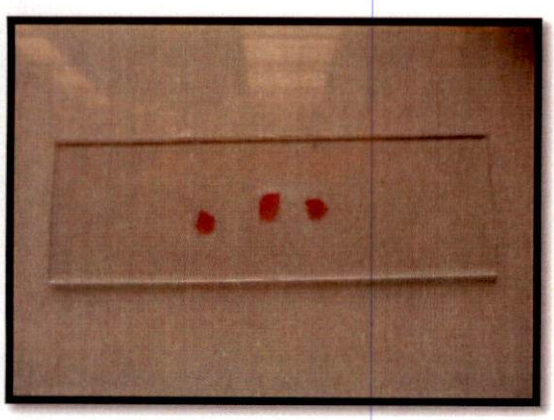
Wax ribbon containing plant material on glass slides

Warming the microslide for proper stretching of the sections

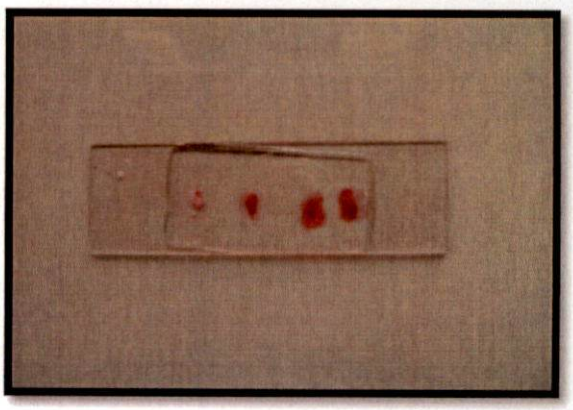
Plate 12. Microtome sectioning of root-shoot transition zone of *in vitro* cocoa plants



Staining



Stained sections with saffranin (0.1%)



Permanent slide prepared with DPX

Plate 12. Continued. Microtome sectioning of root-shoot transition zone of *in vitro* cocoa plants

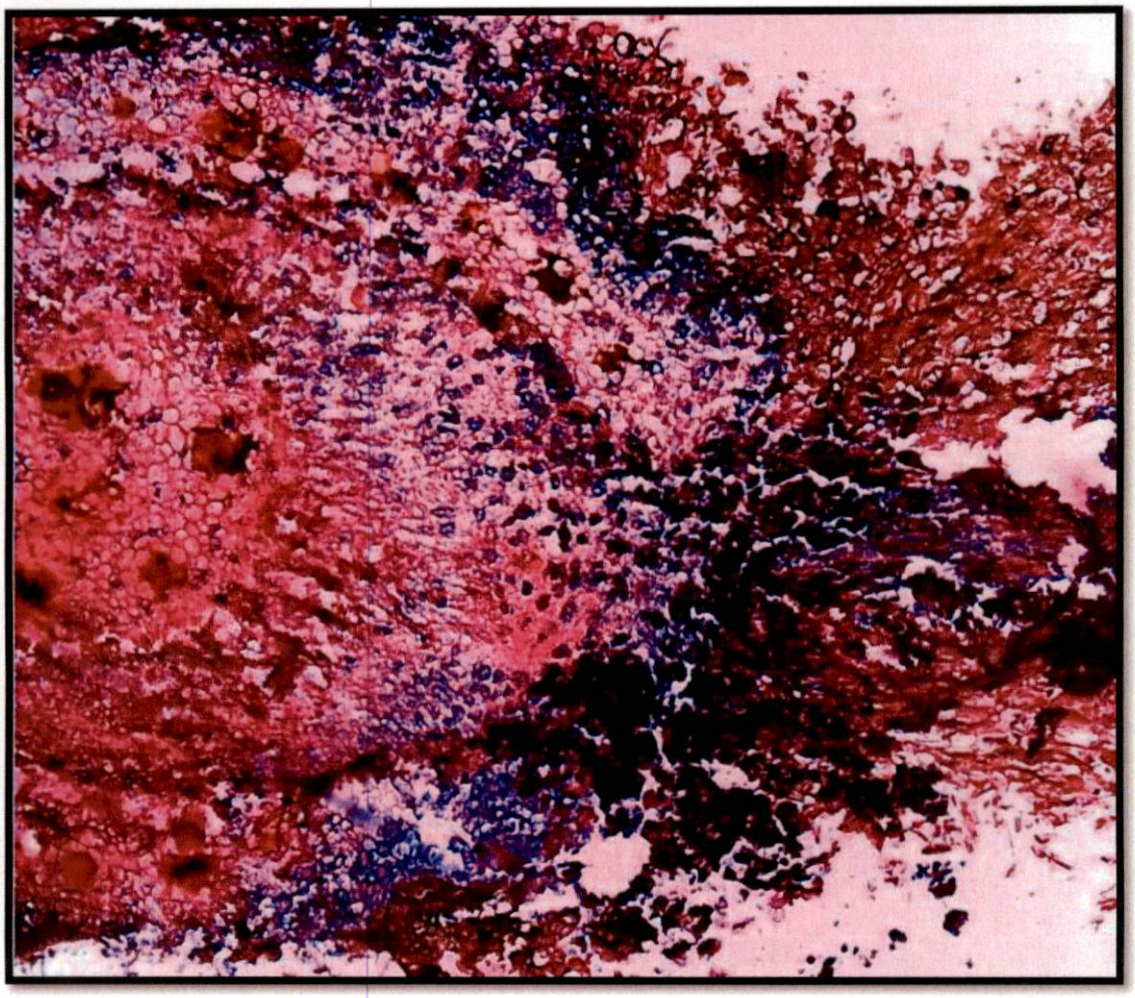


Plate 13. Anatomical section of root-shoot transition zone showing an intervening callus between vascular tissues of root and shoot

DISCUSSION

5. DISCUSSION

Cocoa (*Theobroma cacao* L.) is a major plantation crop of the world and is known as a beverage crop due to the value of its seeds. It shows heterozygosity due to open pollination and is also self-incompatible. Thus, in order to obtain quality seedlings, the seeds should be collected from polyclonal gardens. But even this may not be true to type. In the case of budded plants, those produced from buds of fan shoots require skilled labours for proper pruning operations. Tissue culture is an alternative that allows the production of true to type plants in a short period of time (Levy, 1981). However, for successful plant regeneration through tissue culture, an efficient protocol should be developed for different genotypes. In view of this, an attempt was done to standardize *in vitro* propagation of cocoa using five varieties in Plant Tissue Culture Laboratory, Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara during 2016-18. The result obtained are discussed here.

5.1. SURFACE STERILIZATION

Micropropagation of woody trees are always encountered with the problem of microbial contamination. Surface sterilization of explants helps to eliminate these microbes without causing damage to the explant tissues. The surface sterilization requirements for *in vitro* propagation are different and it varies with the type of tissue and nature of explant used. In cocoa, a high rate of contamination was observed in nodal cuttings than other explants due to the presence of mucilage (Bhavyashree *et al*., 2011).

The regular spraying of explant sources with a systemic fungicide Carbendazim (0.2 %) and a contact fungicide Mancozeb (0.3 %) in an interval of three days to help to brought down the contamination percentage and this is in conformity with Mallika *et al*. (1992) and Bindu (1997).

In present investigation, the most effective surface sterilization treatment was washing the nodal segments in Teepol followed by 0.2 per cent Mancozeb for 30 min

followed by Cefotaxim 0.1 per cent for 10 min outside the laminar air flow chamber. Inside the laminar air flow, Ethyl Alcohol 70 per cent was treated for 3 min followed by $HgCl_2$ 0.1 per cent for 5 min. This treatment gave 91.67 per cent living contamination free cultures and the contaminated cultures were only 8.33 per cent. Bonga (1982) revealed the importance of using alcohol alone or in combination with other chemicals for surface sterilization of explant tissues. Devi (2011) standardized the use of 70 per cent Ethyl alcohol for 3 min followed by 0.1 per cent $HgCl_2$ for 5 min for the nodal segments of *Saraca asoca* and resulted in maximum contamination free cultures.

Silva *et al.* (2008) reported that surface sterilization of immature flower buds of cocoa by immersion in 70 per cent (v/v) ethanol for 1 min and 30 sec followed by 20 min in 2.5 per cent (v/v) sodium hypochlorite solution containing 0.1 per cent (v/v) Tween-20 and then rinsing in sterile water reduces maximum culture contamination.

Legrand and Mississo (1986) suggested the use of 70 per cent alcohol, 10 per cent calcium hypochlorite and 10 per cent hydrogen peroxide for the surface sterilization of cocoa explants. Bhavyashree *et al.* (2011) standardized the surface sterilization of nodal cutttings of cocoa by treating with Bavistin (1 g l^{-1}) for 1 hour followed by two drops of Tween 20 for 15 min outside the laminar air flow chamber. Inside the laminar air flow chamber, surface sterilization was done by using 0.1 per cent $HgCl_2$ for 3-4 min.

5.2. CULTURE ESTABLISHMENT AND SHOOT INDUCTION IN DIFFERENT VARIETIES OF COCOA

Results of the present study indicated that, there was no significant variation in culture establishment percentage with respect to the different media and varieties. Culture establishment percentage in all the five varieties varied from 83.33 per cent to 100 per cent. The culture establishment percentage was cent per cent in medium WPM + 2-ip 1 ppm + IAA 0.02 ppm for varieties CCRP 15 and Scavina, WPM + 2-ip 2 ppm + IAA 0.04 ppm for varieties CCRP 2, CCRP 6 and Scavina, WPM + 2-ip 3 ppm + IAA 0.06 ppm for CCRP 8 and WPM + 2-ip 4 ppm + IAA 0.08 ppm for varieties CCRP 2 and Scavina. In

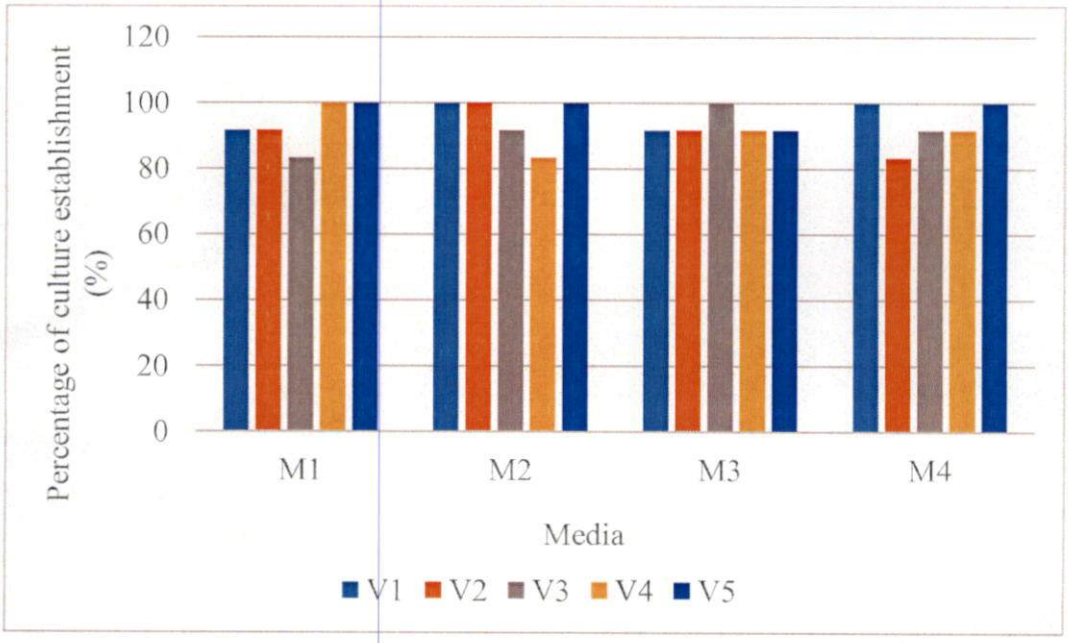
all the treatment combinations, basal media was WPM supplemented with different concentration of 2-ip and IAA. Bindu (1997) reported that the best basal medium for the culture establishment indicated by bud break, shoot elongation and leaf production in cocoa was the full strength WPM. This was also in conformity with the findings of McCown (2013) who used 2-ip in WPM in woody species like *Azalea* and *Rhododendron* for better culture establishment. Mallika *et al.* (1992) reported that in basal WPM, the cultures could be also maintained for a long time without chlorosis and leaf abscission.

Karun and Bhavyashree (2011) reported that bud burst and sustained growth of shoots of cocoa under *in vitro* condition could be achieved when nodal segments were cultured in WPM containing additional supplements. Moreover, the stage of flush at which the explants are harvested also influences the enhanced release of axillary buds.

In the present investigation, there was no significant variation in shoot induction percentage with respect to the different media and varieties. Shoot induction percentage varied from 83.33 per cent to 100 per cent. Shoot induction was recorded cent per cent in medium WPM + 2-ip 1 ppm + IAA 0.02 ppm for varieties CCRP 15 and Scavina, WPM + 2-ip 2 ppm + IAA 0.04 ppm for varieties CCRP 2, CCRP 6 and Scavina, WPM + 2-ip 3 ppm + IAA 0.06 ppm for CCRP 8 and WPM + 2-ip 4 ppm + IAA 0.08 ppm for varieties CCRP 2 and Scavina. WPM fortified with growth regulators like 2-ip and IAA was found be the best for shoot induction in all varieties. Mallika *et al.* (1992) reported that WPM containing kinetin/2-ip 1 mg l^{-1} and IAA 0.02 mg l^{-1} was reported to have successful bud sprout and leaf expansion from pre-existing meristems. Bindu (1997) reported that 2-ip (5 mg l^{-1}) showed the maximum shoot elongation in cocoa. Giridhar *et al.* (2005) confirmed that in *Decaleptis hamiltonii*, the best multiplication under *in vitro* condition was obtained with 2-ip as compared to other sources of cytokinin like kinetin, BAP and thidiazuron.

In culture establishment media, the period of shoot induction for all the five varieties was significantly influenced by the media, varieties and their interactions. Irrespective of the variety, it was recorded lowest in WPM + 2-ip 1 ppm + IAA 0.02 ppm

(8.41). Irrespective of media used, the lowest period of shoot induction was noted in CCRP 2 (7.10) and this was statistically on par with varieties Scavina (8.37). Interaction effect of media and varieties showed that, the period of shoot induction was lowest in variety CCRP 15 and the media WPM + 2-ip 1 ppm + IAA 0.02 ppm (6.33). Bindu (1997) had studied the number of days for bud break in cocoa and stated that the minimum number of days for bud break was 7.33 in WPM supplemented with 2-ip, adenine sulphate, silver nitrate, cycocel and phloroglucinol.



M_1 – WPM + 2-ip 1 ppm + IAA 0.02 ppm

M_2 – WPM + 2-ip 2 ppm + IAA 0.04 ppm

M_3 – WPM + 2-ip 3 ppm + IAA 0.06 ppm

M_4 – WPM + 2-ip 4 ppm + IAA 0.08 ppm

V_1- CCRP 2

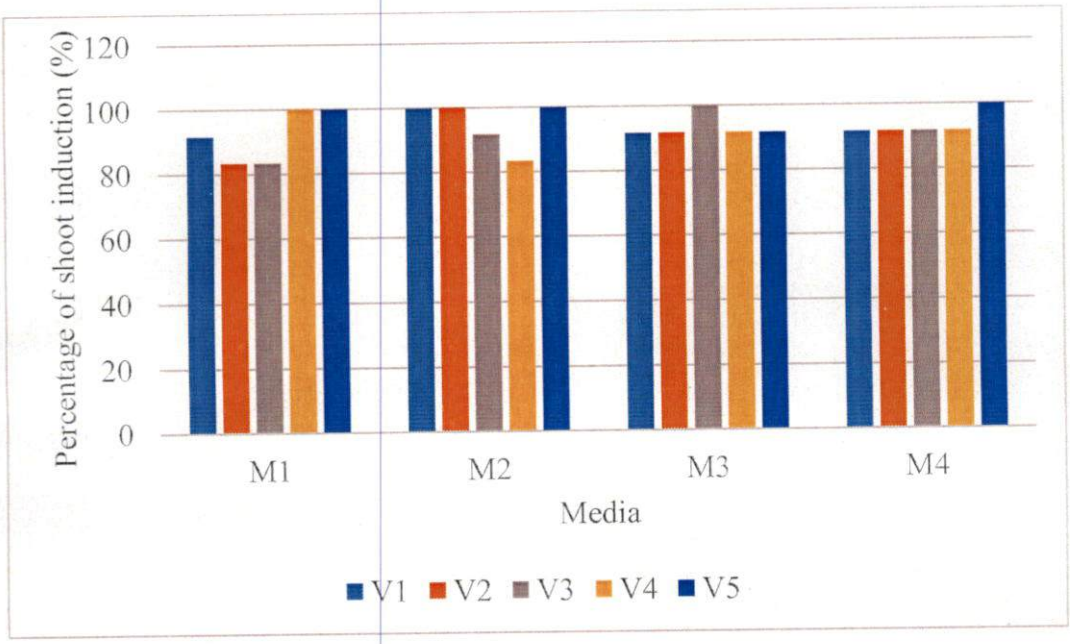
V_2- CCRP 6

V_3- CCRP 8

V_4- CCRP 15

V_5- Scavina

Fig 1. Effect of culture establishment media on percentage of cultures established in different cocoa varieties



M_1 – WPM + 2-ip 1 ppm + IAA 0.02 ppm

M_2 - WPM + 2-ip 2 ppm + IAA 0.04 ppm

M_3 – WPM + 2-ip 3 ppm + IAA 0.06 ppm

M_4 – WPM + 2-ip 4 ppm + IAA 0.08 ppm

V_1- CCRP 2

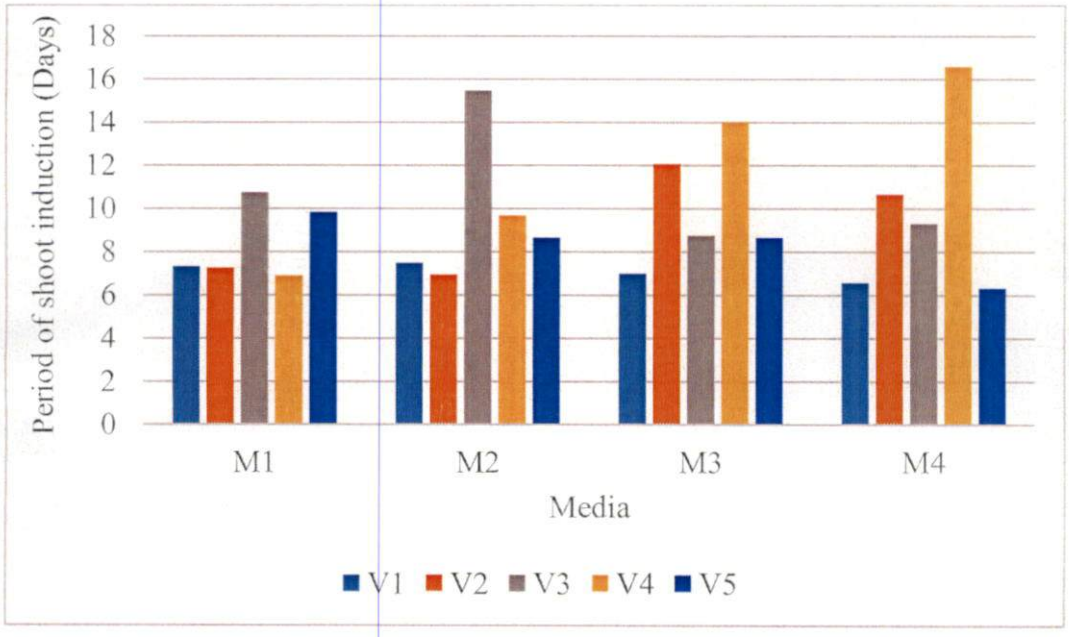
V_2- CCRP 6

V_3- CCRP 8

V_4- CCRP 15

V_5-Scavina

Fig 2. Effect of culture establishment media on percentage of shoot induction in different cocoa varieties



M_1 - WPM + 2-ip 1 ppm + IAA 0.02 ppm

M_2 - WPM + 2-ip 2 ppm + IAA 0.04 ppm

M_3 – WPM + 2-ip 3 ppm + IAA 0.06 ppm

M_4 – WPM + 2-ip 4 ppm + IAA 0.08 ppm

V_1- CCRP 2

V_2- CCRP 6

V_3- CCRP 8

V_4- CCRP 15

V_5- Scavina

Fig 3. Effect of culture establishment media on period of shoot induction in different cocoa varieties.

5.3. MULTIPLE SHOOT INDUCTION

Production of multiple shoots in axillary bud culture was found to be ideal as it provides large number of plantlets from a single explant (Karun and Bhavyashree, 2011).

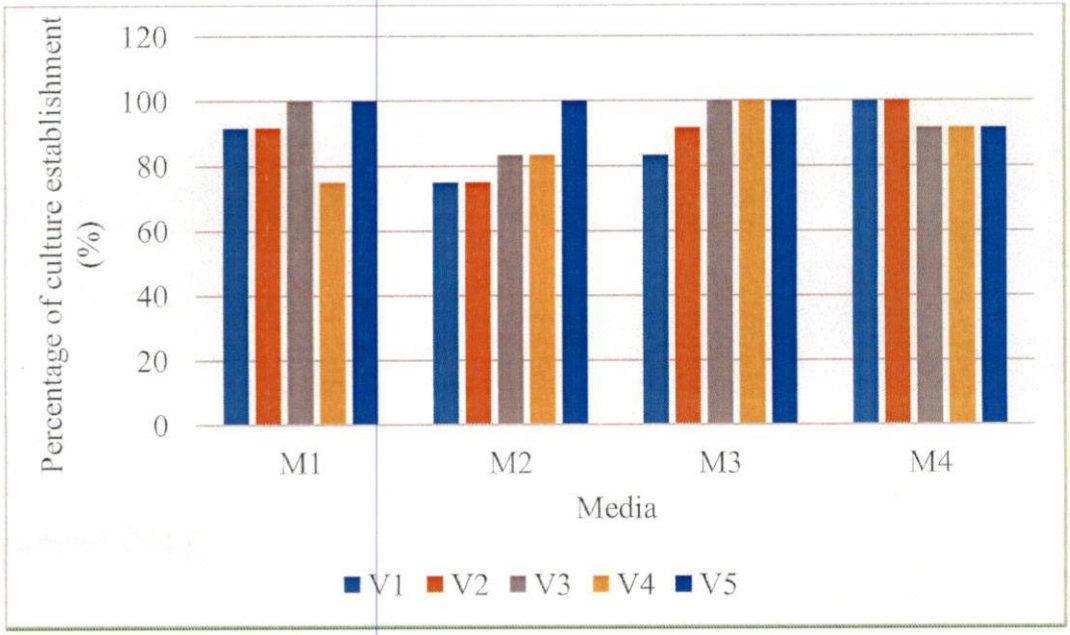
Culture establishment percentage in multiple shoot induction media varied significantly among the media. Highest culture establishment percentage was recorded in media WPM + 2-ip 3 ppm + IAA 0.06 ppm + $AgNO_3$ 5 ppm and WPM + 2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5 ppm (95 %). There was no significant variation in culture establishment percentage of the different cocoa varieties and also among the interactions of media and varieties. In the present study, WPM with different concentration of 2-ip and silver nitrate was used for multiple shoot induction. Similar media was tried by Bindu (1997).

According to Mallika *et al.* (1992), callus formation can be encountered in any explant of cocoa when inoculated in medium without growth regulators. This is due to the presence of high levels of exogenous auxin and gibberellins in the plant. Callusing at the base of explants leads to stunted growth and later, drying of shoots may occur. Addition of silver nitrate reduced the callus formation at the base of nodal stem explants of cocoa and this was also in conformity with the result of Bindu (1997).

Interaction effect of multiple shoot induction media and variety showed cent per cent shoot induction in WPM + 2-ip 1 ppm + IAA 0.02 ppm + $AgNO_3$ 5 ppm and WPM + 2-ip 2 ppm + IAA 0.04 ppm + $AgNO_3$ 5 ppm for Scavina, WPM + 2-ip 3 ppm + IAA 0.06 ppm + $AgNO_3$ 5 ppm for CCRP 8 and WPM + 2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5 ppm for CCRP 2.

Period of shoot induction varied significantly among different media and varieties for multiple shoot induction. The lowest period of shoot induction (14.50) was in variety Scavina when the medium WPM + 2-ip 1 ppm + IAA 0.02 ppm + $AgNO_3$ 5 ppm was used. Interaction effect of media and varieties on number of multiple shoots differed significantly and the highest number of multiple shoots per culture was in variety Scavina

(2.25) when the medium WPM + 2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5 ppm was used. Bindu (1997) stressed the use of WPM supplemented with other media additives like 2-ip, adenine sulphate, cycocel, silvernitrate and phloroglucinol for multiple shoot induction in cocoa. The highest number of multiple shoots produced were 2.66.



M_1 – WPM + 2-ip 1 ppm + IAA 0.02 ppm + $AgNO_3$ 5ppm

M_2 – WPM + 2-ip 2 ppm + IAA 0.04 ppm + $AgNO_3$ 5ppm

M_3 – WPM + 2-ip 3 ppm + IAA 0.06 ppm + $AgNO_3$ 5ppm

M_4 – WPM + 2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5ppm

V_1- CCRP 2

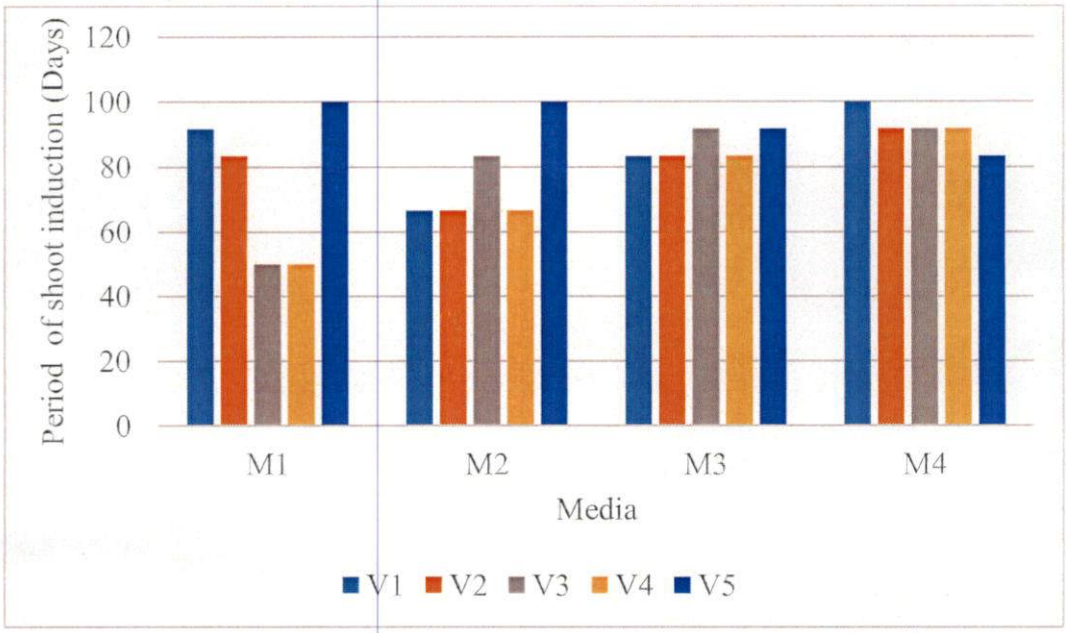
V_2- CCRP 6

V_3- CCRP 8

V_4- CCRP 15

V_5- Scavina

Fig 4. Effect of multiple shoot induction media on percentage of cultures established in different cocoa varieties



M_1 – WPM + 2-ip 1 ppm + IAA 0.02 ppm + $AgNO_3$ 5 ppm

M_2 – WPM + 2-ip 2 ppm + IAA 0.04 ppm + $AgNO_3$ 5 ppm

M_3 – WPM + 2-ip 3 ppm + IAA 0.06 ppm + $AgNO_3$ 5 ppm

M_4 – WPM + 2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5 ppm

V_1- CCRP 2

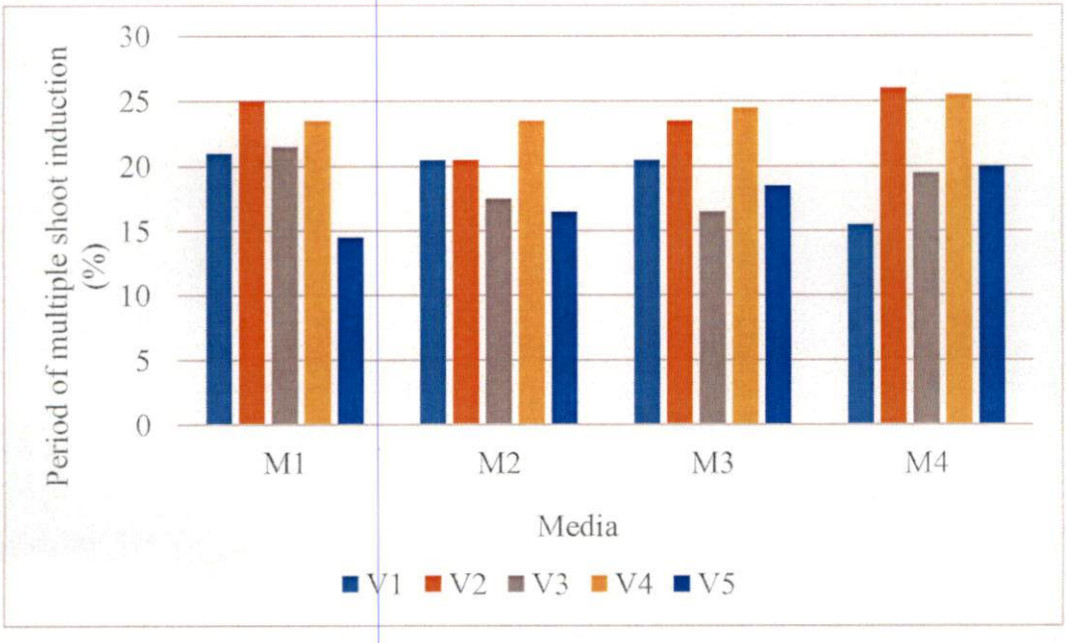
V_2- CCRP 6

V_3- CCRP 8

V_4- CCRP 15

V_5- Scavina

Fig 5. Effect of multiple shoot induction media on percentage of shoot induction in different cocoa varieties



M_1 - WPM + 2-ip 1 ppm + IAA 0.02 ppm + $AgNO_3$ 5 ppm

M_2 - WPM + 2-ip 2 ppm + IAA 0.04 ppm + $AgNO_3$ 5 ppm

M_3 – WPM + 2-ip 3 ppm + IAA 0.06 ppm + $AgNO_3$ 5 ppm

M_4 – WPM + 2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5 ppm

V_1- CCRP 2

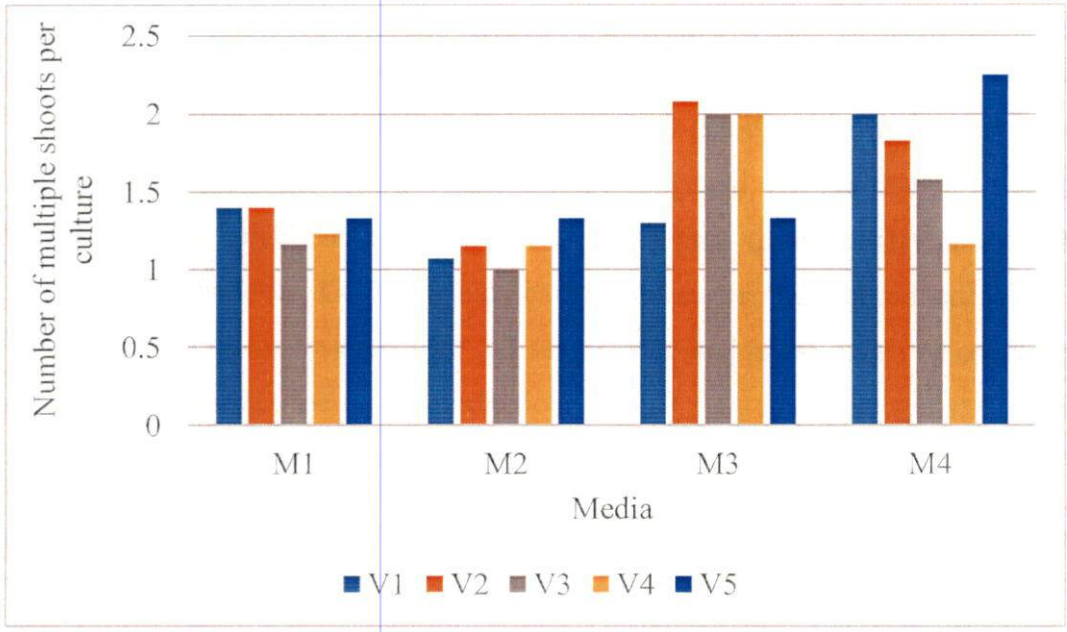
V_2- CCRP 6

V_3- CCRP 8

V_4- CCRP 15

V_5- Scavina

Fig 6. Effect of multiple shoot induction media on period of shoot induction in different cocoa varieties



M_1 – WPM + 2-ip 1 ppm + IAA 0.02 ppm + $AgNO_3$ 5 ppm

M_2 – WPM + 2-ip 2 ppm + IAA 0.04 ppm + $AgNO_3$ 5 ppm

M_3 – WPM + 2-ip 3 ppm + IAA 0.06 ppm + $AgNO_3$ 5 ppm

M_4 – WPM + 2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5 ppm

V_1- CCRP 2

V_2- CCRP 6

V_3- CCRP 8

V_4- CCRP 15

V_5- Scavina

Fig 7. Effect of multiple shoot induction media on number of multiple shoots per culture in different cocoa varieties

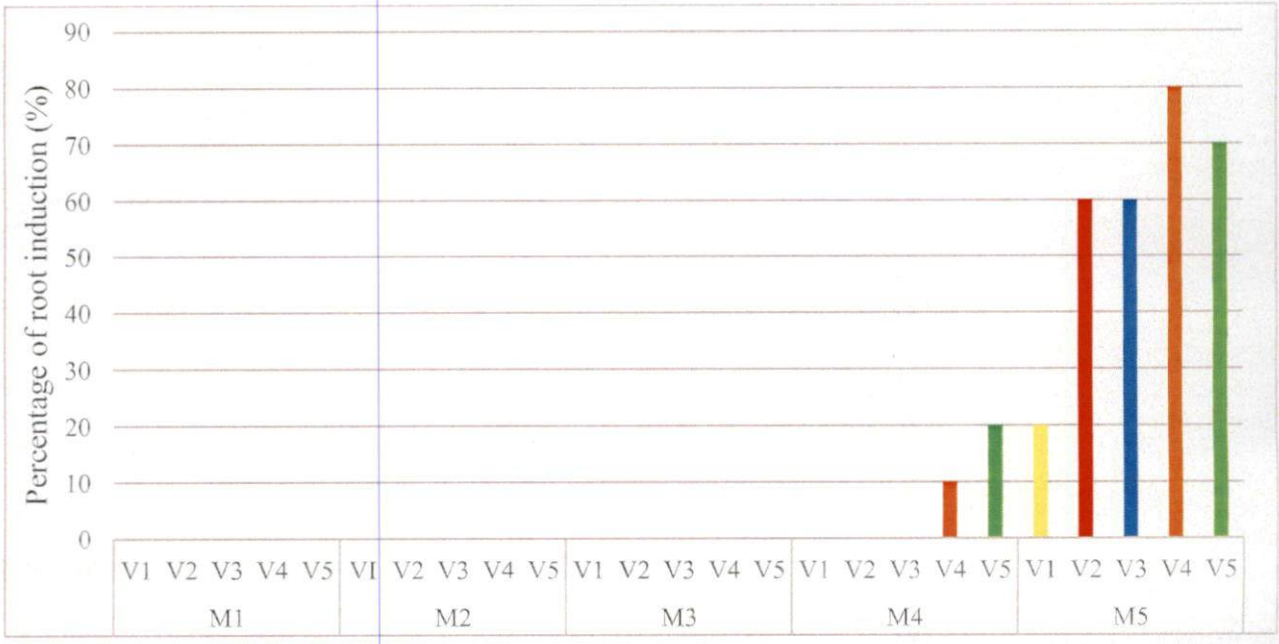
5.4. ROOTING

Rooting percentage was generally low under *in vitro* condition. Among the media tried, ½ MS + IBA 4000 ppm (pulse treatment for 2-3 sec) and ½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec) were found to be congenial. All the five varieties showed successful root induction in ½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec). However, two varieties, CCRP 15 and Scavina, had successful root induction in ½ MS + IBA 4000 ppm (pulse treatment for 2-3 sec). Hence in general, it can be concluded that among all the media tried for root induction, ½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec) proved to be superior.

Nair *et al.* (1993) reported that cocoa shoots shows low root induction percentage under *in vitro* condition (20-30 %). This was also supported by the findings of Bindu (1997).

In the present study, the basal half MS medium with pulse treatment of IBA 5000 ppm showed maximum rooting in cultures. Bindu (1997) highlighted that pretreatment of *in vitro* cocoa shoots with IBA 5000 mg l^{-1} for 3 sec followed by culturing in half strength MS media was ideal for root induction. Studies of Komalavalli and Rao (2000) and Shekhawat *et al.* (2015) also revealed that basal half MS medium supplemented with growth regulators was best for root induction under *in vitro* condition.

Karun and Bhavyashree (2011) reported that rooting of *in vitro* shoots derived from axillary buds could be accomplished by pulse treatment with IBA at various concentrations. The healthy roots were produced when auxin treatment was given to the shoots followed by transferring to an auxin free medium with activated charcoal.



M_1 - ½ MS + 1000 ppm (pulse treatment 2-3 sec)

M_2 - ½ MS + 2000 ppm (pulse treatment 2-3 sec)

M_3 - ½ MS + 3000 ppm (pulse treatment 2-3 sec)

M_4 - ½ MS + 4000 ppm (pulse treatment 2-3 sec)

M_5 - ½ MS + 5000 ppm (pulse treatment 2-3 sec)

V_1- CCRP 2

V_2- CCRP 6

V_3- CCRP 8

V_4- CCRP 15

V_5- Scavina

Fig 8. Percentage of root induction in different cocoa varieties in rooting media

5.5. HARDENING

The tissue culture plants should be treated with a fungicide before transferring to the potting mixture to prevent the fungal attack. Hence, they were treated with Carbendazim (0.1 %) for 5 min and this was found to be effective reducing the rate of contamination. This is in conformity with the findings of Mallika *et al.* (1992).

Hardening of tissue culture cocoa plants were not successful in both Sand: Vermicompost (1:1) and Vermiculite: Perlite: Vermicompost (1:1:1). The Vermiculite: Perlite: Vermicompost (1:1:1) media showed a maximum survival of plantlets for one week. Later, the plantlets showed yellowing and finally the shoots were completely dried off. Similar problem was reported by Bindu (1997).

5.6. ANATOMICAL SECTIONING

The anatomical study on root-shoot transition zone using rotary microtome sectioning revealed lack of vascular connectivity between the root and shoot at the collar region. This was due to the presence of a layer of corky callus in between and this callus inhibits proper vascular connection. Thus, the plants kept for hardening were difficult to get established. This is supported by the findings of Mallika (1992) and Bindu (1997).

Smith *et al.* (1991), reported that the vascular connections between the new *in vitro* roots and the micro cutting stems of maple, bitch and apple were often found to be discontinuous. This was because the *in vitro* roots were often subtended by callus or it may be emerged from callus.

SUMMARY

6. SUMMARY

The present investigation was carried out during the period 2016-2018 in the Tissue Culture Laboratory of Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara with the objective to develop a protocol for *in vitro* regeneration of elite cocoa clones. Nodal segments from grafted plants of cocoa was used as the source of explants. The salient findings of the investigation are presented below:

1. The best treatment for surface sterilization of nodal segments was washing in Teepol followed by 0.2 % Mancozeb for 30 min followed by Cefotaxim 0.1 % for 10 min outside the laminar air flow chamber and inside the laminar air flow, Ethyl Alcohol 70 % was treated for 3 min followed by $HgCl_2$ 0.1 % for 5 min. This treatment showed the highest survival percentage (91.67 %) of cultures with minimum microbial contamination (8.33 %).
2. There was no significant variation in culture establishment percentage with respect to the different media and varieties. Culture establishment percentage in all the five varieties varies from 83.33 % to 100 %. Cent per cent culture establishment was recorded in WPM + 2-ip 1 ppm + IAA 0.02 ppm for varieties CCRP 15 and Scavina, WPM + 2-ip 2 ppm + IAA 0.04 ppm for varieties CCRP 2, CCRP 6 and Scavina, WPM + 2-ip 3 ppm + IAA 0.06 ppm for CCRP 8 and WPM + 2-ip 4 ppm + IAA 0.08 ppm for varieties CCRP 2 and Scavina.
3. There was no significant variation in shoot induction percentage with respect to the different media and varieties. Shoot induction percentage varies from 83.33 % to 100 %. Cent per cent shoot induction was recorded in WPM + 2-ip 1 ppm + IAA 0.02 ppm for varieties CCRP 15 and Scavina, WPM + 2-ip 2 ppm + IAA 0.04 ppm for varieties CCRP 2, CCRP 6 and Scavina, WPM + 2-ip 3 ppm + IAA 0.06 ppm for CCRP 8 and WPM + 2-ip 4 ppm + IAA 0.08 ppm for varieties CCRP 2 and Scavina.

4. In culture establishment media, period of shoot induction for all the five varieties was significantly influenced by media, varieties and their interactions. Irrespective of the variety, it was recorded lowest in WPM + 2-ip 1 ppm + IAA 0.02 ppm (8.41). Irrespective of media used, the lowest period of shoot induction was noted in CCRP 2 (7.10) and this was statistically on par with varieties Scavina (8.37). Interaction effect of media and varieties showed that, the period of shoot induction was lowest (6.33) in variety CCRP 15 when media WPM + 2-ip 1 ppm + IAA 0.02 ppm.
5. Culture establishment percentage in multiple shoot induction media vary significantly among media. Highest culture establishment percentage was recorded in WPM + 2-ip 3 ppm + IAA 0.06 ppm + $AgNO_3$ 5 ppm and WPM + 2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5 ppm (95 %). There were no significant variations in culture establishment percentage among the different varieties and also interaction of media and varieties.
6. Shoot induction percentage vary significantly among media, varieties and their interactions. Irrespective of the media used, highest multiple shoot induction percentage was recorded in WPM + 2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5 ppm (91.67 %). Irrespective of the media used, highest multiple shoot induction percentage was recorded in Scavina (93.75 %). Interaction effect of multiple shoot induction media and variety showed cent per cent shoot induction in WPM + 2-ip 1 ppm + IAA 0.02 ppm + $AgNO_3$ 5 ppm and WPM + 2-ip 2 ppm + IAA 0.04 ppm + $AgNO_3$ 5 ppm for Scavina, WPM + 2-ip 3 ppm + IAA 0.06 ppm + $AgNO_3$ 5 ppm for CCRP 8 and WPM + 2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5 ppm for CCRP 2.
7. Period of shoot induction varied significantly among the different multiple shoot induction media used, varieties and their interactions. Irrespective of varieties, WPM + 2-ip 2 ppm + IAA 0.04 ppm + $AgNO_3$ 5 ppm (19.70). Irrespective of media, lowest period of shoot induction was recorded in Scavina (17.38) which was statistically on par with CCRP 2 (19.38) and CCRP 8 (18.75). Period of shoot

induction varies significantly among different media and varieties for multiple shoot induction. The lowest period of shoot induction (14.50) was noted in variety Scavina when the medium WPM + 2-ip 1 ppm + IAA 0.02 ppm + $AgNO_3$ 5 ppm was used.

8. The number of multiple shoots per culture vary significantly among the different media used. The highest number of multiple shoots per culture was in WPM + 2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5 ppm (1.76). Number of multiple shoots per culture did not vary significanly among varieties. Highest number of multiple shoots per culture was recorded in variety CCRP 6 (1.66). Interaction effect of media and varieties on number of multiple shoots was significantly differed and the highest number of multiple shoots per culture (2.25) was in variety Scavina when media WPM+2-ip 4 ppm + IAA 0.08 ppm + $AgNO_3$ 5 ppm was used.
9. Rooting percentage was generally low under *in vitro* condition. Among the media tried, ½ MS + IBA 4000 ppm (pulse treatment for 2-3 sec) and ½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec) were found to be ideal and two varieties, CCRP 15 and Scavina, had successful root induction in ½ MS + IBA 4000 ppm (pulse treatment for 2-3 sec). Hence in general, it can be concluded that, among all the media tried for root induction, ½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec) proved to be superior.
10. Hardening of tissue culture cocoa plants were not successful in both Sand: Vermicompost (1:1) and Vermiculite: Perlite: Vermicompost (1:1:1). The Vermiculite: Perlite: Vermicompost (1:1:1) media showed a maximum survival of plantlets for one week and later the plant showed yellowing and finally the shoots were completely dried off.
11. The anatomical observation on root-shoot transition zone using rotary microtome sectioning showed the presence of intervening callus between the vascular tissues of root and shoot. The intervening callus inhibits proper vascular connection and thus, the plants kept for hardening were difficult to get established.

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IN VITRO STUDIES ON MORPHOGENESIS AND PLANT REGENERATION IN ELITE CLONES OF COCOA (*Theobroma cacao* L.)

By

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ABSTRACT OF THE THESIS

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ABSTRACT

The experiment entitled "*In vitro* studies on morphogenesis and plant regeneration in elite clones of cocoa (*Theobroma cacao* L.) was carried out in Tissue culture laboratory of Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara during 2016-2018 with an objective to refine/develop a protocol for *in vitro* regeneration and also to determine the variation in morphogenesis and regeneration among elite cocoa clones. The experiment was conducted using five varieties, CCRP 2, CCRP 6, CCRP 8, CCRP 15 and Scavina.

Single nodded cuttings of 2-3 cm collected from the budded plants were used as explants. The best treatment for surface sterilization of nodal segments was washing in Teepol followed by shaking in 0.2 per cent Mancozeb for 30 minutes and then, Streptocycline 0.1 per cent for 10 minutes outside the laminar air flow chamber. Inside the laminar air flow chamber, the explants were treated with Ethyl Alcohol 70 per cent for 3 minutes followed by $HgCl_2$ 0.1 per cent for 5 minutes. This treatment showed the highest survival percentage (91.67%) of cultures with minimum microbial contamination (8.33%).

The medium WPM + 2-ip 1ppm + IAA 0.02ppm showed cent per cent shoot induction in varieties CCRP 15 and Scavina; WPM + 2-ip 2ppm + IAA 0.04ppm in varieties CCRP 2, CCRP 6 and Scavina; WPM + 2-ip 3ppm + IAA 0.06ppm in CCRP 8 and WPM + 2-ip 4ppm + IAA 0.08ppm in varieties CCRP 2 and Scavina. Period of shoot induction for all the five varieties was significantly influenced by media, varieties and their interactions. Among the media, the lowest period of shoot induction was recorded in medium WPM + 2-ip 1ppm + IAA 0.02ppm (8.41). Among the varieties, the lowest period of shoot induction was in CCRP 2 (7.10) and this was statistically on

par with variety Scavina (8.37). Among the interactions, the period of shoot induction was lowest in variety CCRP 15 in medium WPM + 2-ip 1ppm + IAA 0.02ppm (6.33).

Culture establishment percentage in multiple shoot induction media varied significantly among media. Highest culture establishment percentage (95%) was recorded in media WPM + 2-ip 3ppm + IAA 0.06ppm + $AgNO_3$ 5ppm and WPM + 2-ip 4ppm + IAA 0.08ppm + $AgNO_3$ 5ppm. Among the varieties, highest multiple shoot induction percentage was recorded in Scavina (93.75%). Interaction effect of media and varieties on number of multiple shoots per culture was significantly different and the highest number of multiple shoots per culture (2.25) was observed in variety Scavina when medium WPM + 2-ip 4ppm + IAA 0.08ppm + $AgNO_3$ 5ppm was used.

Rooting percentage was generally low under *in vitro* condition. But among the media tried, ½ MS + IBA 4000 ppm (pulse treatment for 2-3 sec) and ½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec) were found to be ideal. All the varieties showed root induction in ½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec) and only two varieties, CCRP 15 and Scavina, had successful root induction in ½ MS + IBA 4000 ppm (pulse treatment for 2-3 sec). Hence in general, it can be concluded that, ½ MS + IBA 5000 ppm (pulse treatment for 2-3 sec) was superior to all the media tried for root induction.

Hardening of tissue culture cocoa plants were not successful in both sand: vermicompost (1:1) and vermiculite: perlite: vermicompost (1:1:1). The vermiculite: perlite: vermicompost (1:1:1) media showed a maximum survival of plantlets for one week. However, later showed yellowing and finally the shoots were completely dried off. The anatomical observation on root-shoot transition zone using rotary microtome sectioning showed the presence of intervening callus between the vascular tissues of root and shoot. This callus inhibits proper vascular connection and thus, the plants kept for hardening were difficult to get established.