SOMATIC EMBRYOGENESIS FROM IMMATURE INFLORESCENCE OF COCONUT (*Cocos nucifera* L.)

by

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(2017-11-097)

THESIS

Submitted in partial fulfilment of the requirements for the degree of
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DECLARATION

I, hereby declare that this thesis entitled "**Somatic embryogenesis from immature inflorescence of coconut (*Cocos nucifera* L.)**" is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis entitled "**Somatic embryogenesis from immature inflorescence of coconut (*Cocos nucifera* L.)"** is a record of research work done independently by Ms. Radhika R (2017-11-097) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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Radhika R

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LIST OF ABBREVIATIONS

%	Per cent
@	At the rate of
2,4-D	2,4-Dichlorophenoxyacetic acid
AC	Activated Charcoal
BA	Benzyl Adenine
°C	Degree Celsius
$CaCl_2.2H_2O$	Calcium chloride dihydrate
CIS	Coconut Inflorescence Sap
cm	Centi meter
CM2	Coconut Medium 2
$CoCl_2.6H_2O$	Cobalt chloride Hexahydrate
CRD	Completely Randomized Design
Cu	Copper
$CuSO_4.5H_2O$	Copper sulfate pentahydrate
dS m^{-1}	Deci siemens per metre
EC	Electrical Conductivity
et al	And others
Fe	Iron
$FeSO_4.7H_2O$	Ferrous sulphate heptahydrate
g	Gram
g/L	Gram per litre
GA	Gibberellic acid
H_2SO_4	Sulphuric acid
H_3BO_3	Boric acid
Ha	Hectare
HCl	Hydrochloric acid

hrs.	Hours
i_b	Inflorescence base
i_t	Inflorescence tip
K	Potassium
KAU	Kerala Agricultural University
KCl	Potassium chloride
kg	Kilogram
kg cm^{-2}	Kilogram per square centimeter
kg ha^{-1}	Kilogram per hectare
KI	Potassium iodide
KNO_3	Potassium nitrate
L.	Linnaeus
Lakh ha^{-1}	Lakh per hectare
m	Metre
Mg	Magnesium
mg	Milligram
mg $100g^{-1}$	Milligram per hundred gram
mg L^{-1}	Milligram per litre
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
min	Minutes
ml	Millilitre
mM	Millimolar
mm	millimetre
$MnSO_4.4H_2O$	Manganese sulfateTetrahydrate
MT/Ha	Million Tonnes per Hectare
N	Nitrogen
Na	Sodium

NAA	Naphthalene acetic acid
$NaH_2PO_4.2H_2O$	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NH_4Cl	Ammonium chloride
$NiCl_2.6H_2O$	Nickel Chloride Hexahydrate
^oN	Degree North
^oS	Degree South
P	Phosphorous
ppm	Parts per million
psi	Pound per square inch
S	Sulphur
t	tonnes
t ha^{-1}	Tonnes per hectare
TDZ	Thidizuron
Zn	Zinc
$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate

1. INTRODUCTION

Coconut (*Cocos nucifera* L.) belonging to the genus *Cocos* and family Aracaceae is cultivated over 93 countries. India holds a unique position in the world accounting 20.96 lakh ha of cultivated area, production of 16.413 MT and productivity of 7.8 MT/Ha (National Horticultural Board, 2018). The coconut is mostly grown in the states of Kerala, Tamil Nadu, Karnataka and Andhra Pradesh. Kerala leads in terms of area and production with 0.771 million ha and 7448.6 million nuts followed by Karnataka (0.514 million ha, 6773.5 million nuts) and Tamil Nadu (0.461 million ha, 6570.6 million nuts) (Coconut Development Board, 2017).

Every part of the tree is useful to human for one or other purpose. Coconut tree provides drink, food, medicine, health, fuel, shelter, wealth and aesthetics. The coconut palm is called as “Kalpavriksha”, reflecting its usefulness to man.

At present the propagation of coconut is exclusively by seed. However, due to the lack of tolerance to biotic and abiotic stresses and limited seed production per palm within its long life cycle, breeding of selected genotypes will be very tough, through natural coconut propagation that ensue only sexually. Though high performing hybrids of coconut can be accomplished through conventional breeding, the features of reproduction of these perennial plants such as long pre-bearing period, great variability among progeny, long interval between generations, low multiplication rate and absence of natural vegetative propagation will challenge the breeding programme.

Fortunately, as an alternate approach coconut can be propagated asexually through *in vitro* culture techniques, predominantly through somatic embryogenesis, a means to obtain identical and high-yielding planting material on a large scale. Unfortunately, coconut is one of the most recalcitrant species to regenerate *in vitro* (George, 1996).

Somatic embryogenesis has been conventionally divided into two main stages, namely induction and expression. In the former one, somatic cells go through complete reorganization of the cellular state, including physiology, metabolism and gene expression to attain embryogenic characteristics (Feher *et al.*, 2002). After a change in one or more culture conditions, the induced cells and tissues reach the expression stage, in which cells exhibit their embryogenic competence and differentiate into somatic embryos. Subsequently, the matured somatic embryo, prepare for germination in the germination stage after a short period of desiccation. Hence, induction of embryogenic calli is the crucial step for the success of plant regeneration.

Foremost challenge in somatic embryogenesis of coconut include lower frequency of development of somatic embryos, abnormalities, genomic variation in culture response of different explants and poor establishment of *in vitro* regenerated plants (Fernando *et al.*, 2010).

Growth and morphogenesis of plant tissue cultures can be amended by adding small amounts of organic nutrients called undefined supplements (George *et al.*, 2007). Coconut Inflorescence Sap is the natural raw material used for the

production of health drink “Neera”. A protocol has been standardised as a part of the Pilot Project under Coconut Mission for the large scale processing of neera. After clarification of raw neera, a semi fluid by-product is obtained. Preliminary studies indicate that, this by-product contains sodium, potassium, calcium, phosphorous, and small amount of sucrose. When added to potting mixture or other medium under *ex vitro* conditions, this by-product was found to promote plant growth. Since neera is a nutritious liquid with a high content of minerals [Na (6.95 mg/100ml), K (3.16 mg/100ml) (Barh and Mazumdar, 2008)], sucrose (Ysidor *et al.,* 2014), hormones, amino acids *etc*., a minor portion of these may also be present in this by-product which might be boosting the plant growth. Since the explant tissue is also from inflorescence, this may have a nursing effect in the tissue culture medium as in the case of endosperm tissue.

Even though laboratories world-wide have been culturing coconut with the aim of attaining clonal plants, progress has been slower than expected. All through the nineties studies using rachillae was carried out, but success achieved was limited (Blake and Hornung, 1995). Infrequent and unsystematic germination, to develop clonal plantlets, from the callus and somatic embryos formed, were observed (Blake, 1990; Blake and Hornung, 1995). Further research using rachilla enabled the development of a reproducible regeneration protocol. But the success in the formation of somatic embryos and plantlet conversion remain irresolute (Verdeil *et al.,* 1994). Somatic embryogenesis using immature inflorescence has still not been completely mastered (Oropeza *et al.,* 2018). On this ground the main objective of the present study is:

- To assess the *in vitro* response of immature inflorescence of coconut explants for induction and germination of somatic embryos by supplementing the medium with by-product of neera processing and neera

2. REVIEW OF LITERATURE

Coconut is a woody tropical palm belonging to Arecaceae family. It is the only species in the genus of *Cocos* with the chromosome number of 2n=32. The coconut palm plays an important socioeconomic role. It is grown in 80 countries over 10 million ha (Pannetier and Buffard-Morel, I986).

2.1 BOTANY OF COCONUT PALM

According to Harries (1991) coconut palm has a robust cylindrical erect single stem with a radiating crown of long pinnate leaves called as 'fronds'; growing to a height of 30 meters or more. Their ringed trunk rises from swollen bole. Flowering usually begins when the palm is 5-6 years old and continues throughout life. The inflorescence, which does not emerge until three years after initiation of the spadix, is normally branched and bears both male and female flowers. After flowering, development and ripening of the fruit (botanically a fibrous drupe) takes another year.

Sholdt & Michell (1967) observed that they are highly heterozygous and generally cross-pollinated, specifically with entomophilous pollination having very lengthy juvenile phase and live long.

Balingasa and Carpio (1983) reported that number of inflorescence produced per year differ between tall and dwarf varieties of coconut. Verdeil and Buffard (1995) reviewed that a fully matured coconut palm has equal number of

opened and unopened leaves, approximately 20- 30 in number, bearing a flower primordium within each axils.

According to Banzon (1990) coconut palms bear fruit continuously for up to 60-70 years, 12 to 13 times a year. Coconut palms are monoecious with both male and female flowers in its inflorescence. The copious male flowers are borne on the top portion of spikelets which are attached to peduncle. The female flowers are positioned at the base of the spikelets.

According to Harries (1991) they live for more than 100 years generally with an economic life of around 60yrs. As reviewed by Niral *et al*. (2017) the protandrous inflorescence of coconut emerges from the axil of each frond every month.

2.2. CONVENTIONAL METHOD OF PROPAGATION

Branton and Blake (1983) observed that the production of good quality planting material, say seed, is a tedious process. Even after this cumbersome procedures, since palm being a cross pollinated and heterozygous crop; it results in variation among the seedlings, which can be evaluated only after five to eight years of growth on reaching maturity.

Assay Bah *et al.* (1991) observed that the planting material collection and exchange encountered problems due to the considerable weight and size of seed nuts, the absence of dormancy and phytosanitary regulations.

According to Rao and Ganapathi (1993) presently in the absence of a commercially viable vegetative propagation technique, coconut is propagated only by seeds so the progeny differ prominently in vigour, yield and resistance to disease.

According to Namboothiri *et al*. (2008) seeds are not suitable to propagate hybrid palms for the reason that they results in segregation.

The morphological and anatomical features of the palm as well as the conventional method of propagation challenge the breeding for crop improvement.

2.3. VEGETATIVE PROPAGATION

Blake and Eeuwens (1980) reported that coconut palm cannot be propagated naturally by vegetative propagation because of the morphological and physiological peculiarities of coconut palm. Reynolds (1982) reviewed that some approaches of vegetative propagation have been attempted with coconut for instance air layering, reversal of coconut spadix from vegetative to vegetative growth, multiple branching of the single shoot by seedling split with a blade, though none were economically successful.

According to Branton and Blake (1983) clonal propagation from high yielding and disease resistant proven hybrids provided uniform material for planting and high quality parental stocks for breeding programmes, but the traditional vegetative propagation techniques such as cutting and grafting fails due

to the single trunked nature and the absence of any adventitious shoots, suckers or tillers in coconut palm.

So *in vitro* propagation techniques is the most appropriate method for culturing coconut to obtain clonal plantlets.

2.4 *IN VITRO* PROPAGATION

Till date many plant species has been propagated vegetatively by tissue culture techniques (Murashige, 1974) but unfortunately only a few reports of successful propagation of woody plants (King, 1974), specifically in palms (Staritsky, 1970).

Vidhanaarachchi and Weerakoon (1997) claimed that for the production of uniform planting material, clonal propagation of selected palms is the only likelihood but without an accepted vegetative propagation technique, *in vitro* culture techniques remains the only methodology for vegetative multiplication of coconut.

Nonetheless the socio-economic importance of *Cocos nucifera.* L and the potentiality of developing large number of plants utilizing micro sized planting material has bring about the use of *in vitro* techniques in exploring methods to propagate elite trees with desirable characteristics.

2.5 SOMATIC EMBRYOGENESIS

Somatic embryogenesis is the method of choice in regeneration of coconut. Explant on culturing develops into a partly dedifferentiated callus, mentioned as "calloid" by Brackpool *et al.* (1986). Blake, 1990; Verdeil *et al.*, 1994 and Blake and Hornung, 1995 reported that the recalcitrant nature of coconut palm for *in vitro* culturing reflects in slow *in vitro* morphogenesis. Embryogenic calli formation was obtained after three to four months, followed by the formation of somatic embryos, which successively germinate and ultimately form clonal plantlets.

2.6 FACTORS INFLUENCING MICROCULTURING A PLANT TISSUES

Factors affecting the success of *in vitro* culture of plant tissues generally includes genetic (genotype of the crop, type of explant, age and developmental stage of an explant), physiological (plant growth regulators), environmental (light, temperature, pH, media composition) (Brown and Thrope, 1986; Gaj, 2004).

2.6.1Genetic Factor: Choice of Explant

2.6.1.1 *Genotype*

The major challenge in tissue culture with coconut seedling or mature palm is the genomic difference in culture response (Iyer, 1993).

Verdeil *et al.* (1993) reported that the three genotypes of coconut palm PB121 (Hybrid Malayan Yellow Dwarf X West African Tall), PB111 (West

African Tall X Malayan Yellow Dwarf) and Malayan Yellow Dwarf Hybrids showed variation in callogenesis responses. PB111 and PB121 gave the best results.

2.6.1.2 *Type of Explant*

Experiments with different types of explants in coconut culturing have been carried out which includes zygotic embryos (Abraham and Thomas, 1962; Gupta *et al.,* 1984; Karunaratne and Periyapperuma, 1989), stem (Apvatjrat and Blake, 1977), leaf (Pannetier and Buffard Morel, 1982; Raju *et al.*, 1984), apical meristem (Hagedon, 1990), young leaf base (Buffard Morel *et al.,* 1992), plumule excised from mature embryos (Hormung, 1995), unfertilized ovary of coconut (Perera *et al*., 2007).

Though various explant have been used for coconut tissue culture for the last five decades, immature inflorescence was the most responsive to callus induction due to the presence of numerous meristematic points (Tisserat, 1981; Zaid and Tisserat, 1983a; Orense *et al*., 1993).

Eeuwens, 1976 used inflorescence stem (rachis) and branches of inflorescence (rachillae) explant enclosed within the outer and inner spathe for testing the mineral requirements of tissue explants excised from mature coconut palms for initiation and proliferation of callus.

According to Braton and Blake (1983) and Gupta *et al.* (1984) rachillae tissues of young inflorescence explant potentially turn out to nodular structures and further developing into leaf and/or root like growth via embryogenesis.

Tixeira *et al*. (1994) reported that immature inflorescence is a promising explant for the propagation of palm trees, as it has been enclosed within leaf- like sheaths; they are protected from unwanted fungal and bacterial infection and can avoid chemical damage during sterilization procedures. According to Vidhanaarachchi *et al.* (1997) immature inflorescence material has shown as a likely source of explant for coconut tissue culture.

Siny (2006) also reported that among the different explant tried, immature rachillae containing anther meristem at the pre meiotic stages were found to be the best for calloid induction and embryo formation.

2.6.1.3 *Age of Explant*

Thanh Tuyen and De Guzman (1983) observed that embryos were readily derived from pollen, from the unopened spadix, subsequent to the newly opened inflorescence as their anthers contain microspores at the developmental stages just before, during or immediately after first pollen mitosis.

Sugimura and Salvana (1989) reported explant taken from inflorescence between 0.8-3.8 cm long showed better callus induction and minimal browning. Immature inflorescence corresponding to the fourth from the youngest open frond has been found most responsive to callus induction (Orense *et al*., 1993).

According to Verdeil *et al*. (1994) callogenesis response is subjective to the age of inflorescence. Four different stages of inflorescence based on age were indexed with respect to the external spathe length (respectively on average 10, 25, 30 and 45 cm) and confirmed youngest stage more reactive than older stage. Inflorescence with external spathe length 10 and 25cm gave the best callusing (Verdeil *et al*., 1994).

Sini (2006) also reported immature rachillae containing anther meristem at the premeiotic stages were best for calloid induction and embryo formation. Nodular callus was induced from seven to nine months old immature zygotic embryos (Fernando and Gamage, 2000).

2.6.1.4 *Establishment of Aseptic Culture – Germ Free Explant*

Explant plant parts is to be surface sterilized for the maintenance of aseptic condition in *in vitro* culture establishment.

Braton and Blake (1983) surface sterilized the explant in 0.1 per cent (w/v) HgCl2 with 0.05 per cent Tween 20 for 10 minutes, rinsed and shaked for two periods of 10 minutes in 10 per cent bleach.

Thanh Tuyen and De Guzman (1983) detached flower buds from floral branches and rapidly immerse in 70 per cent ethanol for sterilization followed by rinsing with sterile glass- distilled water for several times and then anthers were excised aseptically.

Sugimura and Salvana (1989) suggested dipping the inflorescence enclosed with outer and inner spathe in 3 per cent H_2O_2 for 15minutes and 80 per cent alcohol for 5seconds under aseptic condition was better for surface sterilization.

According to Verdeil *et al.* (1994) inner spathe of each inflorescence was immersed in hypochlorite solution for ten minutes for surface sterilisation and then removed under sterile conditions.

2.6.2 Physiological factor: Composition of culture medium

Culture media are largely accountable for the *in vitro* growth and morphogenesis of plant tissues. The accomplishment of culturing depends on the choice of the nutrient medium. *In vitro* grown plants being heterotrophic in nature; should be provided with same nutrients as required by whole plants.

Murashige and Skoog (1962) conducted a methodical study of mineral requirements of plant tissue and organs in *in- vitro* culturing, followed by other scientists, resulting in several media to suit particular needs. According to Eeuwens (1976) growth of *in-vitro* cultured coconut tissues was critically influenced by the mineral composition of the medium. Different constituents of the cultural media include:

2.6.2.1 Inorganic Nutrients: Minerals

Mineral requirement for the initiation of somatic embryogenesis *in vitro* have been established for numerous plant species. Macronutrients (nitrogen,

phosphorus, potassium, calcium, magnesium and sulphur), micronutrients (zinc, boron, iron, copper, molybdenum and cobalt) and aluminum, nickel and iodine are the mineral components of tissue culture media.

According to Eeuwens (1976) growth of leaf, inflorescence and stem explants from coconut palms on a new mineral formulation (Y3) was superior to that on the minerals of other standard medium. Y3 media contain both ammonium and nitrate nitrogen at lower levels and potassium and iodine at higher levels than in MS medium. However, del Rosario (1976) establish that there is no difference between Murashige & Skoog (1962) and Y3 media. Organic forms of nitrogen are also reported to stimulate growth of cultured explants of coconut (Eeuwens, 1978).

Siny (2006) also reported Y3 as the best basal medium for the *in vitro* culture of immature inflorescence of coconut.

2.6.2.2 Organic Supplements: Vitamins

Eeuwens (1976) reported that for the *in vitro* culturing of coconut palm addition of idione to the medium was beneficial.

Eeuwens (1978) reported though myo-inositol and individual vitamins hardly showed any growth in short term cultures, particularly myo-inositol and thiamine would be required during prolonged culture condition.

Tisserat (1979) stated that addition of vitamins namely thiamine- HCl and I-inositol to the nutrient medium facilitated the regeneration of date palm *in vitro*.

Thanh-Tuyen and De Guzman (1983) added vitamins formulated by Bourgin and Nitsch and myo-inositol (100 mgL^{-1}) to the salt solution to make the basal medium. Thiamine (B1), Nicotinic acid (niacin), pyridoxine (B6), biotin, pantothenic acid, riboflavin, folic acid, choline chloride, and para amino benzoic acid are the commonly used vitamins. Myo-inositol is used both as carbon source and as vitamin.

2.6.2.3Growth Regulators

These natural hormones and synthetic compounds supplemented exogenously are the key substances controlling plant growth and development of explant *in vitro.* Positively acting combinations and concentration of PGRs were usually used reliably with small modifications in tissue culture media. Among the five major groups of plant growth regulators, the ratio of auxins to cytokinins is critical in the regulation of *in vitro* morphogenesis. Somatic embryogenesis in coconut is commonly induced by a steady reduction of auxin concentration in the culture media and addition of cytokinin. Shoots and roots were produced from coconut inflorescence explant in response to high cytokinin and high auxin level respectively (Eeuwens and Blake, 1977).

Branton and Blake (1978) reported the production of plantlets from immature rachillae explant by somatic embryogenesis of nodular callus by

decreasing 2, 4-D concentration in Y3 medium followed by addition of Zip and BAP and activated charcoal.

Verdeil *et al*. (1994) stated that 2,4-D concentration significantly influenced the percentage of explants bearing callus. Alternatively auxin, 2, 4, 5-T is also used which has led to the development of nodular calli on inflorescence explants (Buffard-Morel *et al.,* 1988).

Samosir *et al.* (1999) reported that abscisic acid also induce somatic embryogenesis in coconut. The use of ABA for upgraded somatic embryo formation and maturation in mature zygotic embryo-derived callus of coconut. According to Fernando and Gamage, 2000 also ABA can enhanced the production of somatic embryos.

Siny (2006) identified the synthetic auxin 2, 4- D at 15-30 mgL^{-1} as most effective for callus initiation and proliferation. Also observed callus induction from rachillae tissue in Y3 medium containing a combination of 15-30 mgL^{-1} 2, 4-D, 1 mgL^{-1} picloram, 1 mgL^{-1} IAA and 0.1 mgL^{-1} TDZ. y Aké *et al.* (2007) reported the use of GA3 benefited coconut embryos in culture, for GA3 favoured germination and conversion to plants on semi-solid medium.

Montero-Cortes *et al.* (2010) tested the effect of gibberellic acid and reported at 0.5 l M concentration doubled the number of somatic embryos per callus and germinating somatic embryos per callus.

Sandoval *et al.* (2016) tried 25 media in different combination of plant growth regulators 2, 4- D and BAP to induce somatic embryogenesis and observed medium containing no BAP and 0.65 mM 2,4-D most resourcefully induced the formation of callus with embryogenic structures, and sub-culturing these structures as explants, embryogenic callus was formed from them and multiplied.

Sáenz-Carbonell *et al.* (2016) reviewed addition of GA3 in the medium upheld the germination of somatic embryo from the embryogenic calli.

2.6.2.4 Carbon and Energy Source

The standard carbon source is sucrose or glucose. Fructose also can be used. Sucrose is the most commonly used as a source of carbon and as an osmotic regulant (Sopory, 1979) in tissue culture. However del Rosario (1976) reported that glucose was better than sucrose for callus growth. Sometimes a combination of metabolically active (sucrose, glucose) and metabolically inactive (mannose, sorbitol, mannitol) carbohydrates is used for retaining optimum osmotic potential in the medium.

Santamaria *et al*. (1999) suggested that for maintaining chlorophyll concentrations and number of chloroplasts in high, at the initial stages of coconut embryo cultures, the primary requisite is sucrose.

Fuentes *et al*. (2005) found that reducing the concentration of sucrose in the media from 90 to 22.5 g/L or 0 g/L resulted in enhanced photosynthetic response

to light and CO2. *In-vitro* grown plantlets died on transferring *ex vitro* when cultured in media completely devoid of sucrose while those grown with transitional exogenous sucrose exhibited intermediate photosynthetic response, fast growth and high survival rate along with *ex vitro* photosynthesis signifying field establishment and growth of coconut plantlets cultured *in vitro* is influenced by the *in vitro* photosynthesis and exogenous sucrose reserves.

2.6.2.5 Supporting Agents

Growth and morphogenesis of plant tissue cultures can be enriched by adding some undefined supplements (George *et al*., 2007).

Abraham and Thomas (1962) confirmed that unautoclaved coconut milk from young fruit stimulated coconut germination. Studies conducted by Al-Khayri (2010) on date palm, revealed enhanced callus growth and somatic embryogenesis in response to use of coconut water as an additive in culturing shoot tip explant.

Zaid and Tisserat (1983b) suggested the application of an abnormally high concentration of auxin as inclusion of activated charcoal in the media reduces the availability of hormonal substance.

Protein digests like casein hydrolysate is used as a non-specific source of amino acids. In higher concentrations they can influence the osmolarity of the culture medium. Casein hydrolysate and L- Glutamine stimulated growth of callus from inflorescence when added as a supplement in the media. So as to

achieve efficacious callus induction in coconut explants, it is crucial to define the most appropriate combination of 2, 4-D and activated charcoal (Perera *et al*., 2007). Activated charcoal can promote somatic embryogenesis. Contrary to the advantageous effect of activated charcoal like adsorption of phenols and other growth inhibitory substances, they might be a reason for an undefined condition in the culture medium by adsorbing 2, 4-D (Ebert and Taylor, 1990; Verdeil and Buffard-Morel, 1995).

Culture growth was improved by addition of coconut water (Blake and Eeuwens, 1982) as it contain sugars, amino acids, vitamins, plant growth substances and other advantageous growth factors but Gamborg and shuluk (1981) discouraged their use, for the role and composition of natural complexes is highly variable and not always well defined and use in culture medium can interfere with the reproducibility of results.

2.6.2.6 pH of the Medium

pH of the culture medium is vital in ensuring the availability of nutrients to cultures. Street, 1966 reported low pH influence the action of ammonia in the media, that it may either uptake essential inorganic element or boost the outflow of the essential nitrogenous metabolites.

In coconut the pH of the culture medium was adjusted to 5.7-5.8 with 0.1 N NaOH or HCl (Eeuwens, 1978; Braton and Blake, 1983; Karunaratne and Periyapperuma, 1989).

According to Hornung and Verdeil (1999) though pH of the media can be adjusted between 4.5 and 5.8, low browning rate was obtained when adjusted to 4.5.

2.6.3 Environment Factors: Culture Conditions

2.6.3.1 Light

In tissue culture, light is mandatory for synthesis of green pigment (chlorophyll) and development of organs. According to Murashige (1974) the intensity, quality and duration of light stimulate the growth of cultures *in vitro.*

Eeuwens (1976) suggested transferring the coconut cultures to an incubator (31 ± 1 ^{O}C) and retaining all through the culture period in the dark. . Mangesh (1988) reported that light is not required for callus initiation, explant produce calli when incubated at 20 °C in dark.

The coconut cultures should be kept in darkness inside an incubator controlled at 28 ± 2°C until well-developed calluses were obtained (Sugimura and Salvana, 1989).

According to Buffard-Morel *et al*. (1992) and Verdeil *et al*. (1994) culture conditions devoid of light enhance callus initiation and development; hence the cultures were placed in dark at 27°C ± 1°C.

Samosir *et al.* (1998) reported necrosis of explant was more when placed under an illuminated condition than those incubated in the dark concluding

breakdown of components in the media and inhibition of key metabolic process as the reason. Conversely they also discovered that for the further growth and development of callus light is not a limiting factor and for germination light was required.

According to Fernando *et al.* (2010) cultures were incubated at 30 ± 1°C in the dark for two months and progressively exposing to light of 16 h photoperiod for explant initiation and maturation respectively.

2.6.3.2 Temperature

Temperature requirement varies from 20 – 30 °C with respect to species of plants. The optimum temperature for *in vitro* propagation of coconut is reported to be 30^{o}C (Eeuwens, 1976; Braton and Blake, 1983; Sugimura and Salvana, 1989; Fernando *et al.*, 2010)

Gupta (1984) suggested incubating coconut cultures at 27°C for 16 hours under light and at 25^{o}Cin dark for eight hours.

2.6.3.3 Humidity

Humidity is rarely a problem. According to Yeoman (1986) dehumidifier can be used in the culture room in climate with high humidity.

2.7 PROBLEMS ENCOUNTERED DURING SOMATIC EMBRYOGENESIS OF COCONUT

Oropeza, 2005 reviewed that coconut regeneration by somatic embryogenesis from inflorescence explants come across some technical hitches.

2.7.1 Intensive Tissue Browning

During the course of development of *in vitro*, the plant tissues in addition to the depletion of nutrients in the media, also release some growth inhibitory substance into the culture media. The foremost difficulty in coconut tissue culture has been the browning of tissue and its subsequent death. When the tissues are injured while preparing explants, polyphenol oxidases come in contact with phenolic compounds and oxidize polyphenols to form ortho quinones, killing the explant and darkening the medium. To offset this problem, the antioxidant used is activated charcoal (AC). But this will also adsorb the essential auxins and kinetins (2, 4-D and Benzyl Amino Purine) from the culture media after five days of its preparation (Ebert *et al.*, 1993).

According to Zaid (1987) problem of tissue browning in datepalm was rectified by the pre-soaking of explant in an antioxidant solution of 150 mgL^{-1} of citric acid and 100 mgL^{-1} of ascorbic acid.

Zaid and Tisserat (1983b) found that browning could be minimised without the addition of AC by employing small explant and re-culturing them to fresh media after a specific period of inoculation.

Areza *et al*. (1993) suggested that browning of tissues can be reduced by soaking the inflorescence tissue in antioxidants viz., citric acid (50 mgL^{-1}) and ascorbic acid (100 mgL^{-1}), prior to slicing and then culturing the tissues in Y3 medium supplemented with activated charcoal.

2.7.2 Slow *in vitro* Response

Coconut palm is extremely recalcitrant to regenerate *in vitro*. Various hypotheses have been set forth concerning the low reactivity of coconut tissue *in vitro*. Eeuwens (1976) tried to improve callus induction and maturation by manipulating the mineral composition of the MS basal media for coconut tissue culture.

Jesty and Francis (1992) observed a decrease in the nucleocytoplasmic ratio because of disproportionate increase in nucleus size corresponding to rapid increase in cell volume and the recalcitrance of coconut tissues might be due to this cell imbalance.

2.7.3 Low Rate of Somatic Embryogenesis

Embryogenic potentiality of coconut calli are low, usually after six to eight months after culture initiation; furthermore, embryogenesis is often partly expressed, either producing an incomplete or fused embryos embryos with a highly developed cotyledonary part, with or without a root (Branton and Blake, 1983; Blake, 1990). Hunault, 1979 reported shoots were never formed on such

formations; however, the rooting potential is substantial, as with many monocotyledons.

2.7.4 Variation in Tissue Response due to Heterogeneity of Explant

Verdeil and Buffard-Morel (1995) reviewed substantial heterogeneity in tissue reaction as another difficulty uncounted during coconut tissue culture. Ebert and Taylor (1990) reported the varying performance of tissues in callogenesis and the non-repetitiveness of results could be attributed to charcoal under certain medium preparation conditions and utilization. So activated charcoal complements the variability in cultures along with the explant.

2.7 MOLECULAR ASPECTS OF SOMATIC EMBRYOGENESIS IN COCONUT

Coconut being a recalcitrant crop, a different approach to increase coconut micropropagation efficiency, is to improve the embryogenic capacity of coconut tissues by inserting genes related to this capacity. Proteome and transcriptome analysis has led to the identification of certain genes that have been isolated from Arabidopsis such as *BAYBY BOOM* (Boutilier *et al*., 2002) and *LEC1* (Stone *et al.,* 2008) encoding transcription factors involved in the conversion from vegetative to embryonic growth. Such genes are attractive and are promising tools for improving somatic embryogenesis and clonal propagation in coconut.

Among the different gene involved in regulation of somatic embryogenesis, identified till date, *SERK* gene (Schmidt *et al*., 1997) is well studied and

demonstrated to play key role in somatic embryogenesis. *SERK* gene has been known to be a marker for embryogenic competence. *SERK*-like gene in *Cocos nucifera* was sequenced and referred to as *CnSERK*.

Expression analysis by Perez-Nunez *et al*. (2009) demonstrated that its expression could be identified sooner than embryo formation could be observed. Oppositely, low or no expression of the gene was detected in nonembryogenic tissues, indicating the association of *CnSERK* expression and the somatic embryogenesis, on this basis *CnSERK* could potentially be an appropriate marker for competent cells in the formation of somatic embryos from coconut tissues cultured *in vitro*.

The *CDKA* (Cyclin-Dependent Kinases A) gene is associated with plant development from the differentiated tissues as the gene expression enhances cell proliferation and preserves the cell division competence (Martinez *et al.*, 1992; Hemerly *et al*., 1993). Montero *et al*. (2010) conducted a study isolating *CDKA* gene from *Cocos nucifera* L. and a comprehensive expression analysis was performed during somatic embryogenesis and observed a steady increase of the putative *CnCDKA* gene expression during the embryogenic callus formation phase after embryogenic competence is attained. A decrease in the expression of *CnCDKA* was detected when analysing different stages of somatic embryo formation; the lowest level of expression being in germinated somatic embryos.

Rajesh *et al.* (2015) conducted a homology modelling and docking studies in coconut to study molecular interaction of *SERK* gene with plant growth

regulators to establish a basis for its biological role and interaction properties and identified *SERK* to play a key role in somatic embryogenesis by promoting the transition of single somatic cell to an embryogenic stage.

Oropeza *et al*. (2016) demonstrated that with the formation of translucid and embryogenic structures in the culture, there is an elevated expression of *CnSERK* gene (ortholog of *SERK* gene). Rajesh *et al*. (2015) studied the interaction of plant growth regulators with *CnSERK* gene. Furthermore studies on cloning and characterizing and gene-expression of genes involved during somatic embryo formation such as *SERK, MAP* kinase and *BABYBOOM* are, have been in progress at CPCRI (Karun and Rajesh, 2010).

Experiments conducted by Bhavyashree *et al*. (2016) revealed the enhanced expression of *PKL, SERK* and *WUS* in embryogenic calli compared to non-embryogenic calli. Normal somatic embryos showed an elevated expression of *GLP, ECP* and *GST* paralleled to abnormal somatic embryos. Similarly expression of *ECP, LEAFY, GLP* and *WRKY* could be observed in normal meristemoids compared to aberrant meristemoids.

Bandupriya *et aI*. (2016) isolated *AINTEGUMENTA-LiK*E(ANT-like) gene from coconut genome which shows higher conserved domain sequence similarities with *BABYBOOM* gene which has been previously shown a relationship with embryogenesis of brassica (Boutilier *et al*., 2002).

Rivera-Solís *et al.* (2018) conducted a study on the effect of calcium ionophore A23187 to promote somatic embryogenesis and reported that at 1 µM

ionophore there were significant increases in the formation of embryogenic structures per embryogenic callus and also the ionophore treatment promoted a change of pattern of the expression of the *CnSERK* gene during embryogenic callus formation and propose that this coupled effect could potentially increase the efficiency of the formation of somatic embryos and plantlets of coconut.

Montero-Cortes *et al*. (2010) could obtain the complete sequences of two *KNOX*-like genes for coconut *CnKNOX1* and *CnKNOX2* and detected the expression of *CnKNOX1* gene embryogenesis process except in somatic embryos at the pro-globular stage whereas no gene expression occurred in calli with aberrant embryos. *CnKNOX2* expression occurred at all stages peaking at the globular stage, but gibberellic acid treatment decreased the expression.

3. MATERIALS AND METHODS

Studies were conducted at College of Agriculture , Padanakkad during 2017 - 2019 to assess the *in vitro* response of immature inflorescence of coconut explants for induction and germination of somatic embryos by supplementing the medium with by-product of neera processing and neera (Coconut Inflorescence Sap- CIS).

Materials and methods used in the present study on somatic embryogenesis from immature inflorescence of coconut (*Cocos nucifera* L.) are presented here.

3.1. MATERIALS

3.1.1 Explant

Immature inflorescences were collected from the crown region of coconut palms of above 20 yrs from the axils of top four unopened fronts. Inflorescence were separated out using sterile blades and kept in refrigerator till the inoculation by wrapping with plastic bag. The length of the explants varied and hence was classified into four classes ranging from 1-5 cm, 5-10 cm, 10-15 cm and 15-20 cm in length (Plate. 1).

3.1.2 Chemicals

All the media components viz; major and minor nutrients, vitamins, plant growth regulators, activated charcoal and sucrose used for media preparation were

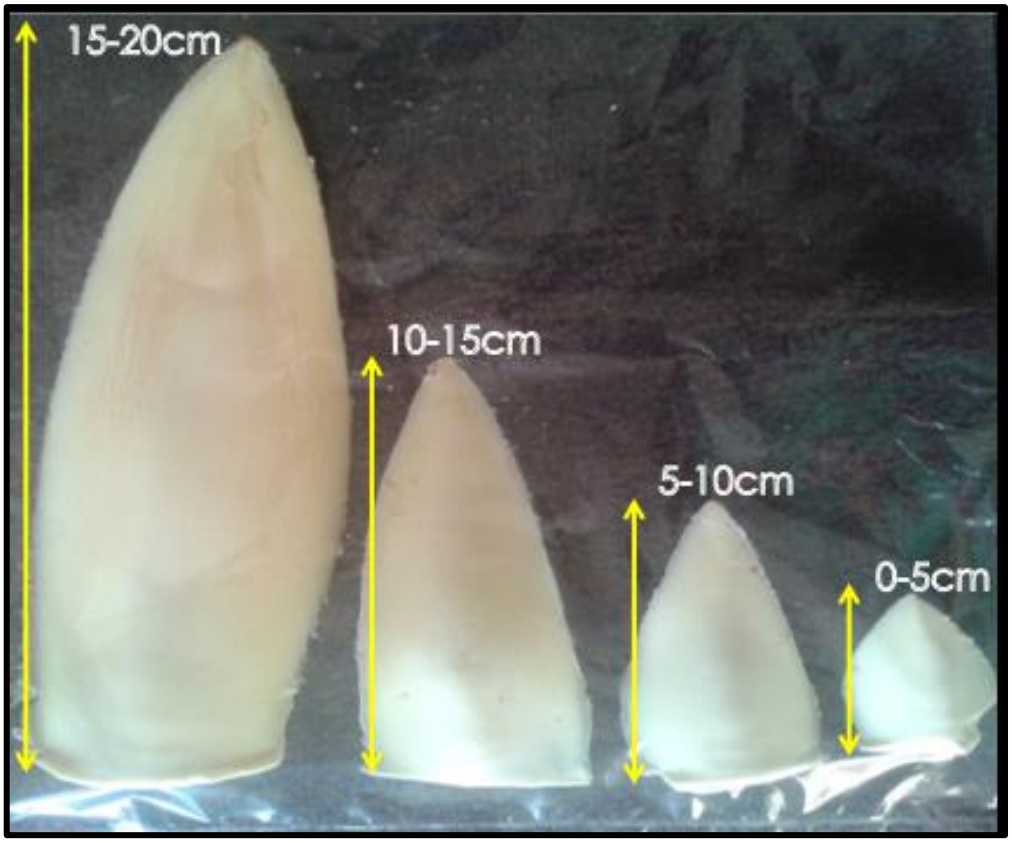

Plate 1. Different developmental stages of immature inflorescence explant

of tissue culture analytical grade purity and procured from Himedia laboratories Pvt. Ltd. The gelling agent used was clerigel from Himedia laboratories Pvt. Ltd.

3.1.3. Glass wares

The borosilicate and reusable large sized test tubes and jam bottles with polypropylene lids were used for tissue culture of immature inflorescence of coconut.

3.1.4 Equipment

Various equipments available in the laboratory viz., refrigerator, electric oven, autoclave, distillation assembly, analytical electronic balance, pH meter, laminar air flow chamber *etc*. were used.

3.2. METHODS

3.2.1 Preparation and Sterilization of Glass wares

Glass ware used for *in vitro* culture were first washed in soap solution followed by thorough washing in tap water and dried in hot air oven at 60^{o}C. Dried glass wares were autoclaved at 121^{o}C for 15 minutes and were stored away from any contaminants.

3.2.2 Preparation of Stock Solution and Culture Media

3.2.2.1 Media

Culture media used in the present study were as detailed below

For culture initiation - standardized CM2 media (Y3 basal media containing a combination of 0.5 mg L^{-1} picloram, 1 mg L^{-1} NAA and 0.1 mg L^{-1} TDZ and 300-600 μM 2,4-D].

For sub-culturing - Ten media combinations supplemented with neera and by-product of neera processing was tested.

For Germination - Y3 + 0.5 mg L^{-1} picloram + 1 mg L^{-1} NAA + 0.1 mg L^{-1} TDZ supplemented with GA (1-2 mg L^{-l}) and BA (10-15mg $L^{-1)}$.

3.2.2.2 Composition of Culture Media

The inorganic components of the media were grouped into four categories and the organic constituents into fifth category as given in the Table.1. Plant growth regulators, casein hydrosylate, L- glutamine, myo-inositol, sucrose, activated charcoal and clerigel were separately added in the medium.

3. 2.2.3 Preparation of Stock Solutions for Basal Medium

Stock solution of macronutrients, micro nutrients and vitamins were separately prepared by dissolving the individual components in sterile water. Major elements were prepared as three stocks.

Stock I consist of NH_4Cl, KNO_3, $NaH_2PO_4.2H_20$, KCl, and $MgSO_4.7H_2O$. Required quantities were separately weighed using an analytical electronic balance and dissolved individually and made up with sterile water using volumetric flask.

Due to the chance of precipitation, $CaCl_2.2H_2O$ was separately prepared as stock II.

Stock III was prepared using hot sterile water. Constituent of the stock, $FeSO_4.7H_2O$ and Na_2EDTA are separately dissolved in hot water. $FeSO_4.7H_2O$ solution is added to Na_2EDTA solution and the volume was made up. Being highly photo-oxidative, the stock was stored wrapping with Aluminium foil or in amber coloured bottles.

Stock IV and stock V are of micro nutrients and vitamins respectively. Elements as in Table 1 were dissolved in sterile water and made up using volumetric flask.

All the stock solution were stored under refrigerated condition at 4 ^oC and brought back to the ambient condition before media preparation.

3. 2.2.3 Preparation of Growth Regulators

Auxins (picloram, NAA and 2, 4-D), cytokinins (TDZ, BA) and gibberellic acid were used as growth regulators in the media. Stock solution of picloram was prepared by dissolving in DMSO and later made up the volume with sterile water.

NAA and TDZ were dissolved using drops of 1N KOH and 1N NaOH respectively and made up with sterile water. 2, 4-D and BA were prepared by first dissolving in 1N NaOH and later made up the volume with sterile water. Stock solution of gibberellic acid was prepared by initially dissolving in ethanol and then the volume was made up using sterile water. Stocks were stored under refrigerated condition (4 ^{o}C).

3. 2.2.4 Preparation of Culture Medium

Beaker was thoroughly washed and rinsed with distilled water and used for the preparation of media. Aliquots from all stock solution of major, minor, vitamins and organic compounds were pipetted into the beaker. To this 0.5 mgL^{-1} picloram, 1 mgL^{-1} NAA and 0.1 mgL^{-1} TDZ and 300-600 μM 2,4-D from the prepared plant growth regulator stock and 0.1 g/L of myo-inositol, along with 4 per cent sucrose were added fresh and made up using distilled water. The pH of the media were corrected to 5.8 using 1N NaOH or 1N HCl before the addition of gelling agent (clerigel 2 gL^{-1}) and activated charcoal (0.3 %)

The media were heated by continuously stirring after the addition of clerigel and charcoal to homogenize the same and were poured to jam bottles and test tubes. All the culture media were autoclaved at 121 ^{0}C at 15 psi for 15 minutes. The media were allowed to cool to room temperature and stored in culture room until used.

Table 1. Composition of the basal Y3 medium used for culturing coconut inflorescence

Stock Solution	Reagents	Amount(mg/l)
Macro Nutrients		
I	NH_4Cl	535
	KNO_3	2020
	$NaH_2PO_4.2H_2O$	312
	KCl	1492
	$MgSO_4.7H_2O$	247
II	$CaCl_2.2H_2O$	294
Micro Nutrients		
III	$FeSO_4.7H_2O$	13.9
	Na_2EDTA	37.3
IV	KI	8.3
	H_3BO_3	3.1
	$MnSO_4.4H_2O$	11.2
	$ZnSO_4.7H_2O$	7.2
	$CuSO_4.5H_2O$	0.16
	$CoCl_2.6H_2O$	0.24
	$NiCl_2.6H_2O$	0.024
Vitamin		
V	Nicotinic acid	1.0
	Pyridoxine HCl	1.0
	Thiamine HCl	1.0
	Glycine	1.0
	Calcium-d pantothenate	1.0
	Biotin	1.0

3.2.3 Explant Preparation

Immature inflorescence were surface sterilized by swabbing with pure alcohol or by dipping in pure alcohol for one minute under the aseptic condition of laminar air flow chamber, followed by flame sterilization for 30- 60 seconds depending on the stage of explant. The rachillae were dissected out of the inflorescences and were sliced to 0.3-0.5 mm thickness for inoculation.

3.2.4 Analysis for Chemical Composition of the Supplement

By- product of neera processing was used as a supplement in the culture media. This semi- solid substance was chemically analysed using the standard procedures as given in Table 2.

3.3 PROCEDURE FOR CULTURE ESTABLISHMENT

3.3.1 Sterilization and Inoculation

Laminar air flow was set with previously autoclaved plates, forceps, blades and media bottles or test tubes by switching on the UV for 30min to ensure complete disinfection before commencement of inoculation. Thoroughly washed and dried hands were wiped with 70 per cent alcohol before inoculation. Surface sterilization of the hood using 70 per cent alcohol and flame sterilization of all the plates, forceps and blades using pure alcohol was also done later.

During the course of inoculation the surgical instruments used where frequently flamed by dipping in pure alcohol and cooled before use.

Table 2. Chemical parameters and methodologies used for analysis of media supplement in tissue culture of coconut

Sl. No	**Parameters**	**Method**	**Reference**
1	pH	pH meter	Jackson (1958)
2	EC	Conductivity meter	Jackson (1958)
3	Organic carbon	Chromic acid wet digestion method	Walkley and Black (1934)
4	Available N	Alkaline permanganate method	Subbiah and Asija (1956)
5	Available P	Bray extraction and photoelectric colorimetry	Jackson (1958)
6	Available K	Flame photometry	Pratt (1965)
7	Available S	Flame photometry	Pratt (1965)
8	Available Na	Flame photometry	Pratt (1965)
9	Available Fe	Atomic absorption spectroscopy	Sims and Johnson (1991)
10	Available Cu	Atomic absorption spectroscopy	Emmel *et al.* (1977)
11	Available Zn	Atomic absorption spectroscopy	Emmel *et al.* (1977)

3.3.2 Callus Initiation

a. Initiation of cultures from immature inflorescence of coconut using standardized medium

- Immature inflorescence after wiping with pure alcohol was flame sterilized.
- The rachillae were separated out of the inflorescence using sterile blade and sliced to 0.3-0.5 mm pieces.
- Four different stages of explant were inoculated directly in the standardized CM_2 medium, ranging from 1-15 cm length. After removing the extreme ends of the inflorescence, both the tip (i_t) and base (i_b) were separately inoculated (Plate 2). The different stages of explant were as follows:
    - i_1 (15-20 cm length)
    - i_2 (10-15 cm length)
    - i_3 (5-10 cm length)
    - i_4 (1-5 cm length)
- The bottles / test tubes were tightly sealed and were kept in complete darkness at 26 ± 2 °C.

3.3.2.1 Observation Recorded

1. Presence /absence of calloid

2. Consistency of calloid (Embryogenic /organogenic)

3. Kind of calloid produced

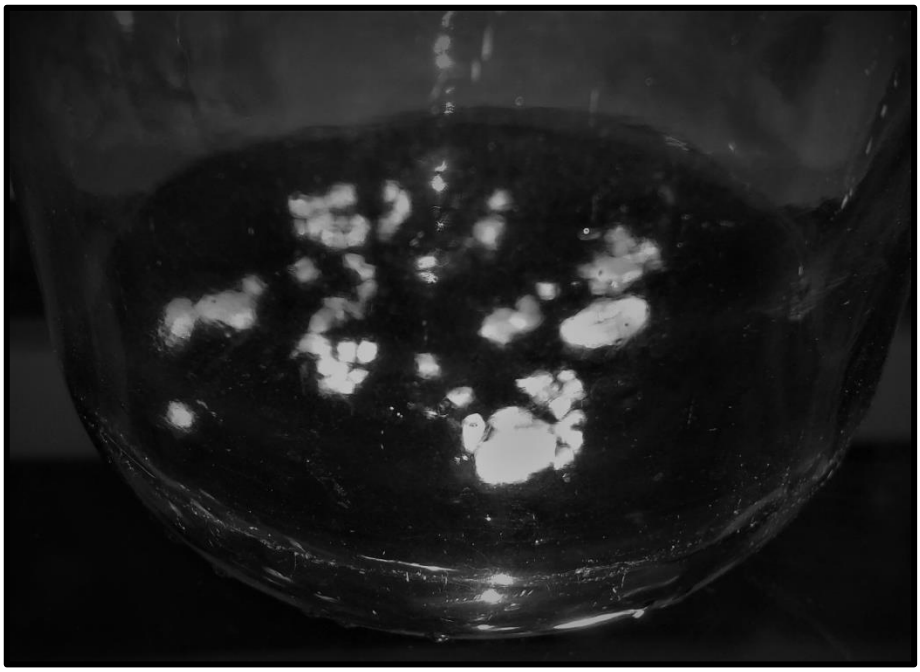

Plate 2a. Inoculation of tip region of immature inflorescence

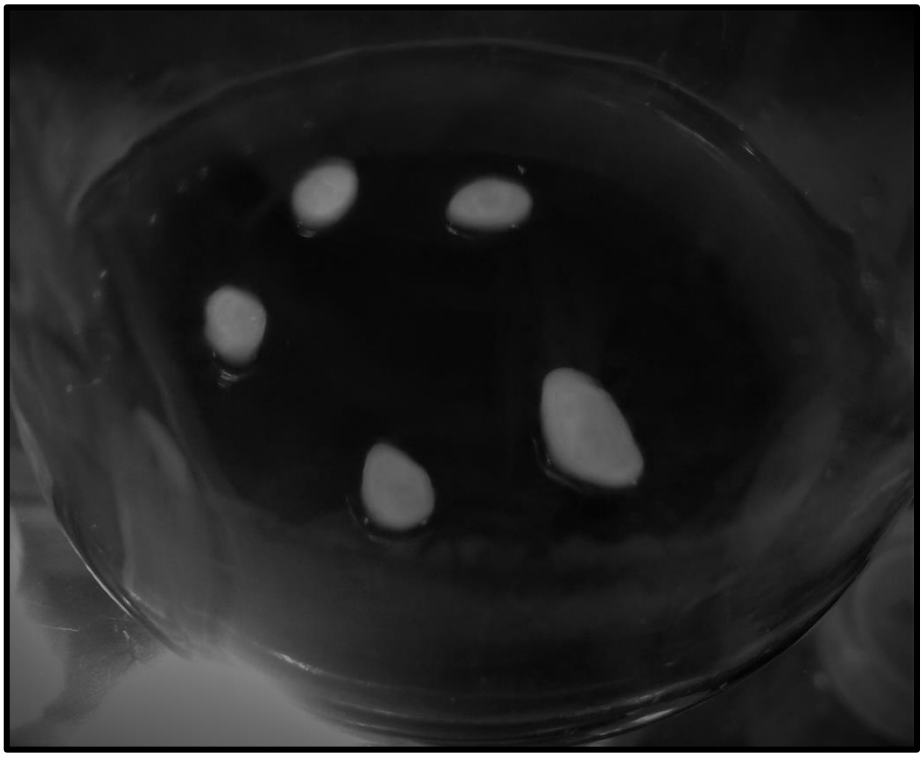

Plate 2b. Inoculation of base region of immature inflorescence

3.3.3 Somatic Embryo Formation

(b) Sub culturing in CM2 by varying the amount of AC, sucrose and different amounts of the 'supplement' and different amounts of neera

- Calloid consisting of pearly white structures and spongy tissues were formed by 90 days.
- These calloids were kept in the same medium for another 60 days, ultimately forming embryogenic calloid.
- Subcultured 10 cultures of embryogenic calloid each into different media composition for maturation of somatic embryos as detailed below.
  - Control: CM2 +0.3 % AC + 4 % Sucrose
  - T1: control +1 % supplement
  - T2: control + 2 % supplement
  - T3: control + 3 % supplement
  - T4: CM2+0.2 % AC+4% sucrose+1 % supplement
  - T5: CM2+0.1 % AC+4% sucrose+2 % supplement
  - T6: CM2+ 4 % sucrose +3 % supplement
  - T7: control + 5 ml / litre CIS
  - T8: control + 10 ml/ litre CIS
  - T9: control + 15 ml/litre CIS

Supplement used in treatments 1 to 6 was the semi-solid compound obtained after neera processing and for treatments 7 to 9, Coconut Inflorescence Sap (CIS) which is the unprocessed neera as the additives.

Table 3. Composition of Coconut Inflorescence Sap (CIS) used in the media for culturing coconut inflorescence (Barh and Mazumdar, 2008)

Sl no:	Parameters	Value(per 100ml sap)
1	pH	7.40
2	Total sugar (g)	9.30
3	Total protein (mg)	13.30
4	Total lipid (g)	0.03
5	Calcium (mg)	1.62
6	Magnesium (mg)	2.15
7	Iron (mg)	1.20
8	Sodium (mg)	6.95
9	Potassium (mg)	3.16
10	Copper (mg)	0.03
11	Zinc (mg)	0.03
12	Phosphorus (mg)	1.55
13	Niacin (mg)	0.02
14	Thiamine (mg)	0.02
15	Riboflavin (mg)	0.03
16	Ascorbic acid (mg)	2.93
17	Vitamin A (IU)	43.0

The nine treatments and control for the media as detailed above with four stages of explant (based on length), each stage with two types (tip or base) resulted in eighty combinations in the inoculated cultures (Table 3).

Table 4. Different combinations of media, developmental stage and type of explant used tissue culture of coconut

Sl. No:	Treatment combinations
1	i_{1t} + 0.3 % AC
2	i_{1t} + 0.3 % AC + 1 % supplement
3	i_{1t} + 0.3 % AC + 2 % supplement
4	i_{1t} + 0.3 % AC + 3 % supplement
5	i_{1t} + 0.2 % AC + 1 % supplement
6	i_{1t} + 0.1 % AC + 2 % supplement
7	i_1 + 3 % supplement
8	i_{1t} + 0.3 % AC + 5 mlL^{-1} CIS
9	i_{1t} + 0.3 % AC + 10 mlL^{-1} CIS
10	i_{1t} + 0.3 % AC + 15 mlL^{-1} CIS
11	i_{1b} + 0.3 % AC
12	i_{1b} + 0.3 % AC + 1 % supplement
13	i_{1b} + 0.3 % AC + 2 % supplement
14	i_{1b} + 0.3 % AC + 3 % supplement
15	i_{1b} + 0.2 % AC + 1 % supplement
16	i_{1b} + 0.1 % AC + 2 % supplement
17	i_{1b} + 3 % supplement
18	i_{1b} + 0.3 % AC + 5 mlL^{-1} CIS
19	i_{1b} + 0.3 % AC + 10 mlL^{-1} CIS
20	i_{1b} + 0.3 % AC + 15 mlL^{-1} CIS
21	i_{2t} + 0.3 % AC
22	i_{2t} + 0.3 % AC + 1 % supplement
23	i_{2t} + 0.3 % AC + 2 % supplement
24	i_{2t} + 0.3 % AC + 3 % supplement
25	i_{2t} + 0.2 % AC + 1 % supplement
26	i_{2t} + 0.1 % AC + 2 % supplement

27	i_{2t} + 3 % supplement
28	$i_{2\,t}$+ 0.3 % AC + 5 mL^{-1} CIS
29	i_{2t} + 0.3 % AC + 10 mL^{-1} CIS
30	i_{2t} + 0.3 % AC + 15 mL^{-1} CIS
31	i_{2b} + 0.3 % AC
32	i_{2b} + 0.3 % AC + 1 % supplement
33	i_{2b} + 0.3 % AC + 2 % supplement
34	i_{2b} + 0.3 % AC + 3 % supplement
35	i_{2b} + 0.2 % AC + 1 % supplement
36	i_{2b} + 0.1 % AC + 2 % supplement
37	i_{2b} + 3 % supplement
38	i_{2b} + 0.3 % AC + 5 mL^{-1} CIS
39	i_{2b} + 0.3 % AC + 10 mL^{-1} CIS
40	i_{2b} + 0.3 % AC + 15 mL^{-1} CIS
41	i_{3t} + 0.3 % AC
42	i_{3t} + 0.3 % AC + 1 % supplement
43	i_{3t} + 0.3 % AC + 2 % supplement
44	i_{3t} + 0.3 % AC + 3 % supplement
45	i_{3t} + 0.2 % AC + 1 % supplement
46	i_{3t} + 0.1 % AC + 2 % supplement
47	i_{3t} + 3 % supplement
48	i_{3t} + 0.3 % AC + 5 mL^{-1} CIS
49	i_{3t} + 0.3 % AC + 10 mL^{-1} CIS
50	i_{3t} + 0.3 % AC + 15 mL^{-1} CIS
51	i_{3b} + 0.3 % AC
55	i_{3b} + 0.3 % AC + 1 % supplement
53	i_{3b} + 0.3 % AC + 2 % supplement
53	i_{3b} + 0.3 % AC + 3 % supplement
54	i_{3b} + 0.2 % AC + 1 % supplement
56	i_{3b} + 0.1 % AC + 2 % supplement

57	i_{3b} + 3 % supplement
58	i_{3b} + 0.3 % AC + 5 mlL^{-1} CIS
59	i_{3b} + 0.3 % AC + 10 mlL^{-1} CIS
60	i_{3b} + 0.3 % AC + 15 mlL^{-1} CIS
61	i_{4t} + 0.3 % AC
62	i_{4t} + 0.3 % AC + 1 % supplement
63	i_{4t} + 0.3 % AC + 2 % supplement
64	i_{4t} + 0.3 % AC + 3 % supplement
69	i_{4t} + 0.2 % AC + 1 % supplement
66	i_{4t} + 0.1 % AC + 2 % supplement
67	i_{4t} + 3 % supplement
68	i_{4t} + 0.3 % AC + 5 mlL^{-1} CIS
69	i_{4t} + 0.3 % AC + 10 mlL^{-1} CIS
70	i_{4t} + 0.3 % AC + 15 mlL^{-1} CIS
71	i_{4b} + 0.3 % AC
72	i_{4b} + 0.3 % AC + 1 % supplement
73	i_{4b} + 0.3 % AC + 2 % supplement
74	i_{4b} + 0.3 % AC + 3 % supplement
75	i_{4b} + 0.2 % AC + 1 % supplement
76	i_{4b} + 0.1 % AC + 2 % supplement
77	i_{4b} + 3 % supplement
78	i_{4b} + 0.3 % AC + 5 mlL^{-1} CIS
79	i_{4b} + 0.3 % AC + 10 mlL^{-1} CIS
80	i_{4b} + 0.3 % AC + 15 mlL^{-1} CIS

[i_{1t} (tip region of i1 stage inflorescence), i_{1b} (base region of i1 stage inflorescence), i_{2t} (tip region of i2 stage inflorescence), i_{2b} (base region of i2 stage inflorescence), i_{3t} (tip region of i3 stage inflorescence), i_{3b} (base region of i3 stage inflorescence), i_{4t} (tip region of i4 stage inflorescence), i_{4b} (base region of i4 stage inflorescence), CIS- Coconut Inflorescence Sap]

3.3.3.1 Observation Recorded

1. Presence /absence of somatic embryo
2. Stage of embryo maturation (globular / heart shaped / torpedo)
3. Main and interaction effect of media and stages and type of explant

3.3.4 Somatic Embryo Germination

(c) Sub culturing into standardised germination medium

Somatic embryos obtained from the various treatments in experiment (b) was sub cultured into standardised germination medium, Y3 + 0.5 mgL^{-1} picloram + 1 mgL^{-1} NAA + 0.1 mgL^{-1} TDZ supplemented with GA (1-2 mgL^{-1} and BA (10-15 mgL^{-1}), 0.1 gL^{-1} of myo-inositol, 0.3 per cent AC and 4 per cent sucrose and made up using distilled water. The pH of the media was corrected to 5.8 using 1N NaOH or 1N HCl before the addition of gelling agent (clerigel 2 g L^{-1}) and activated charcoal.

3.3.4.1 Observation Recorded

1. Stage of embryo maturation (globular / heart shaped / torpedo)
2. Embryo germination / organ formation

3.4. STATISTICAL ANALYSIS

Statistical interpretations of the data obtained from the above experiments were done by adopting Completely Randomised Design, Students' T- test and Factorial CRD using the software OPSTAT. Results were interpreted at 5% significance.

4. RESULT

The results of the experiment on "Somatic embryogenesis from immature inflorescence of coconut (*Cocos nucifera* L.)" conducted during 2017-2019 are presented in this chapter.

4.1 SELECTION OF EXPLANT

Due to the presence of more number of growing points in the immature inflorescence of coconut and also all the cells are in diploid stage due to the very young condition, in the present study, immature inflorescence was preferred as an explant.

4.2 FORMATION OF CALLOID

Different stages of explant were inoculated into the standardized CM2 medium for culture initiation through indirect somatic embryogenesis and were observed frequently for induction of calloid and contamination.

First the inoculated inflorescence tissues showed floral bud swelling as an indication of callogenesis after 60 days of culturing. This was followed by the initiation of calloid consisting of pearly white structures and spongy tissues by 90 days after inoculation. Calloid formation was observed untill 9 months thereafter.

Among all the explants inoculated into the standardized CM2 medium, 80.22 per cent of the explant tissues formed calloids and 10.73 per cent of the inoculated inflorescence explant does not respond to the medium (Table 5). It

was observed that the percentage of calloid formation has increased with time, 37.29 per cent, 65.54 per cent and 80.22 per cent in three month, six month and nine month respectively.

4.2.1 Influence of Developmental Stage of Explant on Calloid Induction and Browning

Immature inflorescences of four different stages, i_1 (15-20 cm), i_2 (10-15 cm), i_3 (5-10 cm) and i_4 (0-5 cm) were inoculated on standardized CM2 media. Explant stage significantly influence calloid initiation. Mean percentage calloid initiation from each explant stage is presented in the Table 6.

There were significant differences among the stages of explant in calloid formation. i_4 with 85.71 per cent calloid initiation was significantly superior to all other stages of inflorescence explant. Data revealed that the percentage calloid initiation decreased with inflorescence stages, i_3, i_2 and i_1 as 82 per cent, 78.57 per cent and 74.42 per cent respectively.

Browning was observed in some of the cultures after around 60 days of inoculation. The percentage response for browning at this time was least recorded in i_4 stages of explant (4.76 %) which differ significantly with i_3 (6.00 %) i_2 (11.90 %) and i_1 (13.95 %) stages (Table 6).

Explant at 1-5 cm and 5-10 cm length showed better calloid induction and minimal browning.

Table 5. Response of cultures in the inoculation medium for coconut

	No. of cultures responded	**Culture response (%)**
Total cultures inoculated	198	
Contamination	21	10.6
Net inoculated	177	89.39
Callus formed	142	80.22
Tissue browning	16	9.04
Culture not responded	19	10.73

Table 6. Rate of calloid induction and browning in explants with different stages of development

Explant portion inoculated	**Response**	
	Calloid induction (%)	**Browning (%)**
i_1	74.42	13.95
i_2	78.57	11.90
i_3	82.00	6.00
i_4	85.71	4.76
CD	**1.88**	**1.88**

4.2.2 Influence of Region of Explant (tip /base) on Culture Response and Browning

Among the different types of inflorescence explant both the tip region ($i_{1t,}$ i_{2t}, i_{3t}, i4t) and base regions (i1b, i2b, i3b, i4b) were separately inoculated. Data on response on calloid induction and browning are presented in table 7.

Both the tip and basal region of the explant showed significant difference in culture initiation media. The percentage of total cultures responding to maximum calloid induction (91.30 %) and minimal browning (0.00 %) was recorded when inoculated the tip region of inflorescence explant whereas least percentage of calloid induction (70.00 %) and maximum browning (20.00 %) was with basal region of inflorescence explant inoculated. However the i_{1t} (tip region) and i_{4b} stage of inflorescence was on par with 78.26 per cent and 78.94 per cent calloid initiation.

4.3. KINDS OF CALLOID FORMATION

Different types of calloids were observed in the cultures such as, friable smooth creamy calloid, spongy calloid and calloid with necrotic tissues (Plate 5). Smooth calloids were formed from the tip of the explant (Plate 3) and spongy calloids from the base (Plate4). The friable, smooth, creamy calloid eventually evolved into embryogenic calloids.

Plate 3. Smooth calloid formed from tip region of immature inflorescence

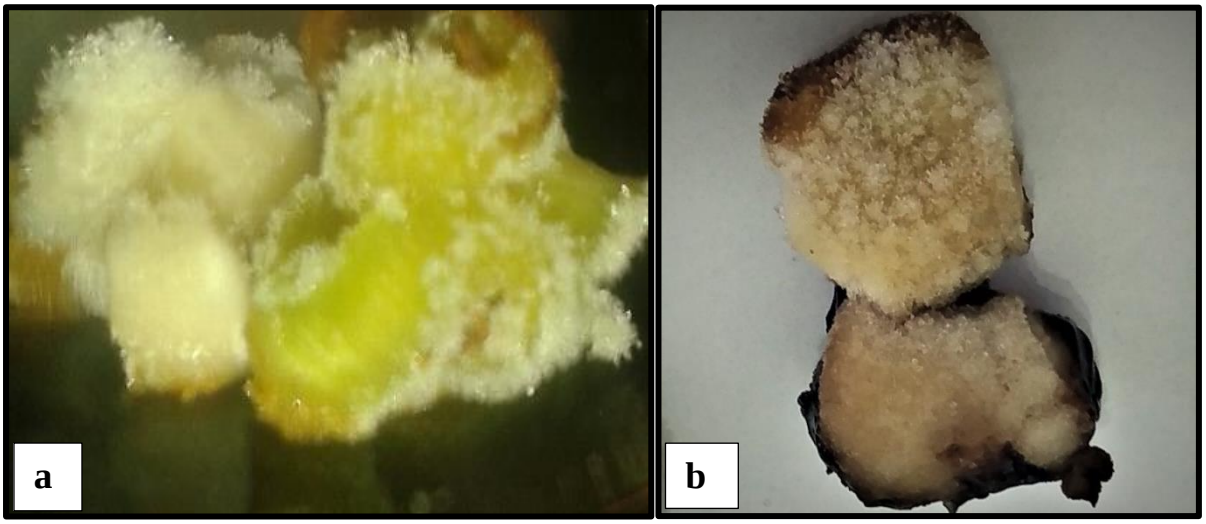

Plate 4. Spongy calloid formed from the (a) base region of inflorescence (b) individual explant

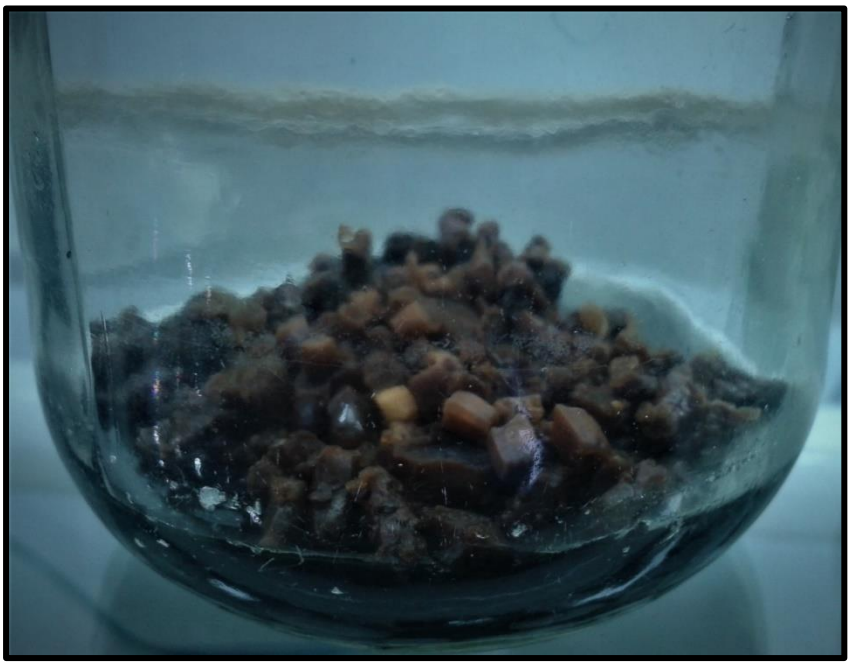

Plate 5. Formation of necrotic tissues without calloid formation

The embryogenic calloids produced in the present study were further sub-cultured in different combination of media for the further development of somatic embryos.

Table 7. Rate of calloid induction and browning in different regions of explants

Explant portion inoculated	**Response**	
	Calloid induction (%)	**Browning (%)**
i_{1t}	78.26	9.000
i_{1b}	70.00	20.000
i_{2t}	83.33	8.000
i_{2b}	72.22	17.000
i_{3t}	87.10	0.000
i_{3b}	73.68	16.000
i_{4t}	91.30	0.000
i_{4b}	78.94	11.000
CD(0.05)	**1.75**	**1.52**

4.4. NUTRITIONAL COMPOSITION OF THE SUPPLEMENTS

By-product of neera processing used as supplement in the culture media was analysed for the presence of important mineral nutrients present using standard procedures. The results are shown in Table 8.

The values were significant and the by- product was found to have a pH 5.78 with 1.16mS electrical conductivity also contain some amount of nutrients, such as organic carbon (7.24 %), available nitrogen (1.3 %), available phosphorous (0.14 %), available potassium (0.11%), available sulphur (0.16 %) and available sodium (0.56%) and available iron (0.71%). The by- product was devoid of available copper and zinc.

4.5 EFFECT OF MEDIA AND EXPLANT IN SUB- CULTURING

For somatic embryo formation, ten cultures with embryogenic calloids were sub- cultured in ten media composition. The results obtained in the sub- culturing are presented below.

4.5.1 Influence of Media on Proliferation of Calloid and Embryo Formation

Effect of altering the concentration of charcoal and additives were examined. The response of cultures with calloids in the sub- culturing media are presented in the Table 9. The effect of activated charcoal, by-product of neera as media supplement and CIS added to the media are detailed below.

4.5.1.1. Role of Activated Charcoal in Calloid Proliferation and Browning of Culture

Percentage of cultures in which there were proliferations of calloid varied significantly between the different treatments. It was highest (92.75 %) in the case of treatment, T2 with activated charcoal at a concentration of 0.3 per cent. However the lowest (9.79 per cent) value was found in the treatment T6, in which there was no activated charcoal.

When reducing the concentration of activated charcoal from 0.3 per cent to no activated charcoal in the media with simultaneous increase in the concentration of supplement showed a decrease in response of calloid proliferation respectively as T4 (86.00%), T5 (59.21%) and T6 (9.79%).

Significant differences were observed with respect to response in browning among the treatments. The highest percentage of cultures showed browning in treatment, T6 with no activated charcoal (80.08%). Treatment T6 differed significantly with all other treatments.

The percentage of cultures showing tissue browning decreases from 57.77 per cent in treatment T5 to 38.63 per cent in treatment T4 and to 15.94 per cent in treatment T3, with the increase in the concentration of activated charcoal in the media from 0.1 per cent, 0.2 per cent and 0.3 per cent respectively.

Table 8. Chemical composition and properties of neera supplement used in the culture media

Sl. No.	Parameter	Value
1	pH(1:2.5)	5.78
2	EC(dS m^{-1})	1.16
3	Organic Carbon (%)	7.24
4	Available Nitrogen (%)	1.30
5	Available Phosphorous (%)	0.74
6	Available Potassium (%)	0.11
7	Available Sulphur (%)	0.16
8	Available Sodium (%)	0.16
9	Available Iron (%)	0.71
10	Available Copper (%)	0.00
11	Available Znic (%)	0.00

4.5.1.2 Effect of Supplement in Calloid Proliferation and Browning

Supplement used in the medium was semi- solid in nature which was obtained as a by- product of neera processing. The result of the present investigation to test the suitability of the by-product from neera processing to culture the immature inflorescence of coconut is presented in the Table 9.

In the treatments control, T1, T2 and T3 the concentration of activated charcoal remained same (0.3 per cent) and the concentration of supplement varied. It was observed that treatment, T2 differed significantly with highest value in percentage of cultures with calloid proliferation (92.75 per cent) compared with other treatments whereas as T1 and T3 were on par.

In the treatments T3, T4, T5 and T6 both the activated charcoal and supplements were varied. The treatments were significantly different from each other with calloid proliferation of 84.41 per cent, 86.00 per cent, 59.21 per cent and 9.79 per cent, respectively. Among the different treatments, response in T4 (0.2 per cent AC and 1 per cent supplement) was better than other treatments and was on par with control (0.3 per cent AC).

Results showed that all the treatments differed significantly in mean percentage browning. Among the treatments with same concentration of activated charcoal (0.3 per cent) and varied concentration of supplement, *viz.*, control, T1, T2 and T3; the treatment T2 differed significantly with least value in percentage browning (11.58) than other treatments. Control (26.95 per cent), T1 (16.83 per cent) and T3 (15.94 per cent) were also significantly different from each other.

Table 9. Effect of different sub culturing media on calloid proliferation and browning in coconut

Additives	**Concentration of Charcoal**	**Response**	
		Calloid proliferation (%)	**Browning (%)**
Control	0.3% AC	86.54 (71.39)	26.95 (30.51)
T1	0.3%AC+1% Supplement	83.75 (73.02)	16.83 (18.56)
T2	0.3%AC+2% Supplement	92.75 (80.05)	11.58 (13.67)
T3	0.3%AC+3% Supplement	84.42 (72.14)	15.94 (17.87)
T4	0.2%AC+1% Supplement	86.00 (73.16)	38.63 (36.39)
T5	0.1%AC+2% Supplement	59.21 (52.26)	57.77 (49.66)
T6	3% Supplement	9.79 (12.51)	80.08 (75.10)
T7	0.3%AC+5ml/l CIS	79.63 (69.10)	24.79 (24.76)
T8	0.3%AC+10ml/l CIS	90.17 (75.09)	22.70 (24.82)
T9	0.3%AC+15ml/l CIS	81.92 67.71	26.25 (25.68)
CD(0.05)		**1.45**	**0.37**
(Arc Sin Transformation**)**			

In the treatments T3, T4, T5 and T6 with varied concentration of activated charcoal and supplements, the treatment, T6 with only 3 per cent supplement showed maximum tissue browning (80.08 per cent). All the treatments are significantly different from each other.

4.5.1.3. Effect of CIS in Calloid Proliferation and Browning

The suitability of CIS in coconut tissue culture medium as an additive was analysed. The result of the experiment was as presented in the Table 9.

The treatments T7, T8 and T9 with 5 mlL^{-1}, 10 mlL^{-1} and 15 mlL^{-1} of CIS respectively were having all other media constituents same as that of control (CM2 + 4 % sucrose + 0.3 % AC). The treatments were significantly different form each other. The treatment T8 (10 mlL^{-1} CIS) recorded the highest value of percentage of cultures with calloid proliferation (90.17 per cent) and cultures were better performing than control (86.54 per cent).

CIS significantly influenced the percentage browning of the culture. The treatment T8 recorded the least value of percentage of cultures showing browning (22.70 %). Treatments T7, T8 and T9 differed significantly with browning response of 24.79 per cent, 22.70 per cent and 26.25 per cent respectively. Treatment with CIS in the media recorded minimal response of browning than control also (26.95 %).

4.5.2 Influence of Stage of Development and Region of Explant on Calloid Proliferation and Browning

The effect of stage of development of explant on the percentage of cultures with calloid proliferation and browning is presented in the Table 10. Explant showed significant influence on calloid proliferation and browning.

The data revealed significant difference between the tip and base region of immature inflorescence in calloid proliferation on sub- culturing. The highest response of calloid proliferation was recorded in case of tip region (91.30 %) than base region of the immature inflorescence.

Among the explants with different stages of development, i_4 stage (1-5 cm) significantly differed from i_3 (5-10 cm), i_2 (10-15 cm) and i_1 (15-20 cm) stage which are on par with percentage of cultures with calloid proliferation during sub-culturing (91.30 %, 87.63 %, 83.57 % and 84.17 % respectively).

Among the different stages of base region of explant, i_{4b} stage recorded highest calloid proliferation (76.23 %) which is significantly different from other stages of base region explant. I_{1b} and i_{3b} stages are on par. Least calloid proliferation on sub- culturing was recorded from i_{1b} stage (59.10 %).

All the explant stages differ significantly from each other in browning of the cultures, with i_{3b} explant recording highest value (56.54 %) and i_4 stage the least value (5.35 %) in browning .

4.5.3 Interaction Effect of Media and Explant Type in Calloid Proliferation in Sub - culturing

Eighty treatment combinations were studied for the percentage calloid proliferation and the following data obtained are presented in Table 11.

The interaction effect of media and explant was significant**.** All the treatment combination with percentage of total cultures showing calloid proliferation above 97.52 per cent was on par. However, numerically the highest value in calloid proliferation was recorded (100 %) with combination T_2i_1, T_2i_2, T_2i_3, T_2i_4 and T_8i_1.

The combination of Treatment T6 with different stage (i_1, i_2, i_3 and i_4) and type of explant (tip and base region) recorded the least calloid proliferation (0.00%).

Table 10. Effect of different sub culturing media on calloid proliferation and browning in coconut

Explant	Response	
	Calloid proliferation (%)	**Browning (%)**
i1	84.17 (71.54)	27.41 (27.29)
i2	83.57 (70.58)	32.76 (30.24)
i3	87.63 (76.45)	19.37 (20.98)
i4	91.3 (79.6)	5.35 (13.53)
i1b	59.10 (51.05)	39.64 (37.29)
i2b	60.97 (52.53)	46.03 (43.87)
i3b	60.37 (50.90)	56.54 (50.17)
i4b	76.23 (64.29)	30.12 (30.28)
CD(0.05)	**1.30**	**0.34**
(Arc Sin Transformation)		

Table. 11. Interaction effects of media and explant region in calloid proliferation (%)

	C	T1	T2	T3	T4	T5	T6	T7	T8	T9
i1	77 (61.34)	99.67 (86.98)	100 (88.35)	80.00 (63.43)	98.67 (84.22)	87.67 (70.80)	25.00 (29.99)	98.67 (84.03)	100 (88.35)	75.00 (60.01)
i2	98.67 (84.22)	99.67 (86.98)	100 (88.35)	99.00 (84.83)	98.33 (83.42)	80.00 (63.43)	0.00 (1.06)	99.00 (84.83)	86.00 (68.03)	75.00 (60.01)
i3	99.67 (86.98)	99.67 (86.98)	100 (88.35)	99.00 (84.83)	98.67 (84.03)	50.00 (45)	33.00 (35.06)	98.67 (84.22)	99.33 (85.62)	98.33 (83.42)
i4	99.33 (85.62)	99.67 (86.98)	100 (88.35)	99.333 (85.62)	98.67 (84.03)	99.00 (84.83)	20.33 (26.81)	99.00 (84.83)	99.33 (85.62)	98.33 (83.42)
i1b	78 (62.04)	99.00 (84.83)	99.33 (85.62)	33.00 (35.06)	0.00 (1.65)	25.00 (29.99)	0.00 (1.65)	98.67 (84.03)	83.00 (65.6)	75.00 (60.01)
i2b	74.333 (59.57)	6.67 (12.84)	75.67 (60.44)	98.67 (84.03)	98.00 (82.79)	50.00 (45)	0.00 (1.65)	32.67 (34.85)	75.00 (60.01)	98.67 (84.03)
i3b	88 (69.75)	67.33 (55.14)	67.67 (55.35)	67.67 (55.35)	97.667 (82.27)	25.00 (29.99)	0.00 (1.65)	50.33 (45.20)	80.00 (63.43)	60.00 (50.76)
i4b	77.33 (61.58)	98.33 (83.42)	99.33 (85.62)	98.67 (84.03)	98.00 (82.79)	57.00 (49.02)	0.00 (1.65)	60.00 (50.76)	98.667 (84.03)	75.00 (60.01)
CD = 4.09 (Arc Sin Transformation)										

4.5.4 Interaction Effect of Media and Explant Region in Browning in Sub – culturing

Data on the interaction effect of media and explant type in browning is presented in the Table 12.

Interaction effects of media and explant significantly influenced the browning of cultures. Treatment combination with base region recorded more browning than with tip region and media T6 recorded more browning than all other treatment. Numerically highest value of 99.67 per cent browning was in combination of T_6i_2, T_6i_{1b}, T_6i_{2b}, T_6i_{3b} and T_6i_{4b}.

However no browning (0.00%) was recorded in combinations of T_1i_2, T_1i_2, T_1i_3, T_1i_4, T_2i_1, T_2i_2, T_2i_3, T_2i_4, T_2i_{4b}, T_3i_1, T_3i_4, T_3i_{1b}, T_3i_{4b}, T_4i_4, T_7i_2, T_7i_3, T_7i_{1b}, T_8i_3, T_8i_4 and T_9i_{4b}.

Table 12. Interaction effects of media and explant region in browning (%)

	C	T1	T2	T3	T4	T5	T6	T7	T8	T9
i1	31.10 (33.90)	0.00 (1.65)	0.00 (1.65)	0.00 (1.65)	20.33 (26.81)	66.99 (54.94)	75.33 (60.22)	40.33 (39.42)	14.62 (22.47)	25.33 (30.21)
i2	9.42 (17.88)	0.00 (1.65)	0.00 (1.65)	43.19 (41.08)	80.33 (63.68)	80.33 (63.68)	99.67 (86.98)	0.00 (1.65)	14.62 (22.47)	0.00 (1.65)
i3	13.66 (21.70)	0.00 (1.65)	0.00 (1.65)	16.99 (24.34)	20.33 (26.81)	50.33 45.20)	66.99 (54.93)	0.00 (1.65)	0.00 (1.65)	25.33 (30.21)
i4	12.83 (20.99)	0.00 (1.65)	0.00 (1.65)	0.00 (1.65)	0.00 (1.65)	20.33 (26.81)	60 (50.76)	20.33 (26.81)	0.00 (1.65)	0.00 (1.65)
i1b	44.78 (42)	33.67 (35.46)	33.67 (35.46)	0.00 (1.65)	66.99 (54.94)	75.33 60.22)	99.67 (86.98)	0.00 (1.65)	16.99 (24.34)	25.33 (30.21)
i2b	25.33 (30.21)	33.67 (35.46)	25.33 (30.25)	33.67 (35.46)	33.67 (35.46)	50.33 45.20)	99.67 (86.98)	66.99 (54.94)	24.67 (29.78)	66.99 (54.94)
i3b	44.778 (42)	33.67 (35.46)	33.67 (35.46)	33.67 (35.46)	66.99 (54.94)	75.33 (60.22)	99.67 (86.98)	50.33 (45.20)	60.33 (50.97)	66.99 (54.9)
i4b	33.67 (35.46)	33.67 (35.46)	0.00 (1.65)	0.00 (1.65)	20.33 (26.81)	43.19 (41.08)	99.67 (86.98)	20.33 (26.81)	50.33 (45.20)	0.00 (1.65)
CD = 1.0 (Arc Sin Transformation)										

4.6 EMBRYOGENESIS FROM CALLOID

4.6.1 Formation of Somatic Embryo in the Sub-culturing Media

Embryogenic calloids (Plate 6) after proliferation started producing the somatic embryos in the subcultured media. It was evident from the experiment that the somatic embryo formed was significantly influenced by the media and explant as well as time. The tissues turned brownish initially after formation of calloids and remain as such for nearly six months and later developed somatic embryos. All the treatment combination showed difference in response. Somatic embryos (Plate 7 & Plate 8) were produced eight to nine months after initiation.

However, though the frequency was less in some combinations viz., Ci4, T1i2, T2i2, T5i3, T6i1, T6i3, T8i1, T8i3, T9i2 and T9i4 the rate of response was higher.

4.7. SOMATIC EMBRYO GERMINATION

Somatic embryos formed on the calloid were transferred to the germination medium, Y3 + 0.5 mgL^{-1} picloram + 1 mgL^{-1} NAA + 0.1 mgL^{-1} TDZ supplemented with GA (1-2 mgL^{-1}) and BA (10-15 mg L^{-1}) (Plate 9 and Plate 10).

Since the number of somatic embryos showing germination potential that could be used for further experiment was limited the work is in progress for further maturation of the germinated somatic embryo. A prolonged growth period is required to develop the germinated somatic embryo to a plantlet.

Plate 6. Development of embryogenic structures from the calloids

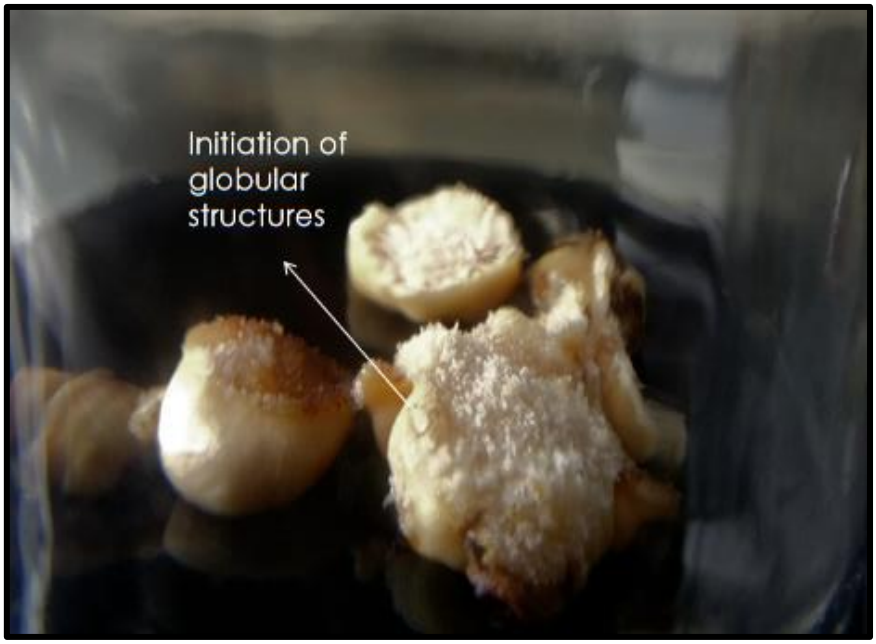

Plate 7. Initiation of globular embryo

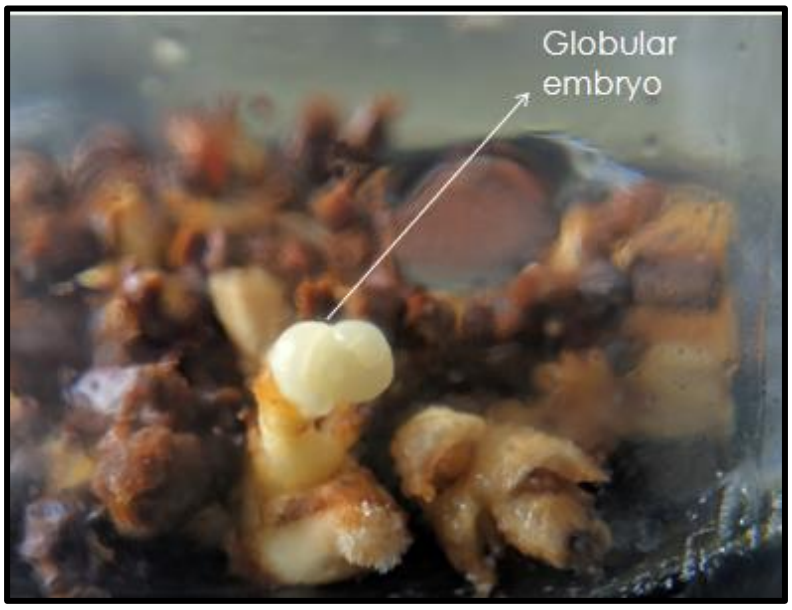

Plate 8. Development of globular stage embryo

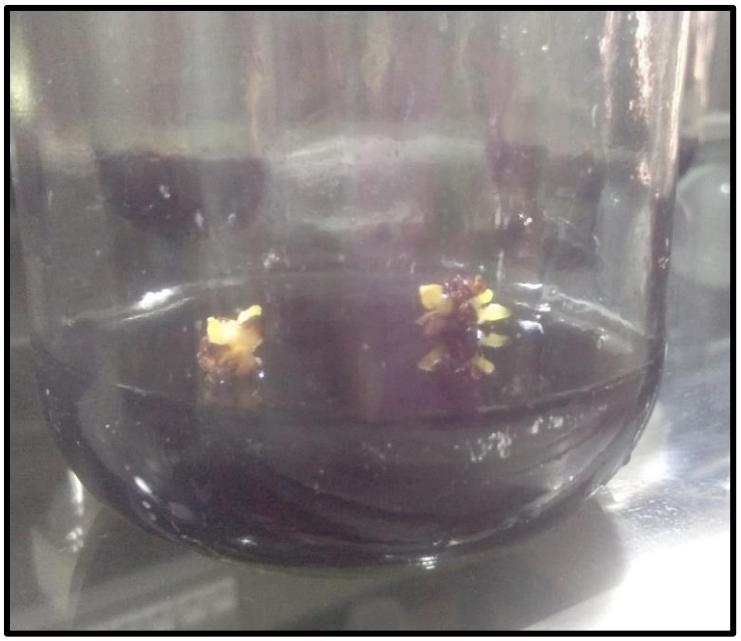

Plate 9. Initiation of germination of somatic embryo

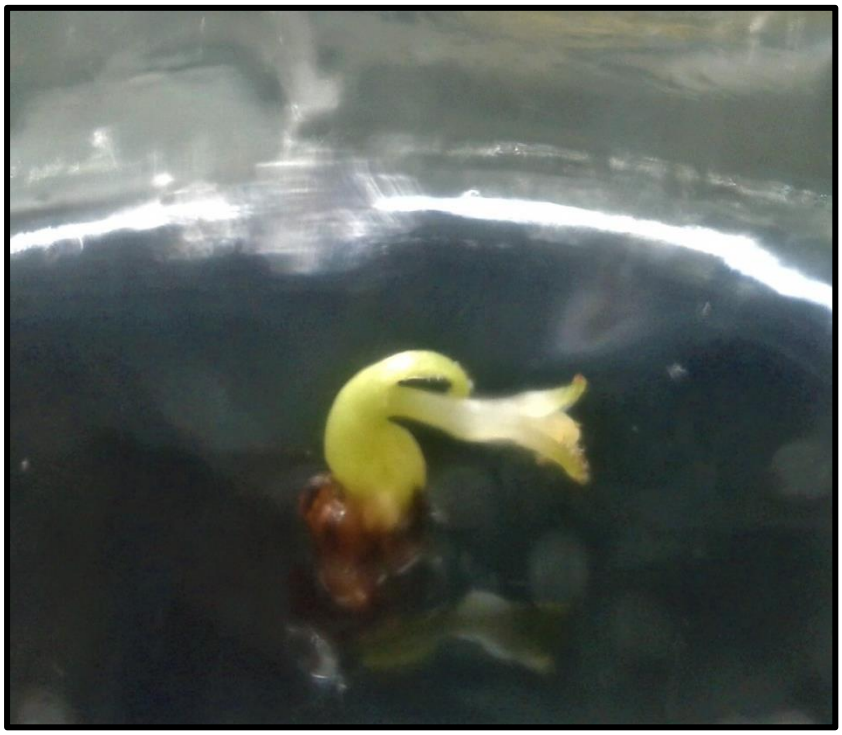

Plate 10. Development of germinated somatic embryos in the germination medium

5. DISCUSSION

Coconut is a versatile tropical plantation crop having multiple uses. Unfortunately, today the coconut industry walks forward freighted with a number of problems. Low yield, pests and diseases, increased hectares of senile or unproductive plantations, scarcity of skilled labour, low and fluctuating price of coconut etc. threats the development of coconut industry. One of the clear-cut ways to solve most of these problems is to provide coconut farmers with early bearing, high yielding, short stature, quality planting materials. But coconut is highly heterozygous and generally cross-pollinated. The features of reproduction of this perennial plant such as long pre-bearing period, great variability among progeny, long interval between generations, low multiplication rate and the absence of commercially viable vegetative propagation techniques challenges the breeding programmes. In view of these facts, vegetative propagation employing tissue culture techniques, offers a means of cloning improved planting material within a short period.

Among the various explants reported, immature inflorescence is the most suitable for micropropagation in coconut (Sandoval *et al*., 2016). Addition of some undefined supplements can improve the growth and morphogenesis of plant tissue cultures (George *et al*., 2007). The present investigation on "Somatic embryogenesis from immature inflorescence of coconut (*Cocos nucifera* L.)" was carried out during 2017-2019 at College of Agriculture, Padannakkad to assess the *in vitro* response of immature inflorescence of coconut explants for induction and germination of somatic embryos by supplementing the medium with by-product of neera processing and the Coconut Inflorescence Sap (CIS). CIS is the natural phloem sap from coconut which is a rich source of minerals and growth promoting substances, used for the production of health drink "Neera". Preliminary studies indicate that after the processing of CIS, the remaining semisolid by-product, promotes plant growth when added to potting mixture. Hence in the present study, CIS and the by-product were tried as the media

supplements for tissue culture of coconut. The results obtained under the research programme are discussed in this chapter.

5.1 CHOICE OF EXPLANT

Selection of appropriate explants is the most important factor which governs the success of regeneration of a crop *in vitro*. In the present study of somatic embryogenesis, immature inflorescence was exclusively used as an explant. Earlier reports in coconut tissue culture revealed that immature inflorescence was the most responsive to callus induction compared to other explant due to the presence of numerous meristematic points (Tisserat, 1981; Zaid and Tisserat, 1983a; Orense *et al*., 1993).

Chan *et al.* (1998) suggested plumules from zygotic embryo, the most responsive explant for formation of embryogenic calloid, production of somatic embryo and its further developent into plantlets. However Namboothiri *et al.* (2008) found propagating hybrid palms using their seeds results in segregation, so to propagate fruit bearing adult palms that are promising require rachillae from immature inflorescences.

Vidhanaarachchi and Weerakoon (1997) have shown immature inflorescence to be a promising source of explants for coconut tissue culture with reproducible formation of embryogenic callus. Investigation by Siny (2006) also revealed that among the different explants tried, immature rachillae were best for calloid induction and embryo formation.

However in the present study emphasis was placed on the effect of stage and type of immature inflorescence in calloid induction and phenolic interference.

5.2 CALLOID INDUCTION AND GROWTH

In the present investigation it was found that on a suitably defined medium and under controlled laboratory conditions, pieces of immature inflorescence proliferate into calloid (partially de-differentiated calli, as there is no true callus phase in coconut regeneration, Brackpool *et al.* (1986)). Calloid has been successfully induced from coconut tissues in many laboratories since Eeuwens (1976) successfully developed Y3 medium modifying nitrogen and micronutrient constituents of MS basal media. Since then many reports have been published on calloid induction and growth from different explants.

In this investigation the explant initially showed floral bud swelling by 60 days followed by formation of calloid by 90 days after culture initiation. In the case of treatment T6 (CM2 + 4% Sucrose + 3% Supplement) the calloid formation was achieved after nine months (270 days). This might be due to the dependence of length of the induction phase on the physiological status of the explant as well as the culture conditions.

Branton and Blake (1983) reported the formation of callus from immature inflorescence was too slow, taking about 3-4 months on Y3 medium. Similarly Verdeil *et al*. (1992) described that the calloid production was achieved after 9 months and subsequently the embryogenic callus after 12 months.

5.2.1 Effect of Stage of Development of Explant on Culture Response

The physiological maturity of the explant plays a significant role in the embryogenic potentiality. Generally younger tissues are best in tolerating the phytohormonal concentration for dedifferentiation. The results of the present study indicate that among the different stages, immature inflorescence with length 1-5 cm responded better as compared to 5-10 cm, 10-15 cm and 15-20 cm long inflorescence. The immature floral primordia contain more active and dividing meristematic points, which are also active source of growth hormones. These

innate growth hormones present in the tissues might have contributed to the higher *in vitro* performance of the 1-5 cm long immature inflorescence.

Sugimura and Salvana (1989) also reported that in coconut, explant taken from inflorescence between 0.8 - 4.1 cm long showed better callus induction and minimal browning. However Verdeil *et al*. (1994) reported that the inflorescence with length 10 and 25cm gave the best callusing.

Karunaratne and Periyapperuma (1989) reported that culture of immature embryo of coconut respond better than mature zygotic embryo. Vidhanaarachchi and Weerakoon (1997) reported tissues from immature inflorescence to be opened in seven and eight month time respectively, has been successfully utilized for micropropagation while embryogenic calli production failed from the mature stage of inflorescence explant.

Sandoval *et al*. (2016) reported that younger inflorescences were more responsive than the more mature explant stages tried. Younger stages formed callus with pearly white tissues whereas no callus were formed when more mature explant stage was used.

5.2.2 RESPONSE OF DIFFERENT REGIONS OF EXPLANTS

Among the two different regions of the inflorescence studied in the experiment, the inflorescence tip region (after removing the extreme tip) explant is better than inflorescence base region in calloid induction with minimum phenolic interference. Sugimura and Salvana (1989) also reported the use of specific portion of rachillae excluding the top and base of immature inflorescence for high frequency of callus induction.

Vidhanaarachchi and Weerakoon (1997) also suggested that the portion of inflorescence explant about 3 cm in length was better than the extreme top and basal portions of the inflorescence.

5.3 POLYPHENOL INTERFERENCE

Like many tropical species, coconut contains large amounts of phenolic compounds. Oxidation and phenolic accumulation has been common in palm tissue cultures (Jones, 1974; Nwankwo and Krikorian, 1983). So for the establishment of coconut *in vitro* some remedial measures should be ensured.

In the present study youngest inflorescence with reduced thickness showed least browning due to phenolic exudation and keeping the culture in dark reduces browning to a certain extent. This result was supported by the findings of Blake (1983), who mentioned size of explant is correlated with browning and survival rate, that is smaller explants have a better survival rate with minimal browning than larger explants.

Sugimura and Salvara **(**1989) reported that irrespective of the stage of the inflorescence, the coarse slices of non-uniform explant size exhibited browning. So minimising the thickness of transverse slices can reduce the tissue browning wherein culturing a 4.6 cm length of inflorescence, one mm slices showed 32% browning while 0.5 mm slices showed 11 % browning.

Samosir *et al.* (1998) reported explant necrosis as one of the major limiting factor in somatic embryogenesis, which was more prominent in mature explant than in immature explant. This findings support the result obtained from the present study that the least browning (4.76 %) was from the i4 stage (length 1-5 cm) followed by decreasing the percentage browning as 6.00 per cent in i_3, 11.90 per cent in i_2 and 13.95 per cent in i_1 stage.

5.4 INFLUENCE OF DIFFERENT MEDIA ADDITIVES

Activated charcoal is presently one of the most essential components in the coconut tissue culture medium to prevent the browning of tissues and adsorb polyphenols released from the cultured tissue (Fernando *et al.*, 2010). The present study was conducted to test the embryogenic potentiality of immature

inflorescence of *C. nucifera* cultured on different nutrient media fortified with different percentages of neera and by-product of processing of neera and to access its feasibility along with or without activated charcoal.

5.4.1 Effect of Activated Charcoal in the Media

Activated charcoal maintains the balance of auxin, which will induce dedifferentiation of the tissues, in the media without any noxious effects of released exudates. The effect of varying concentrations of activated charcoal in calloid proliferation and browning ranging from 0.3 per cent to complete absence of activated charcoal by replacing it with by-product from neera processing (1-3%) and coconut inflorescence sap (5 mlL^{-1} to 15 mlL-1) were studied.

The result revealed that activated charcoal had a key role in *in vitro* propagation of coconut. Among the different concentration of activated charcoal tried it was found that 0.3 per cent activated charcoal in combination with supplement (2%) gives better response (treatment T_2). Decrease in the concentration of activated charcoal from 0.3 per cent to 0.1 per cent (treatment T_3, T_4 and T_5) results in the decrease in percentage number of cultures showing calloid proliferation and increase in browning of the cultured tissues. This difference might be due to inability to define the concentration of auxin available to tissue in the presence of activated charcoal. Medium completely devoid of activated charcoal but was replaced with supplement (treatment T6) showed very low percentage of cultures forming calloid and that too with a time span of nine months after the culture initiation. This response might be due to the lack of equally potent adsorbent to completely adsorb the toxic substance released by the tissues and unbalanced phytohormones in the system.

Similar result was reported by Zaid and Tisserat (1983b) in date palm where medium devoid of activated charcoal exhibited severe browning and growth inhibition whereas inclusion of 3mgL^{-1} of the same in the medium caused considerable growth of the date palm embryo.

5.4.2 Role of Supplement in the Media

As reported by the earlier investigators intense browning of tissues challenges the coconut regeneration by somatic embryogenesis. Presently, this was overcame to a certain extend by the use of activated charcoal (Zaid and Tisserat, 1983b; Areza *et al*., 1993). But there were reports of ill effects of activated charcoal as it adsorb the required nutrients from the medium (Ebert and Taylor, 1990; Ebert *et al.*, 1993) additionally to the unwanted polyphenols. (Weatherhead *et al*., 1978). Constantin *et al*. (1977) also reported that adsorbance of essential phytohormones by activated charcoal from the media might cause the severe inhibition of callus growth.

Variety of charcoal is available in the market. Based on the source and method of preparation, their adsorption property differs. This results in the variation in the level of plant growth regulators in the medium as the type of charcoal differs. So indirectly this phytohormonal adsorption obstacle the mastering of *in vitro* coconut regeneration.

The present investigation was to replace the activated charcoal with the by- product from neera processing. This by- product was chemically analysed and found that this substance with pH- 5.78 and 1.16mS electrical conductivity also contain some amount of nutrients, such as organic carbon (7.24 %), nitrogen (1.3 %), phosphorous (0.14 %), potassium (0.11%), sulphur (0.16 %) and sodium (0.56%) and iron (0.71%).

Significant reduction in browning of tissues (11.52%) and better calloid formation (92.75%) was observed in treatment T2 when adding two per cent by-product and activated charcoal (0.3%) in combination in the media. This positive response might be due to compensative effect of by- product of neera processing along with activated charcoal and the minor portion of the nutrients present in this by-product might be boosting the growth of cultured explants.

But complete replacement of activated charcoal with the supplement is not an appreciable alternative. Activated charcoal free media (treatment T_6) led to the formation of calloid but the percentage calloid formation was very low (9.79 %) that to after a prolonged period of 9 months after culture initiation. This result was in accordance with the results observed in the study of Suranthran *et al.* (2011) on oil palm variety *Dura* and Eeuwens (1978) that organic forms of nitrogen stimulate growth of cultured explants of coconut.

5.4.3 Effect of CIS in the Media

Coconut Inflorescence Sap is extracted from inflorescence of various species of palms. This natural and non- alcoholic beverage was earlier reported as highly nutritive (Devdas *et al*., 1969) with neutral pH containing 16 kinds of amino acid, vitamins *viz.*, vitamin B complex, especially nicotinic acid, vitamin C (Aalbersberg *et al*., 1997) high content of minerals such as Na (6.95mg/100ml) and K (3.16 mg/100ml) (Barh and Mazumdar, 2008) and sucrose (Ysidor *et al*., 2014).

Cavieres *et al*. (2006) defined "nurse plant syndrome", which is the positive influence of the adult plants on seedlings. An adult plant enhances the establishment of new seedlings in its neighbouring area by ameliorating extreme environmental factors. Similarly a nurse effect in the tissue culture medium was expected using inflorescence sap since the explant tissue is also from inflorescence.

In the present investigation treatment, T8 with $10mlL^{-1}$ CIS showed better calloid proliferation response (90.17 %) and minimal browning (22.70 %) than without CIS (control). This might be due to the nursing effect of CIS or buffering of the non-optimal media condition to improve the availability of nutrient to the explant tissues.

Similarly Blake and Eeuwens (1982) reported the improvement in culture growth by the addition of coconut water in the medium as in case of endosperm

tissue. Studies conducted by Al- Khayri (2010) on date palm, revealed improved callus growth and somatic embryogenesis in response to use of coconut water as an additive in culturing shoot tip explant and it was observed that the callus growth was low at five per cent coconut water, which significantly increased when 10- 15 per cent coconut water was used but 20 per cent coconut water in the media appeared to inhibit callus growth. Similar results were shown in case of somatic embryogenesis also with respective concentration of coconut water in the media.

This was in accordance with the findings of the present study that at five mlL^{-1} of CIS in the media (treatment T_7) the calloid proliferation percentage was low (79.63%). As the concentration of CIS in the media increased to 10 mlL^{-1} (treatment, T8) a significantly high calloid proliferation (90.17%) was obtained, which was higher than the control (with no CIS in the medium). But when the concentration of CIS further increased to $15mlL^{-1}$ (treatment T9), the response also reduces as percentage calloid proliferation of 81.92 per cent suggesting treatment T8 (CM2 + 4% Sucrose + 0.3% AC + $10mlL^{-1}$ CIS) the better for calloid proliferation with additive as CIS which was also better than control (CM2 + 4% Sucrose + 0.3% AC).

5.5 FORMATION OF SOMATIC EMBRYO AND MATURATION

The decision whether a particular calloid is a regenerative calloid which eventually might lead to further embryogenic structures and finally to clonal plantlets was made only by visual assessments. In the present investigation it was evident that media, explant as well as time are interdependent for the formation of somatic embryo from embryogenic calloids.

Embryo formed initially was globular in shape. Treatment combination and the developmental stage of the explants showed remarkable responses in somatic embryo formation. In Ci_4, T_1i_2, T_2i_2, T_5i_3, T_6i_1, T_6i_3, T_8i_1, T_8i_3, T_9i_2 and T_9i_4 the rate of somatic embryo formation was higher. But the other combination shows

response slowly. This slow response of the coconut for the formation of somatic embryo might be similar to the prolonged period required for development of zygotic embryo.

This result was in accordance with results presented by Sandoval *et al*. (2016) that sub-culturing initially too scarce, pearly white structures through a stepwise procedure produces copious new calluses and finally obtaining fully embryogenic calluses from rachilla explants.

Oropeza *et al.* (2018) also reported that initially, embryogenic callus structures were scarce, but they are progressively more abundant in the new calli formed.

5.5.1 Stage of Embryo Maturation

In the present study calloids were formed from the explant by 90 days of culture initiation. After 240 days of initiation, somatic embryos at globular developmental stage were obtained. By 270 days of initiation the somatic embryo showed response of germinating potential and transferred to GA_3 medium for maturation.

This was accordance with the experiments of Oropeza *et al.* (2018) that somatic embryos was formed from the embryogenic callus after 30 days of sub-culturing twice and transferring of this somatic embryos to GA_3 for another 30days starts germinating and further culturing for 90 days.

5.6 SOMATIC EMBRYO GERMINATION

Somatic embryos developed on the calloid were transferred to the germination medium (Y3 + 0.5 mgL^{-1} picloram + 1 mgL^{-1} NAA + 0.1 mgL^{-1} TDZ supplemented with GA (1-2 mgL^{-1}) and BA (10-15 mg L^{-1}). Somatic embryo developed in the media combination T1i2, T5i3, T9i2 and T9i4 were sub- cultured to GA_3 medium for germination.

Oropeza *et al.* (2018) reported a reproducible protocol for the germination and maturation of somatic embryos from the embryogenic calli and further development into plantlets by enhancing the medium with $GA_{3,}$ with duration of 20 months.

Since the number of embryos that could be used for further experiment was limited the work is in progress. A prolonged growth period is required to develop the germinated somatic embryo into plantlet.

5.7 CONCLUSION

One of the prerequisite to support the burdened coconut industry and to sustain the current and future demand is to provide high yielding disease resistant elite planting material. The seed propagation of single elite palms identified faces the problem of high genetic variation due to the heterozygosity of the palm. This can be rectified only through *in vitro* clonal propagation by somatic embryogenesis with coconut tissues. Coconut being a recalcitrant crop this method is not yet completely mastered. Morphological, anatomical and physiological characteristics of coconut palm challenges its *in vitro* regeneration.

Present study to assess the *in vitro* response of immature inflorescence explant of coconut for induction and germination of somatic embryos by supplementing the medium with by-product of neera processing and coconut inflorescence sap revealed that tip region of the inflorescence is better than the basal region for dedifferentiation to form calloids. The tip region of i_4 stage of explant with 1-5 cm external spathe length showed high percentage calloid initiation (85.71%), indicating youngest stage respond better than mature stage(i1stage of external spathe length 15-20 cm with 74.42 per cent calloid initiation).

Prolonged period (90 days) of incubation in dark was required for calloid formation. The media additives such as activated charcoal and media supplements influenced the time taken for *in vitro* response and the percentage of cultures showing calloid proliferation and embryo formation, where, culturing in

medium with 0.3 per cent activated charcoal and two per cent supplement (T_2) responded in three months, while treatment devoid of activated charcoal (T_6) responded after nine months in sub-culturing.

Both the media additives (by- product and CIS) had significant role in enhancing calloid proliferation. Media with two per cent by- product and 10 mlL^{-1} CIS respectively (T2 and T8) along with 0.3 per cent activated charcoal showed the highest calloid proliferation than with no additives (control). Media with one per cent and three per cent by- product with 0.2 per cent and 0.3 per cent activated charcoal respectively and 15 mlL^{-1} CIS and 0.3 per cent activated charcoal also showed better response to calloid proliferation.

The somatic embryos formed after proliferation was transferred to germination medium. Though the frequency of somatic embryo formed was less within this period there is chance of increasing the percentage embryo formation with prolonged period, successive sub-culturing for embryo formation and its subsequent germination and finally obtaining a plantlet.

6. SUMMARY

An investigation on "Somatic embryogenesis from immature inflorescence of coconut (*Cocos nucifera* L.)" was conducted during 2017-2019. The salient findings of the study are summarised below.

1. Immature inflorescence (i4- external spathe length of 1-5 cm), most responsive to calloid induction compared to other stages of explant due to the presence of numerous meristematic points.
2. Tip region (excluding the extreme ends) is better for high frequency of calloid induction with minimal browning.
3. Among the different media tested, addition of by- product of neera (0.3% AC + 2% Supplement) and CIS (0.3% AC + 10mlL^{-1} CIS) in the media have positive effect and enhanced the calloid proliferation (92.75% & 90.17%) with minimal browning (11.58% & 22.70%) than Control (0.3% AC only) with 86.54% calloid proliferation 26.95% browning.
4. By- product of neera and CIS cannot completely replace use of AC in the media (T6- 9.79% calloid proliferation & 80.08% browning).
5. Youngest inflorescence with reduced thickness showed least browning due to phenolic exudation.
6. Three per cent supplement is not an equally potent adsorbent to AC for completely adsorbing the toxic substance released by the tissues and unbalanced phytohormones in the system 0.2 per cent by- product of neera (T2) and 10 mlL^{-1} CIS (T8) could reduce the ill effects of AC in the media.
7. Frequency of calloid induction and proliferation and development, germination and maturation of somatic embryos increases with time.
8. Phenolic oxidation can be reduced by keeping the cultures in the dark condition.
9. The somatic embryos formed were sub- cultured to the germination media. Among the eighty combinations tested twelve combinations could support growth and germination of somatic embryos at a faster rate. The somatic

embryos showed potential to germination after a period of nine months. Maturation of germinated embryo requires further time to develop into plantlet.

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SOMATIC EMBRYOGENESIS FROM IMMATURE INFLORESCENCE OF COCONUT (*Cocos nucifera* L.)

by

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ABSTRACT OF THE THESIS

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ABSTRACT

Coconut palm (*Cocos nucifera* L.), the only species of genus *Cocos*, is a major crop plant which is propagated exclusively by seeds presently. Coconut is highly heterozygous, generally cross-pollinated and long lived. So the offspring differ greatly due to segregation, which can be assessed only after a very long juvenile phase. Breeding for crop improvement is thus a difficult and long-term process. So within a short period, *in- vitro* propagation, employing tissue culture techniques, offers a means of cloning improved planting material. So the objective of the study was to assess the *in vitro* response of immature inflorescence of coconut explants for induction and germination of somatic embryos by supplementing the medium with by-product of neera processing and neera.

The media for culture initiation, subculturing and embryo germination were standardised in earlier experiments (Siny, 2006; Sujatha, unpublished data, 2011). In the present experiments, standardised sub-culturing medium with addition of different supplements were tested.

Embryogenic calloids and somatic embryos were induced in standardized CM2 media (Y3 basal media containing a combination of 0.5 mgL^{-1} picloram, 1 mgL^{-1} NAA and 0.1 mgL^{-1} TDZ and 300-600 μM 2,4-D) from the immature inflorescence explant. Among the different stage and region of immature inflorescence tested in standardised CM2 medium, tip region of inflorescence (85.71%) with length 1-5 cm was found to be the best for calloid induction with low browning (4.76%). The adverse effect of exudation of polyphenols was reduced by inoculating youngest inflorescence with reduced thickness.

Embryogenic calloids were sub- cultured into 10 media combinations. With four stages of explant (based on length), each stage with two types (tip or base) there were eighty combinations. The suitability of the by-product from neera processing as well as neera (CIS- Coconut Inflorescence Sap) as supplements for CM2 medium to culture the immature inflorescence of coconut was assessed and

found that CM2 medium with 0.3 per cent activated charcoal, 4 per cent sucrose and 2 per cent supplement and also CM2 medium with 0.3 per cent activated charcoal, 4 per cent sucrose and 10 mlL^{-1} CIS was the best medium for obtaining cultures with calloid proliferation (92.75 % & 90.17 %) with minimal phenolic interference (11.58 % & 22.7%). Calloid proliferation was successfully achieved from all the media combinations.

Chemical properties of neera supplement used in the culture medium were analysed for the presence of important mineral nutrients and found that this pH-5.78 substance with 1.16 mS electrical conductivity also contain some amount of nutrients, such as organic carbon (7.24 %), nitrogen (1.3 %), phosphorous (0.74 %), potassium (0.11%), sulphur (0.16 %) and sodium (0.16%) and iron (0.71%) which might boost the growth of the explant.

Somatic embryo maturation from twelve media combinations was attempted. Somatic embryos showed initiation of germination, when matured somatic embryos were transferred to germination medium Y3 + 0.5 mg L^{-1} picloram + 1 mgL^{-1} NAA + 0.1 mgL-1 TDZ supplemented with GA (1-2 mg L^{-l}) and BA (10-15 mg L^{-1}) for further development. A prolonged growth period is required to develop the germinated somatic embryo into plantlet.

The study could identify the potentiality of by- product of neera and coconut inflorescence sap (neera) as additives in tissue culture media of coconut to enhance the induction, proliferation and germination of somatic embryos and in reducing the polyphenolic interference.