EXPRESSION PROFILING OF *E2F* AND *CDKA* GENES DURING EMBRYO CULTURE IN COCONUT (*Cocos nucifera* L.)

By

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(2020-11-092)

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THESIS

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DEPARTMENT OF PLANT BIOTECHNOLOGY

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2023

DECLARATION

I, K. Mounika Sai (2020-11-092) hereby declare that the thesis entitled **"Expression profiling of *E2F* and *CDKA* genes during embryo culture in coconut (*Cocos nucifera* L.)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Certified that the thesis entitled **"Expression profiling of *E2F* and *CDKA* genes during embryo culture in coconut (*Cocos nucifera* L.)"** is a record of research work done independently by **Ms. K. Mounika Sai (2020-11-092)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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DEDICATED TO

MY FAMILY

MY ADVISOR

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LIST OF ABBREVIATIONS

cm	Centimetre
m	Metre
°C	Degree Celsius
%	Percentage
µM	Micromolar
mm	Millimetre
mM	Millimolar
M	Molar
G1	Gap 1 phase
G0	Quiescent phase
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
mRNA	Messenger RNA
DNA	Deoxyribonucleic acid
S	Synthesis phase
RNase	Ribonuclease
cDNA	Complementary DNA
dNTP	Deoxyribonucleoside triphosphate
Taq	*Thermus aquaticus*
PCR	Polymerase Chain Reaction
bp	Base pair
KNO_3	Potassium nitrate
KCl	Potassium chloride

NH_4Cl	Ammonium chloride
$NaH_2PO_4.2H_2O$	Sodium dihydrogen phosphate
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
$CaCl_2.2H_2O$	Calcium chloride
Na_2EDTA	Disodium Ethylenediaminetetraacetic Acid
$FeSO_4.7H_2O$	Ferrous sulphate heptahydrate
$MnSO_4.4H_2O$	Manganese sulphate tetrahydrate
KI	Potassium iodide
$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate
H_3BO_3	Boric acid
$CoCl_2.6H_2O$	Cobalt chloride hexahydrate
$CuSO_4.5H_2O$	Copper sulphate pentahydrate
$NiCl_2.6H_2O$	Nickel chloride hexahydrate
HCl	Hydrochloric acid
ppm	Parts per million
N	Normal
NaOH	Sodium hydroxide
mL	Millilitre
psi	Pound per square inch
UV	Ultra Violet
NCBI	National Center for Biotechnology Information
µL	Microlitre
TAE	Tris Acetate EDTA
mg	Milligram
g	Gram

V	Volt
nm	Nanometer
$MgCl_2$	Magnesium chloride
rpm	Revolutions per minute
BLASTN	Nucleotide Basic Local Alignment Search Tool
NR	Non-Redundant
ng	Nanogram
T_m	Melting temperature
et al	Co-author/ Co-worker

INTRODUCTION

1. INTRODUCTION

The coconut palm (*Cocos nucifera* L., Family- Arecaceae) is a very versatile crop that is appropriately referred to as *Kalpavriksha*. The coconut, also referred to as the "tree of life," gives us everything we require for survival. India is one of the 94 nations that cultivate coconut. The Indian agrarian economy depends heavily on the coconut industry. At the global level, coconut had a production of about 63.7 million metric tonnes in the year 2021 (STATISTA, 2023). In, the Indian economy for the year 2021-22, the export value of coconuts accounted for 3236.83 crore Indian rupees (IBEF, 2022).

Inadequate management techniques, invasions by pests and diseases and the ageing of coconut palms are the main causes of low production and productivity of coconut globally (Warokka *et al*., 2006). Plantations need to be revitalised with superior seedlings due to the loss of coconut trees caused by the devastating effects of diseases like root wilt disease, lethal yellowing, cadang-cadang disease and pests like rhinocerous beetle, red palm weevil, etc., (Sáenz *et al.*, 2018) and also the natural ageing of the palms. Coconut palm has a single apical meristem and lacks branches and suckers (Rajesh *et al*., 2018). Therefore, it is not suited for vegetative propagation and is propagated only through seed nuts. To address the problem of scarcity of planting materials, traditional propagation methods are insufficient. *In vitro* culture methods, on the other hand, can be employed for the commercial production of planting materials in a shorter span of time and can pave the way for mass multiplication (Sushmitha *et al*., 2019).

Micropropagation is the finest method for meeting the growing need for promising genotypes where vegetative propagation is not an option. In 1954, Cutter and Wilson conducted the first experiment involving embryo culture using endosperm of the coconut. Inflorescence culutres in coconut were successfully established by Blake and Eeuwens (1982). In India, embryo culture was employed as a method of conserving genetic material in coconut (Karun *et al*., 1993). Fernando *et al*. (2004) used the plumule as explant in the local Sri Lankan variety (Sri Lanka Tall) and was successful in developing the *in vitro* propagation method. Despite using a variety of explants, including leaf, inflorescence, zygotic embryos,

etc., only plumular cultures had the highest efficiency and reproducibility, outperforming all other explant cultures in coconut (Sáenz *et al*., 2018).

Somatic embryogenesis is the most studied pathway in coconut compared to organogenic pathway. Direct shoot organogenesis can serve as the alternative *in vitro* technique for clonal propagation. This pathway of regeneration has an advantage of reduced somaclonal variation when compared to the somatic embryogenesis (Kong *et al*., 2021). Although, scientists have been successful in producing callus in coconut using somatic embryogenesis (Pérez-Nuez *et al*., 2006), there is yet no procedure for coconut micropropagation that is economically feasible (Sushmitha *et al*., 2019). Hence, in the present study, we aimed at initiating *in vitro* culture in coconut through the organogenic pathway using embryos as explants.

Coconut is known to be recalcitrant to *in vitro* manipulations. The *in vitro* response depends largely on the growth regulators supplemented with tissue culture media like the auxins, cytokinins and gibberellins. Cell cycle-controlling genes appear to be crucial for controlling germination of the embryos (Montero-Corte´s *et al*., 2011). So, to understand the expression of cell cycle control genes like *E2F* (E2 promoter binding Factor) gene, encoding transcription factor necessary for the G1/S phase transition and *CDKA* (Cyclin Dependent Kinase A) gene, responsible for cell proliferation and preservation of cell division competence during early stages of *in vitro* response of coconut zygotic embryos cultured in the presence of different plant growth regulators, the present study was designed with the following objective:

- Expression profiling of the genes governing organogenic regeneration pathway at different morphogenic response stages in coconut embryo culture.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Introduction

Micropropagation, which occurs in a lab setting with regulated temperatures, intense lighting and a predefined growth substrate, is a rapid method of plant propagation. Today, it is essential to use this method for coconut palm propagation to meet the demand for quality planting materials. The *in vitro* propagation has widespread advantage in different palms like coconut, oil palm, date palm, etc.

Micropropagation techniques have been successfully established in several palms. Alternative seedling development methods, such as the *in vitro* approach of tissue culture, are effective for producing planting material more quickly in arecanut as the seeds must be treated for two to three months in order to break the dormancy and trigger germination (Dinatri *et al*., 2021). Scientists have successfully used a variety of factors that affect the *in vitro* development of date palms in their long-term efforts to micropropagate date palms, including the origin, age, size of explant, quality and intensity of the light, temperature, pH of the medium, the presence of plant hormones, the culture medium and the age of the culture (Mazri *et al*., 2016). Date palm micropropagation is primarily based on somatic embryogenesis and organogenesis (Bhansali, 2010).

The method of tissue culture most frequently used for the micropropagation of oil palm is somatic embryogenesis (indirect and direct). Oil palm micropropagation through somatic embryogenesis has been accomplished effectively using a variety of explants, including mature and immature zygotic embryos, immature male and female inflorescences, mature and immature leaves, young plantlets and shoots (Yarra *et al*., 2019). Usually, the tissue culture-raised oil palms have an increased oil content (Marbun *et al*., 2015).

Micropropagation methods are available for many members of the Arecaceae family, but we still need a robust tissue culture protocol in coconut, the most valued member of the family.

2.2. The coconut palm

Coconut (*Cocos nucifera* L.) is one of the most fascinating and useful trees in the world. It is the most valued and a unique member of Arecaceae family. In Sanskrit, it is known as '*Kalpavriksha*' as every part of the palm from crown to the roots is useful to humans. In India, especially in the state of Kerala, the daily lives of the people completely revolve around this palm. It yields more products of use to mankind than any other tree. In tropical regions, it is having a large socio-economic impact on the livelihood of the rural population depending on them (Nayar, 2018).

2.3. Taxonomy

The detailed taxonomical classification of coconut has been showed in Table 2.1 below.

Table 2.1. Taxonomic classification of coconut (ITIS database, 2010)

S. No.	Taxonomic hierarchy	Name
1	Kingdom	Plantae
2	Subkingdom	Viridiplantae
3	Superdivision	Embryophyta
4	Division	Tracheophyta
5	Subdivision	Spermatophytina
6	Class	Magnoliopsida
7	Superorder	Lilianae
8	Order	Arecales
9	Family	Arecaceae
10	Genus	*Cocos*
11	Species	*nucifera*

2.4. Coconut propagation methods

The propagation of coconut is traditionally done using seeds. It is considered as the most feasible method for the propagation. Since coconut is a perennial crop, its performance can be known only after certain period (around 10-15 years); therefore, selection of good quality materials for planting is essential. So, a three-tier selection system is adopted for the production of the good quality nuts. The process of selection occurs at three different levels and these include mother palm selection, seed nut selection and seedling selection (Shareefa *et al*., 2020).

2.4.1. Mother palm selection

There are certain criteria to be considered while selecting mother palm. These are given as follows:

- Palms should have regular bearing habit and should yield more than 80 nuts/year (rainfed condition) and around 100-120 nuts/year (irrigated condition).
- Palm should be above 22 years of age and free from different pests and diseases.
- Palm should possess distinct characters (nuts, crown, inflorescence, leaf) pertaining to a particular variety.

Palms possessing the desired characters are selected for the purpose of propagation (Ledo *et al*., 2019).

2.4.2. Seed nut selection

Seed nuts are collected from the desired mother palms once they have attained full maturity. Nuts usually take 10-12 months to attain full maturity. The harvested seed nuts are stocked under shade to prevent the drying of husk as well as the water present inside the nuts for few days (Ledo *et al*., 2019).

2.4.3. Seedling selection

The one-year-old seedlings are selected. The robust seedlings with thick leaf stalks, large number of roots and leaves are selected. The seedlings with the

circumference of 10 cm around the collar region are preferred. The seedlings produced from early germinated nuts are desirable (Shareefa *et al.,* 2020).

The main type of seedling production system adopted is seed-bed and nursery. Firstly, the nuts (seeds) are placed in the soil either in vertical or horizontal orientation for the purpose of germination. The vertical orientation is the most preferred one. The seedlings are later transplanted to the nursery after they have reached a certain height of 0.20 m. In nursery, seedlings are either planted in soil or in poly bag. After one year, the seedlings with good performance, germination and vigor are selected for further planting (Ledo *et al*., 2019).

2.5. Coconut *in vitro* culture

Plant tissue culture is one of the basic gadgets in Plant Biotechnology. It is frequently used in studying various plant developmental processes, gene studies, commercial micropropagation, crop improvement and transgenic studies etc. Plant tissue culture is a term used extensively for the culture of any plant part in unnatural and controlled environmental conditions aseptically (Loyola-Vargas and Ochoa-Alejo, 2018).

Coconut tissue culture was first attempted by Cutter and Wilson (1954), where they used liquid endosperm as the medium for the growth of the embryos. Subsequently, the use of different explants for the *in vitro* coconut culture became successful.

2.5.1. Significance of coconut *in vitro* culture

Coconut has immense importance as far as the national economy is considered. Even though this palm has good adaptability, the productivity of coconut is reducing worldwide and this is mainly because of the old and senile palms. The effect of existing pests and diseases also have posed serious problem to the survival rate of the palms. The diseases like root wilt disease, lethal yellowing, viroid disease cadang-cadang, etc., have already caused a lot of economic losses to the farmers and the pests like rhinocerous beetle, red palm weevil, etc., have also caused alot of havoc to the coconut palms world-wide. All these reasons urge us to

take up replanting in order to save the production and to meet the existing and future demands of the public (Sáenz *et al.*, 2018).

Good quality and resistant planting materials are needed for replanting. These can be provided by our traditional breeding practices but the traditional approach of replanting using seed nuts will not be able to produce enough elite seedlings that can meet the global demands (Kong *et al*., 2019). The breeding strategies used in the coconut for the development of the resistance is also time consuming as it takes many years to develop a good variety with resistance to various stresses like drought, pests and diseases. Kong *et al*. (2021) proposed that high-quality seedlings can be obtained from *in vitro* cultures through somatic embryogenesis. Thus, tissue culture can serve the purpose of replanting the coconut palms in large quantities and in a quick span of time when compared to the conventional approaches in order to meet the raising global demands for the coconut products.

2.5.2. Coconut micropropagation studies

As mentioned earlier, Cutter and Wilson were the scientists who tried embryo culture for the first time in 1954. They used the liquid endosperm for the development of the embryos. In 1964, De Guzman along with Del Rosario was successful in establishing the first zygotic embryo culture protocol for Makapuno cultivar. Inflorescence was used as explant by many scientists in different time periods (Davis, 1964; Blake and Eeuwens, 1982; Siny, 2006; Shareefa *et al*., 2019; Radhika, 2019). Davis (1964) used inflorescence as the explant for micropropagation but he couldn't succeed. Later, Blake and Eeuwens (1982) were successful in establishing the shootlets in the culture excised with inflorescence. This work wasn't pursued furthermore as it couldn't produce true-to-type plants. Thanh-Tuyen and De Guzman (1983) and Monfort (1985) used anther as explant and could obtain some direct embryos at a low rate (Perera *et al*., 2008). In India, embryo culture was used as a means of germplasm preservation (Karun *et al*., 1993). Plumule was tried as explant by Fernando *et al.* (2004) in local Sri Lankan variety (Sri Lanka Tall), where they successfully established the *in vitro* propagation

technique. Later, few other scientists tried plumule as explant and were able to regenerate calli.

Even though different explants like inflorescence, zygotic embryos, shoots, etc., were used, only the plumular cultures had shown the maximum efficiency and reproducibility surpassing all other explant cultures (Sáenz *et al*., 2018).

In Kerala Agriculture University (KAU), the tissue culture works were carried out by Siny (2006) and Radhika (2019) on somatic embryogenesis where immature inflorescence was used as the explant and they were successful in establishing the inflorescence cultures in coconut.

2.5.2.1. Embryo culture

Embryo culture is the activity involving the inoculation and culture of the isolated embryo (mature or immature) on suitable artificial medium. This type of culture was first proposed by Cutter and Wilson (1954) in coconut but the generation of embryo culture protocol was possible only after a decade by De Guzman and Del Rosario in 1964 in Makapuno cultivar. This type of culture can be done either using mature or immature embryos. This technique helps in the reduction of the duration of germination and overcomes dormancy when the mature embryo is used whereas in the case of immature embryos this process helps in the embryo rescue where the embryo will otherwise get aborted due to lack of endosperm (Drewes-Alvarez, 2017).

Drewes-Alvarez (2017) reported embryo culture studies in coconut. The embryos collected from 10-14 months old coconuts were inoculated in the basal Y3 media supplemented with additives like activated charcoal, growth hormones, etc. Initially the cultures were kept in dark at 25-31ºC for about 5-8 weeks and then were shifted to the light conditions. Later these plants were sub-cultured based on the requirement (Drewes-Alvarez, 2017).

Nguyen *et al*. (2015) reported that the high content of sucrose (>4 %) and gelrite (gelling agent) had an important role in embryo germination. The addition of activated charcoal to the media had reduced necrosis of tissues. The growth hormones like GA3 (0.5 µM) had promoted the germination of embryos whereas

auxins (Indole Butyric Acid (IBA) and Naphthalene Acetic Acid (NAA)) had increased the root growth in the early seedling stage.

For the purpose of hardening, different potting mixtures have been tried. According to Pech y Ake *et al*. (2004), peat moss and soil in the ratio of 1:1 was the ideal combination for tissue culture raised plants. The survivability of these plants improved when they were exposed to different optimum temperatures beginning from the fogging chamber to a shaded nursery and then finally to the direct sunlight conditions (Talavera *et al*., 2005).

The technique of embryo culture has been successful in coconut *in vitro* multiplication and it is used for germplasm conservation and exchange.

2.5.2.2. Anther culture

Anther culture was first initiated by Thanh-Tuyen and De Guzman (1983) in coconut. Here, both direct and indirect plant regeneration has been observed. The plant growth regulators like 2,4-Dichlorophenoxyacetic acid (2,4-D) and 2-isopentenyladenine (2iP) enhanced the regeneration. Different types of calli were produced out of which the compact ones were commonly seen in the cultures. The anthers would show browning after 10 weeks of culture initiation. Plants with single shoot were obtained in a lesser frequency than the plants with multiple weak shoots (Perera *et al*., 2009).

2.5.2.3. Plumule culture

Sáenz *et al*. (2018) attempted plumule culture in coconut. The zygotic embryos were isolated from the nut and then the plumules were separated from the embryo using a stereoscopic microscope. For three months the cultures were maintained at 27 ± 2 °C in the dark during which callus production was noticed. Somatic embryogenesis and germination were observed when they were kept in another new media provided with the same culture conditions. Similarly, in the further stages shoot growth was seen. The plantlets were finally prepared for *ex vitro* hardening. They were raised in poly bags with potting mixture of peat moss, sand and soil respectively in 1:1:1 ratio for a brief period and then were moved to the shaded nursery before being planted in the main field (Sáenz *et al*., 2018).

The Central Plantation Crops Research Institute (CPCRI) Kasaragod, had experimented on the influence of various auxins on high frequency callus induction from coconut (WCT) plumule explant. They found that mature embryos treated with gibberellic acid (GA3) demonstrated decreased callus initiation (28 %) when compared to directly scooped plumules. Additionally, decreased callus growth and embryogenic calli formation were observed in GA3 treated embryos (60 %) than in control embryos. The 70 per cent of cultures that underwent direct plumule extraction responded well for embryogenic calli after the incubation period of a month and an increased (80 %) embryogenic calli multiplication was seen after four months of culture incubation. They also found that the lowest dose of GA3 (1 mgL^{1}) was shown to be the most effective for embryo germination (Karun and Sajini, 2010).

2.5.2.4. Inflorescence culture

This type of culture was mostly attempted by the scientists in the 1960's but the protocol was not much successful due to less regeneration frequency and lack of development of true-to-type plantlets (Blake and Eeuwens, 1982).

In Centre for Plant Biotechnology and Molecular Biology (CPBMB), KAU, the use of immature inflorescence as explant was experimented. The use of anthers taken at premeiotic stages and immature rachillae showed best results for callus and embryo formation (Siny, 2006). Radhika (2019) proposed that the inflorescence had many meristematic regions which are beneficial for callus induction. The tip region of inflorescence was found to have better calloid differentiation than basal areas during somatic embryogenesis. Furthermore, duration of nine months was required for the initiation of germination of somatic embryos.

In CPCRI Kayankulam, Shareefa and her co-workers (2019) proposed standardized *in vitro* regeneration protocol using rachillae bit as explants. Pieces of rachillae (1.0 to 1.5 mm), from immature inflorescence were inoculated in four different medium mixtures. The maximum response of white translucent outgrowth with the least amount of browning was produced by Y3 medium supplemented with 4.54 µM 2,4-D.

Subculturing to Y3 medium with 4.54 µM 2,4-D and 4.14 µM picloram produced many shoot-like structures during the incubation period. Transferring these shoot-like outgrowths to 1/2 MS (Murashige and Skoog) medium with 5.37 µM NAA and 4.44 µM BAP (6-Benzylaminopurine) under light conditions led to the highest multiple shoot formation. The individual shoots were detached and moved to Y3 medium containing 5 µM 2ip and 5 µM BA (Benzyl Adenine) for shoot regeneration after the cultures were maintained for 4-6 months in 1/2 MS medium with 5.37 µM NAA and 4.44 µM BAP. Using SCoT (Start Codon Targeted) markers, the plantlets' clonal fidelity was ensured (Shareefa *et al*., 2019).

2.5.2.5. Leaf culture

The coconut leaf was used as explant by Jesty and Francis (1992). The basal segments of the immature leaf were used. They treated the sterile explants with citric and ascorbic acid of 100 mM concentration each to reduce browning of tissues. Calluses were obtained which had slight leaf like appearance but morphogenesis was difficult in these cultures (Jesty and Francis, 1992).

2.5.3. Problems in coconut *in vitro* culture

2.5.3.1. Browning of the tissues

According to Zaid (1985), the formation of guinones and the oxidation of polyphenols are the factors causing browning of the tissues and surrounding media. These substances have a high degree of reactivity and are extremely damaging to the tissues. This is the common phenomenon seen during the *in vitro* propagation of woody plant (Block and Lankes, 1996). The browning appears due to the occurrence of phenol oxidation inside the tissues (Alkhateeb and Ali-Dinar, 2002). The addition of sucrose to the media also promotes browning of tissues through the production of ethylene *in vitro*. This can be replaced by maltose which has been proved effective in reducing browning. The immersion solution medium containing maltose of different concentrations were used for the treatment. The embryo explants of coconut were given the treatment of 1 M maltose (60 minutes), followed by 0.05 M maltose (1 minute) and then with 0.01 M maltose (5 minutes). This procedure was found effective in reducing phenolic exudation in coconut (Zuraida *et al*., 2017).

The addition of activated charcoal to the media and washing the explants in antioxidant solution before inoculation can resolve this issue to certain extent (Alkhateeb, 2008).

2.5.3.2. Early rooting

The formation of roots before the appearance of shoot buds is not economical as these roots decrease the nutrient availability to produce shoot buds. The presence of higher auxin concentrations or darkness during culture incubation or low mineral concentrations can be few reasons for early rooting (Alkhateeb, 2008).

2.5.3.3. Fungal and bacterial contamination

Bacteria and fungi are common contaminants seen during incubation. Often, they destroy the cultures completely. Proper surface sterilization can reduce the contamination to certain extent. Maintenance of aseptic conditions is essential. The addition of antibiotics to the media can reduce bacterial contamination. Proper closure of the culture tubes is also important to avoid the spoilage of the cultures (Fernando, 2008).

2.5.3.4. Heterogenous response of the explants

The response of the explants is not uniform. The response of somatic tissues like leaf and inflorescence is not consistent when compared to the zygotic embryos which have above 75 per cent callusing frequency under optimum conditions (Diyasena, 1998). However, the response of cultures depends on many factors like genotype, age of explant, stage of explant, etc., (Fernando, 2008).

2.5.3.5. Effect of activated charcoal

The activated charcoal is added to the medium for the purpose of adsorption of phenols and other inhibitory compounds in the cultures but the addition of this leads to the variable tissue response in the cultures due to the adsorption of essential growth hormones (Pan and Staden, 1998). Polyvinylpyrrolidone or ascorbic acid can be used as alternative to charcoal but the development of callus was difficult in such cultures (Fernando, 2008).

2.5.3.6. Poor quality and quantity of the callus

The callus obtained from the cultures were compact and proportionately in less quantities which makes coconut difficult for clonal propagation (Fernando, 2008). A helpful technique for the quick proliferation of callus is the establishment of cell suspensions, however this requires friable callus. But despite all of the treatments used, the generation of friable callus was difficult in coconut (Fernando, 2001).

2.5.3.7. Slow regeneration of the plantlets

After the development of shoots and roots to a certain stage, the further growth of the young plant is observed to be slow. The time taken to reach the stage needed for the *ex vitro* hardening and subsequent field level planting is also very high (Fernando, 2008).

2.5.3.8. Recalcitrance

In vitro recalcitrance is the inefficacy of plant tissues to respond to tissue culture. This is the common feature seen in many woody perennials. It is a genetic character of certain species and thus it becomes difficult to control this trait in culture conditions (Mc Cown, 2000). It occurs during all stages of culture growth. The slow growth of regenerated plants and slow response towards shoot establishment are few outcomes of recalcitrance in coconut (Benson, 2000).

2.5.4. *In vitro* recalcitrance

The incapacity of plant cells, tissues and organs to respond to tissue culture treatments is known as *in vitro* recalcitrance. Recalcitrance can be a significant barrier to commercial micropropagation as well as the wider adoption of *in vitro* germplasm conservation. It can happen at any point in the development of a culture regime and our understanding of the causes is limited (Benson, 2000). Coconut and oil palm are few palms exhibiting this phenomenon (Engelmann *et al*., 1995).

2.5.4.1. Factors responsible for *in vitro* recalcitrance

2.5.4.1.1. Phylogeny

According to Benson (2000) gymnosperms are more responsive to tissue culture than angiosperms. In angiosperms, monocots and dicots vary in their response towards tissue culture. Monocots are more recalcitrant than dicots. Their shoot meristems are basal in origin and vascular tissues are dispersed at random locations in the stem. These tissues usually lack cambium and meristematic points that are essential for response to the tissue culture (Benson, 2000).

2.5.4.1.2. The choice of explant

The selection of explant from meristematic regions is essential for successful establishment of the cultures. Meristematic regions include basal leaf meristems, seed scutella, basal plates, immature embryos, etc. The zygotic embryos are more responsive than cultures prepared from anthers or any other gamete cells. This is because of the presence of juvenile tissues in the embryo which are more responsive to *in vitro* culture. The physiology of the donor plant and the explant response to *in vitro* cultures should be known in order to reduce the *in vitro* recalcitrance. The selection of explant at a particular responsive stage from a mature tree (donor plant) is essential in overcoming recalcitrance issues (Benson, 2000).

2.5.4.1.3. Lifecycle of the whole plant

Since plants have different patterns of growth, their response to *in vitro* culture also varies accordingly. Life cycle is directly linked to the vegetative growth and morphogenesis of the explant species. Hence, the lifecycle of the species including different stages of growth like reproduction, dormancy and rejuvenation should be well studied to develop strategies for the reduction of recalcitrance (Benson, 2000).

2.5.4.2. *In vitro* manipulations to overcome *in vitro* recalcitrance

2.5.4.2.1. Plant growth regulators

The use of certain compounds like thidiazuron in the media can reduce recalcitrant phenomena. Thidiazuron (TDZ) acts as potent regulator of

morphogenesis and triggers many differentiation and de-differentiation reactions (Benson, 2000).

Compound like jasmonates, ascorbic acid, polyamines, etc., also have profound effect on rooting and shooting when used in the media (responses are based on the type and species of explant) (Benson, 2000).

The interaction between endogenous and exogenous growth substances should be minimal for good culture growth. Ethylene has both positive as well negative effects on the cultures. It has a role in morphogenesis of the explant. The production of ethylene in excess can lead to increase in recalcitrance of the explant (Benson, 2000).

The exposure of the cultures to plant growth regulators for a longer period may lead to recalcitrance due to many genetic and epigenetic changes that hinder the normal development of the explant. Hence, it is necessary to standardize the application of growth hormones for each species (Benson, 2000).

2.5.4.2.2. Solidifying agents

These compounds are used for the solidification of the media. Agar, gelatin, gelrite, etc., are few examples of solidifying agents. Although, different gelling agents have different effects on the explant but the use of particular gelling agent in the media solely depends on the type of response produced by that explant. Sometimes the same gelling agent available in different brands will also vary in their effect on the explant. This changes the growth pattern of explant and thus leads to the varied response (Benson, 2000).

When compared to tissue cultures on agar medium, tobacco, bamboo, carrot, jojoba, etc., cultured on appropriately solidified gelrite media typically displayed greater shoot proliferation, rooting, shoot and root vigour and callus development (Huang *et al*., 1995). Gelrite has become an essential part of coconut culture initiation and has been found to increase the number of embryos that germinate (Adkins, 2016).

2.5.4.2.3. *In vitro* stress physiology

Stress has been advantageous as well as disadvantageous for *in vitro* cultures. The over accumulation of ethylene gas has negative effects. The oxidation of phenols during initiation of cultures have led to the early recalcitrance in greater proportions. This is a major issue in woody perennials as they have a high content of the phenols during the process of secondary thickening and lignification. Since phenol oxidation is a wound response, it is inevitable for *in vitro* propagation. The addition of activated charcoal to the media and proper surface sterilization of the explant may be helpful in overcoming recalcitrance (Benson, 2000).

2.5.4.2.4. Other parameters

- The application of certain suitable amino acids like proline or hydroxyproline is useful (Benson, 2000).
- Proper ventilation to the cultures reduces the ethylene accumulation (Benson, 2000).
- The quality and quantity of light provided also have positive effects on the growth of cultures (Benson, 2000).
- The maintenance of proper temperature influences the *in vitro* responses (Benson, 2000).
- The transition of cultures from *in vitro* to *ex vitro* conditions can lead to late-stage recalcitrance. This leads to the poor acclimatisation of the plantlet towards external conditions and thus culture transfers should be done with great care to overcome recalcitrance at this stage (Benson, 2000).

2.6. Understanding the molecular mechanisms of *in vitro* plant response in coconut

The earliest attempt on coconut somatic embryogenesis was made by Eeuwens and Blake (1977). This regeneration pathway includes the formation of somatic embryos from tissues like immature inflorescences, ovaries or mature and immature zygotic embryos or plumules obtained from embryos. In this process of regeneration, the somatic cells undergo dedifferentiation (Feher *et al*., 2003). According to Antonova (2009) the somatic tissues can be used to generate true-to-

type plants. Scientists have been successful in generating callus using somatic embryogenic regeneration pathway in coconut (Pérez-Nuñez *et al*., 2006). According to Feher (2015), the somatic cells present inside the plant have the ability to resume the embryogenic pathway when they are exposed to abiotic stresses like osmotic pressure, salt, high temperature, etc. Auxin is the key player in this pathway as it enhances the formation of new cells and thus helps in callus development (Ikeda-Iwai *et al*., 2002). The auxin (2,4-D) has been found to have profound impact on the generation of morphogenic structures during plumule culture in coconut (Solís-Ramos *et al*., 2012). Thus, the addition of auxin hormone is essential for the initiation of the embryogenic pathway.

There are several genes involved in the downstream processes that have a key role in the embryogenic pathway. Class I *KNOX* (KNOTTED-like homeobox) gene expression has been found to be crucial for somatic embryogenesis. Two KNOX-like genes, *KNOX1* and *KNOX2* were identified in coconut. The *KNOX1* and *KNOX2* genes were expressed in all stages of embryo development. The *KNOX1* gene expression was highest during coleoptilar stage while *KNOX2* gene expression was maximum at globular stage (Montero-Cortés *et al*., 2010b).

In coconut tissues cultured *in vitro*, the expression of *SERK* (Somatic Embryogenesis Receptor Kinase) was linked to the induction of somatic embryogenesis and it may serve as a marker for cells capable of forming somatic embryos (Pérez-Nunez *et al*., 2009). The other genes like *WUS* (Wuschel)*, BBM* (Baby Boom), *LEC* (Leafy Cotyledon) and *PKL* (Pickle) were also expressed during somatic embryogenesis of coconut *in vitro* culture. The *BBM* was expressed maximum during 90 days after inoculation whereas *WUC* and *LEC* were expressed maximum at 120 days after inoculation. The *PKL* gene had highest expression during 15 days after inoculation (Osorio-Montalvo *et al*., 2020).

2.7. Influence of plant growth regulators

Plant growth hormones like auxins, gibberellins, cytokinins and abscisic acid are used as growth promoting substances. Auxins (indole-3-acetic acid (IAA), 2,4-D, etc.) are used for the induction of callus. Adenine derivatives like kinetin, BA, 2-ip, TDZ, zeatin, etc., are cytokinins used in the media. The auxin to cytokinin ratio

plays an important role in the morphogenesis of the explant in the cultures. High auxin to cytokinin ratio leads to callus initiation, embryogenesis and root formation whereas axillary and shoot growth is expected if their ratio is low. The GA3 is the commonly used gibberellin which also enhances callus growth and elongates the plantlets to certain extent. Abscisic acid (ABA) can either have a positive or negative effect on the callus growth depending upon the species of the explant used in the culture (Bhatia, 2015).

The addition of plant growth regulators like auxins, cytokinins and GA3 to the media showed beneficial effects on the culture growth. The addition of NAA in the range of 100-300 µM resulted in the elevation of primary shoot proliferation (Ashburner *et al*., 1993). The supply of GA3 to the media increased the germination of the explant to a higher rate (Montero-Cortes *et al*., 2011). The TDZ has the capacity to fulfil both the cytokinin and auxin requirements of regeneration responses in a variety of woody plants and resulted in better regeneration rates when compared to purine-based cytokinins (Jones *et al*. 2007).

2.8. Gene expression studies under *in vitro* culture

Coconut is known for its recalcitrance to *in vitro* propagation. The knowledge on genes related to morphogenesis or embryogenesis and their relative expression during *in vitro* propagation enlightens us regarding the role of these genes during certain stages of development. The enhancement and regulation of such genes can help us improve the results of clonal propagation.

In 1992, Jesty and Francis used coconut leaf as explant and found out that most of the cells in immature leaf were present in G1 phase of cell cycle and hence, the process of mitosis occurred at a low rate. This kind of accumulation of cells in G1 phase were seen in both *in vitro* as well as *in vivo* conditions of coconut. They suggested that factors responsible for G1/S phase transition may increase the morphogenic capacity of the *in vitro* cultures by improving the mitotic rate in them.

According to Planchais *et al*. (2000) the cell cycle regulation is related to the morphogenetic stages of the cultures. Sandowal *et al*. (2003) studied the cell cycle

regulation in the coconut *in vitro* cultures. According to him around 90 per cent of cells accumulated in G0/G1 phases of cell cycle.

Somatic embryogenesis is most widely studied regeneration pathway in coconut *in vitro* propagation. Some of the genes like *SERK* (Perez-Nunez *et al*., 2009), *CDKA* (Montero-Cortes *et al*., 2010a) and *KNOTTED* like homeobox gene (Montero-Corte *et al*., 2010b) were studied in coconut somatic embryogenesis. The expression of *SERK* gene was seen in the calluses containing meristematic regions. It was discovered that 2,4-D buildup could cause the expression of several genes, including *SERK*, during somatic embryogenesis (Pérez-Nunez *et al*., 2009).

Rajesh *et al*. (2016) identified 14 different genes in West Coast Tall (WCT) cultivar of coconut during transcriptome analysis of various tissues like somatic embryos and plumular and embryogenic calli. They validated the expression patterns of these genes at six different stages utilizing Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR or qRT-PCR). They also studied the interaction of plant growth regulator with the *SERK* gene. They found that the expression of certain genes (namely, *SERK, LEA* (Late Embryogenesis Abundant), *ANT* (Aintegumenta), *AGP* (Arabinogalactan Protein), *MAPK* (Mitogen Activated Protein Kinase), *AP2* (AP2/ERF domain-containing transcription factor), *SAUR* (Small Auxin Up-regulated RNA) and *ECP* (Embryogenic Cell Protein) was higher in callus than the initial cultures. They concluded that *MAPK* had a specified role in auxin mobilization which is an oxidative stress response. Thus, it controls and regulates the cell cycle.

Bhavyashree *et al*. (2016) reported the differential pattern of expression of genes during two different stages of calli (21 days old and 21 weeks old) obtained from shoot meristem explants and observed that genes like *ECP*, *GST* (Glutathione S-Transferase), *LFY* (Leafy) and *WUS* were strongly expressed in long-term embryogenic calli (21 weeks old), while genes like *SERK*, *GLP* (Germin Like Protein), *WRKY* (WRKY transcription factor) and *PKL* were significantly expressed in early embryogenic calli (21 days old). They also found that these genes were expressed higher in WCT than COD (Chowghat Orange Dwarf) at different stages.

The *ANT-like* (Aintegumenta-like) gene isolated by Bandupriya *et al*. (2013) from the embryogenic callus of coconut, had conserved domains similar to the *BABYBOOM* gene (gene which was related to the embryogenesis of *Brassica* species).

The expression of *CDKA* gene and *E2F* gene was studied by Montero-Cortes *et al*. (2011). These genes had a role in the G1/S transition phase and thus in the control of the cell cycle. The expression of *CDKA* gene was higher than *E2F* gene during first eight days of culture initiation. The addition of GA3 to the media increased the expression of the *E2F* gene two days after inoculation. The expression of *E2F1*, *E2F2* and *CDKA* genes varied with the addition of GA3. The GA3 increased the expression of these genes. The higher *CDKA* gene expression was seen with the onset of germination (Montero-Cortes *et al*., 2011).

2.8.1. qRT-PCR

Analyses of gene expression are necessary for many facets of molecular biology research. Understanding the patterns of gene expression in various biological processes offers understanding of intricate regulatory networks (Dussert *et al*., 2013; Tranbarger *et al*., 2011). The qRT-PCR is an effective approach for analysing changes in gene expression with excellent sensitivity, specificity and a wide quantification range (Garson *et al*., 2005; Ginzinger, 2002). The most routine technique for verifying whole-genome microarray or digital expression data generated from second generation sequencing is qRT-PCR analysis (Andersen *et al*., 2004; Tranbarger *et al*., 2011).

Although qRT-PCR analysis is a strong and well-liked technology, the normalisation step, which uses internal reference genes, has a significant impact on the accuracy and reliability of this method. Housekeeping genes (HKGs) are extensively used reference genes in plant molecular biology. These genes have fundamental cellular functions such as the creation of ribosome subunits (rRNA), actin cytoskeleton elements and enzymes in the glycolytic pathway like glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The HKGs are employed in normalisation since it is assumed that they have constant expression levels. However, multiple studies have shown that under various experimental settings, the

expression levels of these genes change significantly (Chandna *et al*. 2012; Czechowski *et al*. 2005; Dekkers *et al*. 2012; Fan *et al*. 2013; Zhu *et al*. 2013). As a result, reference genes must be evaluated and chosen based on their ability to maintain expression in a variety of experimental conditions, as this is required for the precise normalisation of gene expression research (Xia *et al*., 2014).

In order to choose the most consistently expressed reference genes from qPCR data in a specific biological sample, statistical algorithms like geNorm (Vandesompele *et al*., 2002), NormFinder (Andersen *et al*., 2004) and BestKeeper (Pfaffl *et al*., 2004) have been developed. There are several housekeeping genes that are frequently employed, including *ACT* (β-actin), *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase), 18S (18S rRNA) and 25S (25S rRNA), *TEF* (Translation Elongation Factor), *EF1A* (Elongation Factor1-A), *CYP* (Cyclophylin), *UBQ* (Polyubiquitin) and *TUB* (Tubulin) etc., (Dheda *et al*., 2004; Bustin, 2002; Kim *et al*., 2003).

The *α-tubulin* gene was used as endogenous control for the validation of the expression patterns of different genes (*SERK*, *LEA*, *ANT*, *MAPK*, *AP2*, etc.), in different tissues like, somatic embryos and embryogenic and plumular calli (Rajesh *et al*., 2016). The expression of certain genes like *WRKY*, *WUS*, *GLP*, *GST* and *PKL* were higher during somatic embryo stage whereas genes like *SERK*, *SAUR*, *MAPK*, *AP2*, *ANT*, *AGP*, *ECP* and *LEA* genes were expressed more during the embryogenic calli stage. On the other hand, the expression of the internal control gene was stable at all the stages of development. They also found that *MAPK* gene had a specific role related to auxin mobilization. Bhavyashree *et al*., (2016) also used *α-tubulin* gene as the endogenous control for their transcriptome study on different genes in *in vitro* culture of two cultivars, WCT and COD (Chowghat Orange Dwarf). They observed that the expression of genes related to somatic embryogenesis like *SERK, GLP, GST, PKL, WUS* and *ECP* were higher in WCT than COD.

The 18S rRNA gene was used as internal control for the expression study of *CDKA* gene in coconut. The *CDKA* gene expression was greater during embryogenic calli stage rather than mature embryo stage (Montero-Corte´s *et al*.,

2010a). Therefore, *α-tubulin* gene and 18S rRNA gene can be used as reference genes in coconut for gene expression studies *in vitro*.

2.8.2. Transcriptome sequencing

The fundamental dogma of molecular biology describes the progression of information from DNA encoded by genes to RNA that is then translated into proteins (Crick, 1958; Crick, 1970). The phenotype of an organism is the final manifestation of this genetic information altered by environmental circumstances. A cell's identity is determined by the transcription of a subset of its genes into complementary RNA molecules, which also controls the biological processes taking place inside the cell. These RNA molecules, collectively known as the transcriptome, are crucial for deciphering the functional components of the genome and comprehending development (Kukurba and Montgomery, 2015).

Gene content is revealed by genomics, while gene activity in individual cells is shown by transcriptomics (Skol *et al*., 2020). The total amount of RNA that a single cell or a group of cells has produced during a specific biological state is known as the transcriptome. An organism's informational content is encoded in its genome's DNA and expressed through transcription. In this situation, mRNA operates as a temporary intermediary molecule in the information network (Lowe *et al*., 2017).

Wickramasuriya and Dunwell (2015), studied the global transcriptome analysis of somatic embryogenesis (SE) in Arabidopsis. In the study, 426,001,826 sequence reads in total were obtained and 26,520 genes were mapped to the *Arabidopsis thaliana* reference genome. The 1,195 genes were found to have varied levels of expression in embryonic cultures after 5 and 10 days of inoculation, 778 of which had higher levels of expression after 5 days than after 10 days. Additionally, between 10 and 15 days, 1,718 genes showed differential expression in embryonic cells during *in vitro* culture.

Lin *et al*. (2009) studied multiple genes related to SE in Oil palm. It was found that multiple genes previously linked to SE or zygotic embryogenesis in other species are similarly activated during SE in oil palm. Transcripts encoding proteins

that play a role in auxin response and cellular polarisation processes during early development were specifically found.

In the study conducted by Rajesh *et al*. (2016), high-throughput Illumina RNA sequencing derived a global transcriptome from embryogenic calli taken from plumular explants of WCT cultivar during somatic embryogenesis. Transcripts like *GLP*, *GST*, *PKL*, *WUS* and *WRKY* were highly expressed in the somatic embryo stage and in the embryogenic callus stage, *SERK*, *MAPK*, *AP2*, *SAUR*, *ECP*, *AGP*, *LEA* and *ANT* transcripts showed enhanced expression (Rajesh *et al*., 2016).

Bhavyashree *et al*. (2016) also used transcriptome sequencing for the study of genes in *in vitro* culture of two cultivars, WCT and COD. The expression of *SERK, PKL, WUS, GLP, GST* and *ECP* genes was higher in WCT cultivar than in COD. This indicated a higher rate of somatic embryogenesis in WCT when compared to COD cultivar of coconut.

2.8.3. The coconut *E2F* gene

Cell cycle management, a conserved process in eukaryotes, is crucial for controlling how plant cells develop into organs and how they react to environmental and developmental signals. According to reports, the transcription factor family known as E2Fs in mammals is crucial for the G1/S transition and subsequent advancement of the cell cycle. The discovery of nearly all of the main cell cycle homologs in plants, including E2Fs, strongly suggests that plant cell division is regulated similarly to that of other eukaryotes. From many plant species, homologs of the E2F/RB (retino blastoma) regulation pathway similar to mammalian system have been discovered (Montero-Corte´s *et al*., 2011). As a result, E2F proteins promote the expression of *E2F* responsive genes, which are required for the G1/S transition (Black and Azizkhan-Clifford, 1999).

The E2F transcription factors regulate entry into the S phase of the cell cycle by inducing the transcription of genes necessary for cell cycle progression and DNA replication. Only a small number of *E2F* target genes in plants have undergone experimental validation, despite the fact that the E2F pathway is substantially conserved in higher eukaryotes (Vandepoele *et al*., 2005). Montero-Corte´s *et al*.

(2011) proposed that the expression of *E2F2* genes was accelerated by adding GA3 to the medium during coconut embryo culture. Since cell division is vital for initial response of an explant *in vitro*, the expression of *E2F* gene is important during the early response stages.

2.8.4. The coconut *CDKA* gene

Through regulation of the cell cycle in meristematic tissues, proliferative activity is tightly controlled and coordinated during plant morphogenesis (Sugiyama, 1999; Planchais *et al*., 2000). The creation and establishment of the dedifferentiated, focused meristematic cell require the artificial induction and maintenance of cell division. Diverse developmental routes can be followed by dividing cells in cell cultures, such as uncoordinated callus expansion, root and shoot initiation, or somatic embryo formation for the establishment (Feher *et al*., 2003).

The organised action of cyclin-dependent kinases (CDKs) regulates the eukaryotic cell cycle. The cellular control is connected to the *CDKA* gene. The CDKs are activated by the cyclins, positive regulators that bind periodically during the biological cycle and control the stability and activity of cell division partners (Roudier *et al*., 2000). Numerous CDK-related genes are found in plants, that are engaged in various stages of the cellular cycle (Burssens *et al*., 1998). The plant *CDKA* has a dual role in cell proliferation and the preservation of cell division competence in differentiated tissues throughout plant development (Martinez *et al*., 1992; Hemerly *et al*., 1993), as well as during the progression of both the S and M phases (Colasanti *et al*., 1991; Ferreira *et al*., 1991; Hirt *et al*., 1993).

Montero-Corte´s *et al*. (2010a) characterized the *CDKA* gene from the *in vitro* coconut embryogenic tissues. The *CDKA* gene expressed higher during embryogenic calli stage rather than later stages of somatic embryo formation. Thus, *CDKA* gene has an important role in the cell cycle control and is therefore expressed higher in the actively dividing cells in the early development stages when compared to matured stage (Montero-Corte´s *et al*., 2010a). The expression of *CDKA* gene was found to be higher during germination. The addition of GA3 growth regulator enhanced the expression of this gene when compared to control during embryo culture (Montero-Corte´s *et al*., 2011).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Agriculture, Kerala Agriculture University (KAU), Vellanikkara, Thrissur provided the resources and facilities necessary for the research project entitled "Expression profiling of *E2F* and *CDKA* genes during embryo culture in coconut (*Cocos nucifera* L.)". The materials used for the study and the research procedures are described in depth in this chapter.

3.1. Materials

3.1.1. Explant for tissue culture

Mature coconuts (10-11 months old) of the variety West Coast Tall (WCT) were procured from the Department of Plantation Crops and Spices, College of Agriculture, Vellanikkara. Embryos obtained from the nuts were the explants for initiating *in vitro* culture.

3.1.2. Media components

Major and minor nutrients, vitamins, plant growth regulators, activated charcoal and sucrose were all purchased from M/S Sigma-Aldrich Chemicals Pvt. Ltd. and Himedia Laboratories Pvt. Ltd. They were of tissue culture grade purity. Clerigel, a gelling agent from Himedia Laboratories Pvt. Ltd., was used for the media.

3.1.3. Glassware

For the tissue culture of coconut, reusable, medium-sized test tubes made of borosil were employed. Stock solutions and sterile water were prepared and stored in jam bottles with polypropylene closures. For the media preparation, borosil measuring cylinders and conical flasks were employed.

3.1.4. Equipment for tissue culture experiments

The refrigerator, electric oven, autoclave, distillation assembly, analytical electronic balance, pH metre, laminar air flow cabinet and other laboratory equipment available at CPBMB were used.

3.1.5. Laboratory chemicals and equipment required for gene expression studies

The present research was carried out using pure and approved chemicals that were purchased from Sigma Aldrich Pvt. Ltd. The chemicals, such as DEPC (diethyl pyrocarbonate), RNA Later solution and RNase ZAP were purchased from Sigma Aldrich Pvt. Ltd. The 2x RNA loading dye, the Riboruler RNA low range ladder, the RevertAid first strand cDNA synthesis kit and the RNeasy plant mini kit were brought from ThermofisherScientific™. The SsoAdvanced Universal SYBR Green Supermix used for qRT-PCR analysis was purchased from Bio-Rad Laboratories. The dNTP mix, *Taq* DNA polymerase and *Taq* buffer used in the PCR were procured from GeNei Labs Pvt. Ltd. The PCR grade water was purchased from HiMedia. The study also made use of the StepUp™100 bp DNA ladder from GeNei Labs Pvt. Ltd. and the Biolit ProxiO100 bp DNA ladder from SRL Chemicals Pvt. Ltd.

The equipment used in the study, included electronic balance (Shimadzu), icematic (F 100 compact), fume hood (CAFH 1500), high-speed refrigerated centrifuge (KUBOTA 3500, Japan and Eppendorf), microcentrifuge (SPINWINTM MC-100, SPINWINTM MC-01), micropipettes (Eppendorf) and pH metre (EUTech instrument pH tutor). BioRad thermal cycler was used for the PCR reactions. Nucleic acids were quantified and their purity was checked using a NanoDrop spectrophotometer (IMPLEN NanoPhotometer® NP80), BioRad Gel DocTM XR+ and gel electrophoresis equipment. The StepOnePlus Real-Time PCR machine (Applied Biosystems) at the Kerala Veterinary and Animal Sciences University at Mannuthy, Thrissur, Kerala was used for the qRT-PCR experiment and glassware were purchased from Borosil, while the plastic goods were procured from Tarsons India Ltd.

3.2. Methods

3.2.1. Sterilization of Glasswares

The borosil glasswares were cleaned initially by soaking in potassium dichromate solution for 12 hours. They were thoroughly cleaned in tap water to eliminate all traces of the potassium dichromate solution. They were then thoroughly washed in tap water after being further cleaned with soap solution. The tubes were cleaned and then dried in hot air oven at 60 °C. Glasswares that had been dried were autoclaved at 121 °C for 15 minutes. Later, these were stored away from dirt and contaminants.

3.2.2. Preparation of culture media

The media used for the culture of coconut embryos was modified Y3 media (Eeuwens, 1978). The constituents of the media included the inorganic components, organic components and plant growth regulators like auxin (2,4-D), cytokinin (BA and TDZ) and gibberellic acid (GA3). The other components like sucrose, activated charcoal, clerigel and inositol were also used in the media.

3.2.2.1. Preparation of stock solutions for Y3 media

The constituents of Y3 media were prepared as five different stock solutions as mentioned in Table 3.1. The stock solutions were prepared in sterile distilled water.

Table 3.1. Composition of the basal Y3 media for coconut embryo culture

Stock solutions	Quantity of stock for 1L media (mL)	Reagents	Amount (gL^{-1})
I (50X)	20	KNO_3	50.5
		KCl	37.3
		NH_4Cl	13.38
		$NaH_2PO_4.2H_2O$	7.8
		$MgSO_4.7H_2O$	6.175
II (50X)	20	$CaCl_2.2H_2O$	7.35
III (100X)	10	Na_2EDTA	1.86
		$FeSO_4.7H_2O$	1.39
IV (100X)	10	$MnSO_4.4H_2O$	2.24
		KI	1.66
		$ZnSO_4.7H_2O$	1.44
		H_3BO_3	0.62
		$CoCl_2.6H_2O$	0.048
		$CuSO_4.5H_2O$	0.032
		$NiCl_2.6H_2O$	0.0048
V (100X)	10	Thiamine HCl	0.05
		Pyridoxime HCl	0.005
		CaPanthothenate	0.005
		Nicotinic acid	0.005
		Biotin	0.005

The macronutrients, KNO_3, NH_4Cl, KCl, $NaH_2PO_4.2H_2O$ and $MgSO_4.7H_2O$ constituted Stock I. Using a volumetric flask and an analytical electronic balance, the necessary portions were separately measured, dissolved and the volume was made up with sterile water.

The $CaCl_2.2H_2O$ was prepared separately as Stock II because of the possibility of precipitation of the chemical.

The Stock III was iron stock. Hot sterile water was used to make Stock III. The $FeSO_4.7H_2O$ and Na_2EDTA, two stock ingredients, were individually dissolved in hot water. The Na_2EDTA solution was made up by adding the solution of $FeSO_4.7H_2O$. Due to its strong photo-oxidativeness, the stock was kept in amber-coloured bottles.

Micronutrients and vitamins were included in Stocks IV and V, respectively. The stock solutions were stored at 4 °C and brought back to room temperature before preparing the media.

3.2.2.2. Preparation of growth regulators

Growth regulators used in the media included auxins (2,4-D), cytokinins (BA and TDZ) and gibberellic acid (GA3). The 1000 ppm (1000 mgL^{-1}) stock solutions were prepared for each growth regulator. The 100 mg of 2,4-D and BA each were first dissolved in a few drops of 1N NaOH solvent. Then the volume was made up to 100 mL with sterile water once the substance has completely dissolved. Similarly, TDZ stock was also prepared. But in the preparation of gibberellic acid stock solution, solvent was not used as the chemical dissolved directly in the sterile water. One mL from the 1000 $mgmL^{-1}$ stock was added to the media to get a concentration of 1 mgL^{-1}. Stocks were stored at 4 °C.

3.2.2.3. Preparation of basal Y3 media

The 500 mL of sterile water was taken in a beaker. Then, 20 mL, 20 mL, 10 mL, 10 mL and 10 mL each were pipetted from Stock I to Stock V respectively. Sucrose (60 gL^{-1}) and myoinositol (0.1 gL^{-1}) were added to the solution and mixed well until they dissolved completely. The required amount of plant growth regulators was added. The pH of the media was adjusted to 6.2 using 1N NaOH and the volume was made up to 1L using distilled water. After adding gelrite (2.0 gL^{-1}) and activated charcoal (1.0 gL^{-1}), the media was heated while being constantly stirred to homogenise its contents. All of the culture media was poured into a conical flask and then autoclaved for 20 minutes at 121 °C and 15 psi.

The growth regulators like GA3 and TDZ are heat labile; so, they were added to the media after autoclaving. The addition of 1 gL^{-1} cephotaxim, an

antibacterial agent was practiced as a precaution to avoid bacterial contamination during the culture establishment. Since, cephotaxim is also thermolabile it was added to the media after being autoclaved similar to GA3 and TDZ.

After autoclaving, the media was taken to the laminar airflow cabinet and was allowed to cool to a temperature of around 50 °C. The plant growth regulators (GA3 and TDZ) and cephotaxim were added to the media separately through filter sterilisation and 0.22 µm sized filters were used for this purpose. The autoclaved media was dispensed into test tubes only after the addition of growth regulators (GA3 and TDZ) and cephotaxim into it. The test tubes were then plugged with non-absorbent cotton (to avoid the absorption of the moisture from the external environment).

3.2.2.4. Media combinations used for coconut embryo culture

The different combinations of the culture media used in the present study are detailed below in Table 3.2.

Table 3.2. Different media combinations

S.no	Media	2,4-D ($2\ mgL^{-1}$)	BA ($5\ mgL^{-1}$)	GA3 ($1.6\ mgL^{-1}$)	TDZ ($33\ mgL^{-1}$)
1	Basal	-	-	-	-
2	A	✓	✓	-	-
3	B	✓	✓	✓	-
4	C	-	-	✓	-
5	D	-	-	-	✓

3.2.3. Establishment of coconut embryo culture

3.2.3.1. Preparation and surface sterilisation of the explant

The mature coconuts of the variety West Coast Tall (WCT) were collected as per the requirement. The embryo plugs were scooped out from the broken half of the coconut using the plug remover. The embryo plugs were collected in an autoclaved jam bottle (Plate 3.1).

3.2.3.1.1. Sterilization outside laminar airflow cabinet

The embryo plugs were washed thoroughly with tap water twice. They were treated with fungicide (0.2 % bavistin) for five minutes. Then they were washed with tap water to remove the traces of the chemical. The embryo plugs were then washed with Tween 20 (detergent) and thoroughly rinsed with tap water to remove the traces of Tween 20. The embryo plugs were treated with 1 per cent sodium hypochlorite solution for five minutes. Then, they were immersed in 70 per cent ethanol for three minutes. After that, they were treated with 2.5 per cent sodium hypochlorite solution for twenty minutes. After the stipulated time, they were thoroughly washed with sterile water thrice.

3.2.3.1.2. Sterilization inside laminar airflow cabinet

Before beginning further surface sterilisation treatments, the laminar airflow cabinet was set with autoclaved plates, forceps, blades, sterile water, tissue paper and filter paper required for the further processes. The UV was turned on for 20 minutes to assure full disinfection. Hands were thoroughly cleaned and wiped with 70 per cent alcohol before proceeding with the next steps. The hood was later sterilised with 70 per cent alcohol and all the plates, forceps and blades were flame sterilised with 70 per cent alcohol.

Coconut embryo plugs were treated again with 70 per cent ethanol for three minutes. Then, they were treated with 2 per cent sodium hypochlorite solution for twenty minutes. They were thoroughly rinsed with sterile water thrice to remove all the traces of the chemical. The coconut embryo plugs were kept on filter paper and the embryo was separated by removing the endosperm tissue with scalpel blade.

After removing the whole embryo from the plugs, they were taken in a jam bottle and treated with 0.6 per cent sodium hypochlorite solution for ten minutes. They were thoroughly washed with sterile water thrice to remove all the traces of the chemical. Finally, the explant was treated with antioxidants like citric acid (0.1 %) and ascorbic acid (0.15 %) solution for ten minutes. Then the explant was dried completely by placing on sterile tissue paper before inoculation.

3.2.3.2. Inoculation

The forceps and blade used during inoculation were routinely flamed by soaking them in 70 per cent alcohol and cooling them before use. The explant (embryos) which were previously surface sterilised and dried were inoculated vertically into the culture medium (Plate 3.2).

The inoculated cultures were kept in culture room in dark provided with the temperature of 28 °C.

3.2.3.3. Subculturing

Once in a month the embryos were transferred to a fresh tube containing the same previous media in order to reduce the polyphenol effect, to avoid the drying of the media and to ensure that all media components are provided sufficiently to the embryos. The unwanted brown portion was removed to reduce the interference of polyphenols (Plate 3.3). The sprout formed was not cut immediately, it was kept as such for two to three months for its growth. Once the sprout grew long it was cut and the embryo was split into two. These split portions were inoculated separately in two different tubes containing the media same as initial stages (Plate 3.4)

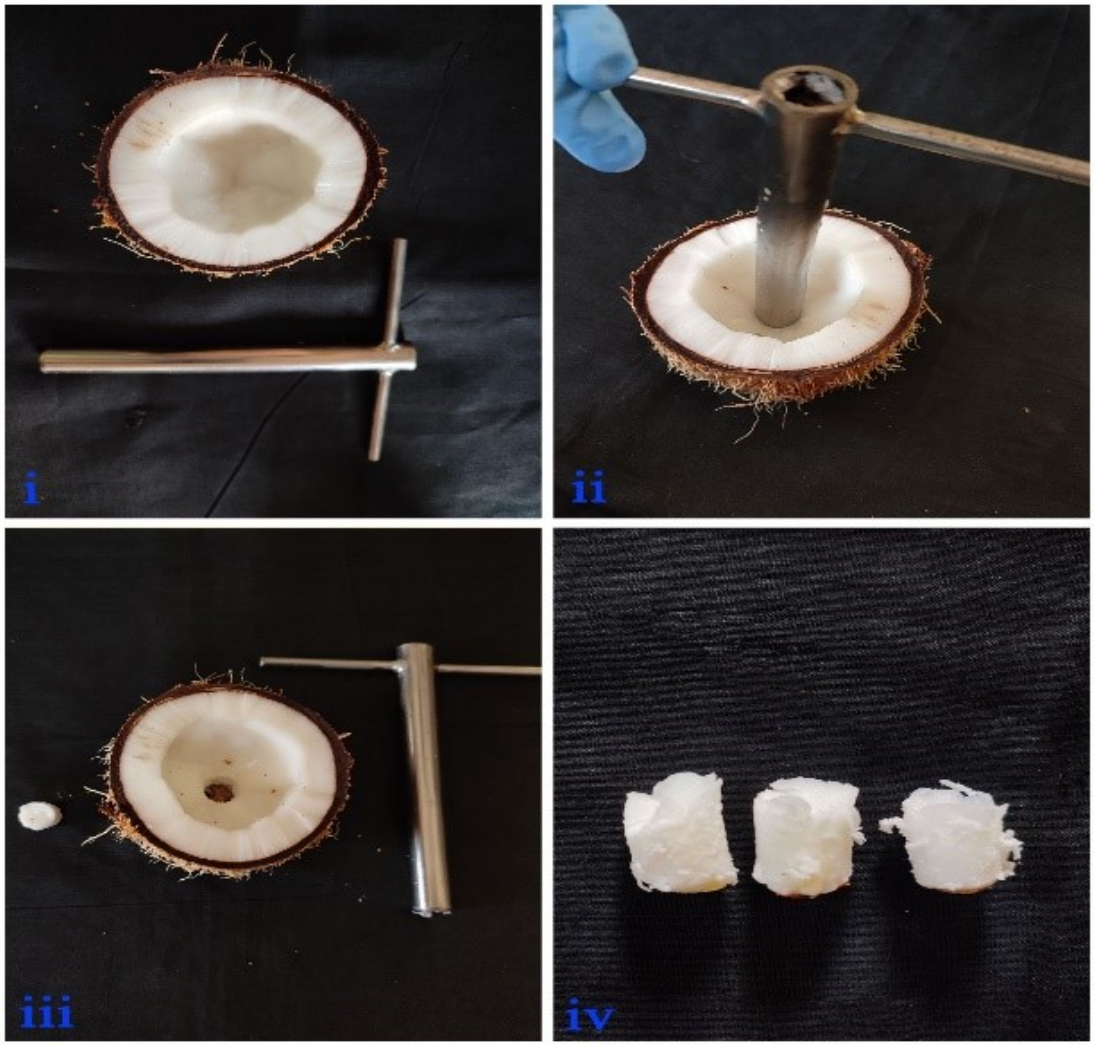

Plate 3.1. Removal of embryo plugs from coconut

i- Broken coconut along with plug remover; ii- Removal of coconut embryo plug using plug remover; iii- Coconut after the removal of embryo plug;
iv- Embryo plugs

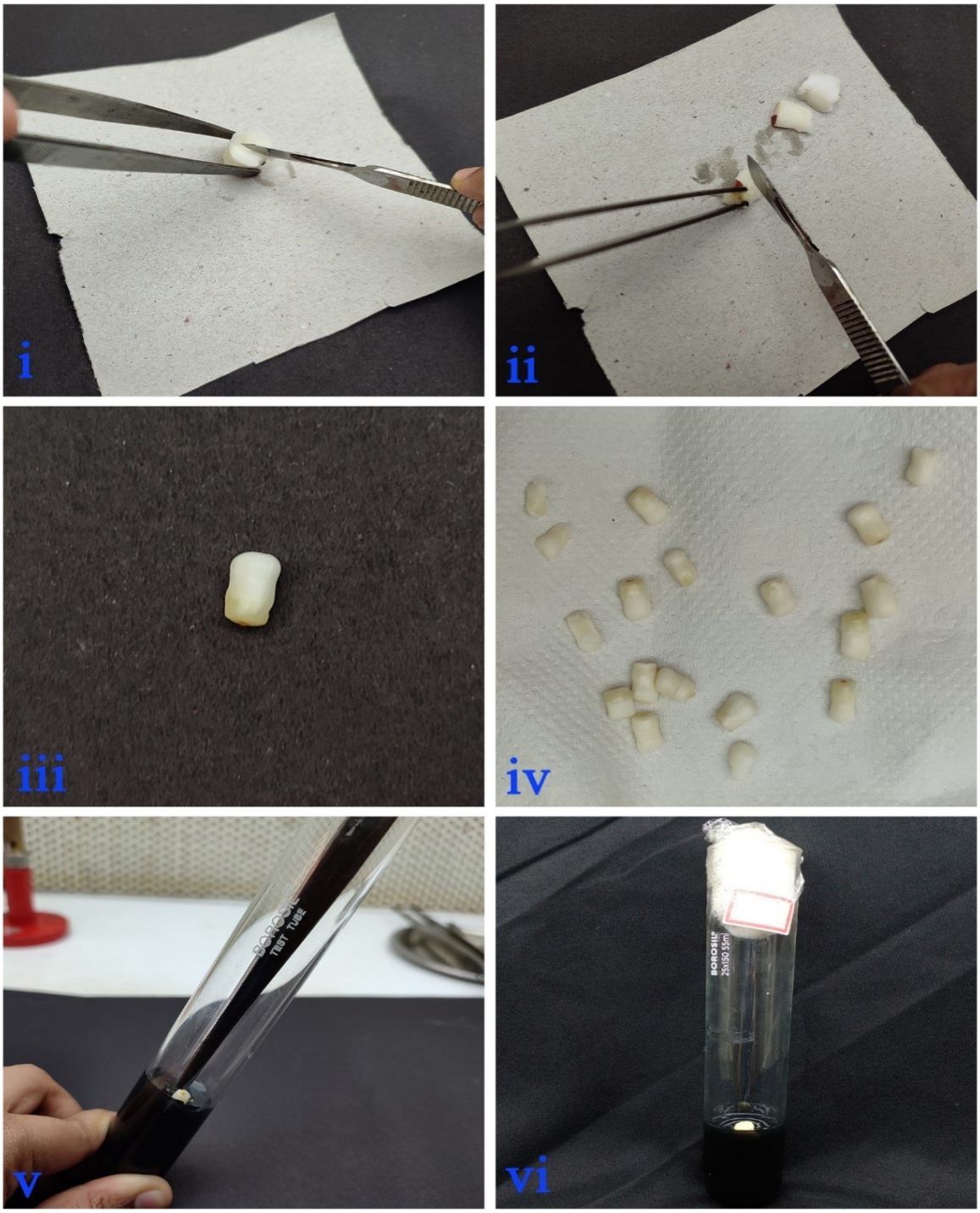

Plate 3.2. Separation and inoculation of coconut embryo

i and ii- Removal of embryo from the embryo plug; iii and iv- Coconut embryos;

v- Inoculation of embryo; vi- Inoculated tube

Plate 3.3. Transferring of the embryo to fresh media

i- Removal of media and brown portion surrounding the embryo; ii- Embryo after removal of surrounding portion; iii- Inoculation of embryo into fresh medium

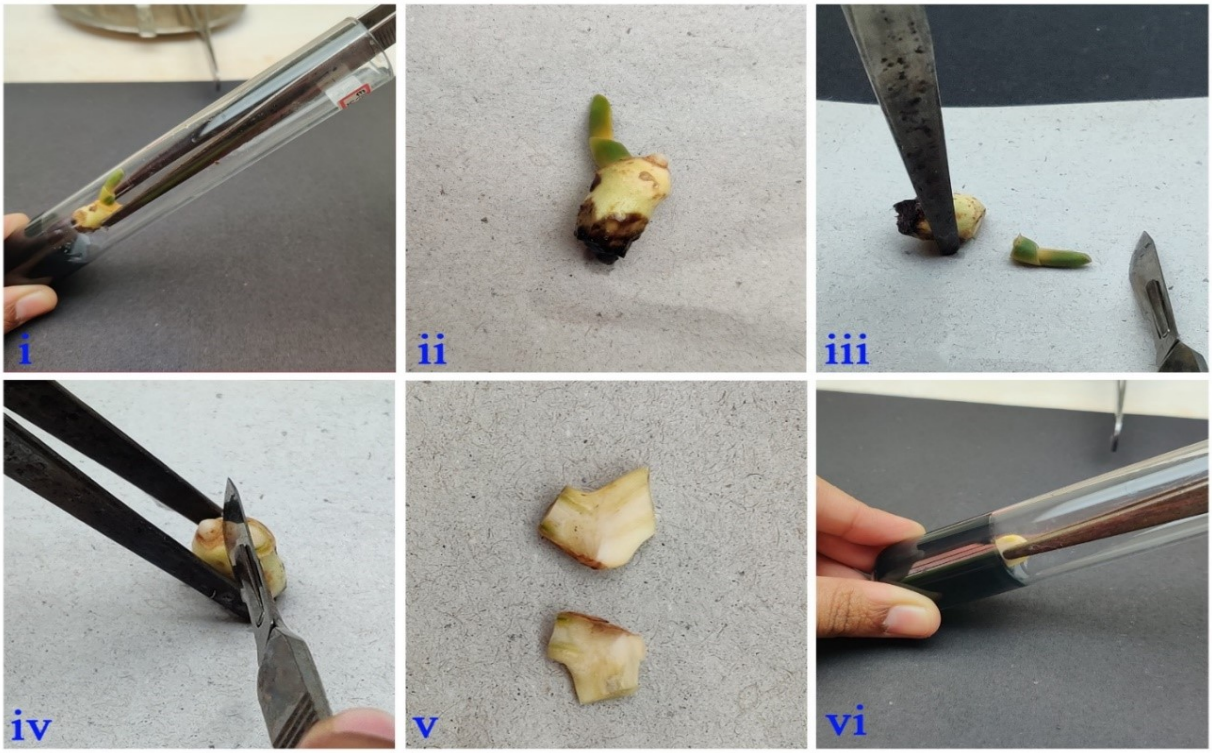

Plate 3.4. Subculturing of the embryo

i- Removal of the embryo with sprout; ii- The embryo with sprout; iii- Removal of sprout from the embryo; iv- Splitting the embryo base into two; v- The split embryo; vi- Inoculation of each split portion into the fresh medium

3.3. Expression profiling of *E2F* and *CDKA* genes in coconut embryo culture

Expression profiling of *E2F* and *CDKA* genes was done during early stages of *in vitro* response in coconut. The stages selected were: 5 days after inoculation (DAI), 10 DAI and 20 DAI. For each selected stage, three embryos (biological replicates) were sampled from each media combination for RNA isolation.

3.3.1. Primer designing

Primers were designed to study expression of *E2F* and *CDKA* genes. The α-*tubulin* and 18S rRNA genes were used as reference genes. Primers were designed for each of the test and control genes using Primer3 software (https://primer3.ut.ee/). The available sequence information at NCBI was used as a source for primer designing. Primers were synthesized by Sigma-Aldrich Pvt. Ltd.

3.3.2. Isolation of RNA

Three different stages of cultured coconut embryos were utilised for RNA isolation and gene expression study. They were,

a. 5 days after inoculation (5 DAI)

b. 10 days after inoculation (10 DAI)

c. 20 days after inoculation (20 DAI)

3.3.2.1. General precautions taken before RNA isolation

The substances necessary for the RNA isolation like microtips (10 µL, 100 µL, 200 µL and 1000 µL), 1.5 mL microcentrifuge tubes, mortar and pestle were treated with 0.1 per cent DEPC overnight. They were double autoclaved after the overnight treatment and dried in hot air oven before use. The distilled water was also treated with 0.1 per cent DEPC overnight and double autoclaved before use. The solutions like 75 per cent ethanol and 50X TAE buffer were prepared using DEPC treated double autoclaved water. The working area and all the things necessary for the isolation like pipettes, spatula and forceps were wiped with 75 per cent ethanol and RNase ZAP solution before use. The gel electrophoresis unit including the gel

tank, gel tray and comb were also thoroughly wiped with 75 per cent ethanol and RNase ZAP solution before use. All the procedures related to the isolation were performed carefully wearing the hand gloves (wiped with 75 % ethanol and RNase ZAP solution) to prevent RNase action.

3.3.2.2. Extraction of total RNA

The extraction of RNA from the coconut embryos was done using RNeasy plant mini kit from Qiagen. The manufacturer's instructions in the protocol were followed.

3.3.2.2.1. Reagents required

- RLC buffer, RW1 buffer, RPE buffer and RNase free water (available in the kit)

- β-mercaptoethanol, 100 per cent ethanol, liquid N_2 and RNase ZAP

3.3.2.2.2. Procedure

3.3.2.2.2.1. Measures taken before RNA isolation:

- RLC buffer (450 µL) was taken in a 1.5 mL microcentrifuge tube. To this, 10 µL of β-mercaptoethanol was added and mixed immediately using a pipette in a fume hood.

- The required quantity of 100 per cent ethanol was added to the RPE buffer as mentioned in the manufacturer's protocol before its usage. This process was done at the beginning of the use of the kit.

- The centrifuge was set to 4 $^{\circ}$C for pre-cooling.

3.3.2.2.2.2. RNA Isolation protocol

- The sample (100 mg) necessary for the isolation was collected.

- The pre-chilled mortar and pestle were wiped with 75 per cent ethanol and RNase ZAP solution.

- The required quantity of the liquid N_2 was taken in a pre-chilled mortar and pestle. Using that the embryo was ground into fine powder. Immediately, the ground sample was transferred to the 1.5 mL microcentrifuge tube (containing RLC buffer 450 µL and β-mercaptoethanol 10 µL) using a spatula.

- The contents were vortexed vigorously until they were mixed properly.

- The sample was centrifuged at 5000 g for 2 minutes. The lysate was transferred to the QIAshredder spin (lilac) column.

- The QIAshredder spin column was centrifuged at 12000 g for 2 minutes. The supernatant of the flow-through was transferred to a new microcentrifuge tube (1.5 mL) without disturbing the cell-debris pellet.

- To this, 0.5 volume of 100 per cent ethanol was then immediately pipetted.

- All the contents were added to the RNeasy Mini spin column (pink) and centrifuged for 1 minute at 12000 g. The flow-through was discarded and the RNeasy Mini spin column was placed back after the collection tube was dried on the tissue paper for few seconds.

- Then, 700 µL of the RW1 buffer was added to the column and it was centrifuged for 1 minute at 12000 g. The flow-through was discarded and the RNeasy Mini spin column was placed back after the collection tube was dried on the tissue paper for few seconds.

- The 500 µL of the RPE buffer was added to the column and it was centrifuged for 1 minute at 12000 g. The flow-through was discarded and the RNeasy Mini spin column was placed back after the collection tube was dried on the tissue paper for few seconds.

- The 500 µL of the RPE buffer was added to the column and it was centrifuged for 3 minutes at 12000 g. The column was placed in a new collection tube and it was centrifuged for 1 minute at 12000 g.

- The RNeasy Mini spin column was placed in a new 1.5 mL tube (provided in the kit) and 30 µL of RNase-free water was added directly to the column. The lid of the tube was closed and it was kept aside for 1-2 minutes without disturbing. Later, it was centrifuged for 2 minutes at 12000 g.

- The RNA sample was stored in -80 °C.

3.3.3. Total RNA analysis

An agarose gel electrophoresis was used to assess the quality of the isolated RNA. The RNA was quantified and purity was checked using a NanoDrop spectrophotometer.

3.3.3.1. Quality analysis using Agarose gel electrophoresis

Agarose gel electrophoresis (1.2 %) was used to evaluate the quality of the total RNA that was extracted.

3.3.3.1.1. Reagents required

- Agarose, ethidium bromide, 50X TAE buffer prepared in DEPC treated water, DEPC treated water, 100 bp DNA ladder and RNA loading dye

3.3.3.1.2. Procedure

- The 1.2 per cent gel was prepared in the gel tray. The gel casting tray was inserted into the gel caster with the sides closed, the tray and comb were then cleaned with 70 per cent ethanol and RNase ZAP. The comb was kept in the gel tray about an inch from one end and held vertically so that the teeth are about 1-2 mm above the tray's surface.

- The 0.48 g of agarose was added to conical flask containing 40 mL of 1X TAE buffer to prepare 1.2 per cent agarose gel. Until the agarose has been dissolved completely and the solution has become clear, it was boiled in a microwave. Before pouring, the solution was allowed to cool (between 42 °C and 45 °C) and at this time, ethidium bromide at a concentration of 0.5 μgmL^{-1} was added. This warm gel

mixture was poured into the gel tray. The gel was kept aside (30 to 45 minutes) for solidification at room temperature.

- After the gel had solidified, the comb was carefully removed and the gel tray was kept in the electrophoresis tank with 1X TAE buffer. The gel tray was placed in the gel tank in such a way that the wells were positioned at the cathode (negative electrode) end.

- The DNA ladder and RNA loading dye were taken in a minicooler and thawed before loading. In the first well 3 µL of the 100 bp DNA ladder (along with dye) was loaded. In the subsequent wells, RNA samples (3 µL) were loaded after being blended with RNA loading dye (3 µL).

- The gel tank's cover was closed and the electrical wires were connected. The gel was run at 80V until the bromophenol blue and xylene cyanol migrated three fourth distance. After the run was complete, the current was turned off and the gel was observed using gel documentation system (BioRadGelDoc^{TM}XR system using Quantity One software).

3.3.3.2. RNA quantity and quality analysis using a nanodrop spectrophotometer

Utilizing a NanoDrop spectrophotometer (IMPLEN NanoPhotometer® NP80), the isolated RNA was subjected to quality analysis and quantification. Purity of the sample was assessed using the A_{260}/A_{280} ratio because proteins and nucleic acids have absorption maxima at 260 nm and 280 nm, respectively. For an RNA sample solution containing 40 μgmL^{-1}, the absorbance at optical density 260 nm equals 1.0.

The pedestal and sampling arm were thoroughly cleaned using nuclease-free water and wiped with tissue paper before loading the blank and sample. The spectrophotometer was set according to the guidelines given by manufacturer. The 1.0 µL of nuclease-free water was used to set the blank and 1.0 µL of the RNA sample was added for quality and quantity analysis.

3.3.4. First strand cDNA synthesis

The RevertAid first strand cDNA synthesis kit (Thermofischer) was used to synthesize the first strand of cDNA using the RNA sample as the template. The manufacturer's protocol for the reaction was followed.

3.3.4.1. Reagents required

- 5X reaction buffer, Oligo (dT) primer, 10 mM dNTP mix, RevertAid M-MuLV RT (200 U/µL), Ribolock RNase inhibitor (20 U/µL)

3.3.4.2. Procedure for cDNA conversion

- The kit's components were briefly centrifuged at 3000 g for 30 seconds and were kept on ice after thawing.
- The 20 µL reaction was prepared in a sterile, nuclease-free, 0.2 mL microcentrifuge tube placed on ice:

i. 4.0 µL of template RNA (2 µg)

ii. 1.0 µL of Oligo (dT) primer

iii. Nuclease-free water to make the total volume to 12.0 µL

- The quantity of water was adjusted according to the template volume.
- The contents of the tube were then mixed properly using pipette and were briefly centrifuged. The tube was incubated at 65 °C for 5 minutes and then kept back on ice
- The following components were added to the tube in the order listed below:

i. 4.0 µL of 5X reaction buffer

ii. 1.0 µL of Ribolock RNAase inhibitor

iii. 2.0 µL of 10 mM dNTP mix

iv. 1.0 µL of RevertAid M-Mul VRT

- The contents of the tube were gently mixed and briefly centrifuged. The tube was incubated at 42 °C for 60 minutes for reverse transcription and were kept back on ice after enzyme inactivation at 70 °C for 5 minutes.

- The cDNA sample was stored in -20 °C.

3.3.5. Reverse Transcription Polymerase Chain Reaction

3.3.5.1. Reagents required for PCR amplification

- 10X *Taq* buffer A (with 1.5 mM $MgCl_2$), dNTP mix (2.5 mM each), forward and reverse primers and *Taq* DNA polymerase

3.3.5.2. Procedure for PCR amplification

- The gradient PCR was followed with annealing temperature ranging in between 55-65 °C.

- Master mix was prepared according to the requirement. The components added in the master mix are shown in Table 3.3. The cDNA template was added to the respective 0.2 mL microcentrifuge tubes after the addition of aliquots of the master mix.

- The reaction mixture was mixed and briefly spun down for uniform mixture of the components and the following program was set in the thermocycler (Table 3.4).

Table 3.3. Constituents of PCR reaction mixture

Constituents	Quantity (µL)
10X *Taq* buffer A (with 1.5 mM $MgCl_2$)	1.5
dNTP mix (2.5 mM each)	1.5
Forward primer	1.0
Reverse primer	1.0
Taq DNA polymerase	0.5
Template (cDNA)	2.0
Nuclease free water	7.5
Total	**15**

Table 3.4. Thermal profile for gradient PCR

Steps	Temperature (˚C)	Time	No. of cycles
Initial denaturation	95	4 minutes	1
Denaturation	95	30 seconds	35
Annealing	55-65	30 seconds	35
Extension	72	30 seconds	35
Final extension	72	8 minutes	1

3.3.6. Analysis of PCR amplicons

3.3.6.1. Quality analysis using Agarose gel electrophoresis

The PCR amplicons were analysed on 1.2 per cent agarose gel. The 50X TAE was prepared using distilled water. The DNA gel loading dye and 100 bp DNA ladder was used. The gel electrophoresis was performed as mentioned in section 3.3.3.1.2.

3.3.6.2. Gel purification of amplicons

The PCR amplicons of the desired size were cut from the gel and eluted using the NovagenSpinPrep^{TM} Gel DNA kit as per the manufacturer's protocol.

3.3.6.2.1. Reagents required

- SpinPrep Wash buffer (B), SpinPrep Elute buffer (C) and SpinPrepGelMelt^{TM} solution (A)

3.3.6.2.2. Procedure

- The gel was placed on a long-wavelength UV light box and the desired DNA band was sliced with the help of a clean scalpel. The sliced portion of the gel was taken in a pre-weighed 2 mL tube and its weight was recorded.

- The 300 µL of SpinPrepGelMelt Solution (A) was added (per 100 mg of gel slice) and the contents were vortexed briefly. The tube was kept in water bath at 50 $^{\circ}$C for 10 minutes (tube was inverted once in 2 minutes).

- The completely dissolved gel solution was transferred to SpinPrep filter kept in a 2 mL receiver tube. The contents were centrifuged for 1 minute at 10000 rpm (room temperature). The 700 µL of solution was transferred at a time and the flow-through was discarded. The procedure was repeated until the entire gel solution was passed through the filter.

- The 400 µL of fresh SpinPrepGelMelt solution (A) was added to the filter and contents were centrifuged for 1 minute at 10000 rpm. The flow-through was discarded.

- The 650 µL of SpinPrep Wash Buffer (B) plus ethanol was added. The tube was centrifuged for 1 minute at 10000 rpm. The flow-through was discarded and it was centrifuged additionally for 2 minutes at 10000 rpm to remove the excess SpinPrep Wash Buffer (B) plus ethanol.

- The SpinPrep filter was transferred to a new 1.5 mL Eluate Receiver Tube.

- The 22 µL of nuclease free water was added to the filter and incubated at 50°C for 3 minutes. The contents were centrifuged at 10000 rpm for 1 minute at room temperature. The filter was discarded.

- The eluted PCR amplicon was stored in -20 °C.

The final eluted product was checked on 1 per cent agarose gel by electrophoresis. Here, 3 µL of the sample was loaded in the gel.

3.3.7. Sequencing of the PCR amplicons

The eluted PCR amplicons were sequenced at AgriGenome Pvt. Ltd., Kochi. Sequencing was done using the respective forward primers.

3.3.7.1. Similarity search

The similarity search was done using BLASTN. The sequences were searched against the NCBI NR database.

3.3.8. Expression profiling of *E2F* and *CDKA* genes

The qRT-PCR was performed to study the expression pattern of the *E2F* and *CDKA* genes during early stages of *in vitro* response of coconut embryos. The α-*tubulin* and 18S rRNA genes were used as endogenous controls. Embryo samples from four treatments (plant growth regulator combinations) and one control (basal medium) were taken for study. For each treatment, we selected three stages 5 DAI, 10 DAI and 20 DAI. There were three biological replicates and three each technical replicates for each stage and treatment. The SYBR Green real time PCR chemistry was used for the study. The StepOne Plus Real-Time PCR system at Kerala Veterinary and Animal Sciences University at Mannuthy, Thrissur, was used for the experiment. The SsoAdvanced Universal SYBR Green Supermix was used for the reaction. The reaction mixture and the thermal profile for qRT-PCR are mentioned in Table 3.5 and Table 3.6 respectively.

Table 3.5. Components of qRT-PCR reaction

Components	Quantity (µL)
SsoAdvanced™Universal SYBR® Green Supermix 2x	10.0
Forward primer	1.0
Reverse primer	1.0
Template (DNA)	5.0
Nuclease free water	3.0
Total	**20.0**

Table 3.6. Thermal profile for qRT-PCR

Steps	Temperature (°C)	Time (seconds)	No. of cycles
Initial denaturation	95	30	
Denaturation	95	15	40
Annealing	60	30	
Extension	72	30	
Melt curve analysis	95	15	
	60	60	
	95	15	

The melt curve analysis was performed at the end of the reaction. This was done for the confirmation of fidelity of the amplicons. The relative expression of *E2F* and *CDKA* genes was normalized with the expression of endogenous controls α-*tubulin* and 18S rRNA genes.

The fold change in expression was calculated using the method of geometric averaging of Ct (cycle threshold) values proposed by Vandesompele *et al.* (2002) and Hellemans *et al.* (2007). The SYBR Green fluorescence data gathered after the run were utilised to create an amplification plot for each reaction and the Ct values were obtained directly from the machine. The average obtained from three technical replicates was used to calculate the mean Ct value for each gene in each sample. The relative expression values of target genes and reference genes were calculated using the following equation as a relative quantity (RQ),

$$RQ = 2^{-\Delta Ct}; \text{ where } \Delta Ct = \text{Mean Ct (Treated)} - \text{Mean Ct (Control)}$$

The α-*tubulin* and 18S rRNA, two reference genes, were used to normalise RQ for target genes. By dividing the RQ of the target genes by the geometric mean of the RQ of the reference genes, normalised relative quantities (NRQ) were computed.

$$NRQ = \frac{RQ\ (Target)}{\sqrt{[RQ\ (\alpha\text{-}tubulin) \times RQ\ (18S\ rRNA)]}}$$

The mean NRQ values for three biological replicates for each treatment were calculated. Then, the scale values were calculated for each biological replicate in each treatment taking control as the base value. Finally, the graphs were plotted with the average of the relative expression values (scale values) of three biological replicates. Then, the standard deviation of expression values was calculated for each sample and with the standard deviation values, standard error of the mean (SEM) was calculated using the formula given below,

$$SEM = SD/\sqrt{\text{sample size}}$$

where, sample size is the number of biological replicates

The error bar in the graph was added using SEM values obtained.

RESULTS

4. RESULTS

The results of the study on "Expression profiling of *E2F* and *CDKA* genes during embryo culture in coconut (*Cocos nucifera* L.)" conducted during 2021-2023 are presented in this chapter.

4.1. Selection of explant

The coconut variety WCT (West Coast Tall) was selected for the study. The mature nuts, 10-12 months old, with sufficient amount of water were procured from the Department of Plantation Crops and Spices, College of Agriculture, Vellanikkara and the embryos were carefully extracted, surface sterilized and inoculated in Y3 media supplemented with growth regulators.

4.2. *In vitro* response in coconut

The embryos were inoculated on modified Y3 medium with four different combinations of growth regulators including 2,4-D, BA, GA3 and TDZ. The basal Y3 medium was the control. The initial response obtained during the embryo culture was bulging of the embryos and formation of sprout. The embryo response in the different media combinations used in the present study are described below.

4.2.1. Effect of basal Y3 medium on the embryo

The size of the embryo at 5 days after inoculation (DAI) was identical to the day of inoculation as there was no significant change in the embryo. There was slight increase in the size of the embryo at 10 DAI. After 10 DAI the increase in the width of the embryo was seen which led to the bulging of the embryo gradually at 15 DAI. At 20 DAI sprout initiation point was observed which would develop into shoot at about 75 DAI.

4.2.2. Effect of A medium (2 mgL^{-1} 2,4-D and 5 mgL^{-1} BA) on the embryo

The size of the embryo 5 DAI was similar to the day of inoculation whereas bulging of embryo was observed at 10 DAI. There was increase in the size as well as the bulging of embryo at 15 DAI. At 20 DAI sprout initiation point became visible

similar to that of the basal medium. The development of roots was found in few of the embryos three months after inoculation. These roots were removed during subculturing as they would affect the growth of the sprout.

4.2.3. Effect of B medium (2 mgL^{-1} 2,4-D, 5 mgL^{-1} BA and 1.6 mgL^{-1} GA3) on the embryo

The embryo size at 5 DAI was same as that of the day of inoculation. Embryo at 10 DAI showed expanded growth in terms of its size and width. At 15 DAI the embryo showed increased bulging compared to 10 DAI. At 20 DAI sprout initiation point was observed as seen in previous media combinations. Further growth of the embryos in this medium was found to be slow compared to other media combinations.

4.2.4. Effect of C medium (1.6 mgL^{-1} GA3) on the embryo

The embryos showed enlargement in their size at 5 DAI when compared to the day of inoculation. The sprout initiation point was seen earlier at 10 DAI indicating positive impact of the growth regulator. At 15 DAI, extended growth of the sprout initiation point was observed. Finally, the development of the sprout was seen at 20 DAI which was not observed in 20 DAI in previous media combinations.

4.2.5. Effect of D medium (33 mgL^{-1} TDZ) on the embryo

The bulging of the embryo was seen at 5 DAI. The sprout initiation point was seen at 10 DAI and the extended growth of that point was observed at 15 DAI. The development of the sprout was seen at 20 DAI, as seen in the C medium.

The change in size of the embryos under different media compositions has been represented graphically in Plate 4.1. The overall response of the embryo in different media combinations including basal medium is shown in Plate 4.2. The details regarding the total number of cultures initiated and their response have been summarized in Table 4.1.

Table 4.1. Observations of the culture tubes inoculated

S.No	Observations	Basal	A	B	C	D
1	No of embryos inoculated	30	30	30	30	30
2	No of cultures used for RNA isolation	9	9	9	9	9
3	No of cultures for observation	21	21	21	21	21
4	No. of cultures contaminated	2	3	5	2	1
5	No. of cultures without any response	2	1	1	0	0
6	No. of cultures showing bulging	12	11	11	12	12
7	No. of cultures with sprout initiation point	5	6	4	7	8
8	No. of cultures showing browning	11	13	13	12	11
9	Percentage of cultures contaminated (%)	9.5	14.2	23.8	9.5	4.7
10	Percentage of cultures without any response (%)	9.5	4.7	4.7	0.0	0.0
11	Percentage of embryos showing bulging (%)	57.1	52.3	52.3	57.1	57.1
12	Percentage of cultures with sprout initiation point (%)	23.8	28.5	19.0	33.3	38.0
13	Percentage of cultures showing browning (%)	52.3	61.9	61.9	57.1	52.3

4.3. Growth of embryos upto 60 DAI

The increase in the length of the developed sprout was observed. After sprout emergence, the cultures were changed from dark to light conditions. This was the reason for the development of green colour in the sprout tissues that were observed at 35 DAI. This greening of the tissues continued until the entire sprout became complete green in colour. In few embryos (without any sprout development) the entire embryo would develop green colour. Few embryos showed further bulging and increase in size. The bulging was observed to be in a circular fashion beginning at the bottom of the embryo and moving up towards the top. The development of pale pink colour at the sprout initiation point along with the increase in its prominence was observed in few embryos. The sprout initiation point alone would show increase in size. Browning and drying of the tissues were observed for embryos in which the proper sprout did not emerge. In few embryos, browning developed around the sprout region alone. Thus, the pattern of development of browning was different in different embryos. There were a small number of embryos that would respond very slowly. Few of the embryos remained dormant. The growth stages of embryo upto 60 DAI in cultures which showed positive response is shown in Plate 4.3.

4.4. Growth of embryos after 60 DAI

The embryos which showed proper sprout formation were subcultured at 60 DAI. The sprout was cut and the embryo was split into two and inoculated in the same medium. Four embryos in total displayed well-developed shoot length at 60 DAI. Two embryos from TDZ medium and one embryo each from GA3 and basal Y3 media had a well-developed sprout. The number of embryos that were subcultured in different media combinations 60 DAI is given in Table 4.2. The development of new tissues at the region of split was observed 20 days after subculturing (Plate 4.4). These tissues would either be in pale yellow or green in colour. The growth of these tissues was observed to be slow compared to the initial growth of the embryo as it took three to four months for the development of shoot. In some cases, browning and drying was also observed in the entire tissues of the split portion of embryo. Sometimes, both the split portions would respond and

sometimes both would show drying up of the tissues. In some cases, any one of the split portions would respond. The further growth of such responded portions would lead to the development of a small plantlet after eight to nine months after inoculation (Plate 4.5).

Table 4.2. The number of embryos that were subcultured

S. No	Media	No. of embryos subcultured
1	Basal	1
2	A	0
3	B	0
4	C	1
5	D	2

4.5. Constraints faced during coconut embryo culture study

Browning of the tissues was the most common problem during embryo culture. The oxidation of phenols led to the browning of the tissues (Plate 4.6). Out of 150 tubes initiated, 60 tubes showed browning in the entire tissues. Another major constraint was the fungal (Plate 4.7) and bacterial contamination (Plate 4.8). The 13 tubes were contaminated during the study out of which 9 tubes showed fungal contamination and 4 tubes showed bacterial contamination. Fungal contamination was observed more after inoculation than after subculturing but bacterial growth was visible both after inoculation and subculturing.

The response of the embryos in the same medium was not uniform. Sometimes, the embryos which showed bulging, did not show sprout initiation. The growth of the embryos was very slow. Even after subculturing, embryos would not show uniform growth. The generation of the shoot was very slow. If two tubes were inoculated with the same split embryo only one would respond sometimes and the other would either dry up quickly or would exhibit sprout development very slowly compared to the other. Sometimes both the split portions of the same embryo would die due to drying (Plate 4.9) and browning of the tissues.

The response of the embryos was not uniform during the *in vitro* culture. The size and shape of the embryos also varied during the same stage of development (Plate 4.10). There were few embryos that would remain dormant without responding to the media and growth regulators (Plate 4.11). The size of such embryos was the same as the day of inoculation even after a month. Thus, the growth of the embryos was uncertain even though all the culture conditions were maintained same without any deviation.

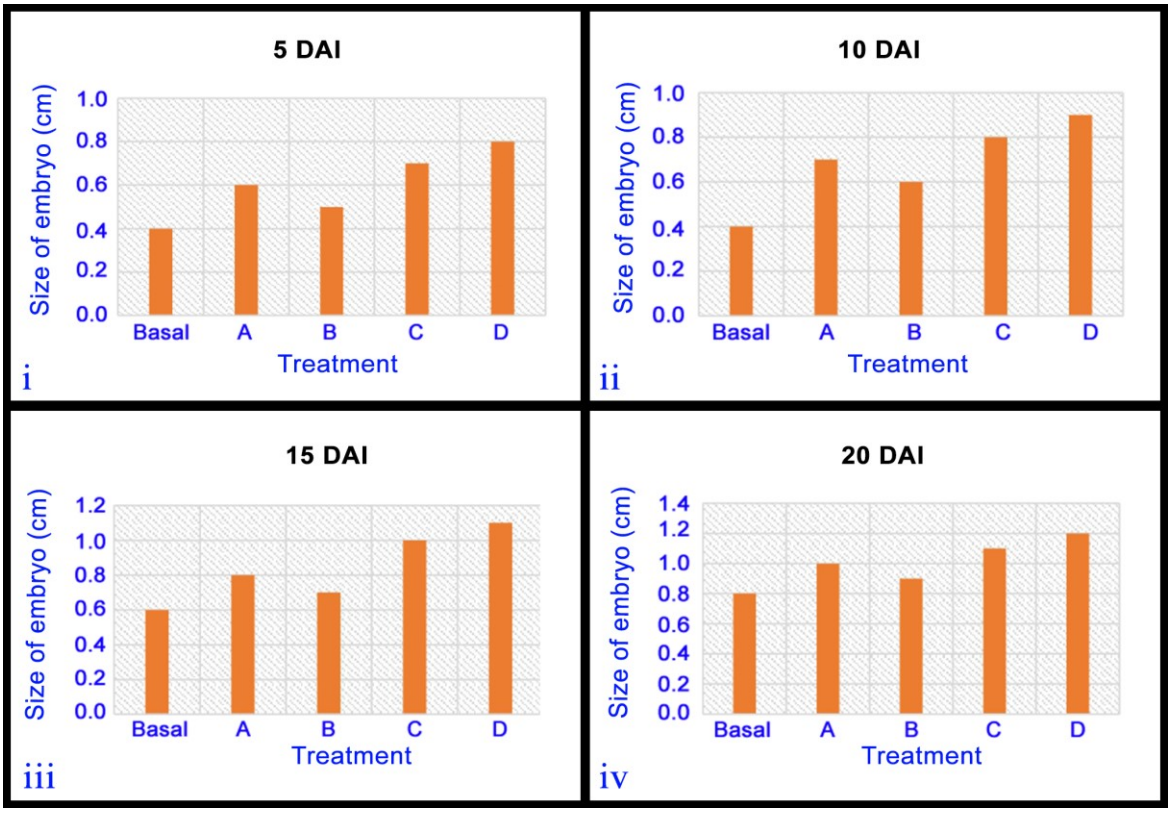

Plate 4.1. Change in embryo size over time in different media combinations

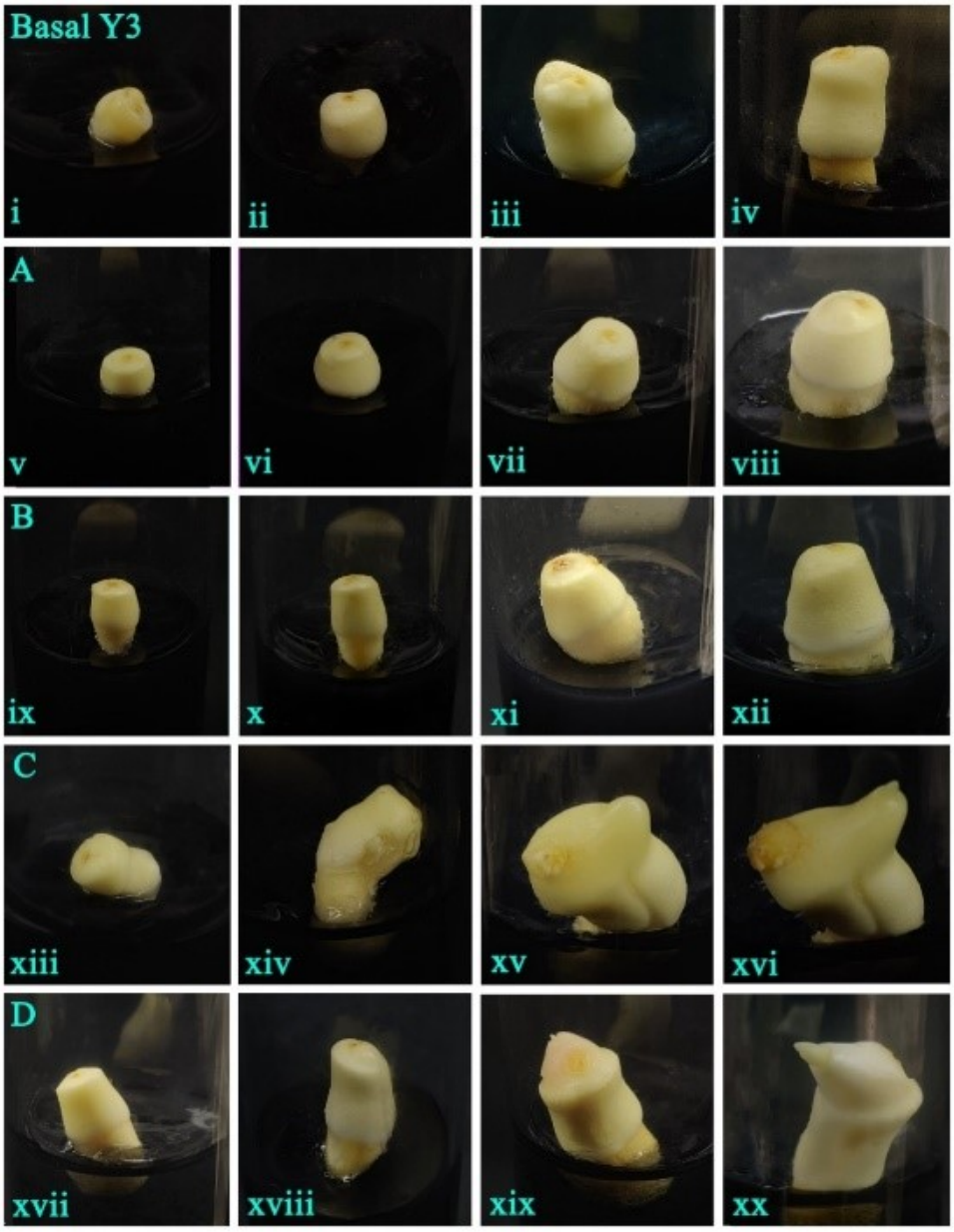

Plate 4.2. Response of embryos to different media combinations

Response of embryo to basal medium- i (5 DAI), ii (10 DAI), iii (15 DAI), iv (20 DAI); Response of embryo to A medium- v (5 DAI), vi (10 DAI), vii (15 DAI), viii (20 DAI); Response of embryo to B medium- ix (5 DAI), x (10 DAI), xi (15 DAI), xii (20 DAI); Response of embryo to C medium- xiii (5 DAI), xiv (10 DAI), xv (15 DAI), xvi (20 DAI); Response of embryo to D medium- xvii (5 DAI), xviii (10 DAI), xix (15 DAI), xx (20 DAI)

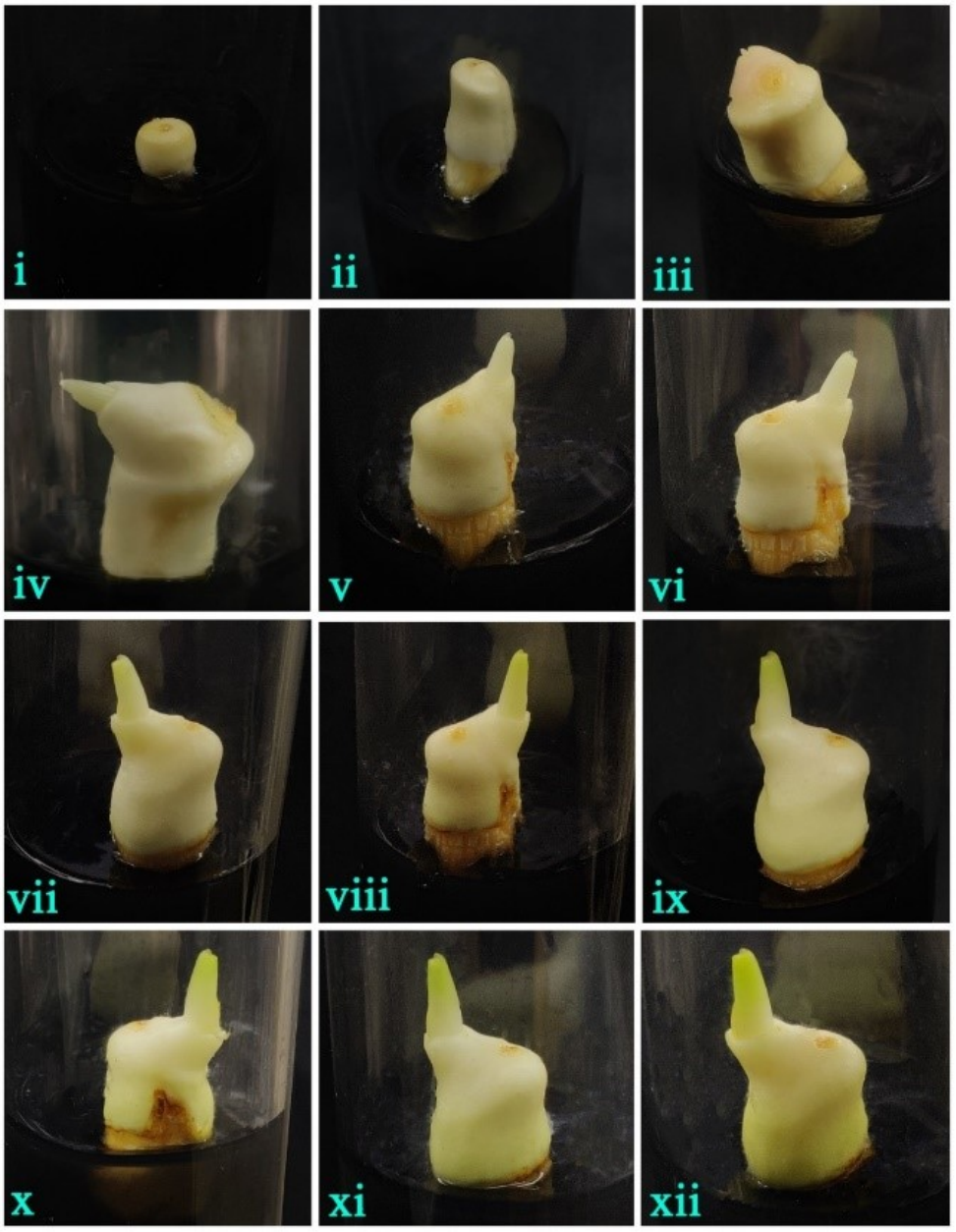

Plate 4.3. Various growth stages of coconut embryo in Y3 supplemented with 33 mgL^{-1} TDZ culture

i-5 DAI; ii-10 DAI; iii-15 DAI; iv-20 DAI; v-25 DAI; vi-30 DAI; vii- 35 DAI; viii- 40 DAI; ix- 45 DAI; x- 50 DAI; xi- 55 DAI; xii- 60 DAI

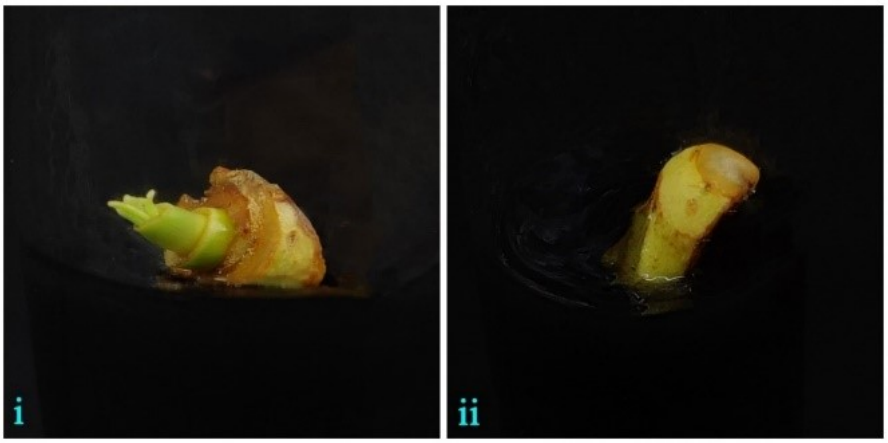

Plate 4.4. Heterogenous response after subculturing

i-Split portion with new tissue growth 20 days after subculturing;

ii. Split portion without any response

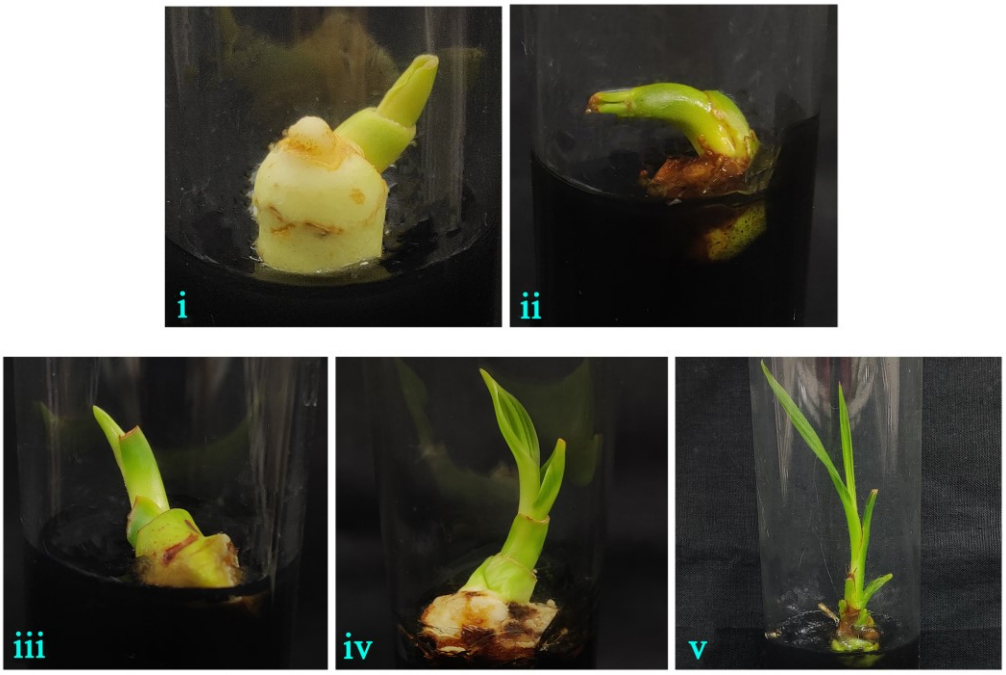

Plate 4.5. Stages of response of embryo after subculturing

i- Response at 30 days after subculturing (total 90 days); ii- Response at 60 days after subculturing (total 120 days); iii- Response at 90 days after subculturing (total 150 days); iv- Response at 120 days after subculturing (total 180 days);

v- Response 150 days after subculturing (total 210 days)

Plate 4.6.
Browning of tissues

Plate 4.7.
Fungal contamination

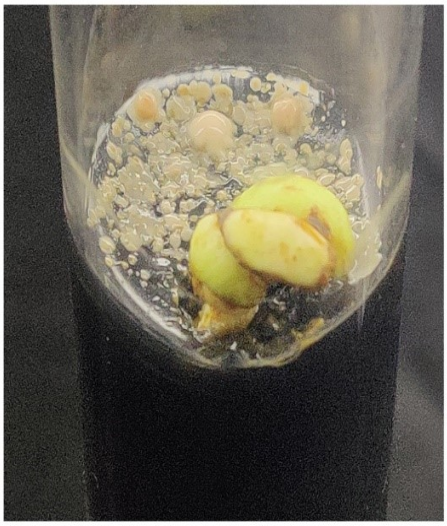

Plate 4.8.
Bacterial contamination

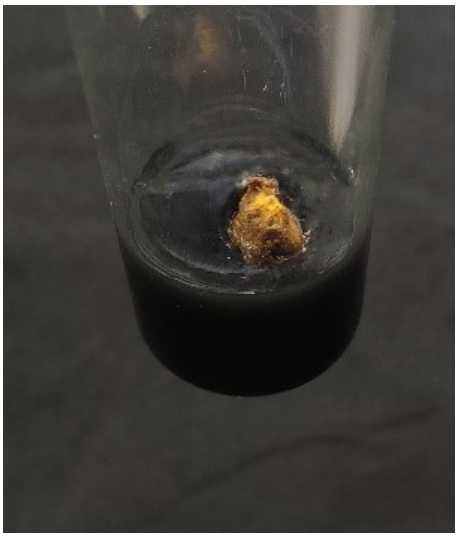

Plate 4.9.
Drying of embryo after subculturing

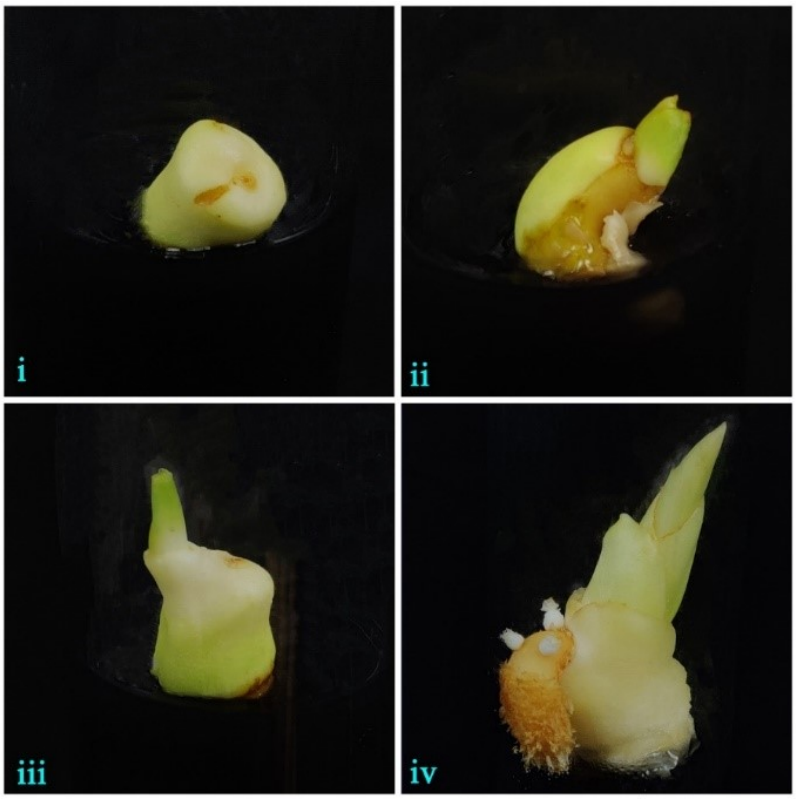

Plate 4.10. Heterogenous response of embryo from the same treatment

i, ii, iii and iv- Different embryos at 70 DAI in Y3 medium with 33 mgL^{-1} TDZ

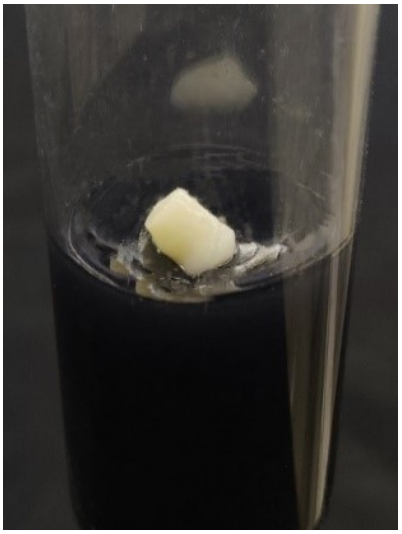

Plate 4.11. Dormant embryo

The embryo without any response 60 DAI

4.6. Expression study of *E2F* and *CDKA* genes

Quantitative Real Time PCR based expression profiling of *E2F* and *CDKA* genes was undertaken during early response stages of coconut embryo culture.

4.6.1. RNA Isolation

Total RNA was isolated during early response stages like 5 DAI, 10 DAI and 20 DAI. Good quality RNA was obtained using RNeasy plant mini kit (Qiagen).

4.6.2. RNA quantification and quality analysis

The quality analysis of the RNA was done using NanoDrop spectrophotometer (IMPLEN NanoPhotometer® NP80). The A_{260}/ A_{280} ratio was found to be between 1.9 and 2.2, indicating good RNA quality. The concentration of RNA was between 362 and 2104 ngμL^{-1}. The two intact bands corresponding to 18S rRNA and 28S rRNA was observed as indicated in Plate 4.12.

4.6.3. First strand cDNA quantification and quality assessment

When the cDNA was subjected to quality analysis and quantified using NanoDrop spectrophotometer (IMPLEN NanoPhotometer® NP80), the concentration was found to be between 689.65 and 3669.4 ngμL^{-1}.

4.6.4. Primer designing for qRT-PCR analysis

One pair of primer was designed for each of the test genes (*E2F* and *CDKA*) and control genes (*α-tubulin* and 18S rRNA). The information related to the primers designed for qRT-PCR analysis are given in the Table 4.3. The primers were all synthesized using Sigma -Aldrich Pvt. Ltd.

Table 4.3. Primers for qRT-PCR

S.No	Gene	Primer name	Sequence (5' to 3')	Expected PCR amplicon size
1	*α-tubulin*	coctbf	GTCCTACTGGCTTCAAGTGTGGC	214
		coctbr	AACTCTCCCTCCTCCATGCCCT	
2	18S rRNA	coc18f	CGGCTACCACATCCAAGGAA	200
		coc18r	GCTGGAATTACCGCGGCT	
3	*E2F*	Cce2f2f	AGTGGGATGCAATGGGCAGA	181
		Cce2f2r	GGGGAAAAAGTTGCCGACCTG	
4	*CDKA*	coccdkaf	GCTCGGAATGCTCTGGACCA	230
		coccdkar	TCCCAGTTCCCAGCTCAGGA	

4.6.5. Amplification and sequencing of the test and control genes

Amplicons of expected size were obtained for all the four primer pairs studied. In the gradient PCR, optimum annealing temperatures were obtained for all the four primer pairs (Table 4.4) and respective agarose gel profiles are shown (Plate 4.13). The BLASTN analysis of the sequences confirmed that they corresponded to the selected genes (*α-tubulin,* 18S rRNA, *E2F and CDKA*). Representative sequences were submitted to NCBI database (GenBank accession number OQ999406, OQ423173 and OR067378).

Table 4.4. Optimum annealing temperature for the primers

Primer	Annealing temperature (°C)
α-tubulin (coctbf and cocotbr)	56.9, 59.0 and 59.6
18S rRNA (coc18f and coc18r)	55.3, 56.9, 58.1 and 59.0
E2F (Cce2f2f and Cce2f2r)	60.0 and 60.4
CDKA (coccdkaf and coccdkar)	55.0, 56.5, 58.1, 59.9 and 61.4

4.6.6. qRT-PCR analysis

Initially, the housekeeping gene, α-*tubulin,* specific primers were used for the amplification of all the cDNA samples through RT-PCR. Amplicons of the anticipated size (214 bp) were obtained for all samples.

The qRT-PCR was used to examine the differential expression of the *E2F* and *CDKA* genes in the early response phases of coconut embryo culture in the presence of different combinations of growth regulators. The melting temperature in the melt curve analysis was found to be 82.44 ± 0.5 for *E2F* amplicons (Fig 4.1), 83.79 ± 0.5 for *CDKA* amplicons (Fig 4.2), 86.47 ± 0.5 for α-*tubulin* amplicons (Fig 4.3) and 85.27 ± 0.5 for 18S rRNA amplicons (Fig 4.4). The expression of endogenous control genes like α-*tubulin* and 18S rRNA was used to normalise the expression levels of the test genes, *E2F* and *CDKA*. The fold change in expression was calculated using the method of geometric averaging of Ct values proposed by Vandesompele *et al.* (2002) and Hellemans *et al.* (2007). The qRT-PCR amplicons were analyzed on 1 per cent agarose gel. The representative samples are shown in Plate 4.14. The qRT-PCR study revealed the following results.

4.6.6.1. Expression profiling of *E2F* and *CDKA* genes during different stages

4.6.6.1.1. Five days after inoculation (5 DAI)

When different media combinations were considered during 5 DAI, the expression of *E2F* gene was highest in C (1.6 mgL^{-1} GA3) medium followed by its expression in D (33 mgL^{-1} TDZ) medium. The fold change values were 245.5 and 3.67 in GA3 and TDZ media respectively over the basal Y3 medium. In A medium (2 mgL^{-1} 2,4-D and 5 mgL^{-1} BA), the expression was found to be lower than the control Y3 medium; the fold change being 0.39. In B (2 mgL^{-1} 2,4-D, 5 mgL^{-1} BA and 1.6 mgL^{-1} GA3) medium, the fold change in expression of *E2F* was observed to be 1.78.

The expression of *CDKA* gene in D medium was observed to be highest with the fold change value of 4.23, succeeded by its expression in C medium with a fold change value of 1.50. In A medium, the expression was found to be lower; the fold

change being 1.10. In B medium, the fold change in expression of *CDKA* was observed to be 1.20. The graphical representation of gene expression analysis during 5 DAI is shown in Plate 4.15.

4.6.6.1.2. Ten days after inoculation (10 DAI)

The expression of *E2F* gene was highest in C medium and then in B medium with a fold change value of 68.45 and 7.46 respectively. In A medium, the expression was found to be lower than the control Y3 medium; the fold change being 0.80. The fold change value of *E2F* gene in D medium was found to be 4.83.

The *CDKA* gene expression was maximum in C medium during 10 DAI and 19.82 was the fold change value followed by its expression in D medium with fold change value of 1.36. In B and A medium, the expression was found to be lower than the control Y3 medium; the fold change being 0.77 and 0.24 respectively. The graphical representation of gene expression analysis during 10 DAI is shown in Plate 4.16.

4.6.6.1.3. Twenty days after inoculation (20 DAI)

The *E2F* gene expression in C medium was found to be maximum with the fold change value of 4.19 followed by its expression in D medium with a fold change value of 1.22. The least expression was found in A medium with fold change value of 1.09 preceded by its expression in B medium with fold change value of 1.13.

The *CDKA* gene was expressed most in D medium and the fold change value was 3.85, followed by C medium with the fold change value of 2.41. The fold change value in A medium was found to be 1.12. In B medium, the expression of *CDKA* gene was found to be lower than the control Y3 medium and the fold change was obtained as 0.38. The graphical representation of the gene expression analysis during 20 DAI is shown in Plate 4.17.

Finally, on the whole, expression of *E2F* and *CDKA* genes were higher in GA3 provided medium followed by TDZ medium, which indicated that GA3 and TDZ had a positive impact on the expression of these genes. The application of

these growth regulators to the media alone was enough to shoot up the expression of these cell-cycle regulating genes. The heat map representing the expression of *E2F* and *CDKA* genes during early embryogenic stages is shown in Plate 4.18.

The expression of *E2F* and *CDKA* genes was normalized with the expression of endogenous control genes, α-*tubulin* and 18S rRNA.

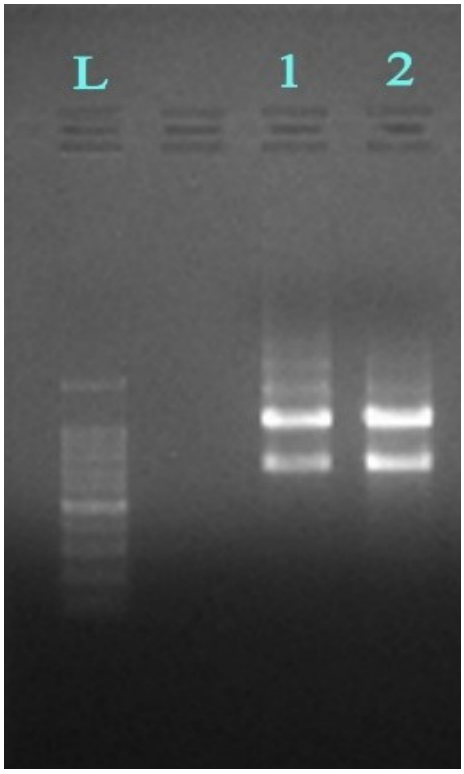

Plate 4.12. Agarose gel profile of RNA sample

L- RNA Ladder; 1 and 2- RNA samples isolated from coconut embryos using RNeasy plant mini kit (Qiagen)

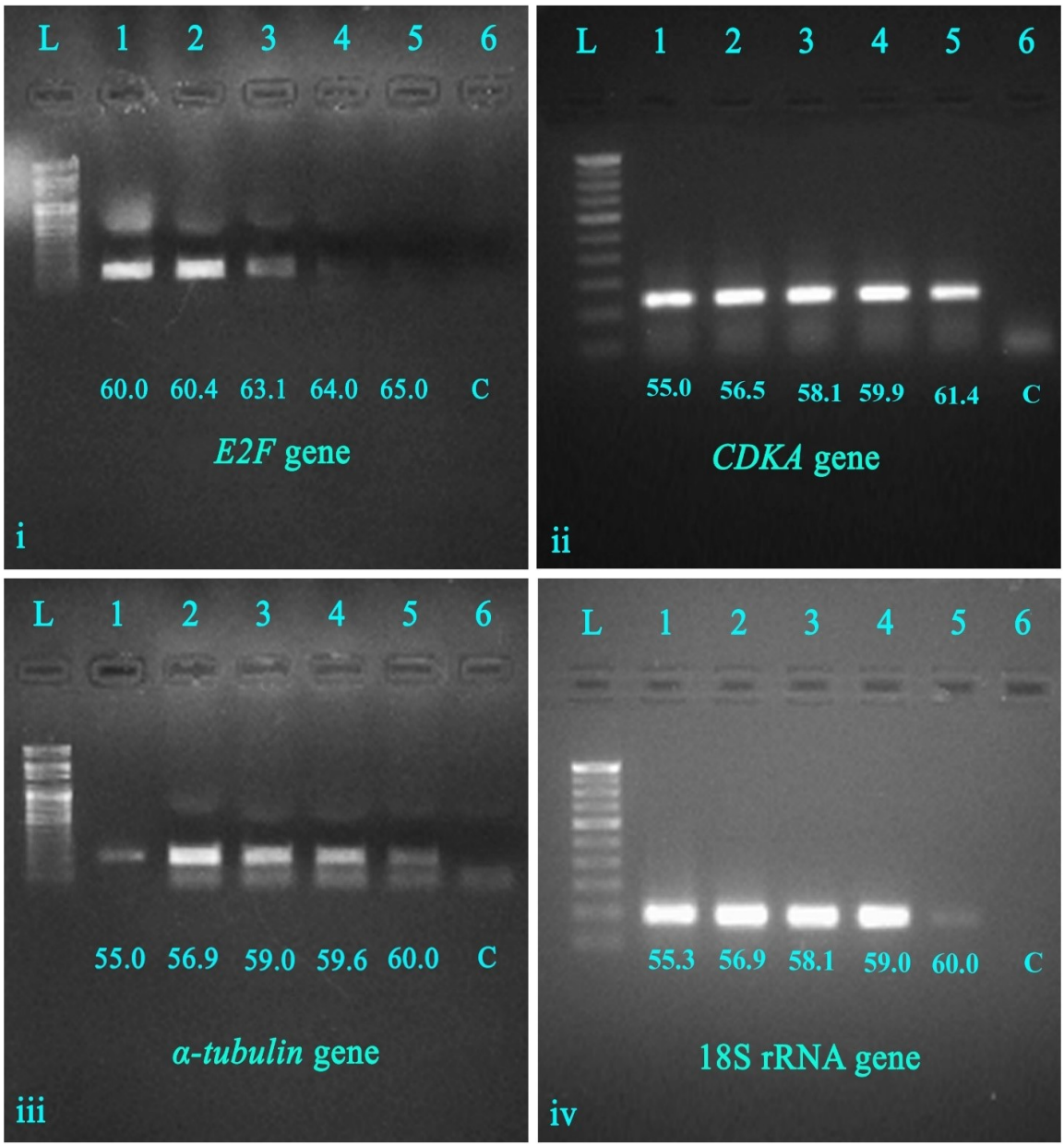

Plate 4.13. Agarose gel profiles of gradient PCR for the four genes studied

i. *E2F* gene- L- DNA ladder; 1,2,3,4 and 5- PCR amplicons; 6- NTC

ii. *CDKA* gene- L- DNA ladder; 1,2,3,4 and 5- PCR amplicons; 6- NTC

iii. α-*tubulin* gene- L- DNA ladder; 1,2,3,4 and 5- PCR amplicons; 6- NTC

iv. 18S rRNA gene- L- DNA ladder; 1,2,3,4 and 5- PCR amplicons; 6- NTC

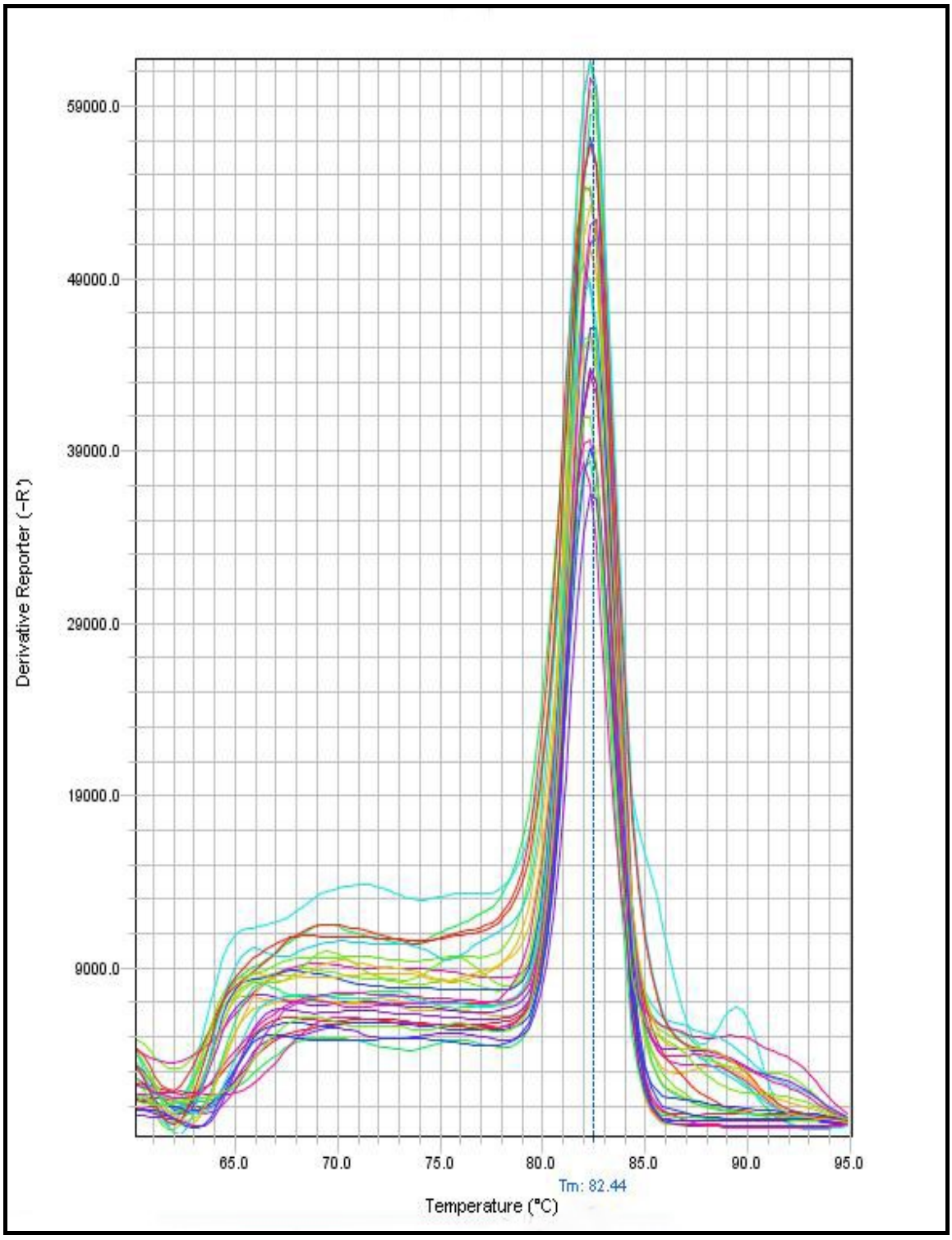

Fig 4.1. Melt curve analysis of *E2F* gene

The T_m was obtained to be 82.44 ± 0.5 ºC

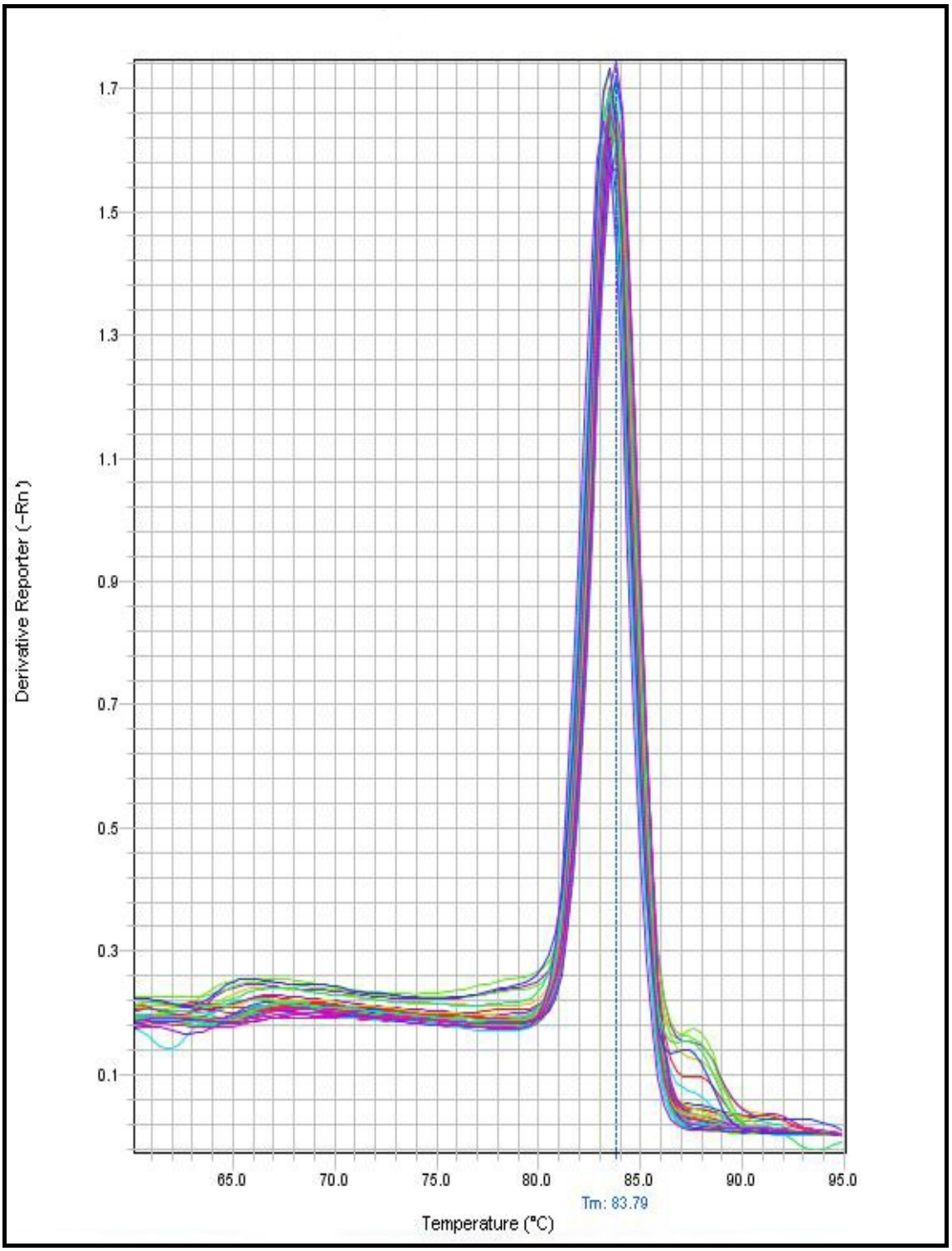

Fig 4.2. Melt curve analysis of *CDKA* gene

The T_m was obtained to be 83.79 ± 0.5 ºC

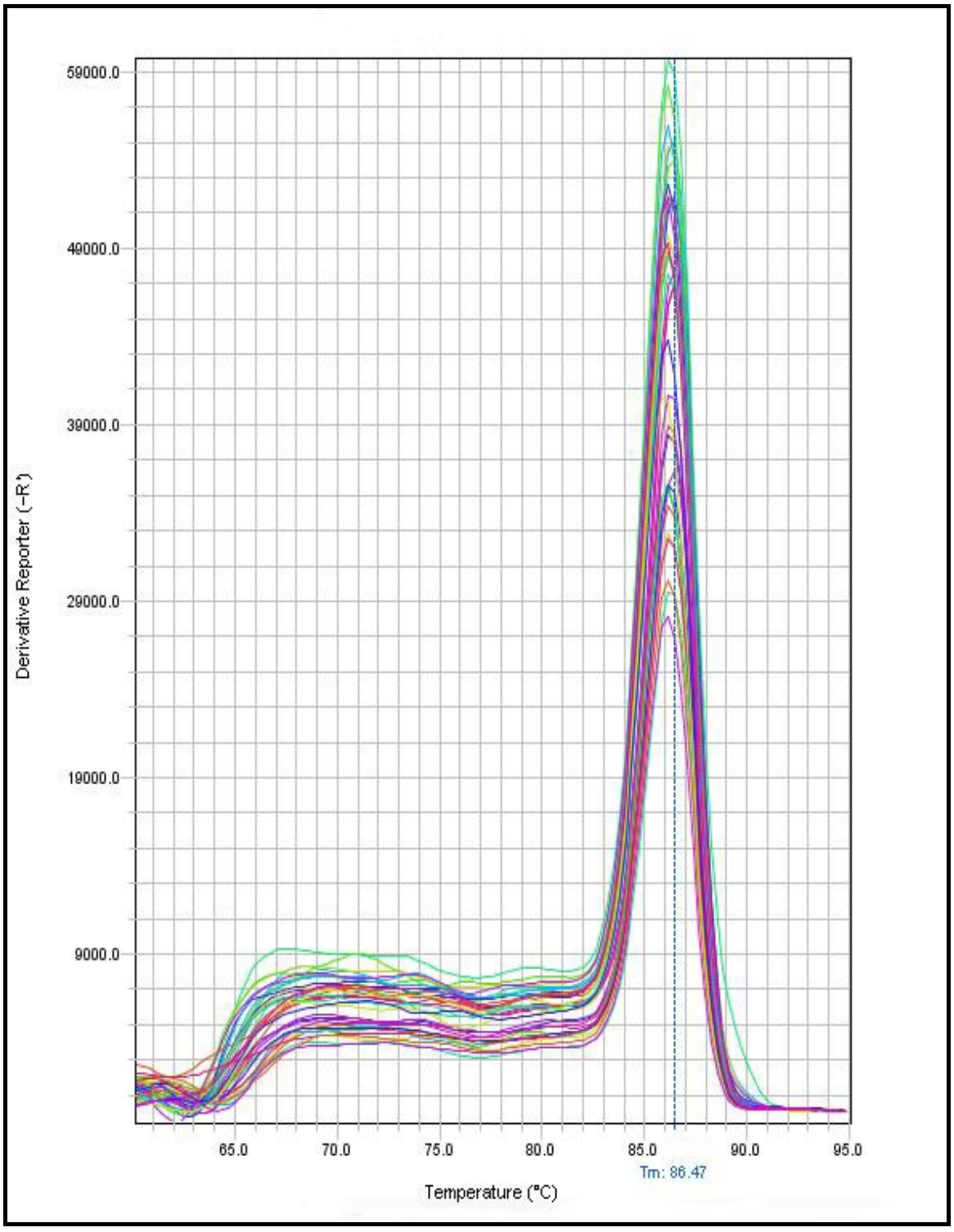

Fig 4.3. Melt curve analysis of *α-tubulin* gene

The T_m was obtained to be 86.47 ± 0.5 ºC

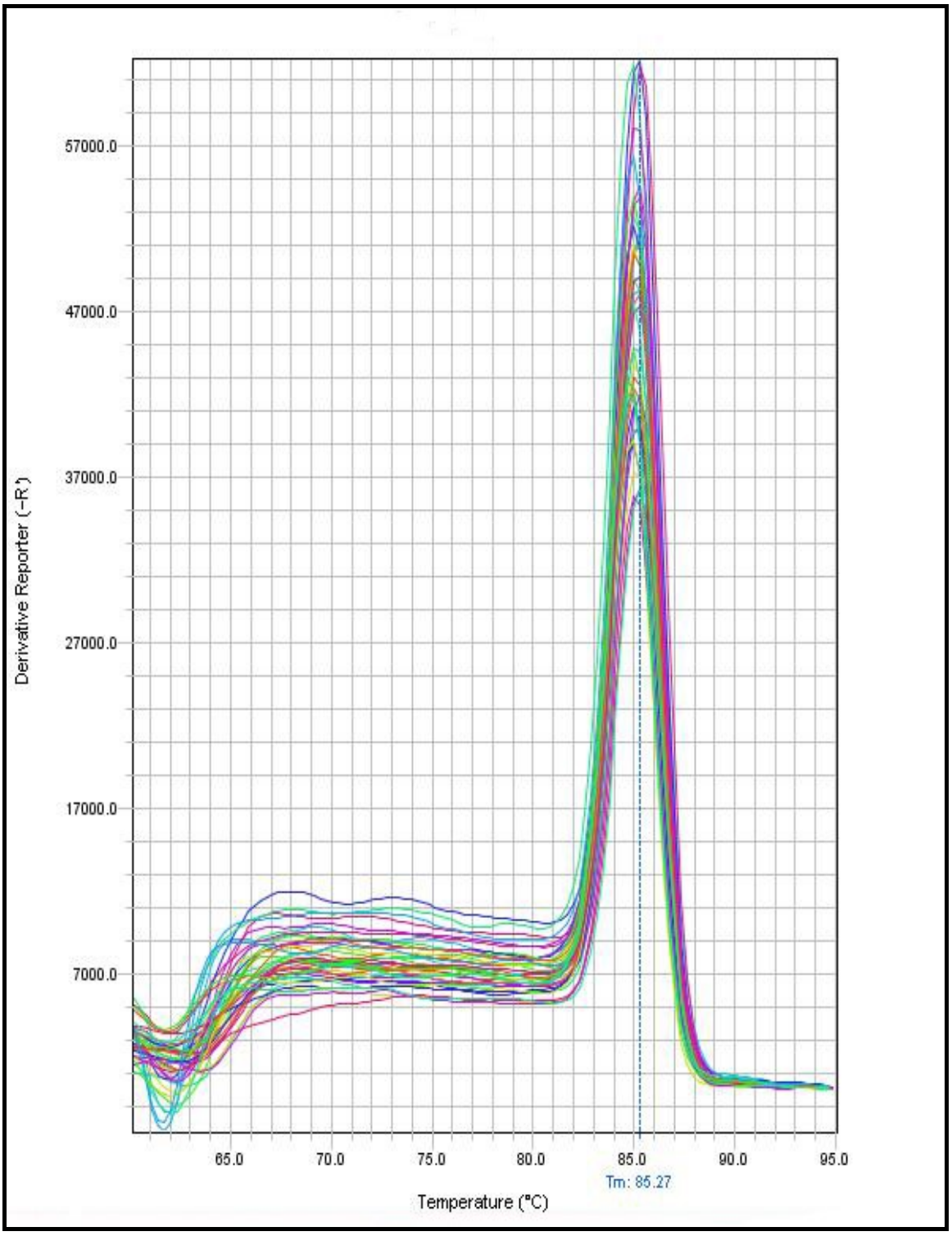

Fig 4.4. Melt curve analysis of 18S rRNA gene

The T_m was obtained to be 85.27 ± 0.5 ºC

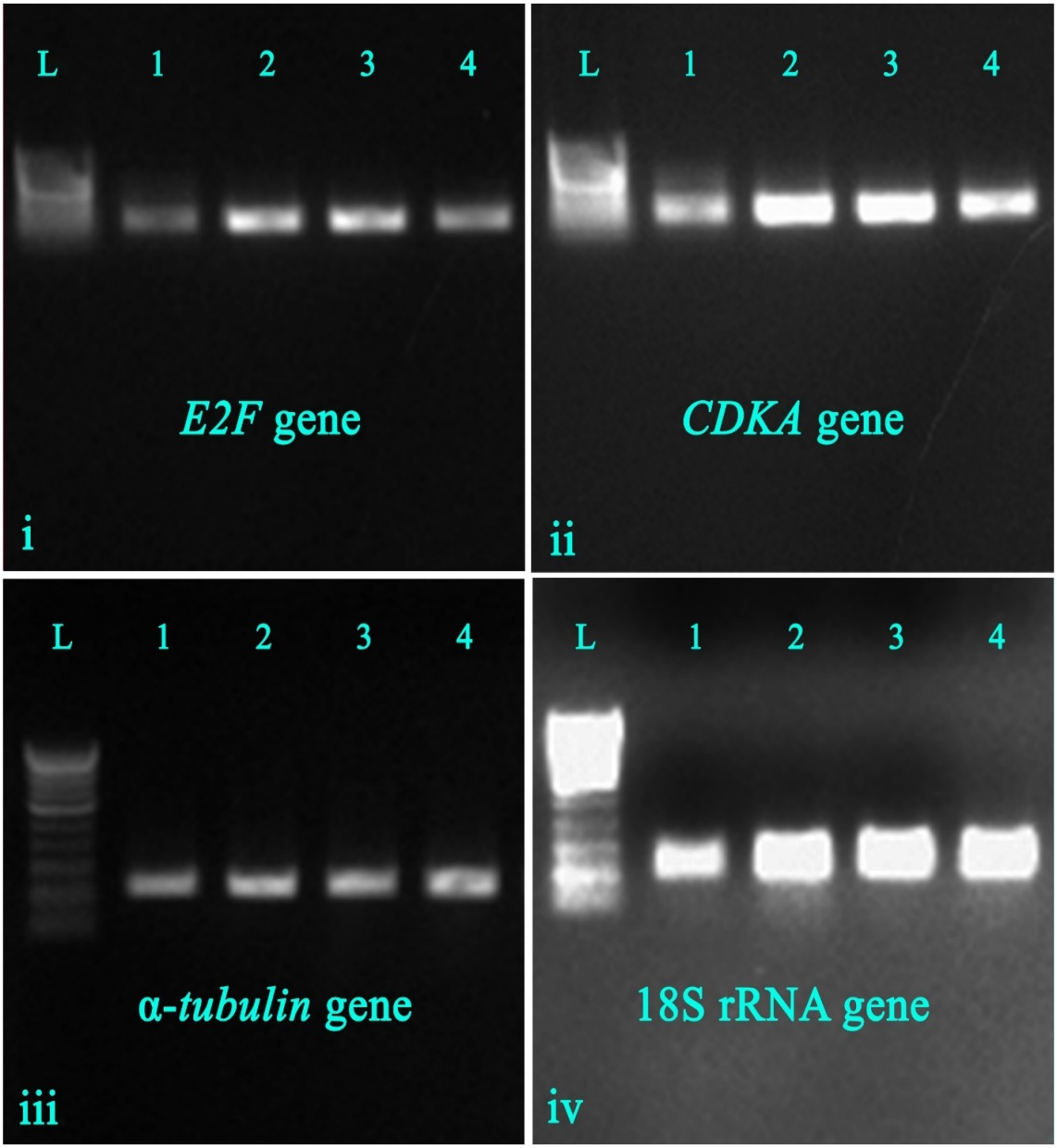

Plate 4.14. Agarose gel profile of qRT-PCR amplicons

i. *E2F* gene; ii. *CDKA* gene; iii. α- *tubulin* gene; iv. 18S rRNA gene

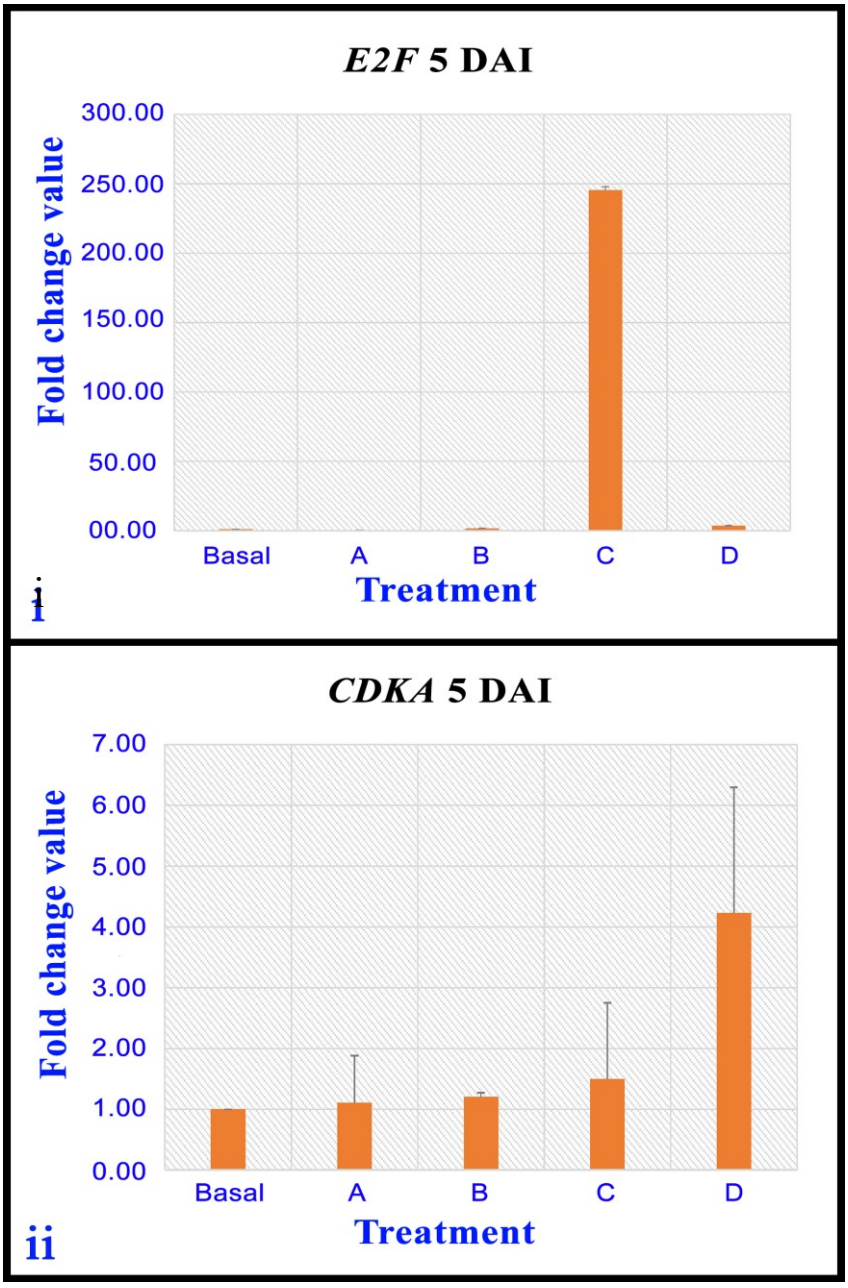

Plate 4.15. Fold change in the expression of *E2F* and *CDKA* genes at 5 DAI

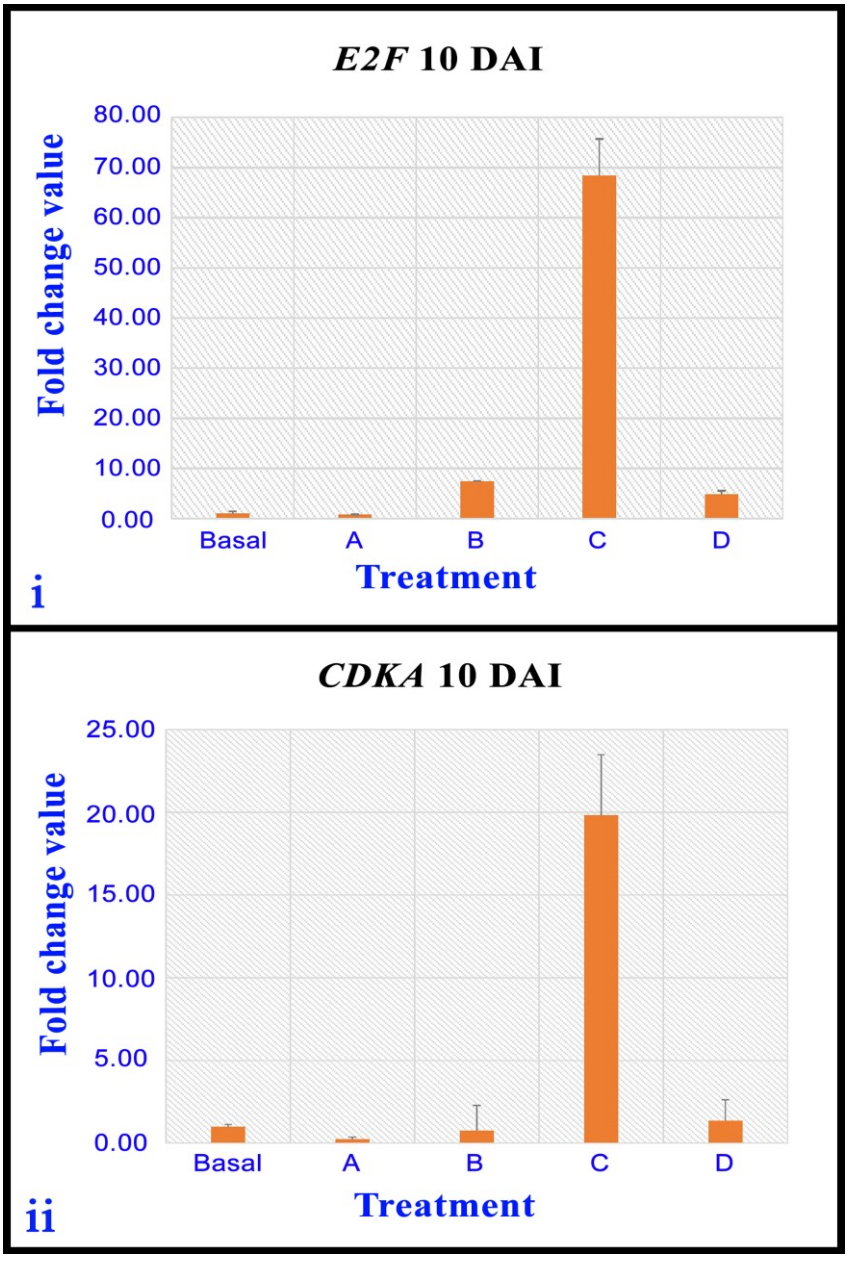

Plate 4.16. Fold change in the expression of *E2F* and *CDKA* genes at 10 DAI

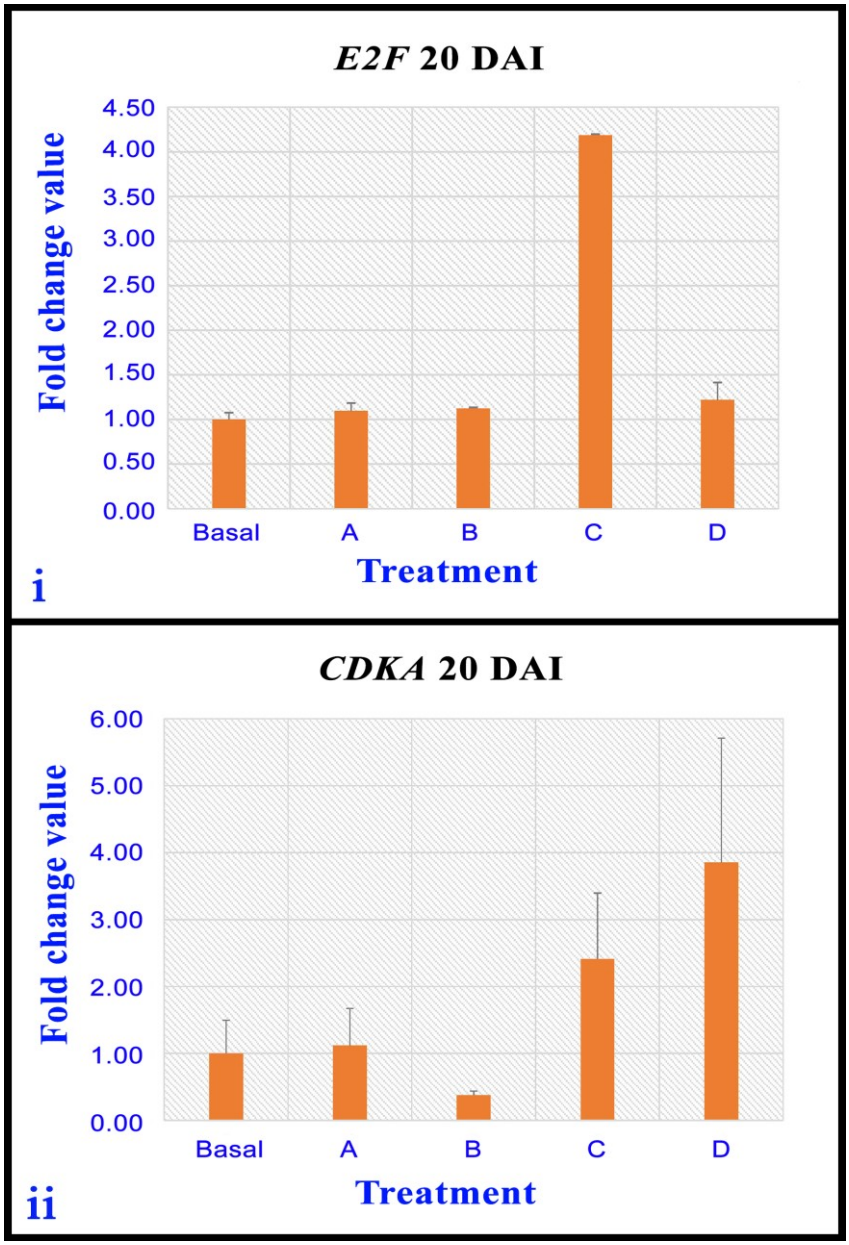

Plate 4.17. Fold change in the expression of *E2F* and *CDKA* genes at 20 DAI

Gene	Stages	Basal Y3	A (2,4-D and BA)	B (2,4-D, BA and GA3)	C (GA3)	D (TDZ)
E2F gene	5 DAI	1	0.39	1.78	245.5	3.67
	10 DAI	1	0.8	7.46	68.45	4.83
	20 DAI	1	1.09	1.13	4.19	1.22
CDKA gene	5 DAI	1	1.1	1.2	1.5	4.23
	10 DAI	1	0.24	0.77	19.82	1.36
	20 DAI	1	1.12	0.38	2.41	3.85

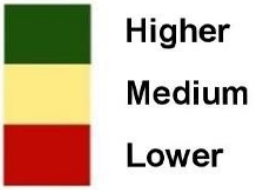

Plate 4.18. The summary of gene expression data of *E2F* and *CDKA* during early response stages

DISCUSSION

5. DISCUSSION

Coconut (*Cocos nucifera* L.) is a major plantation crop of the tropics. A variety of issues are weighing down the coconut business today. The occurrence of pests and diseases has posed a significant threat to the survival rate of the palms. Farmers have suffered significant financial losses as a result of diseases like root wilt, lethal yellowing, cadang-cadang, etc. Pests like the rhinocerous beetle and red palm weevil have also wreaked havoc (Sáenz *et al.*, 2018).

As far as our national economy is concerned, coconut is quite important. To improve its productivity, the old and senile plantations have to be replanted. Replanting requires a lot of quality seedlings. Traditionally, coconuts are propagated only by seed nuts because of single apical meristem and lack of branches and suckers (Rajesh *et al*., 2018). In order to keep up the coconut production, coconut farmers should be provided with high yielding quality planting materials. Hence, appropriate breeding strategies along with the *in vitro* multiplication techniques will help in the production of the quality planting material within a shorter span of time. Many works have been done on coconut micropropagation (Cutter and Wilson, 1954; De Guzman and Del Rosario, 1964; Davis, 1964; Blake and Eeuwens, 1982; Siny, 2006; Karun and Sajini, 2010; Radhika, 2019; Sushmitha *et al*., 2019); but still there is no viable protocol for commercial micropropagation in coconut. Researchers mostly focused on somatic embryogenesis till now. So, direct organogenesis was tried as an alternative to somatic embryogenesis in the present study.

The current study entitled, 'Expression profiling of *E2F* and *CDKA* genes during embryo culture in coconut (*Cocos nucifera* L.) was conducted during 2021–2023 at the College of Agriculture, Vellanikkara to initiate the embryo culture *in vitro*. The *in vitro* response of zygotic embryos to various plant growth regulators was evaluated. Coconut is recalcitrant to *in vitro* culture and the cell cycle control genes have a major role in initial *in vitro* response. Therefore, the cell cycle control genes like *E2F* and *CDKA* were used in the study and their expression levels were analyzed during early response stages. In this chapter, the findings from the research are discussed.

5.1. Explant selection

The most essential aspect governing the efficacy of *in vitro* crop regeneration is the selection of appropriate explants. In the present study, the coconuts of the WCT (West Coast Tall) variety were used. The WCT is one of the popular varieties in the coastal India with higher yields. It can tolerate the burrowing nematode in a moderate way. However, it is vulnerable to basal stem rot, bleeding disease, leaf blight and root wilt in Kerala (Karun *et al*., 2014). Since, WCT is popular among farmers in Kerala, we selected it for our study.

The embryos obtained from the mature nuts were used as the explant in the study as mature embryos would reduce dormancy and enhance germination (Drewes-Alvarez, 2017). De Guzman and Del Rosario (1964) reported the *in vitro* embryo culture protocol for Makapuno cultivar in coconut. Although somatic embryogenesis was the most studied method of regeneration, a commercial procedure for mass-producing coconuts has not yet been standardized. According to Bhatia and Bera (2015), generally the development of shoots was possible directly without the intermediate callus formation in direct shoot organogenesis. The risk of somaclonal variation was also less in direct organogenesis (Kong *et al*., 2021). Direct organogenesis also has a lesser chance of development of putative off types (Wilms *et al*., 2021). With these advantages, direct organogenesis can be used as an alternative to somatic embryogenesis. So, the present study concentrated on the response of zygotic embryos through direct shoot organogenesis in *in vitro* culture.

5.2. *In vitro* response of coconut embryos during embryo culture

In the current study the embryo culture was initiated in four different growth regulator combinations along with the basal Y3 medium as control. The responses of the embryo to various plant growth regulators were observed. Sushmitha *et al.* (2019) initiated the embryo culture using sliced embryos through direct organogenesis in COD (Chowghat Orange Dwarf) variety of coconut using modified Y3 medium. The use of GA3 (Montero-Cortes *et al.*, 2011) and TDZ (Sushmitha *et al*., 2019) showed good response in coconut embryo culture, so we selected these plant growth regulators for our study.

In our study, the explant showed increase in size after 10 DAI and the sprout initiation was observed at 20 DAI in TDZ and GA3 supplemented media. These findings were in contrast with the work done by Bett (2021), where the indicators of growth like enlarged embryos, cracking and colour change were observed from 8th to 16th weeks using Y3 supplemented with growth regulators such as GA3, BAP and 2,4-D. In another study, Muhammad *et al*. (2013) found that MS media did not show any evidence of development until the embryos were moved to liquid media. Danson *et al*. (2009) revealed that plumule and signs of radicle emergence were seen from 16 to 24 weeks after inoculation during embryo culture but this was in contrast to the results obtained during our study where the sprout (plumule) initiation was observed from 2 to 8 weeks after inoculation. Root structures were observed 12 to 14 weeks after initiation of the cultures in few embryos supplemented with 2,4-D and BA. Fernando and Gamage (1995) reported that auxin can induce rhyzogenesis. Alkhateeb (2008) reported that greater auxin concentrations was one of the reasons for early rooting. These root structures either developed earlier to shoot directly from the embryo without shoot formation or emerged after sprout initiation. The development of root before the proper establishment of shoot during the embryo culture was considered undesirable and hence was removed during subculturing. The sprout was encouraged to grow further until it reached the desired length.

The sprout that had initiated was allowed to grow for two to four months. When the sprout reached a precise length (1.5 to 2 cm) it was cut. This practice was done to break the apical dominance. According to Bhatla (2018) the term "apical dominance," was the persistent meristematic activity in the shoot apices that prevented the axillary bud growth. The removal of the shoot apices can break this process. Later, the remaining embryo was divided into half and then, each section was inoculated into a different tube that contained the same medium. The beginning of the new shoot apex was observed after three weeks of clipping. However, only a single shoot emerged from each split section. Usually, in the normal coconut propagation we get a single seedling from an embryo. Here, we are trying to get two seedlings from a single embryo with one round of subculture which can be further increased to four, if we can go for another round of subculture.

5.3. Effect of different media combinations on the explant

Plant growth regulators (PGRs) are described as naturally occurring or manufactured chemicals that, typically at low concentrations, influence the metabolic and developmental processes in higher plants (Bhatla, 2018). Plant growth regulators used in the study were 2,4-D (auxin), BA (cytokinin), TDZ (cytokinin) and GA3 (gibberellin). The effect of different growth regulators on the explant are discussed below.

The 2,4-D being a synthetic auxin has a role in enhancing rooting at lower concentrations (Bhatla, 2018). The BA is a cytokinin and has a role in cell division and proliferation (Kalra and Bhatla, 2018). Auxin and cytokinin are the two plant growth regulators that work in tandem to control plant development. Shoot bud growth is induced by an increase in cytokinin concentration, whereas root formation is induced by an increase in auxin concentration (Kalra and Bhatla, 2018). We observed shoot induction 65 DAI during the embryo culture supplemented with 2,4-D and BA. In some of the tubes root initiation was seen prior to shoot and 2,4-D might be the reason for this kind of root development.

In second medium, we had used three different phytohormones namely, 2,4-D, BA and GA3. Usually, the combination of the three phytohormones like 2,4-D (auxin), BA (cytokinin) and GA3 (gibberellic acid) is expected to have better effect on the tissue growth but unfortunately the existence of cross-talk between BA and GA3 was found to have negative impact on the growth of the tissues. According to Greenboim-Wainberg *et al*. (2005) the two phytohormones, GAs (gibberelins) and cytokinins have antagonistic effect on the developmental processes due to which, the growth of the embryos was slower compared to the embryos inoculated in other media combinations.

The media exclusively provided with GA3 and TDZ separately had positive effects on the growth of the embryos. The phytohormone GA3 is known to encourage seed germination both naturally (Evans *et al*., 1996; Gaspar *et al*., 1996; Yoshida and Hirawasa, 1996; Kaur *et al*., 1998) as well as *in vitro* (Geerts *et al*., 1999; White and Rivin, 2000). According to y Aké *et al.* (2007), the introduction of

GA3 enhanced the percentages of germination, conversion and developmental regularity in coconut embryo culture, especially when semi-solid medium was employed. The response obtained from the embryos during the embryo culture was in accordance with the findings of Montero-Cortes *et al.* (2011) where they proposed that the addition of GA3 to the media accelerated the germination of the zygotic embryos. Therefore, the growth of the embryos was accelerated with the presence of phytohormone where the development of sprout initiation point was obtained earlier, within 10 DAI, which was not seen in other phytohormonal combinations, except TDZ. In a variety of plant species, it was reported that TDZ was the most potent cytokinin for inducing adventitious shoot development (Lu, 1993). The TDZ was proved to have a role in enhancing the *in vitro* morphogenesis of the tissues as well as in the induction of differentiated and dedifferentiated responses *in vitro* (Murthy *et al*., 1998). Novikova and Zaytseva (2018) mentioned that TDZ was effective at breaking monopodial growth pattern and also promoted the production of axillary shoots in *in vitro* cultures of various recalcitrant woody species. However, we could not obtain multiple shoots after subculturing. Only one shoot emerged from each split section after subculturing. Al-Mayahi (2014) demonstrated that the greatest response for *in vitro* bud organogenesis in date palm cv. was recorded at a TDZ dosage of 0.5 mgL^{-1}. According to the results of the investigation performed by Sushmitha *et al*. (2019) in coconut, TDZ supplementation at 150 µM (33 mgL^{-1}) concentration successfully promoted the shoot percentage (90.91 %) through direct organogenesis from sliced mature embryos.

5.4. Constrains in coconut embryo culture

The common problem faced during coconut *in vitro* culture was browning. According to Block and Lankes (1996) browning was typically observed in woody plant *in vitro* culture. The tissues turned brown as a result of the oxidation of phenols (Alkhateeb and Ali-Dinar, 2002). Elmaataoui *et al*. (2020) had put forward that tissue browning and hyperhydricity occurred frequently during the *in vitro* culture of date palm. During embryo culture, browning was observed in 40 per cent of the total cultures initiated. Sixty tubes out of one hundred and fifty tubes inoculated showed browning during the study. Since, browning was a common

problem reported in tree crops, we did ascorbic acid and citric acid treatment due to which we could obtain 60 per cent of cultures without browning.

Contamination was also one of the common issues observed during *in vitro* culture. Totally, thirteen tubes out of hundred and five were contaminated during *in vitro* culture. Nine tubes were of fungal contamination and rest four were of bacterial contamination. The fungal contamination was observed more after inoculation but less after subculturing whereas bacterial contamination was observed after inoculation as well as after subculturing.

The responses from the explants were not uniform and their responses varied greatly even within the same treatment. Some of the embryos remained dormant also. The WCT is a tall and cross-pollinated cultivar. Due to cross-pollination, there is significant genetic variation among cultivars in terms of the fruit's colour, size and shape (Sudha *et al*., 2019). Hence, WCT is heterozygous. So, embryos from different palms and different embryos from the same bunch of a palm respond differently in the same media.

The regeneration of new tissues after subculturing was found to be slow and difficult. According to Fernando (2008) the subsequent growth of shoots and roots after a certain growth stage has shown to be very slow in *in vitro* cultures of date palm. This was observed during the embryo culture in coconut where the split portions took longer time to regenerate new tissues in the cut portions. The beginning of sprout in the embryo could be seen from 20 DAI in GA3 and TDZ media. The shoot took two to four months to grow to certain height before subculture, whereas the development of shoots after subculture took more than three months.

5.5. Expression study of *E2F* and *CDKA* genes

In the present study, the expression of the cell cycle control genes *E2F* and *CDKA* were analysed during early response stages of coconut embryo culture. The total RNA was extracted from embryo tissues of coconut during three different stages of embryo culture. Coconut is recalcitrant to tissue culture and hence the effect of different plant growth regulators on expression of the two cell cycle control

genes and in turn on the early *in vitro* response was studied. This study examined the expression of the *E2F* and *CDKA* genes at various developmental stages. The understanding of genes and their relative expression during *in vitro* propagation sheds light on the function of specific genes throughout particular developmental stages. There are many genes governing cell cycle, like genes encoding various cyclin dependent kinases (*CDKA*s and *CDKB*s), cyclins (*CYCA*s and *CYCB*s), *E2F1*, *E2F2* etc., (Komaki and Sugimoto, 2012) and the study of different genes is important for revealing their role during various developmental stages. In this present work only two genes of cell cycle were studied.

Using, coconut leaf as an explant, Jesty and Francis (1992) discovered that mitosis occurred at a low rate under *in vitro* conditions since the majority of the cells in the juvenile leaf were in the G1 phase of the cell cycle. They proposed that the mitotic rate of the *in vitro* cultures may be improved by the variables causing the G1/S phase transition, thereby increasing their capacity for morphogenesis. The morphogenetic stages of the cultures are connected to the cell cycle regulation (Planchais *et al*., 2000). The *in vitro* cell cycle regulation in coconut cultures, was studied by Sandowal *et al*. (2003). They observed that 90 per cent of cells accumulated in the G0 and G1 phases of the cell cycle. In our study, we wanted to check the effect of different plant growth regulators on early *in vitro* response which includes cell division and multiplication. So, we selected *E2F* and *CDKA* genes in the present study. However, to get a clear picture we need to study other genes as well in future.

In the current work, qRT-PCR analysis was used to examine the expression of these genes at various early response phases under the influence of different plant growth regulators. Errors in qRT-PCR experiments can arise from minor differences in the initial amount of RNA used, the quality of the RNA, variances in the efficiency of cDNA synthesis and PCR amplification. A specific gene is amplified together with the target gene to serve as an internal reference against which gene expression data can be normalised, reducing such mistakes and correcting the variation between samples (Gilliland *et al*., 1989). For normalisation, house-keeping genes are frequently utilised. Several internal control genes, such as the 18S rRNA, 28S rRNA, *ACT*, *eEF1*-, *eIF4*-, *GAPDH* and *UBC10* genes, can typically be

employed in coconut (Xia *et al*., 2014). For the expression study of 14 different genes in tissues of somatic embryos and embryogenic and plumular calli, Rajesh *et al*. (2016) used the *α-tubulin* gene as the endogenous control due to its stable expression. The 18S rRNA gene was used as internal control for the expression study of *CDKA* gene *in vitro* by Montero-Corte´s *et al*. (2010a). In our study, we have used two endogenous controls genes, α-*tubulin* and 18S rRNA genes to normalise the expression of the test genes (Vandesompele *et al*., 2002; Hellemans *et al*., 2007).

Real-time PCR based on SYBR Green chemistry was used for the gene expression study. The Sso Advanced Universal SYBR Green Supermix (Bio-Rad) was used for the real-time PCR experiments. According to Dragan and his co-workers (2012) the fluorescence of SYBR Green I changed more than 1000-fold after binding to double stranded DNA. Employing SYBR Green I was preferable to TaqMan probes for quantifying nucleic acids because the method is simple and less expensive (Ponchel *et al*., 2003). The amount of amplified product produced after each amplification cycle directly correlates with the amount of fluorescence generated during that cycle (Makkouk and Kumari, 2006). The fractional PCR cycle number at which the fluorescence exceeds the threshold level is known as the cycle threshold (Ct) value. To determine the threshold cycle for a sample, a fluorescent signal that is detected above threshold is considered a true signal (Arya *et al*., 2005). In the current research work, the relative gene expression study was conducted using the approach of geometric averaging of Ct values described by Vandesompele *et al*. (2002) and Hellemans *et al*. (2007).

The melt curve analysis was also carried out to verify the fidelity of the amplicons. A melting curve analysis was required to confirm whether the fluorescent signal received was from the targeted PCR product itself or not (Arya *et al*., 2005). Melt curve was produced when fluorescence was plotted as a function of temperature. The unique melting temperatures for *E2F* amplicons was 82.44 ± 0.5 °C, *CDKA* amplicons was 83.79 ± 0.5 °C, α-*tubulin* amplicons was 86.47 ± 0.5 °C and 18S rRNA was 85.27 ± 0.5 °C, confirming the fidelity of the amplicons.

The expression of endogenous control genes for α-*tubulin* and 18S rRNA was used to normalise the expression levels of the *E2F* and *CDKA* genes (Vandesompele *et al*., 2002). In the current investigation, it was discovered that the expression of the *E2F* gene was higher in GA3 medium whereas the expression of the *CDKA* gene was higher in TDZ medium during 5 DAI. In GA3 medium, the expression of both *E2F* and *CDKA* genes were greater at 10 DAI. At 20 DAI, TDZ medium had the highest levels of *CDKA* gene expression, while *E2F* expression was highest in GA3 medium compared to other media combinations. The fold change values for *E2F* gene at 20 DAI and *CDKA* gene at 5 DAI and 20 DAI were found to be statistically significant in T-test.

The *E2F* transcription factors control cell cycle progression and DNA replication by promoting the transcription of genes required for the S phase of the cell cycle (Vandepoele *et al*., 2005). In 2011, Montero-Corte's and his colleagues proposed that the addition of GA3 to the culture media during the growth of coconut embryos would enhance the expression of the *E2F2* gene. Our gene expression studies during 5 DAI, 10 DAI and 20 DAI stages were in agreement with the findings of Montero-Corte's *et al*. (2011) where the expression of *E2F* gene was higher in the media supplemented with GA3 compared to the other growth regulators. The morphological response of the cultures was on par with the gene expression pattern. The embryos in the GA3 media showed early response. The transcription factor encoded by the *E2F* gene is important for the advancement of the S phase and is necessary in the early stages of tissue formation (Vandepoele *et al*., 2005). As a result, *E2F* expression was higher at the early stages of development (5 DAI and 10 DAI) and then the expression reduced at 20 DAI in the GA3 supplemented medium.

Cyclin-dependent kinases (CDKs) control the eukaryotic cell cycle. The CDKs are activated with the help of positive regulators known as cyclins. Cyclins regulate the stability and activity of CDKs (Roudier *et al*., 2000). According to Martinez *et al*. (1992) and Hemerly *et al*. (1993), the plant *CDKA* played a dual role in cell division competence preservation and cell proliferation in differentiated tissues during the evolution of S and M phases in the cell cycle (Colasanti *et al*., 1991; Ferreira *et al*., 1991; Hirt *et al*., 1993). It was discovered that *CDKA* gene

expression was greater during germination and formation of somatic embryos (Montero-Corte's *et al*., 2010a). The addition of GA3 growth regulator increased the expression of *CDKA* gene (Montero-Corte's *et al*., 2010a) which was observed at 10 DAI, where its expression was higher in GA3 medium. The expression of *CDKA* gene was higher in TDZ supplemented medium at 5 DAI and 20 DAI. The increase in expression of *CDKA* gene during the early growth stages increase the cell division competence which is essential for the faster cell division and finally, better response of the explant.

The growth of the embryos during the culture was also observed to be faster in GA3 and TDZ supplemented media. Hence, we can conclude that the application of GA3 and TDZ increased the expression of *E2F* and *CDKA* genes which led to the increase in cell cycle progression and thus cell division. This led to the faster growth of the embryos that was observed during culture development. The recalcitrant behaviour of the coconut tissues was reduced with the application of plant growth regulators like GA3 and TDZ.

5.6. Conclusion

The provision of high yielding, disease-resistant elite planting material is one of the requirements for assisting the struggling coconut sector and meeting the existing and future demand. Only seed nuts are used for the purpose of propagation in coconut and these alone are not sufficient to meet the supply of good quality planting materials. Hence, micropropagation is the best way for expanding the supply of promising genotypes for cultivation. Compared to the organogenic pathway, somatic embryogenesis is the pathway that has been explored most in coconut. Although somatic embryogenesis has been reported in coconut from different explants, there is yet no commercially viable protocol for coconut micropropagation. The alternate *in vitro* method for clonal propagation is direct shoot organogenesis.

The coconut palm faces difficulties with *in vitro* regeneration due to its morphological, anatomical and physiological features and thus becomes recalcitrant to *in vitro* modification. Genes that regulate the cell cycle are known to be essential for germination. Two important genes controlling cell cycle are *E2F* and *CDKA*.

The expression of these genes in response to plant growth regulators were observed in the present study. Finally, the results revealed that the provision of growth regulators like GA3 and TDZ had immense effect on the expression of these genes. The expression of these genes during different response stages were higher in GA3 and TDZ media. The effect of GA3 and TDZ growth regulators on the growth of the embryos during the zygotic embryo culture were also positive where the development of sprout was earlier in these media. Based on the gene expression study, the GA3 medium was found to be favourable for early embryogenic response followed by TDZ medium. However, observations of the cultures showed that both TDZ and GA3 favoured early embryogenic response. We have selected only two important genes in the cell cycle for the present study. So, expression studies of more genes related to cell cycle will give a clear picture on the role of TDZ and GA3 in promoting early embryogenic response.

SUMMARY

6. SUMMARY

The study entitled "Expression profiling of *E2F* and *CDKA* genes during embryo culture in coconut (*Cocos nucifera* L.)" was undertaken at the Department of Plant Biotechnology, College of Agriculture, Kerala Agriculture University, Vellanikkara, Thrissur. The objective of the study was, expression profiling of the genes governing organogenic regeneration pathway at different morphogenic response stages in coconut embryo culture. This chapter provides the key insights of the study.

- Coconut cultivar West Coast Tall (WCT) was used in the present work

- Zygotic embryos were used as the explant in the study to initiate direct shoot organogenesis through embryo culture

- On Y3 medium, the embryo was inoculated with four distinct combinations of growth regulators and basal Y3 medium was used as control

- The different media combinations used were:

a. Basal medium (control)

b. A medium with 2,4-D (2 mgL^{-1}) and BA (5 mgL^{-1})

c. B medium with 2,4-D (2 mgL^{-1}), BA (5 mgL^{-1}) and GA3 (1.6 mgL^{-1})

d. C medium with GA3 (1.6 mgL^{-1})

e. D medium with TDZ (33 mgL^{-1})

- According to the observations obtained from the study, the embryos showed better response in GA3 and TDZ supplemented media compared to other media combinations

- In A and B media, the initiation of sprout development point was observed at 20 days after inoculation (DAI). The further sprout development was observed much later

- In C and D media, the development of sprout was observed earlier at 15 DAI and the sprout initiation was seen at 20 DAI early

- There was heterogenous response of cultures. Embryo inoculated on same media showed different response

- Since, WCT is a cross pollinated variety there is variation in the *in vitro* response

- Cultures showing sprout initiation were decapitated and split into two for subculturing

- Expression profiling of *E2F* and *CDKA* genes was done during early response stages of zygotic embryo including 5 DAI, 10 DAI and 20 DAI

- Using the RNeasy plant mini kit (Qiagen), the total RNA was extracted from the tissues of the embryo initiated in *in vitro*

- The reverse transcriptase enzyme and Oligo(dT)18 primer were used to convert the total RNA to cDNA

- One pair of primer each was designed for the test genes (*E2F* and *CDKA*) and endogenous control genes (α-*tubulin* and18S rRNA)

- Gradient PCR was used to identify the ideal annealing temperature for the primers

- The PCR amplicons of the predicted size were purified using Novagen SpinPrep^{TM} Gel DNA kit and sequenced

- All the cDNAs were analysed by RT-PCR with the α-*tubulin* primers

- The differential expression of *E2F* and *CDKA* genes in early response stages (5 DAI, 10 DAI and 20 DAI) during coconut embryo culture was studied using qRT-PCR analysis

- Using the approach of geometric averaging of the Ct values as described by Vandesompele *et al*. (2002) and Hellemans *et al*. (2007), the relative expression level of the test genes was normalised with the expression of the endogenous controls

- It was found that, the expression of *E2F* gene was higher in GA3 provided medium in all stages. It showed 245.5, 68.45 and 4.19-fold over expression in GA3 medium compared to basal Y3 during 5, 10 and 20 DAI respectively

- The expression of *CDKA* gene was higher in TDZ provided medium during 5 DAI and 20 DAI where the fold change in expression was 4.23 and 3.85 respectively. At 10 DAI, the expression of *CDKA* gene was higher in GA3 provided medium with the fold change value of 19.82

- Thus, the media with GA3 and TDZ showed faster growth of the embryos as well as a higher expression of *E2F* and *CDKA* genes indicating their positive role of the growth regulators in enhancing the expression of *E2F* and *CDKA* genes which led to better growth of the embryos

Future line of work

The GA3 and TDZ are possible growth regulators for initiating coconut embryo culture. The further study on the expression pattern of other genes controlling cell cycle like cyclins, CDK-activating kinases, CDK- inhibitors and the hormone signalling pathways is necessary to get deeper insights on the molecular mechanism of recalcitrance in coconut.

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ABSTRACT

EXPRESSION PROFILING OF *E2F* AND *CDKA* GENES DURING EMBRYO CULTURE IN COCONUT (*Cocos nucifera* L.)

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Coconut (*Cocos nucifera* L., Family Arecaceae) is widely cultivated in the humid tropics of the world and is rightly known as *Kalpavriksha* meaning wish-fulfilling tree, since it provides almost all essentials for life. Coconut cultivation is affected by factors like abiotic and biotic stresses and the old age of palms, causing a major loss to coconut growers worldwide. Hence, replanting of old and senile plantations with high yielding variety seedlings is required to increase coconut productivity. Coconut is propagated through seed nuts but this conventional method of propagation is insufficient to provide adequate planting materials. The *in vitro* culture techniques can serve as suitable alternative to accelerate the commercial production of planting materials in coconut. Tissue culture techniques are not novel in the propagation of palms. They have been tried in different palms like oil palm, date palm, arecanut and coconut, but a commercially viable protocol for coconut micropropagation is still not available.

Coconut is known to be recalcitrant to *in vitro* manipulations. Since the *in vitro* response largely depends on the growth regulators used in the tissue culture media, the present study was undertaken to understand the influence of various plant growth regulators on the early *in vitro* response of coconut embryos. The expression profiling of cell cycle controlling genes like *E2F* (E2 promoter binding Factor) and *CDKA* (Cyclin Dependent Kinase A) during early response stages was done using qRT PCR.

We used zygotic embryos collected from mature nuts of coconut variety WCT as the explant. The embryos were inoculated in Y3 media supplemented with four combinations of plant growth regulators: first medium with 2,4-D (2 mgL^{-1}) and BA (5 mgL^{-1}); second medium with 2,4-D (2 mgL^{-1}), BA (5 mgL^{-1}) and GA3 (1.6 mgL^{-1}); third medium with GA3 (1.6 mgL^{-1}); fourth medium with TDZ (33 mgL^{-1}) and the basal Y3 medium as the control.

Embryo bulging and sprout initiation were the initial responses observed. Among the different media combinations tried, the media supplemented with GA3 alone and TDZ alone had rapid response in terms of sprout initiation and its

development. The sprout was seen as early as 20 days after inoculation (DAI) in these media The embryos showing good sprout development were later subcultured.

During the culture growth, the zygotic embryos at different early developmental stages were selected for gene expression study. Total RNA was isolated at three different growth stages namely 5 DAI, 10 DAI and 20 DAI The qRT PCR based on SYBR Green chemistry was performed to examine the expression of *E2F* and *CDKA* genes in the embryos. For the gene expression study, three biological replicates and three each technical replicates were sampled from the three selected early response stages in all the five media. The relative expression of *E2F* and *CDKA* genes was normalised with that of *α tubulin* and 18S rRNA genes using the method proposed by Vandesompele *et al* (2002) and Hellemans *et al* (2007). It was found that the expression of *E2F* was higher in Y3 medium supplemented with GA3 alone, throughout the period of observation. For *CDKA* gene, during 5 DAI and 20 DAI, highest expression was seen in TDZ medium; while its highest expression during 10 DAI was observed in GA3 medium.

These results indicate that the presence of growth regulators like GA3 and TDZ upregulated the expression of genes controlling cell cycle like *E2F* and *CDKA* during *in vitro* culture. However, when GA3 was provided along with 2,4 D and BA, we observed lower expression of these genes compared to the medium supplemented with GA3 alone. This was probably due to the cross talk between cytokinins and gibberellins. The *E2F* and *CDKA* genes are known to have a role in the cell cycle progression. Therefore, due to the application of GA3 and TDZ, the expression of these genes increased and thus, the rate of cell division increased resulting in better morphogenesis of the embryos. The hormones GA3 and TDZ can therefore be used to reduce the recalcitrant behaviour of the coconut embryos to *in vitro* culture in the early stages.