EFFECT OF HISTONE DEACETYLATION IN THE REGULATION OF SOMATIC EMBRYOGENESIS RELATED GENES IN COCONUT (*COCOS NUCIFERA* L.)

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By

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THESIS

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KERALA, INDIA

2025

DECLARATION

I hereby declare that the thesis entitled "**Effect of histone deacetylation in the regulation of somatic embryogenesis related genes 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 of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: Vellayani

Date: 12/05/2025

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Certified that this thesis entitled "**Effect of histone deacetylation in the regulation of somatic embryogenesis related genes in coconut (*Cocos nucifera* L.)**" is a record of research work done independently by **Ms. BEEMA Y. BASHER** (2018 - 09 - 024) under my guidance and supervision and this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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LIST OF ABBREVIATIONS

SE	Somatic embryogenesis
PGR	Plant growth regulators
HDAs	Histone deacetyl transferases
WCT	West coast tall
2,4-D	2,4-Dichlorophenoxyacetic acid
TDZ	Thidiazuron
BA	Benzyl Adenine
2ip	2-isopentenyl adenine
NAA	Naphthalene acetic acid
IAA	Indole acetic acid
SB	Sodium butyrate
GA	Gibberellic acid
AC	Activated Charcoal
℃	Degree Celsius
Cm	Centi meter
et al	And others
g/L	Gram per litre
L.	Linnaeus
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
µM	Micro molar
%	Percent
ABA	Abscisic acid
TCPP	Tris (1,3-dichloro isopropyl) phosphate
mM	Milli molar
M	Molar
w/v	Weight by volume
5-AZaC	5-Azacytidine

TSA	Trichostatin A
NaOH	Sodium hydroxide
ppm	Parts Per Million
mg	Milli gram
HCl	Hydrochloric acid
CIM	Callus induction medium
DEPC	Diethyl pyrocarbonate
β- ME	β-mercaptoethanol
µL	Micro litre
rpm	Revolutions per minute
ml	Milli litre
ng/ µL	Nanogram per microlitre
TBE	Tris Borate EDTA
EDTA	Ethylenediaminetetraacetic acid
RT PCR	Real-time Polymerase Chain Reaction
cDNA	Complementary Deoxyribonucleic acid
BLAST	Basic Local Alignment Search Tool
Ct	Threshold cycle
PCR	Polymerase Chain Reaction

Introduction

1. INTRODUCTION

Coconut (*Cocos nucifera* L.) is a palm species characterized by a single-species classification, encompassing various ecotypes and hybrids, each with valuable agricultural traits. Its significant genetic diversity is reflected in the wide range of coconut-based products and uses (Foale, 2005). Commonly referred to as the "tree of life," the coconut palm is a perennial, versatile tree that offers a broad spectrum of products with both domestic and industrial purposes (Arunachalam and Rajesh, 2017). Coconut cultivation is prominent in tropical and subtropical regions, playing a crucial role in rural economies and supporting millions globally. It is anticipated that demand for coconut-based products like coconut water, milk, and oil will rise sharply in the coming years (Rodrigues *et al.*, 2018). At a recent COGENT meeting, it was noted that increasing coconut production on a large scale is essential for meeting the growing global demand (Bourdeix and Prades, 2018).

A single coconut tree can produce 50 to 80 fruits per year, but propagation from seed is a slow process, often taking 12-16 years. This slow growth particularly affects coconut farming, as new plantings from seeds are genetically diverse, and phenotypic variations from this hinder efficient farming (Yousefi, K. *et al.*, 2023). Vegetative propagation offers a solution by bypassing these limitations and ensuring more uniformity in coconut plants. Furthermore, hybrid coconuts demonstrate greater heterozygosity, which is an advantage in breeding hybrid progenies (Perera et al., 2009; Nair, K. P. 2017). Thus, clonal propagation, especially through somatic embryogenesis (SE), is considered a superior method for large-scale production to meet rising demand (Smertenko, S. et al., 2014).

Somatic embryogenesis, an asexual reproduction technique, is already employed in various species like coconut, oil palm, and date palm, offering high efficiency and long reproductive cycles from a single explant (Feher, 2019; Veluru *et al.*, 2022; Ferreira *et al.*, 2022; Solangi *et al.*, 2023). This method facilitates the mass production of genetically uniform plants while eliminating the need to wait

for the next reproductive season. A wide array of explants, such as immature leaves, roots, shoot apical meristems, and zygotic embryos, are utilized for initiating SE (Fernando *et al*., 2003; Loyola-Vargas *et al.,* 2018; Aparna V. *et al.,* 2023).

Kalaipandian *et al*. (2021) and Nwite *et al.* (2023) revealed that in the somatic embryogenesis (SE) process, the transformation from cells to plantlets is influenced by various factors such as the medium's composition, explant type, plant growth regulators (PGRs), heterogeneous responses, and the acclimatization process. Over the past two decades, there have been significant efforts to refine the propagation methods of coconut plantlets through SE (Sandoval Cancino, 2016). Researchers are utilizing *in vitro* techniques to investigate how somatic cells can grow into new, independent clonal organisms (Elhiti *et al*., 2022). During SE, the reprogramming of cells into whole plants requires the regulation of gene expression and the activation of specific signaling pathways. The first stage in coconut SE involves competent cultured cells responding to signals (PGRs or stress) to initiate dedifferentiation. The endogenous level of PGRs rises during the early phases of SE. The combination of gene activation and undifferentiated cells transitioning leads to embryogenic development (Sáenz-Carbonell, 2020).

Recent insights into the molecular mechanisms underlying somatic embryogenesis (SE) reveal that, in addition to plant growth regulators, various factors, including genetic and epigenetic mechanisms, also play a role in this process (Kalaipandian *et al*., 2021). The specific epigenetic mechanisms involved in coconut SE have yet to be explored, suggesting they may be crucial for better controlling the process. Prior studies have indicated that epigenetic mechanisms are active in the SE of multiple plant species (Feher, 2015; Ikeuchi *et al*., 2015), with DNA methylation particularly important in the initiation and progression of SE (Kumar and Van Staden, 2017). Recent findings suggest that modifying the methylation and deacetylation states of genes within the somatic embryogenesis pathway using targeted compounds can trigger early SE in recalcitrant plants (Nguyen *et al.,* 2015; Bandupriya *et al.,* 2016). These compounds work by inhibiting methylase in methylation pathways or histone deacetylase in

deacetylation pathways, leading to increased gene acetylation and subsequently enhanced gene expression (Zhou *et al*., 2017; Wang *et al*., 2018).

Among various histone deacetylase inhibitors (HDAs), Trichostatin A (TSA) is the most commonly utilized. According to Finnin *et al*. (1999), TSA functions by inserting its aliphatic chain into the hydrophobic cleft within the catalytic domain of HDAs. This specific inhibition of HDAs results in an accumulation of acetylated histones and a decrease in DNA methylation within cells. Studies have demonstrated TSA's potential for inducing callus formation and regeneration in several crops, including wheat (Bie *et al*., 2020) and maize (Hu *et al.,* 2020).

Given this background, the current study aims to investigate the effects of TSA on embryogenic callus induction in coconut using immature ovary explants. The primary objective is to analyse the effect of histone deacetylation in the regulation of somatic embryogenesis related genes (SERK, BBM, WUS) and histone deacetylation gene (HDAC) in coconut (Cocos nucifera L.) using Trichostatin.

Review of literature

2. REVIEW OF LITERATURE

2.1 Somatic embryogenesis in coconut

SE is a method of asexual reproduction widely used for large-scale clonal propagation in various tree species (coconut, oil palm, date palm) with long reproductive cycles from one single explant (Feher, 2019; Veluru *et al*., 2022; Ferreira *et al.,*2022; Solangi *et al.,* 2023). In coconut, somatic embryogenesis has garnered increasing attention due to its implications for improving yield and quality. Research has demonstrated that various factors, including the choice of explant, culture medium composition, and environmental conditions, significantly influence the success of somatic embryogenesis (Srinivasan *et al*., 2019; George *et al*., 2020). Recent studies have also explored the molecular underpinnings of somatic embryogenesis in coconut, revealing important insights into the signaling pathways and gene expressions involved (Sundararajan *et al*., 2023). Understanding these mechanisms can aid in the refinement of culture protocols and the enhancement of embryogenic potential.

In coconut palm, SE involves three main stages; the induction of embryogenic callus (cell cycle, dedifferentiation, and totipotency), somatic embryo development (meristem maintenance), and plantlet maturation (Biddle *et al*., 2020). The stimulation of cell proliferation, acquisition of embryogenic cell competence, and induction of somatic embryos undergo a series of development events (Feher *et al*., 2019). According to Reinert (1959), somatic embryogenesis, which forms the basis of cellular totipotency, is the process by which already differentiated somatic cells reverse their developmental program and become embryogenic, form somatic

embryos, which develop into complete plantlets via characteristic morphological and biochemical changes.

Smertenko and Bozhkov (2014) note that somatic embryogenesis (SE) is a highly effective biotechnological method, particularly for species that have long reproductive cycles or low seed production. While SE is a valuable technique for plant propagation, the varying levels of totipotency among different plant species can lead to differences in their embryogenic responses. Over the past two decades, considerable efforts have been made to develop and refine techniques for propagating coconut plantlets through SE (Sandoval-Cancino *et al*., 2016).

2.1.1. Key factors affecting somatic embryogenesis in coconut

Somatic embryogenesis (SE) in coconut (*Cocos nucifera*) is influenced by a variety of factors that play crucial roles in the efficiency and success of the process. Understanding these factors is essential for optimizing protocols and enhancing the yield of plantlets through SE.

2.1.1.1. Explant Selection

There are several reports on induction of callus or development of organized structures from different sources of explants, such as meristematic shoot and root tissue, inflorescence, leaf, zygotic embryo, endosperm tissue, anther and plumule (Branton and Blake (1983); Verdeil *et al*., (1993); Pannetier and Buffard-Morel (1982); Buffard-Morel *et al.,* (1988); Karunaratne and Periyapperuma (1989); Blake and Hornung (1995); Chan *et al.,* (1998); Fernando and Gamage (2000); Fernando *et al.,* (2003); Oropeza *et al*., 2005; Rajesh *et al*., 2005, 2014; Perez-Nunez *et al*., 2006). The choice of explant significantly impacts the induction of embryogenic callus. The developmental stage of the explant is critical; younger tissues tend to have a greater potential for totipotency and responsiveness to culture conditions (Feher *et al*., 2019).

Adkins *et. al.,* (1999) found that immature zygotic embryos were superior explant tissues for SE, with a callus induction rate of 50%, compared to just 3% from mature embryos. Similarly, Karunaratne and Periyapperuma (1989) reported that embryos over 8 months of age were already undergoing germination and therefore they were not suitable for callus formation.

The plumular tissue was found to be the most suitable for SE (Chan *et. al.,* (1998); Perera *et al*., (2007); Saenz *et al*., (1999)). Perez-Nunez *et al.,* 2006 hypothesized that 98,000 somatic embryos could be produced from a single zygotic plumule. The technique would need to use the repeated subculture of plumular callus, which could rapidly increase the total cell biomass and promote the formation of secondary somatic embryos (Raemakers, 1995).

Table 1. Studies on coconut *in vitro* culture using different explants.

Explant	Method of micropropagation	Regeneration (%)	Reference
Immature embryo	Callus induction	70 %	Fernando and Gamage, 2000
	Somatic embryo induction	50 %	Adkins *et al.,* 1999
Plumules	Callus induction	40 %	Perez-Nunez *et al.,* 2006
	Somatic embryo induction	5.16 %	Perez-Nunez *et al.,* 2006
Immature inflorescence	Callus induction	30 %	Vidhanaarchchi and Weerakoon, 1997
	Multiple shoot induction	92 %	Shareefa *et al.* (2019)
Anthers	Callus induction	22 %	Perera *et al.,*2008
	Somatic embryo induction	44 %	Bandupriya and Waidyarathne, 2021
Unfertilized ovaries	Callus induction	41 %	Perera *et al.,*2007
	Somatic embryo induction	50 %	Perera *et al.,*2009

2.1.1.2. Culture Medium Composition

The Y3 medium was specifically developed for coconut tissue culture (Euwens, 1976) and is now widely used for most coconut work (Adkins, S. 2016; Ledo, 2019). According to Adkins, S. (2016), within this medium, a high concentration of sucrose (usually > 4%) is used as the main carbohydrate source. However, this high sugar content can result in an increased risk of microbial contamination, allowing the contaminants to compete with the explant for nutrients in the medium (Salo, E. N. *et al*., 2020).

Activated charcoal (AC) is also commonly used in coconut tissue culture (Bhat, S.R., *et al*., 1991; Thomas, T.D., 2008). This substance has a matrix structure with very fine pores that gives it a large surface-to-volume ratio. The main purpose of AC is to absorb and therefore remove inhibitory substances (e.g., phenolic compounds) that are produced by the explant tissue and that accumulate in the media. Variation in particle size of AC, which occurs between batches, can affect the frequency of SE callus formation.

2.1.1.3. Growth Regulators

Plant growth regulators are necessary to regulate callus formation, multiplication, somatic embryo formation, and germination responses. The explant tissue's sensitivity towards each specific group of PGRs determines SE outcomes (Jiménez *et al.,* 2005). Auxins, particularly 2,4-dichlorophenoxyacetic acid (2,4-D), are commonly used to initiate callus formation, while cytokinins, such as benzyl aminopurine (BAP), can promote embryo differentiation (Srinivasan *et al.,* 2019). The concentration and combination of these regulators must be carefully optimized, as they can greatly influence the transition from callus to somatic embryos.

Nguyen *et. al.,* (2015) reported that for coconut SE, embryogenic callus induction and proliferation can be promoted by a high concentration of auxin (2,4-D at 24 to 600 µM in the presence of AC), whereas a subsequent reduction in 2,4-D concentration has been shown to aid the maturation and germination of the

somatic embryos formed. In the same report, it is said that, embryogenic callus induction from zygotic embryos required a lower concentration of 2,4-D (24 µM), whereas a high 2,4-D concentration (600 µM) was necessary for embryogenic callus induction from plumule and young inflorescence tissues.

A combination of a cytokinin, such as kinetin and 2iP (6-(γ,γ-Dimethylallylamino) purine; both at 10 µM), and 2,4-D (100 µM), have been found to increase the production of callus and somatic embryos from microspores in coconut (Perera, *et al.,* 2009). Cytokinins, such as 6-benzylaminopurine (BAP), thidiazuron (TDZ), kinetin, or 2iP, are common additions to the medium for the maturation of coconut somatic embryos (Nguyen, *et al.,* 2015). A combination of TDZ and 2,4-D has been shown to enhance coconut callus induction and to decrease tissue browning (Karun, 2017).

Brassinosteroids promote cell division and differentiation of tissues of many species. They have been found to increase the production of primary callus, embryogenic callus, and somatic embryos from the plumular tissue of coconut (Azpeitia, A. *et al.,* 2003). Abscisic acid (ABA; 2.5 to 5.0 µM) has been shown to promote somatic embryo development in coconut when applied to nodular calli derived from immature zygotic embryos (Samosir, *et al.,* 1999; Fernando, S. *et al.,* 2000).

Gibberellic acid (GA3) interacts with many plant processes, including the regulation of proteins that result in changes in gene expression, cellular behavior, and metabolism (Ree, J. F. *et al.,* 2003). According to Montero-Córtes *et. al.* (2010), GA3 (0.5 µM) improved the formation and germination of coconut somatic embryos, with the maturation rate increasing 1.5-fold. GA3 has also been included in a recent protocol developed for the germination of coconut somatic embryos derived from plumular tissues at concentration of 2.8µM (Saenz, 2016).

Table 2. List of *in vitro* protocols for somatic embryogenesis in different varieties of coconut.

Explant	Basal media	PGR (Callus induction)	PGR (Embryo maturation)	Variety /Hybrid	Reference
Rachilla, stem, and foliage	Eeuwen's Y3, sucrose (5%), and AC (0.25%), and agar (0.6%)	2,4-D (452 µM), NAA (2.69 µM), BAP (8.88 µM), Kinetin (4.65 µM)	2,4-D (2.3 µM)	West Coast Tall	Gupta *et al*., 1984
Young foliage tissue	Eeuwens' salts, Morel's Vitamin, sucrose (30 g/L) and agar (0.8%)	2,4-D or TCPP	BAP	Malayan Yellow Dwarf (MYD) × West African Tall (WAT)	Pannetier and Buffard-Morel, 1982
Young embryo	Gamborg's B5 medium, and agar (0.7%)	IAA, NAA, 2,4-D, BA or Kinetin (0.5 mg/L to 5 mg/L)	IAA (2 mg/L)	West Coast Tall (WCT)	Bhalla-Sarin *et al*., 1986
Immature embryo	BM72, sucrose (40 g/L) and AC (0.25%), and agar (0.8%)	2,4-D (24 µM), and ABA (2.5–7.5 µM)	2,4-D and cytokinin (2–10 µM)	Sri Lanka Tall	Fernando and Gamage, 2000
	Eeuwen's Y3, Morel and Wetmore (MW) Vitamins, sucrose (116.8 mM) and AC		2,4-D and BAP (10-5 M)	MYD × WAT, WAT × MYD and MYD	Verdeil and Buffard-Morel, 1995

	(2 g/L)				
Immature inflorescence	Modified MS macro nutrients, Nitsch micronutrients, MW Vitamins, EDTA (26 mg), iron (24.9 mg), sucrose (20 g/L), ascorbic acid (100 mg/L), malic acid (100 mg/L), adenine sulfate (30 mg/L), agar (7.5 g/L) and AC (3 g/L)	2,4-D (100 mg/L) and BAP (1 mg/ L)	2,4-D (130 mg/L) and BAP (140 mg/L)	PB 121 (MYDx WAT)	Magnaval *et al*., 1997
	Eeuwen's Y3, sucrose (30 g/L) and AC (2.5 g/L)	Spermine (0.01 µM), Smoke-saturated-water (10%) and auxin (500 µ M).	2,4-D and PGR free no PGR and	Malayan Yellow Dwarf	Antonova, 2009
Plumule	Eeuwen's Y3, AC (2.5 g/L) and gelrite (3 g/L)	2,4-D (0.1 mM)	2,4-D (1 µM) and BA (50 µM)	Malayan Dwarf	Chan *et al.*, 1998
	BM72, sucrose (4% w/v), and agar (0.8%)	2,4-D (24 µM)	N/A	Sri Lanka Tall	Fernando *et al*., 2003
	Eeuwen's Y3, sucrose (30 g/L), AC (2.5 g/L), gelrite (3	2,4-D (600 µM)	2,4-D (6 µM), and BA (300 µM)	Green Malayan Dwarf	Pérez-Núñez *et al*., 2006

	g/L)				
Unfertilized ovary	CRI 72 and agar (2%)	2,4-D (100 µM)	2,4-D (66 µM) and ABA (5 µM)	Sri Lanka Tall	Perera *et al.*, 2008

2.1.1.4. Genotypic variation

Genotypic variation among coconut cultivars is a significant determinant of their embryogenic potential. Studies have indicated that hybrid and improved varieties of coconut often show enhanced responsiveness in somatic embryogenesis compared to traditional landraces (Sandoval-Cancino *et al.*, 2016). Genetic factors influencing totipotency include the expression of specific genes involved in cell cycle regulation and differentiation. Research has identified several key transcription factors, such as those belonging to the APETALA2/ETHYLENE RESPONSE FACTOR (*AP2/ERF*) family, that regulate embryogenic competence in plants. In coconut, the expression of these genes is positively correlated with the formation of somatic embryos (Sundararajan *et al.*, 2023). According to Chaturvedi *et al*., (2022), Transcriptomic analyses have shown that genes associated with embryogenesis, such as those involved in stress responses, signaling pathways, and hormone metabolism, are expressed at different levels depending on the genotype. Understanding these expression profiles can aid in identifying molecular markers for selecting genotypes with high embryogenic potential.

2.2 Molecular Pathways Regulating Somatic Embryogenesis in Coconut

2.2.1 Diverse Gene Expression Patterns in the Somatic Embryogenesis Pathway

Somatic cells attain embryogenic potential by reprogramming of gene expression (Chugh and Khurana, 2002; Solis-Ramos *et al.*, 2012). Various genes are induced during somatic embryogenesis which results in the synthesis of new mRNA and proteins; some of well-studied genes include *SERK* (Somatic Embryogenesis Receptor Kinase), LEC (Leafy Cotyledon), *GLP* (Germin-like Protein), *GST* (Glutathione-S-transferase), *ECP* (Extracellular Protein), *WUS*

(Wuschel) and *PKL*(Pickle) (Solis-Ramos *et. al*., 2012; Smertenko and Bozhkor, 2014).

In recalcitrant species like coconut, research on differential gene expression during somatic embryogenesis is limited and primarily focuses on a few specific genes, including the cyclin-dependent kinase gene (*CDKA)* (Montero-Cortes *et al.,* 2010), the KNOTTED-like homeobox gene (Montero-Cortes *et al.,* 2010), and the SERK gene (Perez-Nunez *et al.,* 2009). A transcriptome analysis of embryogenic calli obtained from plumular explants of the West Coast Tall (WCT) cultivar identified fourteen genes, and the expression patterns of these genes were confirmed at six developmental stages using RT-qPCR (Rajesh *et al.,* 2015). One approach to enhancing the embryogenic potential of resistant tissues involves altering the expression of genes linked to embryogenic competence, including BABY BOOM (*BBM*), Somatic Embryogenesis Receptor Kinase (*SERK*), Leafy Cotyledon (*LEC*), and Wuschel (*WUS*)-related homeobox (*WOX*) genes (Martínez *et al.,* 2021).

SERK genes are characterized by their receptor-like protein kinase domains, which are implicated in various signal transduction pathways related to plant development and stress responses. In the context of somatic embryogenesis, *SERK* proteins have been shown to be involved in the promotion of embryogenic competence and the transition from somatic cells to embryogenic cells (He *et al.,* 2009). Studies by Perez-Nunez *et al.* (2009) identified the *SERK* gene as a key player in the embryogenic pathway, with its expression correlating positively with embryogenic callus formation and somatic embryo development.

WUS genes are essential for maintaining the meristematic stem cell population in plants. In coconuts, their expression can promote the formation of embryogenic callus, which is critical for successful somatic embryogenesis (Zhang *et al.,* 2017). According to Huang *et al.,* 2017, The expression of *WUS* genes is often associated with the ability to regenerate plant tissues an in coconuts, manipulating *WUS* expression can enhance the efficiency of somatic embryogenesis *WUS* genes interact with other regulatory pathways, such as those

involving auxins and cytokinins, to coordinate the developmental processes leading to somatic embryogenesis (Fambrini *et al*., 2022).

BBM genes are pivotal in inducing embryogenic competence in coconut cells. Their expression leads to the formation of embryogenic callus, which is essential for successful somatic embryogenesis (Jha and Kumar, 2018). *BBM* genes interact with other transcription factors and hormonal pathways, particularly auxins and cytokinins, to regulate the embryogenic process. Khan *et al.*, (2023) reported that the expression of *BBM* genes promotes cell proliferation and the maintenance of pluripotency in embryogenic cultures, facilitating higher regeneration rates in coconut tissue culture. Manipulating *BBM* gene expression through genetic engineering techniques has shown promise in enhancing somatic embryogenesis efficiency and developing superior coconut varieties with desirable traits (Fambrini *et al.*, 2022).

The Cyclin-Dependent Kinases A (*CDKA*) gene is associated with the growth of plants derived from differentiated tissues, as it facilitates cell proliferation and preserves the ability for cell division (Martinez *et al.*, 1992; Hemerly *et al.*, 1993). The AINTEGUMENTA-LIKE (*ANT*-like) gene was discovered in the coconut genome by Bandupriya *et al.* (2016). This gene exhibits significant sequence similarity in conserved domains with the BBM gene, which has been associated with brassica embryogenesis (Boutilier *et al.*, 2002). Additionally, Montero-Cortes *et al.* (2010) obtained the complete sequences of two *KNOX*-like genes in coconuts, named *CnKNOX1* and *CnKNOX2*.

Table 3. List of genes and their gene expression pattern in somatic embryogenesis in plants.

Gene Family and Other Genetic Mechanisms	Gene(s)	Gene Expression Pattern
Somatic Embryogenesis Receptor-like Kinase	*CnSERK*	Highly expressed in the callus tissues at the

(*SERK*)		initiation stage of SE
Cyclin-Dependent Kinases (*CDK*s)	*CnCDKA*	Increased expression during the formation of embryogenic callus and decreased in the germinated somatic embryos
KNOTTED-like homeobox (*KNOX*)	*CnKNOX1*	Highly expressed at the coleoptile somatic embryo growth
	CnKNOX2	Highly expressed at the globular stages of somatic embryo growth
CLAVATA (*CLV*)	*CLV*	Increased at the early stages of callus formation
SERK, Mitogen-Activated Protein Kinase (*MAPK*), *APETALA2*/Ethylene Responsive Factor (*AP2/ERF*), *SAUR* family protein, Embryogenic Cell Protein (*ECP*), Late Embryogenesis-Abundant protein (*LEA*), Arabinogalactan Protein (*AGP*) and AINTEGUMENTA (*ANT*)	*SERK, MAPK, AP2/ERF, SAUR, ECP, LEA, AGP,* and *ANT*	Highly expressed in embryogenic calli
Germin-Like Protein (*GLP*), Glutathione S-Transferase (*GST*), PICKLE (*PKL*), WUSCHEL (*WUS*)	*GLP, GST, PKL, WUS,* and *WRKY*	Highly expressed during the somatic embryo development stage
Micro RNAs (miRNAs)	Several miRNAs, and their targets	Found in embryogenic and non-embryogenic calli produced from plumular explants

2.2.2. Epigenetic regulation of somatic embryogenesis

Epigenetic regulation plays a crucial role in somatic embryogenesis by influencing gene expression without altering the underlying DNA sequence. This regulation involves mechanisms such as DNA methylation, histone modification, and small RNA pathways, which collectively modulate developmental processes and cellular differentiation. Methylation of cytosine residues in DNA can silence genes that inhibit embryogenesis. Changes in DNA methylation patterns have been observed during the induction of somatic embryos, suggesting that demethylation is essential for reactivating developmental programs (Liu *et al.*, 2014). Post-translational modifications of histones, such as acetylation and methylation, are important for the regulation of chromatin structure and gene accessibility. Increased histone acetylation has been linked to the activation of embryogenic genes during somatic embryogenesis (Choi *et al*., 2019).

2.2.2.1 Histone modifications

Histone modifications play a critical role in the regulation of gene expression during somatic embryogenesis by altering chromatin structure and accessibility. These modifications can influence the activation or repression of genes necessary for the developmental processes involved in embryogenesis. Common types of histone modifications include acetylation, deacetylation, methylation, demethylation (Zhou *et al.*, 2017), which alters somatic embryogenesis capacity during tissue culture (Wang *et al.*, 2018).

During somatic embryogenesis, specific patterns of histone modifications can activate the expression of key embryonic genes while silencing those that promote differentiation. According to Liu *et al.*, 2014 changes in histone acetylation and methylation are critical for reprogramming somatic cells into embryogenic cells. The transitions between different stages of somatic embryogenesis are accompanied by specific histone modification signatures that regulate gene expression profiles necessary for each stage (Bie *et al.*, 2020).

Acetylation of histones, mainly on lysine residues, is facilitated by histone acetyltransferases (*HAT*s), leading to a more open chromatin structure that promotes transcription (Choi *et al.,* 2021). In coconut somatic embryogenesis, increased histone acetylation is associated with the activation of embryonic development-related genes, which is essential for reprogramming somatic cells into embryogenic cells (Liu *et al.,* 2014). Histone deacetylases (*HDAC*s) remove acetyl groups from histones, resulting in a compact chromatin structure and repression of gene transcription (Temman *et al.,* 2023). During coconut somatic embryogenesis, *HDAC* activity is crucial for silencing genes that promote differentiation, thus ensuring proper embryonic development (Bie *et al.,* 2019).

2.2.2.2. Histone modifiers

According to Shahbazian & Grunstein (2007), the acetylation of histone lysine residues produces a relaxation of the chromatin structure, and this phenomenon is associated with increased gene activity. The activity of histone acetyltransferases (*HAT*s) and histone deacetyl transferases (*HDA*s), regulates the balance between histone acetylation and deacetylation respectively. Among this during germination, *HDA6* redundantly regulates the repression of embryonic properties directly or indirectly via the repression of embryo-specific gene function (Martinez *et al.,* 2021).

Increased histone acetylation and changes in physiological functions have also been associated with HDAC inhibitor treatments (Wakeel *et al.,* 2018). Trichostatin A (TSA) and sodium butyrate (SB) are among the most commonly used inhibitors of histone deacetylases. When TSA and SB were added to the induction medium, they significantly enhanced the embryogenic responses in grapevine and newly germinated somatic embryos (Martínez *et al.,* 2021). Furthermore, the inclusion of 0.5 µM TSA substantially improved the induction rates of embryogenic callus and green shoots in wheat (Bie *et al.,* 2020).

Finnin *et al.*, (1999) reported that TSA is known to insert its aliphatic chain into the hydrophobic cleft located within the catalytic domain of *HDA*s. TSA specifically inhibits *HDA*s, which results in the accumulation of acetylated histones and a corresponding reduction in DNA methylation within cells. This inhibition can lead to various cellular effects, including induced differentiation, cell cycle arrest, changes in cell morphology, apoptosis, and alterations in transcription, ultimately affecting gene expression on a genome-wide scale. Treatment with TSA has been shown to reduce the mitotic index in maize (Zea mays L.) root tips, suggesting that DNA and *H3K9* methylation, as well as H4 deacetylation, play significant roles in triggering mitosis (Yang *et al.*, 2020).

Table 4. List of histone modifiers used in *in vitro* culture.

Epigenetic regulators	Crops	Use in tissue culture/purpose	Epigenetic modification	Reference
5-Azacytidine	*Cocos nucifera* L.	Somatic embryogenesis	DNA methylation	Nic-Can *et. al*., 2013
	Arabidopsis thaliana	Somatic embryogenesis	DNA methylation	Nic-Can et. al., 2013
Trichostatin A	*Triticum aestivum*	Somatic embryogenesis	Histone acetylation	Bie *et. al.*, 2020
	Vitis vinifera	Somatic embryogenesis	Histone acetylation	Martínez *et. al.*, 2021
Sodium butyrate	*Vitis vinifera*	Somatic embryogenesis	Histone acetylation	Martínez *et. al.,* 2021
	Triticum aestivum	Somatic embryogenesis	Histone acetylation	Bie *et. al.*, 2020
	Oryza sativa	Callus induction	Histone acetylation	Liang and Fang, 1986

Materials and methods

3. MATERIALS AND METHODS

The study entitled Effect of histone deacetylation in the regulation of somatic embryogenesis related gene expression in coconut (Cocos nucifera L.) was conducted at the Department of Molecular Biology and Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2023-2024. The details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

3.1 Pretreatment of explant (plumule)

3.1.1 Preparation of explant

Coconuts (11 months old) of variety West Coast Tall (WCT) was collected from Instructional Farm, College of Agriculture, Vellayani. It was then dehusked and cut open. The plumule containing endosperm part of the coconuts are scooped out using a cork borer.

The laminar air flow (LAF) was prepared by switching on the UV light for 30 minutes to ensure complete disinfection of previously autoclaved plates, forceps, blades, and media bottles before inoculation. Hands were sanitized with 70% alcohol prior to handling the samples. The hood was sterilized with 70% alcohol, and all plates, blades, and forceps were flame sterilized using pure alcohol. The endosperms were transferred to the LAF, and the plumules were extracted using a scalpel. The plumules were then surface sterilized by immersion in 20% sodium hypochlorite for 20 minutes, with intermittent rinsing in sterile water (Rajesh *et. al*., 2016).

3.1.2 Preparation of culture medium

Basal Y3 medium was prepared using the standard protocol (Appendix I). Standard procedures were followed for the preparation of media.

Auxin (2,4-D) and cytokinin (TDZ) were used as plant growth regulators in the medium (Rajesh et. al., 2016). To prepare 1000 ppm stock solution 1000 mg of PGR dissolved in 1000 ml of water. TDZ and 2,4-D were dissolved in 1N NaOH and made up with sterile water. TDZ is heat labile, thus it was filter sterilized after the preparation of stock and were added only after autoclaving the culture medium. All the stock solutions were stored under refrigerated conditions at 4^{o}C and brought back to the ambient condition before media preparation.

For the preparation of the media, aliquots from all stock solutions of major nutrients, minor nutrients, vitamins, and organic compounds were pipetted into the beaker. To this 74.6µM 2,4-D, 4.5µM TDZ was added from the prepared plant growth regulator stocks and Trichostatin of concentrations 0µM (control), 0.5µM, 1.0µM, 1.5µM and 2.0µM was added from the 5mM stock of Trichostatin (Sigma) and 0.1 g/L Myo-inositol, 50mM spermidine along with 4% sucrose was added fresh and made up using distilled water. The pH of the media was corrected to 5.8 using 1N NaOH or 1N HCl before the addition of gelling agent (3%) and activated charcoal (0.1%).

All the culture media were autoclaved at 121^{o}C for 15 min. The media were allowed to cool at room temperature and stored in the culture room until used.

3.2 Embryogenic calli induction in coconut variety WCT in presence of histone deacetylase inhibitor – Trichostatin

After the pretreatment, the plumular explants were transferred to Callus Induction Medium (CIM) (Rajesh *et. al.*, 2005) (Y3 medium containing 2,4-D 74.6µM+TDZ 4.5µM+spermidine 50mM) supplemented with Trichostatin at different concentrations (0, 0.5, 1.0, 1.5 & 2.0µM) and incubated in dark for 4 weeks. The experiment was performed in a completely randomized design (CRD) with four treatments each of four replications. Number of embryogenic calli formed and the time taken for embryogenic calli induction were observed.

Table 5. Different concentrations of TSA used to check the response of plumules.

Treatments	Culture medium
T1	CIM + 0.5µM TSA
T2	CIM + 1.0µM TSA
T3	CIM + 1.5µM TSA
T4	CIM + 2.0µM TSA

3.3 RT-qPCR of somatic embryogenesis related genes in coconut

Total RNA was extracted from embryogenic calli using Trizol reagent (HiMedia). Three genes, Somatic Embryogenic Receptor Kinase (SERK), BABY BOOM (BBM) and WUSCHEL (WUS) that are found to play an important role in somatic embryogenesis in coconut were selected for gene expression studies (Osorio-Montalvo *et al.*, 2020) and GAPDH used as the reference gene.

3.3.1 RNA isolation

RNA was isolated from the plumules inoculated in control (CIM) and treatments. The precautions taken during the RNA isolation are listed below:

- The workbench was cleaned twice with ethanol and RNase reagent (Ambion, USA). All reagents and solutions were made using DEPC-treated distilled water.
- Mortars, pestles, spatulas, and scissors were made RNase-free by immersing all in DEPC (Sigma, USA) overnight. They were dried and wrapped in aluminum foil and baked at 160 °C at least for 5 h in a hot air oven. RNase-free tips and microcentrifuge tubes were used.
- Centrifuge along with its rotor was wiped with RNase Zap reagent before being used for analysis.

- Powder-free, RNase-free sterile gloves were used.

RNA was isolated using the standard protocol with Trizol reagent (Appendix II).

3.3.2 Quantification of RNA

The purity and concentration of the isolated RNA were checked using a NanoDrop spectrophotometer (DeNovix DS-11).

Quantity of RNA (ng/µL) = A260 × 40 × Dilution factor

3.3.3 Quality Determination of RNA

The integrity and quality of RNA thus isolated were checked using Agarose gel electrophoresis. 5µl sample along with 1µl gel loading dye was loaded on 1% agarose gel. Electrophoresis was carried out at 70 V using 1 X TBE tank buffer. Ultimately, the gel was taken and viewed in a gel documentation system (BioRad inc, USA) using the software "Image Lab".

Purity of RNA = A260 / A280 (ratio should be 2 for pure RNA)

3.3.4 cDNA synthesis

RNA was converted to complementary DNA (cDNA) by an RNA-dependent DNA polymerase enzyme called Reverse Transcriptase. cDNA synthesis was done by using 2XRT Easy Mix from G-Biosciences (Table 6). The reaction mixture was incubated in a thermal cycler (Bio-Rad T100 Thermal Cycler) as directed by the manufacturer (Table 7).

Table 6. Reaction mix used for cDNA synthesis.

Reagent	Volume (µl)
2XRT Easy Mix	5
Random primer	0.5
Oligo (dT) primer (50µM)	0.5

RNA template	X
RNase-Free ddH2O	4.5-X
Total volume	10

Table 7. Thermal profile used for cDNA synthesis.

Step	Temperature (^{o}C)	Time (min)
Reverse transcription	42°C	20 min
RT inactivation	85°C	5 min
Optional Step	Hold at 4°C	∞

3.3.5 Confirmation of cDNA

In order to confirm the synthesis of cDNA, PCR amplification was performed using a PCR master mix provided by GBiosciences. The reaction mixture for cDNA confirmation is given below in Table 8. The primers used for cDNA confirmation were all previously reported (Bhavyasree *et. al.*, 2015). The list of the sequence of primers are shown in Table 9.

Table 8. The reaction mixture for cDNA confirmation.

Reagent	Volume (µl)
PCR Master Mix	5
Forward primer	1
Reverse primer	1
cDNA (1:4)	1
RNase-Free ddH2O	2
Total volume	10

Table 9. Sequence of primers (Bhavyasree *et. al.*, 2015).

Target gene	Orientation	Primer sequence (5'-3')	Annealing Temperature
SERK	F	GCCGTACATCGAAACTTGCT	56ºC
	R	GGATCACAATGATCATGCAAA	
WUS	F	TGTACTGCCTGCCTGGTTC	52ºC
	R	CCACCTGATAAGAGGGACGA	
BBM	F	GAGGCCTATGACATTGCAGC	52ºC
	R	CACAGTCATGGCGTCAGATG	

3.3.6 Relative expression of genes

The expression of the genes was analyzed using Real-Time qPCR. The cDNA from the enlarged plumules obtained from control (CIM) and enlarged plumule obtained from CIM with 0.5µM TSA were amplified and the Ct values were obtained using the CFX96 Real-Time system (BIO-RAD) and 2XAB HS SYBR Green qPCR mix (G-Bioscience). The reaction mix and the thermal profile of cDNA are shown in Table 10 and Table 11 respectively.

Table 10. Reaction mix used for Real-Time qPCR.

Reagent	Volume (µl)
SYBR Green qPCR mix	5
Forward primer (2µM)	0.5
Reverse primer (2µM)	0.5

Template (1:4)	2.5
Water, nuclease-free	1.5
Total	10

Table 11. Thermal profile for Real-Time qPCR.

Stage	Temperature	Time	Repeat
Initial denaturation	95	5 min	1
Denaturation	95	45 s	34X
Annealing	Specific to genes	30 s	
Extension	72	45 s	
Final extension	72	1 min	1

3.4. RT-qPCR of histone deacetylase gene

Histone deacetylase gene expression in control (CIM) treatment and plumule of all 0.5µM TSA treatment were analysed to see the change in deacetylation in the presence and absence of Trichostatin.

3.4.1 RNA Isolation

RNA was isolated from the plumules inoculated in control (CIM) and treatments.

3.4.2 Quantification of RNA

The purity and concentration of the isolated RNA were checked using a NanoDrop spectrophotometer (DeNovix DS-11).

Quantity of RNA (ng/µL) = A260 × 40 × Dilution factor

3.4.3 Quality Determination of RNA

The integrity and quality of RNA thus isolated were checked using Agarose gel electrophoresis. 5µl sample along with 1µl gel loading dye was loaded on 1% agarose gel. Electrophoresis was carried out at 70 V using 1 X TBE tank buffer. Ultimately, the gel was taken and viewed in a gel documentation system (BioRad inc, USA) using the software "Image Lab".

Purity of RNA = A260 / A280 (ratio should be 2 for pure RNA)

3.4.4 cDNA synthesis

RNA was converted to complementary DNA (cDNA) by an RNA-dependent DNA polymerase enzyme called Reverse Transcriptase. cDNA synthesis was done by using 2XRT Easy Mix from G-Biosciences (Table 6). The reaction mixture was incubated in a thermal cycler (Bio-Rad T100 Thermal Cycler) as directed by the manufacturer (Table 7).

3.4.5 Confirmation of cDNA

In order to confirm the synthesis of cDNA, PCR amplification was performed using a PCR master mix provided by G-Biosciences. A known gene-specific primer was used for confirmation. The reaction mixture for cDNA confirmation is given below in Table 9.

3.4.6 Primer designing

Primers for the amplification of the histone deacetylase gene *(HDA6)* in coconut were designed by using *in silico* tools based on the exon sequence retrieved from GenBank. Primers were designed using the NCBI Primer-BLAST tool. The primers satisfying the following criteria were selected;

- Primer length: 17 to 20 bases
- G: C content: 50 to 60 %
- Primer Tm: 55 - 65°C

Due to the unavailability of genome sequences of the *HDA6* gene in the coconut genome, sequences of the *HDA6* gene from Arabidopsis were taken for the primer design. This sequence was compared with the whole genome sequence of *Cocos nucifera* using BLAST and the result obtained after this is used for primer designing. Primer was designed using the Primer Probe Test Tool of Primer Express Software Version 3.0. This helps to test self-dimer, cross-dimer, and hairpin structures. The actin gene was used as a reference gene in Real-Time qPCR experiments.

3.4.7 Annealing temperature standardization for gene-specific primers

Gradient PCR using CFX96 Real-Time system (BIO-RAD) was carried out with a temperature range of 54.0 °C, 54.7°C,56.0°C, 57.9°C, 60.4°C, 62.3°C, 63.5°C, 64 °C for *ACTIN and HDA6* genes. The melt curve was analyzed for each gene at each temperature and the optimum annealing temperatures were finalized.

3.4.8 Relative expression of *HDA6* gene

The expression of the *HDA6* genes was analyzed using Real-Time qPCR. The cDNA from the fresh immature ovary, callus obtained from CIM with SB, and callus obtained from CIM without SB were amplified and the Ct values were obtained using the CFX96 Real-Time system (BIO-RAD) and 2XAB HS SYBR Green qPCR mix (G-Bioscience). The reaction mix and the thermal profile of cDNA are shown in Table 10. and Table 11 respectively.

After the completion of the Real-Time qPCR reactions, the threshold cycle (Ct) was recorded and the gene expression level was calculated using the comparative Ct method or delta-delta Ct method. To assess the product specificity, melt curve analysis was performed. The relative gene expression level of control and treated cells are represented as the $2^{-\Delta\Delta Ct}$ method.

$\Delta Ct = Ct\ (target\ gene) - Ct\ (reference\ gene)$

$\Delta\Delta Ct = \Delta Ct\ (sample) - \Delta Ct\ (control)$

Results

4. RESULTS

The study entitled "Effect of histone deacetylation in the regulation of somatic embryogenesis related gene expression in coconut (*Cocos nucifera* L.)" was conducted at the Department of Molecular Biology and Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram, during 2023-2024. The objective of the research was to analyse the effect of histone deacetylation in the regulation of somatic embryogenesis related genes (*SERK, BBM, WUS*) and histone deacetylation gene (*HDAC*) in coconut (Cocos nucifera L.) using Trichostatin. In this study, an attempt was made to initiate embryogenic calli from plumular explants of coconut cultivar WCT. The first part of the study analysed the effect of a histone deactelyse inhibitor, Trichostatin on embryogenic calli initiation. The second part of the study analyse the relative expression levels of *SERK, WUS, BBM,* and *HDAC* genes to understand the molecular mechanisms underlying histone deacetylation-mediated somatic embryogenesis in coconut.

4.1. Pretreatment of explant

The plumule was preconditioned by inoculating it in the dark for 3–4 weeks in basal Y3 medium, following the method described by Rajesh et al. (2005).

A total of 45 explants were inoculated in Y3 medium for preconditioning, out of which 66.6% survived. The surviving explants appeared enlarged, while the remaining explants showed browning and no visible growth.

Table 12. Percentage of explants survived in pretreatment

Media	No. of explants inoculated	Explants survived
Y3 Medium	45	66.6%

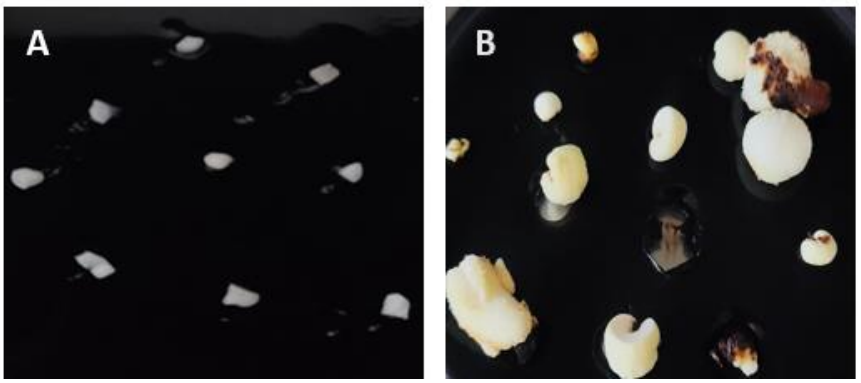

Plate 1. Response of plumules to standardized callus induction medium without TSA treatment. A. Plumules in CIM (on day 0); B. Plumules in CIM (on 4th week).

4.2 Embryogenic calli induction in coconut variety WCT in presence of histone deacetylase inhibitor – Trichostatin

The effect of Trichostatin (TSA), a histone deacetylase inhibitor, on embryogenic callus induction in the coconut variety WCT was analyzed by culturing plumular explants on Callus Induction Medium (CIM) supplemented with various concentrations of TSA (0, 0.5, 1.0, 1.5, and 2.0 µM). The explants were incubated in darkness for 50 days, and the response was evaluated in terms of embryogenic callus formation.

The control treatment (CIM without TSA) resulted in the formation of embryogenic calli in 20.3% of the inoculated explants. However, explants treated with different concentrations of TSA (0.5–2.0 µM) exhibited a distinct response. Instead of callus formation, all TSA-treated explants showed noticeable enlargement of the plumule.

Table 13. Response of plumular explants in CIM with different concentrations of Trichostatin

Sample	No. of explants inoculated	Percentage of embryogenic calli formed
Control	26	20.3%
		Percentage of enlargement of explants
CIM+ 0.5µM TSA	26	18.6%
CIM+ 1.0µM TSA	26	15.8%
CIM+ 1.5µM TSA	26	15.62%
CIM+ 1.5µM TSA	26	14.57%

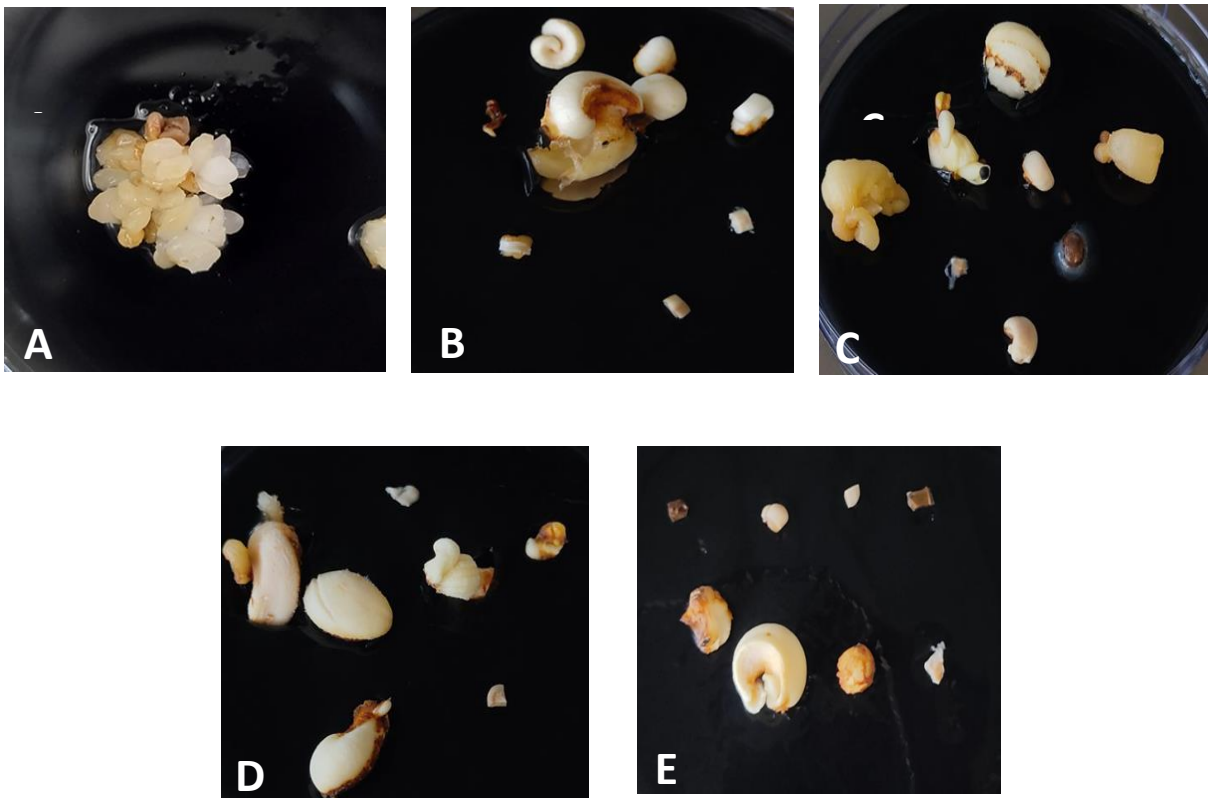

Plate 2: Embryogenic calli induction from the plumule of the coconut variety WCT in CIM with different concentrations of histone deacetylase inhibitor – Trichostatin A. Control B. 0.5µM Trichostatin (50th day) C. 1.0 µM Trichostatin (50th day) D. 1.5µM Trichostatin (50th day) E. 2.0 µM Trichostatin (50th day)

4.3. RT-qPCR of somatic embryogenesis related genes in coconut

4.3.1. RNA isolation

RNA was isolated from the control sample (CIM without TSA) and sample obtained from CIM with 0.5 µM TSA. Samples were eluted out to a final volume of 30 µl and stored at -80 °C.

4.3.2. Quantity and quality of RNA

The purity and concentration of the isolated RNA were checked using NanoDrop (DeNovix DS-11). The integrity and quality of RNA thus isolated were checked using Agarose gel electrophoresis.

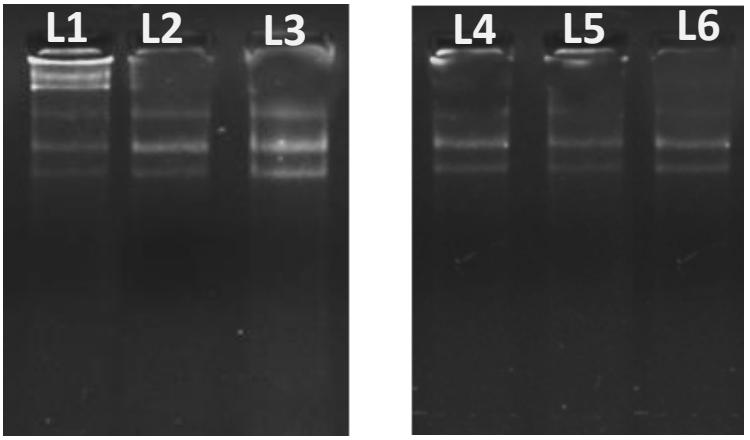

Plate 3. Gel profile of RNA isolated from coconut plumule. L1-L3: RNA isolated from control sample (CIM); L4-L6: RNA isolated from CIM+0.5 µM TSA

Table 14. Concentration and purity of isolated RNA

Sample	OD at 260 nm	260/230 value	260/280 value	Quantity of RNA (ng/µL)
Control: CIM	10.678	1.53	2.07	427.12
T1: CIM+0.5TSA	13.986	1.17	2.03	559.44

4.3.3. Expression profile/analysis of gene

The cDNA synthesized was subjected to Real-Time qPCR using primers of the *SERK* gene. Expression analysis of the gene was studied using GAPDH as the reference gene. The melting curve analysis was performed to assess the specificity of the amplified products. The resulting melt peak plot displayed no distinct peak within the expected temperature range. Instead, multiple curves were remained above and below the baseline, indicating the absence of a specific amplification product (Fig.1 & Fig. 2). Similar patterns were observed for *BBM* and *WUS*, suggesting non-specific amplification or the lack of target gene expression.

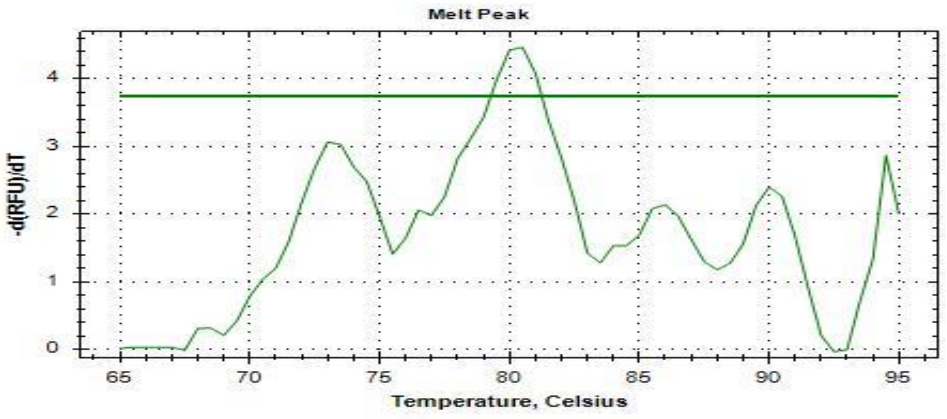

Fig 1. Melt curve of SERK gene in embryogenic calli grown in CIM

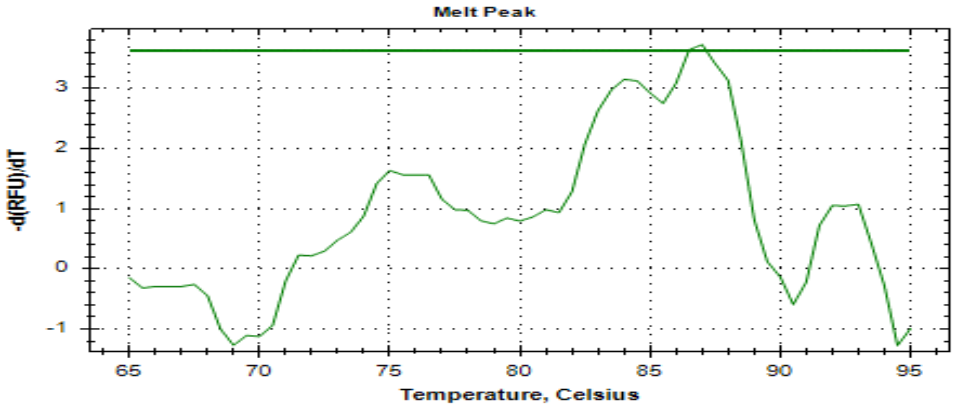

Fig 2. Melt curve of SERK gene in embryogenic calli grown in CIM+0.5µM TSA

4.4. RT-qPCR of histone deacetylase gene

4.4.1 Primer designing

Primers for the amplification of the histone deacetylase gene *(HDA6)* in coconut were designed by using *in silico* tools based on the exon sequence retrieved from GenBank. Due to the unavailability of genome sequences of the *HDAC* gene in coconut genome sequences of *HDAC (HDA6)* gene from Arabidopsis was taken initially. According to Tanka *et al. (*2017) *HDA6* gene in the *HDAC* family has a specific influence in the regulation of genes in the somatic embryogenesis pathway. Hence, the *HDA6* gene in the *HDAC* family was taken for the gene expression analysis. The gene-specific primers for *HDA6* in coconut were designed using primer 3 software. Already reported primers of reference gene Actin were used for Real-Time qPCR.

Arabidopsis thaliana histone deacetylase 6 (HDA6), mRNA

>NM_125705.4 Arabidopsis thaliana histone deacetylase 6 (HDA6), mRNA
GTGCCCACAACTCCTAGTAATGACTTTCTCAGGCATTGTTGACACAAATTTTGCTCTGAGTAAAACTTGG
GAATAGAGAGAGACTCTGAGTGAGAGAGAGATTCTGAGTGAGAGACGGAGATGGAGGCAGACGAAAGCGG
CATCTCTCTGCCGTCGGGACCCGACGGACGTAAGCGGCGAGTCAGTTACTTCTACGAGCCGACGATCGGA
GACTACTACTACGGTCAAGGCCACCCGATGAAGCCTCACCGGATCCGTATGGCTCATAGCCTAATCATTC
ACTATCACCTCCACCGTCGCTTAGAAATCAGTCGCCCTAGCCTCGCTGACGCCTCCGATATCGGCCGATT
CCATTCGCCGGAGTATGTTGACTTCCTCGCTTCCGTTTCGCCGGAATCTATGGGCGATCCTTCCGCTGCA
CGAAACCTAAGGCGATTCAATGTCGGTGAGGATTGTCCTGTCTTCGACGGACTTTTTGATTTTTGCCGTG
CTTCCGCCGGAGGTTCTATTGGTGCTGCCGTCAAATTAAACAGACAGGACGCTGATATCGCTATCAATTG
GGGCGGTGGGCTTCACCATGCTAAGAAAAGCGAGGCTTCTGGGTTTTGCTATGTAAACGACATCGTGCTA
GGGATTCTGGAGTTGCTCAAGATGTTTAAGCGGGTTCTCTACATAGATATTGATGTCCACCATGGAGATG
GAGTGGAAGAAGCGTTTTACACCACTGATAGAGTTATGACTGTTTCTTTCCACAAATTTGGGGACTTTTT
CCCAGGAACTGGTCACATAAGAGATGTTGGCGCTGAAAAAGGGAAATACTATGCTCTAAATGTTCCACTA
AACGATGGTATGGACGATGAAAGTTTCCGCAGCTTGTTTAGACCTCTTATCCAGAAGGTTATGGAAGTGT
ATCAGCCAGAGGCAGTTGTTCTTCAGTGTGGTGCTGACTCCTTAAGTGGTGATCGGTTGGGTTGCTTCAA
CTTATCAGTCAAGGGTCACGCTGATTGCCTTCGGTTCTTAAGATCTTACAACGTTCCTCTCATGGTGTTG
GGTGGTGGAGGGTATACTATTCGAAATGTTGCCCGTTGCTGGTGTTATGAGACTGCAGTTGCTGTTGGAG
TAGAGCCGGACAACAAACTCCCTTACAATGAGTATTTTGAGTATTTCGGCCCAGATTATACGCTTCATGT
CGACCCAAGTCCTATGGAGAATTTAAACACGCCCAAAGATATGGAGAGGATAAGGAACACGTTGCTGGAA
CAACTTTCGGGACTAATACACGCACCTAGCGTCCAGTTTCAGCACACACCACCAGTCAATCGAGTTTTGG
ACGAGCCGGAAGATGACATGGAGACAAGACCAAAACCTCGCATCTGGAGTGGAACTGCGACTTATGAATC
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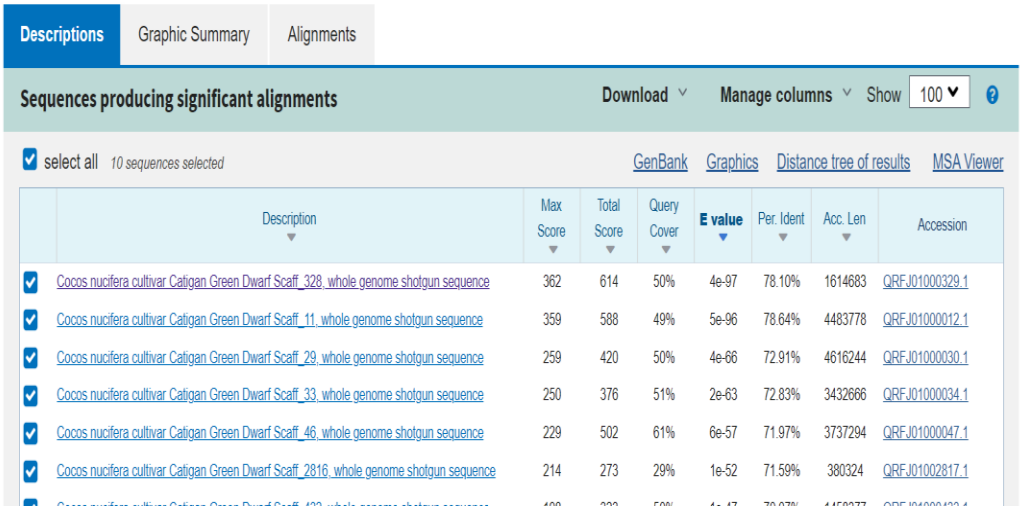
TCTACCGGTGAAGATGAAATGGATGACGATAACCCAGAGCCAGACGTGAATCCTCCATCGTCTTAAACCA
GCTTGATGGTTTGGTGTCTCTTTTGCCATATGATAATGTCGGCAGATTTAAGAAACAAGTTAGGGGAATG
AATGATTCTTTGATGTTTTTTCAGCAACCTTTTGAGTTCTGTGAAAACGCTGCATTGATTAGAACAGTGA
CAACTGACTAGTATTTTGGCCCAAGTTAGAAAATCAGAATATGTGAAAAATGCAAAGAAACTTTTTAAAA
AAAATATGGGCTTTTACTTCTTCTTTTTTTTTTACTTTCTCCGAAGTTGTTGTTACCAAACCGAAGAAAA
CCGATTGCAATAGACCCTAAAATTAAACCATTGAAGCTGTTACT

Fig. 3 Genome sequence of *Arabidopsis thaliana* histone deacetylase 6 gene *(HDA6)*.



Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Cocos nucifera cultivar Catigan Green Dwarf Scaff_328, whole genome shotgun sequence	362	614	50%	4e-97	78.10%	1614683	QRFJ01000329.1
Cocos nucifera cultivar Catigan Green Dwarf Scaff_11, whole genome shotgun sequence	359	588	49%	5e-96	78.64%	4483778	QRFJ01000012.1
Cocos nucifera cultivar Catigan Green Dwarf Scaff_29, whole genome shotgun sequence	259	420	50%	4e-66	72.91%	4616244	QRFJ01000030.1
Cocos nucifera cultivar Catigan Green Dwarf Scaff_33, whole genome shotgun sequence	250	376	51%	2e-63	72.83%	3432666	QRFJ01000034.1
Cocos nucifera cultivar Catigan Green Dwarf Scaff_46, whole genome shotgun sequence	229	502	61%	6e-57	71.97%	3737294	QRFJ01000047.1
Cocos nucifera cultivar Catigan Green Dwarf Scaff_2816, whole genome shotgun sequence	214	273	29%	1e-52	71.59%	380324	QRFJ01002817.1

Fig. 4 Output of nucleotide BLAST of *Arabidopsis thaliana* histone deacetylase 6 gene with whole genome sequence of *Cocos nucifera* L.

Fig. 5 Whole genome shotgun sequence of *Cocos nucifera* Catigan Green Dwarf Scaff_328.

Primer3 Output

```
PRIMER PICKING RESULTS FOR cocos

Template masking not selected
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm      gc%  any_th  3'_th  hairpin  seq
LEFT PRIMER        116   20   58.86   55.00    0.00   0.00     0.00 GGGACAGGGCATATCAAGGA
RIGHT PRIMER       354   20   59.06   55.00   16.68   0.00     0.00 CGTAGACAATCTGCATGCCC
SEQUENCE SIZE: 442
INCLUDED REGION SIZE: 442

PRODUCT SIZE: 239, PAIR ANY_TH COMPL: 6.12, PAIR 3'_TH COMPL: 6.12

    1 GCGTGTGCTCTACGTAGACATTGATGTCCATCATGGAGATGGTGTTGAAGAGGCTTTTTT


   61 CACAACAGATAGAGTCATGACTGTCTCATTCCACAAATATGGGGACTTCTTTCCTGGGAC
                                                              >>>>>

  121 AGGGCATATCAAGGACAATGGTTTTGGGAAAGGAAAGTACTATGCTTTGAATGTTCCTTT
      >>>>>>>>>>>>>>>

  181 GAATGATGGCATGGATGATGATAGCTTCCGTGGACTATTTAGGCCAATCGTTCAAAAAGT


  241 AATGGAGGTTTATCAGCCAGATGCAGTGGTTCTCCAGTGTGGTGCGGATTCCTTGGCTGG


  301 AGATAGGCTAGGTTGCTTCAACTTGTCAGTGAAGGGGCATGCAGATTGTCTACGATATCT
                                      <<<<<<<<<<<<<<<<<<<


  361 CAGATCTTTTAATGTACCTCTGATGGTTTTGGGAGGTGGAGGTTATACTATTAGGAATGT


  421 TGCTCGCTGCTGGTGCTATGAG
```

Fig. 6 Primer designed for the amplification of coconut *HDA6* gene using PRIMER3 software.

Blast analysis of the genome sequence of *Arabidopsis thaliana* histone deacetylase 6 *(HDA6)* was done with the whole genome sequence of *Cocos*

*nucifera***.** From this whole genome sequence, *Cocos nucifera* Catigan Green Dwarf Scaff_328 was taken for primer designing.

Subject:

AGCGTGTGCTCTATGTAGACATTGATGTCCATCATGGAGATGGTGTTGAAGAGGCTTTTT

TCACAACAGATAGAGTTATGACTGTCTCATTCCACAAATATGGGGACTTTTTTCCTGGGA

CGGGGCATATCAAGGACAATGGTTTTGGGAAAGGAAAGTACTATGCTTTGAATGTTCCTT

TGAATGATGGCATGGATGATGATAGCTTCCGTGGACTATTTAGGCCAATCATTCAAAAAG

TAATGGAGGTTTATCAGCCTGATGCAGTGGTTCTCCAGTGTGGTGCGGATTCCTTGGCTG

GAGATAGGCTAGGTTGCTTCAACTTGTCAGTGAAGGGGCATGCAGATTGTCTACGATATC

TCAGATCTTTTAATGTACCTCTGATGGTTTTGGGAGGTGGAGGTTATACTATTCGGAATG

TTGCTCGCTGCTGGTGCTATGAG

Primer was designed from this subject using PRIMER3 software.

Table 17. Sequence of primer designed to amplify coconut *HDA6* gene.

Gene	Primer	Primer sequence (5'-3')	Length
HDA6	FP	GGGACAGGGCATATCAAGA	19
	RP	CGTAGACAATCTGCATGCCC	20

4.4.2. Optimization of annealing temperatures of primers

Gradient PCR using CFX96 Real-Time system (BIO-RAD) was carried out with a temperature range of 55°C, 55.5 °C,56.6 °C, 58.2 °C, 60.1 °C, 61.7 °C, 62.6 °C, 63 °C for ACTIN and HDA6 genes. Based on the results, 56˚C was selected as the annealing temperature at which a high intensity of band amplification was observed.

Table 18. Standardized annealing temperature of Actin and *HDA6* gene primers.

Gene	Temperature
ACTIN	56ºC
HDA6	56ºC

4.4.3. Expression profile/analysis of gene

The cDNA synthesized was subjected to Real-Time qPCR using primers of the *HDA6* gene. Expression analysis of the *HDA6* gene was studied using actin as the reference gene. The melting curve of the *HDA6* gene has shown a single peak in calli grown in CIM and calli grown in CIM+TSA represented in Fig. 7 and Fig. 8 respectively.

The study revealed that, in callus obtained from CIM without TSA and with TSA, relative expression of *the HDA6* gene was found to have a foldchange of 15.23 and 10.08 respectively. Relative expression of the *HDA6* gene was seen downregulated in callus obtained from CIM with TSA.

Table 19. Relative fold change of *HDA6* gene expression.

Treatments	Relative fold change
CIM	15.23±0.16
CIM+TSA	10.08±0.43

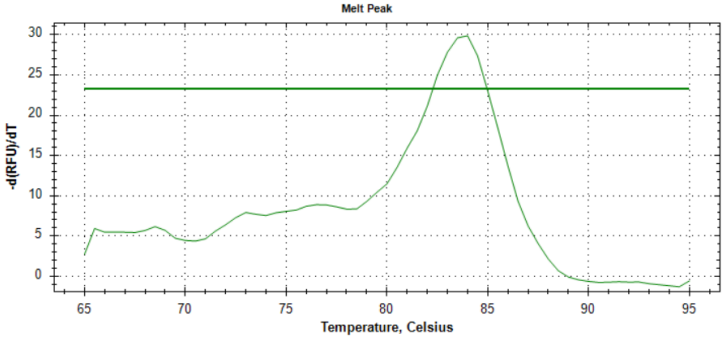


Fig. 7 Melt curve of *HDA6* gene in embryogenic calli grown in CIM

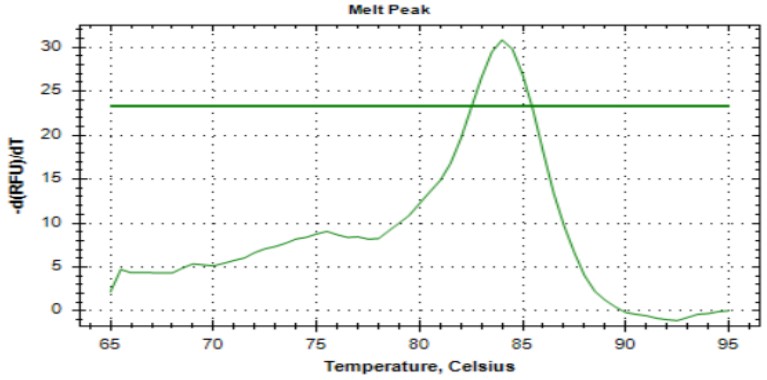


Fig. 8 Melt curve of *HDA6* gene in embryogenic calli grown in CIM+TSA.

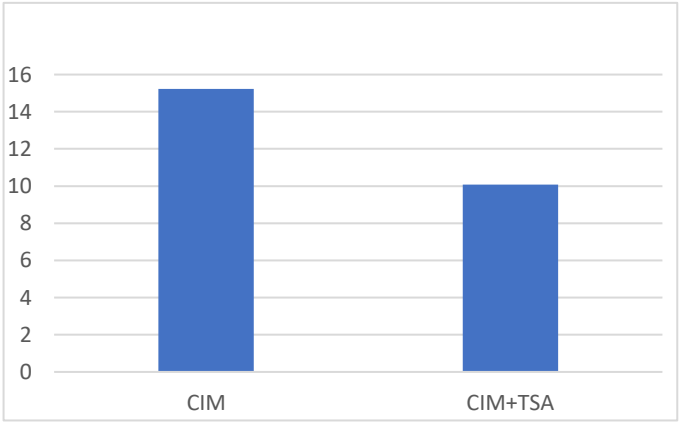


Fig. 9 Relative fold change in expression of *HDA6* gene in embryogenic calli grown in CIM and CIM with TSA.

The gene expression of the *HDA6* gene was reduced in CIM with TSA when compared to CIM. That is, *HDA6* expression showed downregulation in

CIM+0.5µm TSA compared to CIM. In conclusion, treatment with the histone deacetylase inhibitor, TSA did not result in callus induction. Furthermore, expression analysis of somatic embryogenesis-related (*SERK*, *BBM*, and *WUS*) genes showed no specific amplification, as indicated by the absence of distinct melt peaks in the melting curve analysis. These findings suggest that TSA treatment did not activate the expression of key regulators associated with somatic embryogenesis under the given experimental conditions.

Discussion

5. DISCUSSION

Coconut (Cocos nucifera L.) is a crop grown traditionally in India which has a great economic value in many other nations worldwide. Since coconut trees can only be grown from seeds, they are not suitable as high-quality planting material for commercial large-scale production. Coconut palm is difficult to improve since it is heterozygous, has a long juvenile stage, takes a long time between generations, and requires a lot of land even for testing. Therefore, tissue culture was considered as a substitute method for propagating genotypes of coconut that are high-yielding and disease-free.

Coconut tissue culture research was initiated about four decades ago in several laboratories worldwide, but limited success has been achieved so far due to its inherent recalcitrant nature (Fernando et al., 2010). The multiplication and regeneration rate has generally remained relatively low and an economically viable protocol is yet to be developed (Fernando et al., 2010). In coconut, somatic embryogenesis has been reported from different explants (Oropeza et al., 2005; Rajesh et al., 2005, 2014; Perez-Nunez *et al.*, 2006). But the efficiency and reproducibility are very low and highly recalcitrant to *in vitro* culture. Hence it is important to study the molecular events behind embryogenic induction in coconut. Somatic embryogenesis (SE) is an intricate molecular and biochemical process which involves many gene networks. Some of the genes that have been directly linked to zygotic and somatic embryogenesis in coconut are LEAFY COTYLEDON (*LEC*), BABYBOOM (*BBM*), and WUSCHEL (*WUS*) (Rajesh, M. K. et al., 2016). *SERK* overexpression is observed during embryogenic induction till the globular stage and together with other genes like *BBM* and *LEC* promotes the transition to embryogenic cells from non-embryogenic tissues (Gulzar et al., 2020).

In many recalcitrant species, it was reported that deacetylation of genes controlling somatic embryogenesis can increase the gene expression and subsequently increase the rate of somatic embryogenesis in plants (Abrahamsson et al., 2017; Martinez et al., 2021). Histone acetylation modification is an important

form of epigenetic inheritance and a key regulatory scheme in regulating overall gene expression level. The acetylation of genes can be increased by treatment with histone deacetylase inhibitors (HDAs). Trichostatin A (TSA) is the most frequently used histone deacetylase (HDAs) inhibitor (Gorisch et al., 2005). Blocking HDAC activity with Trichostatin A (TSA) in cultured cells leads to a large increase in the proportion of embryogenic growth (Wojcikowsika et al., 2018). TSA specifically inhibits HDAs, which can lead to the accumulation of acetylated histones and a corresponding decrease in DNA methylation in cells and an increase gene activity (Wojcikowsika et al., 2018).

The present study aimed to investigate the effect of histone deacetylation on the regulation of somatic embryogenesis-related genes in coconut (*Cocos nucifera* L.), particularly in the West Coast Tall (WCT) variety. The study focused on the use of Trichostatin (TSA), a known histone deacetylase (*HDAC*) inhibitor, to determine its role in embryogenic calli formation and the expression of key genes involved in somatic embryogenesis.

The results of this study indicate that the application of TSA at varying concentrations (0.5–2.0 µM) had a significant impact on the response of plumular explants. Unlike the control group, where 20.3% of the explants formed enlargement of plumule. TSA-treated explants did not exhibit typical callus formation. Instead, the treated explants showed marked enlargement, suggesting that TSA might have influenced cellular expansion rather than callus induction. This observation does not align with previous findings that histone deacetylation plays a crucial role in cellular differentiation and tissue-specific gene regulation.

The molecular aspect of the study involved the relative expression analysis of *SERK*, *WUS*, *BBM*, and *HDAC* genes in response to TSA treatment. The melting curve analysis of *SERK* and *WUS* genes displayed multiple curves above and below the baseline, indicating the absence of specific amplification. This result suggests that these genes may not have been actively transcribed under the given experimental conditions or that their expression was not significantly influenced by

TSA treatment. The lack of amplification could also be attributed to inefficiencies in primer binding or low transcript abundance.

However, the expression analysis of the HDAC (*HDA6*) gene revealed a distinct peak, confirming its transcriptional activity in the coconut explants. This result is significant, as it supports the hypothesis that *HDA6* plays a pivotal role in regulating gene expression during somatic embryogenesis. The upregulation or downregulation of *HDA6* in response to TSA treatment may indicate its involvement in modulating the chromatin state, which in turn could influence the embryogenic potential of coconut explants. Similar findings have been reported in other species, where *HDA6* was shown to be involved in the regulation of genes associated with developmental transitions.

Summary

6. SUMMARY

The study entitled “Effect of histone deacetylation in the regulation of somatic embryogenesis-related genes in coconut (*Cocos nucifera* L.)” was conducted at the Department of Molecular Biology and Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram, during 2023-2024. The objective was to analyze the effect of histone deacetylation on the regulation of somatic embryogenesis-related genes (*SERK, BBM, WUS*) and the histone deacetylation gene (*HDAC*) in coconut using Trichostatin (TSA), a histone deacetylase inhibitor.

In this study, embryogenic calli initiation from plumular explants of the West Coast Tall (WCT) coconut variety was attempted. Coconuts (11 months old) of variety West Coast Tall (WCT) were collected from Instructional Farm, College of Agriculture, Vellayani. The plumule containing endosperm part of the coconuts was scooped out using a cork borer and was then surface sterilized by immersion in 20% sodium hypochlorite for 20 minutes, with intermittent rinsing in sterile water. Plumules were preconditioned in basal Y3 medium for 3–4 weeks. A total of 45 explants were inoculated, with a 66.6% survival rate. The effect of TSA (0, 0.5, 1.0, 1.5, and 2.0 µM) on embryogenic callus induction was assessed. The control (CIM without TSA) induced 20.3% callus formation, whereas TSA-treated explants exhibited only plumule enlargement instead of callus formation.

Expression analysis of the SERK, WUS, BBM and HDAC genes was carried out by doing Real-time PCR after RNA isolation, quantification, and cDNA conversion. RNA was isolated from control and TSA-treated samples using Trizol method. The purity and concentration of the isolated RNA were checked using NanoDrop (DeNovix DS-11). The integrity and quality of RNA thus isolated were checked using Agarose gel electrophoresis. RNA was converted into complementary DNA (cDNA) by an RNA-dependent DNA polymerase enzyme called Reverse Transcriptase.

The primers for the amplification of *SERK*, *BBM*, and *WUS* genes were obtained from previously reported studies, while primers for the amplification of

the histone deacetylase gene (*HDA6*) in coconut were designed by using in silico tools based on the exon sequence retrieved from GenBank. Optimum annealing temperatures were finalized at 56°C by Gradient PCR using the CFX96 Real-Time system (BIO-RAD). Melting curve analysis showed no specific amplification for *SERK* and *WUS*, indicating potential low transcript levels or TSA-induced transcriptional suppression. However, *HDAC* gene expression exhibited a distinct peak, confirming its transcriptional activity. Relative expression analysis of the *HDAC* gene was performed to assess the impact of TSA treatment. The results indicate that *HDAC* expression was significantly reduced in the CIM + TSA condition compared to CIM alone. This downregulation suggests that TSA, a histone deacetylase inhibitor, effectively inhibited *HDAC* activity, leading to decreased gene expression.

TSA did not induce embryogenic callus formation and its effects on plumule enlargement and *HDAC* expression suggest a complex regulatory mechanism. Further research on optimal TSA concentrations and transcriptomic analysis could provide deeper insights into the molecular basis of somatic embryogenesis in coconut.

This study found that TSA treatment did not lead to embryogenic callus formation under the tested conditions. Further investigations are required to refine TSA concentrations and examine the synergistic effects of other histone-modifying agents on callus induction. Additionally, transcriptomic analyses could be utilized in future studies to identify downstream genes regulated by HDA6 activity.

In summary, this research highlights the potential role of histone deacetylation in the regulation of somatic embryogenesis in coconut. Although TSA treatment did not directly induce embryogenic calli, its impact on cellular expansion and *HDAC* gene expression suggests a complex epigenetic regulation of the embryogenic process.

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Appendices

8. APPENDICES

Appendix I

Composition of the basal Y3 medium used for culturing coconut inflorescence

Stock Name		Compounds	mg/l of media	Stock (g/l)	Volume of stock used per liter of media
Macronutrients (40X)					
A		NH_4Cl	535	21.4	25ml
		KNO_3	2020	80.80	
		KCl	1492	59.68	
		$MgSO_4.\ 7H_2O$	247	9.88	
		$NaH_2PO_4.\ 2H_2O$	312	12.48	
B		$CaCl_2.\ 2H_2O$	294	11.76	25ml
Micronutrients (200X)					
C	C1	$CoCl_2.\ 6H_2O$	0.24	0.05	5ml
		$NaMoO_4.\ H_2O$	0.24	0.05	
		$NiCl.\ 6H_2O$	0.024	0.005	
	C2	$CuSO_4.\ 5H_20$	0.25	0.05	5ml
		$MnSO_4.\ 4H_2O$	11.2	2.24	
		$ZnSO_4.\ 7H_2O$	7.2	1.44	
	C3	H_3BO_3	3.1	0.62	5ml
		KI	8.3	1.66	
D		Na_2EDTA	55.8	5.58	5ml
		$FeSO_4.\ 7H_2O$	41.7	4.17	
Organic supplement (200X)					
E		Glycine	1.0	0.2	5ml
		Pyridoxine HCl	0.05	0.01	
		Thiamine HCl	0.05	0.01	
		Biotin	0.05	0.01	
		Nicotinic acid	0.5	0.1	
F		L-Asparagine	44	8.8.	5ml
		L-Arginine	50	10	
		L-Glutamine	50	10	

Appendix II

Protocol used for RNA Isolation

- 100 mg of the sample was ground using liquid nitrogen.
- After obtaining a fine powder, 1 g of polyvinylpyrrolidone (PVP) was added using a spatula, and the sample was immediately transferred to 1 mL of Trizol-containing tubes.
- To the mixture, 100 µL of chloroform and 50 µL of β-mercaptoethanol were added, and the sample was mixed thoroughly.
- The mixture was then kept at room temperature for 5 minutes.
- Following this, the sample was centrifuged at 15,000 rpm for 10 minutes at 4°C.
- The aqueous phase was carefully collected, and 0.5 mL of chilled isopropanol was added.
- The sample was incubated at room temperature for 5 minutes before being centrifuged at 12,000 rpm for 10 minutes at 4°C.
- The resulting pellet was washed with 70% ethanol and centrifuged again at 7,500 rpm for 5 minutes at 4°C.
- The pellet was air-dried for 20 minutes and then dissolved in 100 µL of RNAse-free water.
- The dissolved pellet was incubated in a water bath at 60°C for 15 minutes.
- Immediately after incubation, the sample was placed on ice to prevent the formation of secondary structures.

Appendix III

Reagents used for Agarose Gel Electrophoresis

1. Components of TBE buffer (5X) (pH: 8)
   - Tris-base 54 g
   - Boric acid 27.5 g
   - 0.5 M EDTA 20 mL

2. Components of Gel loading dye (6X)
   - Bromophenol Blue 0.2 g
   - 50 % glycerol 6 mL
   - Milipore water 4 mL

Abstract

EFFECT OF HISTONE DEACETYLATION IN THE REGULATION OF SOMATIC EMBRYOGENESIS RELATED GENE EXPRESSION IN COCONUT (*Cocos nucifera* L.)

by

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ABSTRACT OF THESIS

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9. ABSTRACT

The study entitled **"**Effect of histone deacetylation in the regulation of somatic embryogenesis-related genes in coconut (*Cocos nucifera* L.)**"** was conducted at the Department of Molecular Biology and Biotechnology, College of Agriculture, Vellayani, during 2023-2024. The objective of this study was to know the effect of histone deacetylation in the regulation of somatic embryogenesis related genes (*SERK, BBM, WUS)* and histone deacetylation gene (*HDAC*) in coconut (*Cocos nucifera* L.) in presence of HDAC inhibitor Trichostatin.

Coconut somatic embryogenesis holds significant promise for cultivating superior coconut plants. Currently, no repeatable and efficient protocol exists for inducing somatic embryogenesis in this crop. Epigenetic regulators have been found to enhance cell differentiation and their role in promoting embryogenic induction has been observed in various recalcitrant crops (Abrahamsson *et al.*, 2017). In many such species, histone acetylation modification of genes that control somatic embryogenesis has been reported to increase gene expression and subsequently improve the rate of somatic embryogenesis (Martinez *et al.*, 2021).

The acetylation of genes can be enhanced by using histone deacetylase inhibitors (HDAs). Trichostatin A (TSA), the most used histone deacetylase inhibitor (Görisch *et al*., 2005), blocks HDAC activity in cultured cells, leading to a significant increase in embryogenic growth (Wójcikowska *et al*., 2018). By specifically inhibiting HDAs, TSA causes an accumulation of acetylated histones, a corresponding reduction in DNA methylation, and an increase in gene activity (Wójcikowska *et al*., 2018). However, how HDAC inhibitors affect the acetylation of genes related to somatic embryogenesis in coconut is still not well explored. Keeping this in view, the present study was planned to find out the effect of Trichostatin in embryogenic callus induction.

For analysing the effect of Trichostatin on callus induction, plumules from 11-month-old West Coast Tall (WCT) coconuts were scooped out, surface sterilized, and pre-cultured in Y3 basal medium for one month. The pre-cultured

plumules were then inoculated into Callus Induction Medium (Y3 + 2,4-D (74.6µM), TDZ (4.5µM), spermine (50µM)) with varying concentrations of TSA (0.5µM to 2µM). The results showed no change in the rate of callus induction with Trichostatin treatment. In the control, a 20% embryogenic callus induction was achieved in 50 days. However, in the treatments with TSA, instead of callus development, enlargement of plumules was observed at 50 days of inoculation, with no change in status even after that period. The percentage of enlargement of plumules varied with TSA concentration and maximum was observed in 0.5µM TSA.

Following the Trichostatin treatment, an analysis of the expression of somatic embryogenesis (SE) related genes and HDAC gene in control and treated samples was conducted. For gene expression analysis, RNA was isolated from plumules inoculated in 0.5µM TSA and control cultures using Trizol. Complementary DNA (cDNA) was synthesized by reverse transcription mix and subjected to quantitative real-time PCR (RT-qPCR) to analyze the expression levels of somatic embryogenesis marker genes (SERK, WUS, BBM) and histone deacetylase genes (HDAC). The results showed no significant change in the expression of the HDAC gene in both the control and the CIM with 0.5µM TSA. Additionally, the results revealed no expression of somatic embryogenesis-related genes in Trichostatin-treated samples.

This finding suggests that Trichostatin treatment does not influence embryogenic callus induction, indicating a need to fine-tune the concentration and conditions of the treatment to optimize its effects. Conclusively, further optimization is required to achieve desirable results in somatic embryogenesis induction in coconut.