FEDERAL UNIVERSITY OF CEARA

CENTER OF SCIENCES

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

POSTGRADUATE PROGRAM IN BIOCHEMISTRY

MAQSOOD ALAM

PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES IN *Calotropis procera* CALLUS UNDER SALT STRESS AND SIGNALLING INDUCERS

FORTALEZA

2024

MAQSOOD ALAM

PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES IN *Calotropis procera* CALLUS UNDER SALT STRESS AND SIGNALLING INDUCERS

A thesis submitted to the Postgraduate Program in Biochemistry and Molecular Biology of the Federal University of Ceara as a partial requirement to obtain the title of Doctorate in Biochemistry. Concentration area: Plant Biochemistry

Supervisor: Prof. Dr. Márcio Viana Ramos.
Co-supervisor: Prof. Dr. Cristina Paiva da Silveira Carvalho.

FORTALEZA

2024

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Sistema de Bibliotecas
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

A274p Alam, Maqsood.
Physiological and biochemical responses in Calotropis procera callus under salt stress and signalling inducers / Maqsood Alam. – 2024.
157 f. : il. color.

Tese (doutorado) – Universidade Federal do Ceará, Centro de Ciências, Programa de Pós-Graduação em Bioquímica , Fortaleza, 2024.
Orientação: Prof. Dr. Márcio Viana Ramos.
Coorientação: Profa. Dra. Cristina Paiva da Silveira Carvalho.

1. PR-proteins. 2. Salt stress. 3. Salicylic acid. 4. Methyl jasmonate. 5. Calotropis procera. I. Título.
CDD 572

MAQSOOD ALAM

PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES IN *Calotropis procera* CALLUS UNDER SALT STRESS AND SIGNALLING INDUCERS

A thesis submitted to the Postgraduate Program in Biochemistry and Molecular Biology of the Federal University of Ceara as a partial requirement to obtain the title of Doctorate in Biochemistry. Concentration area: Plant Biochemistry

Approved on: 15/03/2024.

EXAMINATION BOARD MEMBERS

_____

Márcio Viana Ramos (Supervisor)
Universidade Federal do Ceará (UFC)

_____

Cléverson Diniz Teixeira de Freitas
Universidade Federal do Ceará (UFC)

_____

Jeniffer Johana Duarte Sanchez
Universidade Federal do Ceará (UFC)

_____

Felipe Domingos De Sousa
Universidade de Fortaleza (UNIFOR)

_____

João Pedro Brasil De Oliveira
Secretaria da Educação (SEDUC)

Success is not measured by wealth alone but by the happiness, fulfilment, and positive impact you bring to yourself and others.

ACKNOWLEDGMENTS

I would like to begin by expressing my gratitude and praise to **Almighty Allah (SWT)** for the countless blessings I have received while completing this mega assignment.

This dissertation marks the culmination of a remarkable journey, replete with beautiful and challenging moments that fostered my growth as an independent individual and a scientist. The support of numerous individuals made this journey possible. Although expressing my gratitude comprehensively is challenging, I sincerely thank everyone who contributed to achieving this significant milestone. While it is impossible to acknowledge everyone, several people genuinely deserve heartfelt thanks.

My most profound appreciation goes to my esteemed supervisor, **Prof. Dr. Márcio Viana Ramos** and co-supervisor **Prof. Dr. Cristina Paiva da Silveira Carvalho** for all the guidance, support, and instruction they provided me throughout my doctoral studies. Without their encouragement and guidance, this thesis would not have materialized.

I want to express my gratitude to **Dr. Emanuel Alves Bezerra**, **Misrael Vieira Sales**, **Brandon Ferraz e Sousa**, and **Dr. Thais Andrade Germano** for their fruitful discussions during my research. Special thanks to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) – for financial support. I also appreciate the Department of Biochemistry and Molecular Biology (DBBM) and all the staff for their helpful guidance and cooperation throughout this study. A big thanks to the members of my thesis defence committee.

Lastly, I am deeply thankful to my parents, family, and friends for their continuous prayers, care, and encouragement that supported my journey abroad.

MAQSOOD ALAM

ABSTRACT

The latex of *Calotropis procera* has been previously documented to contain pathogenesis-related (PR) proteins, specifically osmotin, chitinase, and protease known for their defensive functions in the plant. This study examined the temporal expression profiles of *CpOsm*, *CpChit*, and *CpProt* transcripts in *C. procera* callus under salt stress and signalling inducers, Salicylic acid (SA) and Methyl jasmonate (MeJA), aiming to elucidate their roles in physiological aspects in connection with plant defence mechanisms. Under salt stress, calli showed reduced water content, growth, and fresh weight (FW), with the strongest effects observed at 100 mM NaCl. Catalase (CAT) and ascorbate peroxidase (APX) activities were decreased at 80 mM NaCl, while peroxidase (POX) was lowest in control and 60 mM NaCl, and superoxide dismutase (SOD) decreased at 100 mM NaCl. Proteolytic activity and soluble proteins increased at 80 mM NaCl, and hydrogen peroxide activity peaked at 100 mM NaCl. In particular, the levels of chlorophyll a, b, and carotenoid were increased at 80 mM NaCl. Confocal microscopy revealed reduced chlorophyll in 100 mM NaCl, low phenols in 80 mM NaCl, and high lignin in 100 mM NaCl. DNA integrity was preserved, indicating stimulated cellular proliferation under salt stress. SDS-PAGE and Zymogram showed increased protein levels at 100 mM NaCl. Transcriptional analysis revealed an upregulation of *CpOsm*, *CpChit*, and *CpProt* in response to salt stress, which peaked at different time points, indicating their involvement in stress adaptation. The SA treatments 200 and 400 µM reduced the water content in the callus, in contrast to the MeJA treatments, which increased the water content. SA 400 µM inhibited growth, with 100 µM leading to low dry weight (DW). The MeJA treatments did not affect growth, but all treatments reduced DW. SA 400 µM decreased CAT and APX, while MeJA 50 µM increased them. POX and SOD levels increased in both 400 µM SA and 50 µM MeJA-treated callus compared to the control. The proteolytic activity and the soluble proteins decreased with SA 400 µM, while MeJA 50 µM showed remarkably low values. The chlorophyll content increased in the SA treatments compared to the calli treated with MeJA. SDS-PAGE and zymogram evaluations showed increased protein concentrations in the presence of 400 µM SA and 200 µM MeJA. Transcriptional analysis revealed the upregulation of *CpOsm*, *CpChit*, and *CpProt*, providing insights into the responsiveness of these genes under SA and MeJA treatments. The study enhances comprehension of the intricate interaction between salinity and signalling molecules in *C. procera* callus culture. Osmotin, chitinase, and protease genes are highly responsive, indicating

their role in combating both biotic and abiotic stress. Furthermore, their expression is enhanced during signalling molecule-mediated responses.

Keywords: PR-proteins; salt stress; salicylic acid; methyl jasmonate.

RESUMO

O látex de *Calotropis procera* foi previamente documentado como contendo proteínas relacionadas à patogênese (PR), especificamente osmotina, quitinase e protease conhecidas por suas funções defensivas na planta. Este estudo examinou os perfis de expressão temporal dos transcritos *CpOsm*, *CpChit* e *CpProt* em calos de *C. procera* sob estresse salino e indutores de sinalização, ácido salicílico (SA) e jasmonato de metila (MeJA), com o objetivo de elucidar seu papel na fisiologia vegetal nos mecanismos de defesa de plantas. Sob estresse salino, os calos exibiram redução no teor de água, crescimento e peso fresco (PF), com os efeitos mais pronunciados observados em 100 mM de NaCl. As atividades de catalase (CAT) e ascorbato peroxidase (APX) diminuíram com NaCl 80 mM, enquanto a peroxidase (POX) foi mais baixa no controle e NaCl 60 mM, e a superóxido dismutase (SOD) diminuiu com NaCl 100 mM. A atividade proteolítica e as proteínas solúveis aumentaram com NaCl 80 mM, e a atividade do peróxido de hidrogênio atingiu o pico com NaCl 100 mM. Notavelmente, os níveis de clorofila a, b e carotenóides foram elevados em NaCl 80 mM. A microscopia confocal revelou clorofila reduzida em NaCl 100 mM, fenóis baixos em NaCl 80 mM e lignina elevada em NaCl 100 mM. A integridade do DNA foi preservada, sugerindo proliferação celular estimulada sob estresse salino. SDS-PAGE e Zimograma demonstraram níveis elevados de proteína em NaCl 100 mM. A análise transcricional mostrou regulação positiva de *CpOsm*, *CpChit* e *CpProt* em resposta ao estresse salino, com pico em diferentes momentos, indicando seu envolvimento na adaptação ao estresse. Os tratamentos SA 200 e 400 µM diminuíram o teor de água nos calos, diferentemente dos tratamentos MeJA que aumentaram o teor de água. SA 400 µM prejudicou o crescimento, com 100 µM resultando em baixo peso seco (PS). Os tratamentos com MeJA não afetaram o crescimento, mas todos os tratamentos reduziram a (PS). SA 400 µM diminuiu CAT e APX, enquanto MeJA 50 µM os aumentou. Em ambos, os calos tratados com 400 µM SA e 50 µM MeJA, os níveis de POX e SOD aumentaram em comparação com o controle. A atividade proteolítica e as proteínas solúveis diminuíram com SA 400 µM, enquanto MeJA 50 µM exibiu níveis notavelmente baixos. Os níveis de clorofila aumentaram nos tratamentos com SA em comparação com os calos tratados com MeJA. Avaliações de SDS-PAGE e zimograma demonstraram aumento dos níveis de proteína na presença de 400 µM SA e 200 µM MeJA. A análise transcricional revelou a regulação positiva de *CpOsm*, *CpChit* e *CpProt*, fornecendo *insights* sobre a natureza responsiva desses genes sob tratamentos com SA e MeJA. O estudo aumenta a compreensão da intrincada interação entre

salinidade e moléculas sinalizadoras na cultura de calos de *C. procera*. Os genes de osmotina, quitinase e protease são altamente responsivos, indicando o seu papel no combate ao estresse biótico e abiótico. Além disso, a sua expressão é aumentada durante respostas mediadas por moléculas de sinalização.

Palavras-chave: PR-proteínas; estresse salino; acido salicílico; metil jasmonato.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABA	Abscisic acid
ANOVA	Analysis of Variance
APX	Ascorbate peroxidase
ASA	Acetylsalicylic acid
ATPase	Adenosine triphosphatase
AU	Activity unit
BA	Benzoic acid
BSA	Bovine serum albumin
CAT	Catalase
CBMs	Carbohydrate-binding modules
CCs	Callus cultures
cDNA	Complementary DNA
CHS	Chalcone synthase
CLP	Caseinolytic protease
CpChit	Chitinase from *Calotropis procera*
CpOsm	Osmotin from *Calotropis procera*
CpProt	Protease from *Calotropis procera*
C-terminus	Carboxyl terminus
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
dUTP	Deoxyuridine Triphosphate
DW	Dry weight
EC	Enzyme commission number
EDTA	Ethylene diamine tetraacetic acid

EF1α	Elongation factor 1-alpha
FW	Fresh weight
G-POD	Guaiacol peroxidase
GR	Growth rate
JA	Jasmonic acid
kDa	kilodalton
KIN	Kinetin
LSM	Laser scanning microscope
MeJA	Methyl jasmonate
MM	Molecular markers
mRNA	Messenger RNA
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
NBT	Nitro blue tetrazolium
N-terminus	Amino terminus
*o*HCA	*ortho*-hydroxy-cinnamic acid
OLPs	Osmotin-like proteins
PAL	Phenylalanine ammonia-lyase
PBS	phosphate-buffered saline
PEG	Polyethylene glycol
PGRs	Plant growth regulators
PLAs	Phospholipases
PMSF	Phenylmethylsulfonyl fluoride
PR-proteins	Pathogenesis-related proteins
PVPP	Polyvinylpolypyrrolidone
RGR	Relative growth rate
RLT	RNA lysis buffer

RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPIs	Recombinant protease inhibitors
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SA	Salicylic acid
SAR	Systemic acquired resistance
SD	Standard deviation
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SMD	Standardized mean difference
SOD	Superoxide dismutase
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TBARS	Thiobarbituric acid reactive substances
TdT	Terminal deoxynucleotidyl transferase
TLPs	Thaumatin like proteins
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
TWC	Tissue water content
UPS	Ubiquitin-proteasome system
UTR	Untranslated region
Wa	Weight After
Wi	Weight initial

CONTENTS

1. INTRODUCTION

1.1. Laticiferous plants: characteristics and phylogenetic relationship

Latex-bearing plants have a long history of benefiting human health and medicinal use in many regions and cultures worldwide (JESUS et al., 2021). The laticifers exist irregularly throughout the plant kingdom, it has been proposed that the laticifers and latex have independently evolved many times (FARRELL et al., 1991). Laticifers were reported in at least 43 families of vascular plants. Angiosperm is the major group with 41 families, followed by ferns and gymnosperms with one family in each one. The laticifers are present in almost all the groups of angiosperms, in the basal clades (ANA-grade), magnoliids, monocots, basal eudicots, rosids and asteroids (PRADO and DEMARCO., 2018). Although the occurrence and distribution of laticifers in plants are considered by many authors to be diagnostic for several taxonomic groups, few studies establish relationships between the laticifer anatomy and ontogeny and the taxa in which they occur. It is most likely that different types of laticifers can be found in different species of the same family (EVERT., 2006; HAGEL et al., 2008).

In most angiosperms, laticifers are found externally associated with the phloem, but they may also occur in the cambium and xylem. In general, laticifers accompany the vascular bundles along the plant organs (EVERT., 2006). Leaf and stem are the organs with the highest number of records. On the contrary, floral organs are less recorded. The studies with laticifers especially occurring in the vegetative organs of the plant body showed the efficient roles exerted by them in sealing wounds by coagulating, blocking microorganism invasions and avoiding herbivory (DEMARCO and CASTRO., 2008; DEMARCO et al., 2013).

An impressive number of laticiferous plant species distributed in diverse taxonomic groups share a common and intriguing property. These plants exude a milky fluid when they undergo a mechanical injury caused by various biological aggressors (KONNO., 2011). This fluid is frequently white and has a coagulation-like effect after release, sealing the injury and then becoming sticky. This milky fluid is called latex and is the product of the metabolic activity of specialized cells called laticifers (HAGEL et al., 2008). As a large part of laticifer cells are occupied by big vacuoles that develop during laticifer formation (CAI et al., 2009), the majority of latex secreted is likely to originate in vacuoles. Sometimes the exuded latex may also comprise fluid from the cytoplasm of laticifer cells. This is due to the degradation and mixing of the cytoplasm of

laticifer cells with vacuoles in the finishing stage of laticifer development (ZHOU and LIU., 2011). Latex has been reported to occur in around 900 genera and 12000 species (CHAVAN et al., 2015). Like the other plant parts, latex is also rich in many phytochemicals.

Latex flows more quickly from leaves and petioles (green parts), but can also leak from fruits, roots, or stems. Adjacent cells composing the laticifers can have their walls totally or partially dissolved. This observation and the branching and anastomosing events serve as the basis to classify different types of mature laticifers and to study their evolutionary aspects (HAGEL et al., 2008).

Latex has a plastic nature, showing that its production varies according to the surrounding environment. The biotic and abiotic variations, such as drought, light, inter and intra-specific competition, pathogens, and herbivory attack, resulting in the final concentration of compounds in latex (DICKE and VAN LOON., 2000). In some groups, such as Apocynaceae, the production of latex is an ancestral condition, since it is conserved in all species of the family (METCALFE., 1967). However, latex is usually present in only a few taxa of each family (PRADO and DEMARCO., 2018). Latex occurs in the palaeontological record starting in the latest Cretaceous and has been commonly reported in ancient members of the plant families Apocynaceae, Eucommiaceae, and Moraceae (MCCOY et al., 2022).

Latex and the related insect adaptations used to circumvent latex and effectively attack laticiferous plants are major components of plant-insect interactions in modern ecosystems (AGRAWAL and KONNO., 2009). However, little is known about the origin and evolutionary development of latex or about the role latex has played in ancient ecosystems. Notably, despite being one of the plant chemical defences most preserved in the fossil record (MCCOY et al., 2021; MCCOY et al., 2022), little chemical study has been performed on fossil latex, except latex from the Geiseltal fossil site.

1.2. *Calotropis procera* (Aiton) R. Br.

1.2.1. Geographical distribution, morphology, and habitat

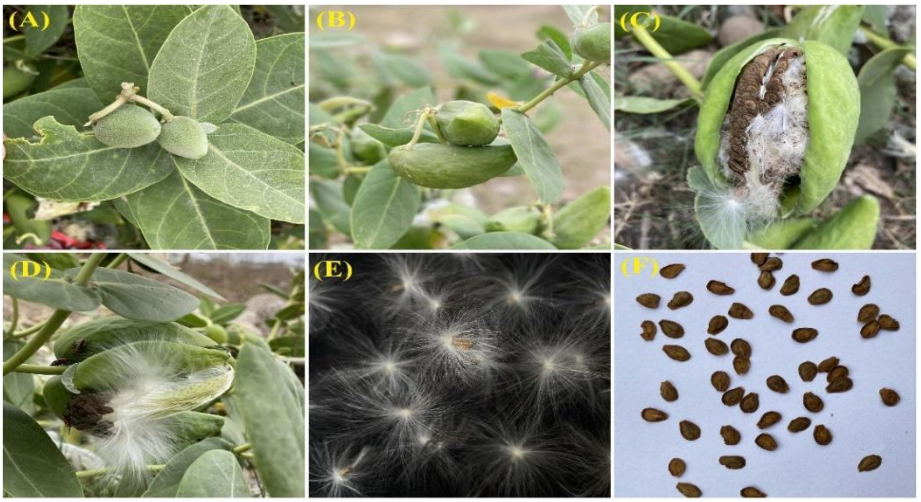
Plants are the basis of sophisticated traditional medicine approaches that have been used for thousands of years and continue to provide mankind with new remedies. Although some of the therapeutic properties attributed to plants have been proven to be erroneous, medicinal plant therapy is still supported by empirical discoveries (GURIB-FAKIM., 2006). For example, the non-cultivated shrub *Calotropis procera* (*C. procera*) is a reliable source of useful, albeit toxic, molecules (KUMAR and ARYA., 2006; AL-QURA., 2009).

C. procera is distributed in different tropical and subtropical countries of the world. The plant is known by different names like Sodom apple, dead sea apple, milkweed, Madar, (English), Usher or Oshar (Arabic), Arka, Ak (Hindi), Kharak (Persian), and Spalmai (Pushto) amongst others (PATHANIA et al., 2020; AMINI et al., 2021). *C. procera* is adapted to various ecological adversities and is recognized as invasive where it is found (RAMOS et al., 2019). The basis for the ecological classification of *C. procera* as invasive is related to seed size, dispersal mode, and germination traits (LEAL et al., 2013). Biometric studies with fruits and seeds of *C. procera* showed that approximately 350–500 seeds are found in a single fruit and that almost all seeds germinate until day 10 after sowing.

The word "*Calotropis*" is derived from Greek, meaning "beautiful" which refers to its flowers; whereas "*procera*" is a Latin word referring to the cuticular wax present on its leaves and stem (HASSAN et al., 2015). *C. procera* is a stout shrub or small tree of the family Apocynaceae and subfamily Asclepiadaceae (the milkweed family). It is an evergreen xerophytic plant which can grow up to 6 m in height (AL-ROWAILY et al., 2020). Leaves of the plant are slightly leathery, simple, entire, sub-sessile, pubescent when younger but glabrous on both sides upon maturity, with broadly ovate or oblong lamina up to 19 cm long and 5−13 cm broad and are oppositely arranged on the branches (RATHORE and MEENA., 2010).

KAUR et al., 2021

Figure 1- *Calotropis procera*: flowering plant **(A)**; phyllotaxy **(B)**; reproductive buds **(C)**; inflorescence **(D)**; individual flower **(E)**; and latex oozing out of the wounded stem **(F)**.



KAUR et al., 2021

Figure 2- Fruit characteristics of *Calotropis procera*: immature fruits **(A)**; mature fruits **(B)**; dehisized fruits **(C, D)**; seeds with pappus **(E)**; seeds without pappus **(F)**.

1.2.2. Phytochemistry, pharmacological applications and cytotoxicity

C. procera is rich in a diverse array of phytochemicals such as cardiac glycosides, flavonoids, terpenoids, steroids/ phytosterols, phenolics, tannins, proteins, amino acids, resins, enzymes, and fatty acids (MOHAMED et al., 2015; VIANA et al., 2017; ORAIBI and HAMAD., 2018). Based on previous literature, different phytochemicals like flavonoids (ORAIBI and HAMAD., 2018), alkaloids, cardiac glycosides (SWEIDAN and ZARGA., 2015), steroids, triterpenoids (ANSARI and ALI., 1999), saponins, protein, proteolytic enzymes (AWORH and NAKAI., 1988), carbohydrates, and fatty acids (PHOO et al., 2014) have been isolated and identified from different parts and latex of *C. procera* collected from various countries.

The latex of *C. procera* has been reported to comprise chitinases, proteinases and anti-oxidative enzymes (FREITAS et al., 2007). Further studies have shown that laticifer proteins are involved in plant defence against crop pests (RAMOS et al., 2007; RAMOS et al., 2010). Combined analyses of electrophoresis and mass spectrometry led to the identification of two osmotins in laticifer proteins of *C. procera,* named *CpOsm*. Laticifer cells have been described in vegetative and reproductive organs of Apocynaceae, including leaves, stems, roots and floral buds, but not in seeds and undifferentiated cells (calli) (TEIXEIRA et al., 2011; DGHIM et al., 2015).

Moreover, the application of *C. procera* latex proteins to various crop pests reduced their growth and survival (RAMOS et al., 2007). In *C. procera* more than 30 different structurally diverse cardenolides have been reported. Although these cardenolides are highly toxic to many insects through inhibition of the Na+/K^+ATPase pump, *C. procera* serves as food for at least three insect pests (Lepidoptera: *Danaus plexippus*; Hemiptera: *Aphis nerii* and *Oncopeltus fasciatus*) that are insensitive to cardenolides (AGRAWAL et al., 2012). These species evolved to overcome the toxicity of cardenolides and are capable of ingesting and storing them in their body thus using latex-borne cardenolides in their defence against predators (AGRAWAL et al., 2012). However, many aspects underlying the structural diversity of cardenolides and the ability of insects to store them are unknown.

The plant is well-documented in Ayurveda, Siddha, and Sudanese traditional medicines, and has been used also in Unani and Arabic folk and traditional medicines (PATHANIA et al., 2020; AMINI et al., 2021). Despite the severe toxicity that can be caused by the latex of *C. procera*

and the possibility of allergic skin reaction in the form of blisters or rash in sensitive individuals (PARROTTA., 2001), the tissues of *C. procera,* especially the root phloem, are widely described in the preparation of traditional medicine for treating a wide range of diseases such as dysentery, diarrhoea and other gastrointestinal disorders (SADAT-HOSSEINI et al., 2017), skin diseases (IDM'HAND et al., 2020), respiratory diseases (YEBOUK et al., 2020), scorpion bites, rheumatism, jaundice (KARAR and KUHNERT., 2017), malaria (TRAORE et al., 2013), and diabetes (DANTON et al., 2019) amongst others. Oral mucositis, an intense inflammatory reaction that can lead to tissue damage and ulceration, was found to be curable using PII-IAA, a homogenous cocktail of laticifer proteins of *C. procera* (RAMOS et al., 2020). Similarly, intestinal mucositis is observed to be abolished by latex proteins of *C. procera* (DE ALENCAR et al., 2017). An earlier retrieval of sensorimotor activities, reduced ROS, and increased total antioxidant activity (particularly, the enhanced activities of arylesterase and paraoxonase), suggested a positive impact of roots of *C. procera* on functional recovery upon a nerve injury (ZAFAR et al., 2020).

Considering these properties of *C. procera* molecules, it would be not only of botanical interest but also of biotechnological relevance to establish an *in vitro* protocol to produce these molecules. Therefore, an alternative protocol to obtain active molecules implicated in such important activities would be useful as an alternative to save natural resources and avoid extensive collection of latex from native specimens.

1.2.3. Stress physiology of *Calotropis procera*

Calotropis procera has an exceptional ability to adapt and maintain productivity in severe arid conditions (RAMADAN et al., 2014). It is a C_3 plant that can survive drought, salinity, extreme temperatures, high vapour pressure deficit, and high photosynthetic active radiations (RIVAS et al., 2020). It can easily thrive in prolonged dry seasons with rainfall >150 mm per year (DHILEEPAN., 2014). The plant grows abundantly in xerophytic conditions on a variety of soils, without irrigation or application of fertilizers (HASSAN et al., 2015). The plant has a great potential to endure stress caused by roadside pollutants and contaminated soils (KHALID et al., 2018; ULLAH and MUHAMMAD., 2020).

Plants surviving in the hostile environment of arid/semi-arid regions have advanced morpho-physiological adaptations and special defence mechanisms. So is the case of *C. procera*, in which multiple processes contribute to the resistance, resilience, and recovery of individuals growing under abiotic stress conditions (RIVAS et al., 2017). The stems and leaves of *C. procera* are characterized by thick cuticles, lactiferous canals, and low specific leaf area (HASSAN et al., 2015). Leaves are found to be narrower and thicker under optimum moisture conditions, whereas they are broader and thinner under dry conditions (POMPELLI et al., 2019). These factors help in the conservation of acquired resources and create a water permeability barrier, thereby reducing the transpiration rate (POMPELLI et al., 2019).

Endophytic microbes such as *Pseudomonas stutzeri* and *Virgibacillus koreensis* are reported to be associated with *C. procera* under salt-stressed conditions, which may facilitate its survival under harsh conditions (AL-QUWAIE., 2020). Similarly, endophytic fungal species, *Phaeoramularia calotropidis*, *Guignardia bidwellii*, *Curvularia hawaiiensis*, *Cochliobolus hawaiiensis*, *Alternaria alternata*, *Mucor circinelloides*, *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., *Chaetomium* spp., and *Candida* spp. are isolated from *C. procera*, which protects the plant from pests, pathogens, and herbivores (NASCIMENTO et al., 2015; RANI et al., 2017).

1.3. Biotic and abiotic stress and their effects on plants

Plants are exposed to a variety of stress factors, either biotic or abiotic during different developmental phases and their response to such factors receives increasing scientific attention. Numerous research annually is dedicated to this topic, but relatively few deals with the combination of stresses (PANDEY et al., 2017) as most researchers focus on a particular stress factor, which is not completely relevant to the actual situation in nature (MUKHOPADHYAY et al., 2021). The biotic stress factors include bacteria, fungi, and viruses, while drought, salinity, high or low temperature, ultraviolet radiations, and hypoxia are the abiotic factors that provoke stress conditions in plants (BASHIR et al., 2020).

Plants can sense and respond to different biotic and abiotic stresses that are complex and integrative. Consequently, an array of cascade interactions evolves in the plants (ATKINSON and

URWIN., 2012). Biotic stresses activate many intracellular defence signals leading to the production of antimicrobials and proteins, particularly the synthesis of pathogenesis-related (PR) proteins (VERONESE et al., 2003). PR proteins act as the first line of plant defence and are induced in response to not only biotic but also to abiotic stresses (VAN LOON., 1997). They were first observed in tobacco infected with tobacco mosaic virus and high levels of PR-5 proteins were detected in young leaves when exposed to salt stress (VAN LOON and KAMMEN., 1970). Plants defend themselves by initiating different stress response mechanisms, for example, the structural response that involves cell wall strengthening and waxy epidermal cuticle development. These responses deliver enough strength and rigidity to the plants that enable them to decrease the effects of damage caused by biotic and abiotic stresses (BASHIR et al., 2020).

Plants inherently have complex defence systems against pathogens. Plants can recognize phytopathogens, transmit signals, and activate their defence response systems (ZAHID et al., 2015). The balance between synthesis and degradation of proteins plays an important role in a plant's survival during abiotic adaptation (HINKSON and ELIAS., 2011). Plant's defence response mechanisms involve the stimulation of phenylpropanoid, fatty acids, and specific chemical messengers such as salicylic and jasmonic; the reinforcement of the cell walls by the lignification; and the productivity of secondary products such as PR proteins, induced enzymes of glucanase and chitinase, which possessed the hydrolytic activity against microorganism cells (ZAHID et al., 2015). Chitinase activity has been considered a verifiable indicator for plant disease-resistant induction (HIRANO et al., 2001; SHARMA et al., 2011). The natural polysaccharides of chitin, chitosan, and its derivatives are inherent components in the cell membranes of many microorganisms but are not present in plants. Therefore, once treated by these substances, plants can recognize and activate the defence systems in a mode like being attacked by microbial pathogens (YIN et al., 2016).

The use of suitable crops with improved tolerance to abiotic stresses could be one of the suitable options to cope with changing climatic conditions (SCHEBEN et al., 2016). The physiological and metabolic adaptations of plant's responses to abiotic stress vary at the molecular, cellular, and organism levels. Understanding these mechanisms requires suitable stress models and stress markers (HINOJOSA et al., 2018). A plant's response to synthesizing and accumulating specific bioactive compounds is likely to be affected by a combination of different stresses rather

than by a single factor (NAMDEO., 2007). Understanding abiotic stress responses and signal transduction to control adaptive pathways is a crucial step in determining the plant resistance exposed to unfavourable environments (KHAN and HAKEEM., 2014).

1.4. Salt stress and the complexities of salinization in plant ecosystem

Salinity is a major environmental stressor, particularly in arid and semi-arid regions of the world, causing substantial crop losses (ZULFIQAR and ASHRAF., 2021; ASHRAF and MUNNS., 2022). A high percentage of cultivated land worldwide is affected by salt stress, increasing daily (PANDOLFI et al., 2012; ZAKARIA et al., 2012). The world loses about ten million hectares of arable land annually due to salinization (MUNNS., 2002). According to some reliable estimates, more than 6% of the world's land is considered saline (RENGASAMY., 2016), and of the total irrigated lands, 20% are saline, resulting in estimated agricultural losses of US$27.3 billion annually (MUNNS et al., 2020a).

Salt stress affects the physiology of the whole plant and cellular levels from seed germination to maturity. During salt stress plants may suffer four types of stress: osmotic conductance, specific ion toxicity, ion imbalance, and oxidative stress, involving the production of reactive oxygen species (TESTER and DEVENPORT., 2003). Two factors impact plant growth, the presence of salt in the soil decreases the capacity of the plant to raise water and leads to reduce the plant growth rate, when the amount of salt is in the plant through transpiration flows there that will decrease in growth this is called the ion-excess impact of salinity (PANDOLFI et al., 2012; AL-ISSA et al., 2020).

Salt stress occurs in plants due to the cultivation of plants in Na^+-enriched soils (KANDHOL et al., 2022). Under severe saline conditions, the plant photosynthesis and respiration processes are badly impacted and ultimately result in plant death (PASSIOURA and ANGUS., 2010). Additionally, salinity stress disrupts nutrient uptake, K^+/Na^+ ratios, membrane integrity, and water movement, and damages the photosynthetic activities in plants. Salinity was found to decrease the contents of dry matter, chlorophyll, and soluble proteins, but to enhance those of free amino acids, such as proline. This free amino acid is one of the potential biochemical indicators of

salinity tolerance in plants and is involved in plant protection (ASHRAF and HARRIS., 2004). Salinity also alters the phytohormone content (JAVID et al., 2011). Salt stress induces oxidative damage and alters the amounts and activities of the enzymes involved in scavenging oxygen radicals (HERNANDEZ et al., 1993; BORSANI et al., 2001).

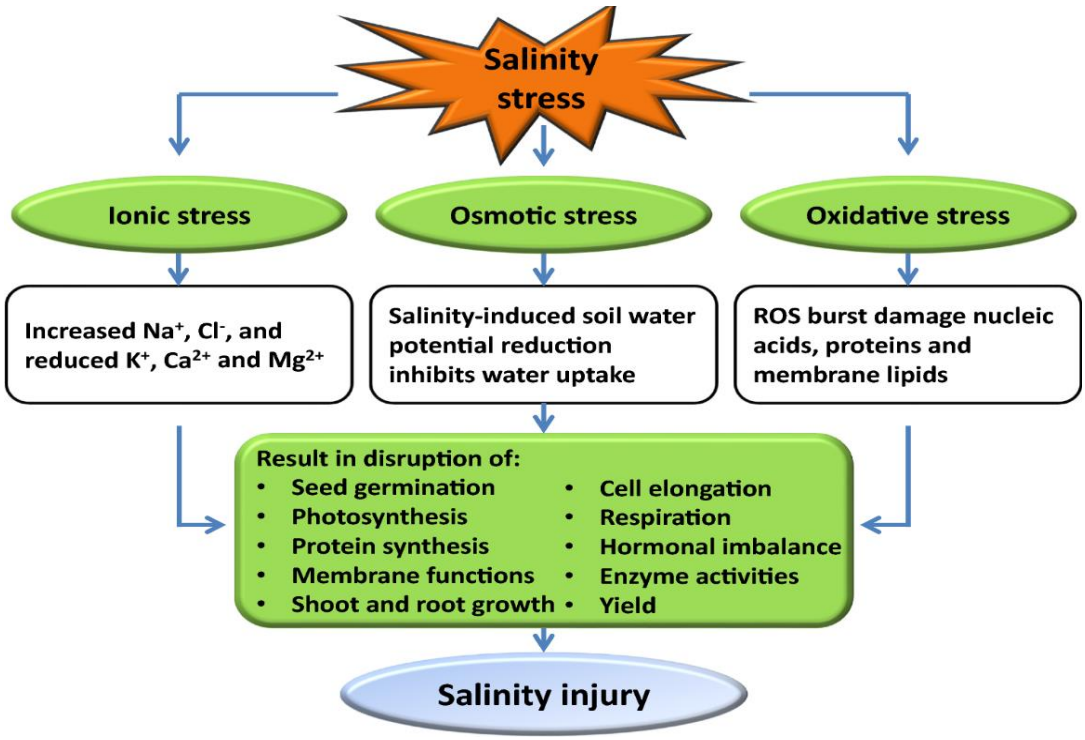


Figure 3- Overview of salinity stress effects on plants. Salinity-induced ionic, osmotic, and oxidative stresses affect numerous metabolic and physiological aspects resulting in plant injury (MANSOUR., 2023).

The management of salt stress in plants entails a complex phenomenon that elicits notable transformations in multiple aspects, including the vegetative parts, physiochemical properties, biochemical processes, metabolic pathways, and gene expressions. This intricate response underscores the comprehensive nature of plant adaptation and resilience to salt stress (GUPTA et

al., 2021). The plants growing in salt-affected soils exhibit two distinct phases- osmotic (water) stress and salt stress- of growth inhibition. The water potential in surrounding soils is less than the water potential in root cells of plants this osmotic stress inhibits water uptake, which results in a water deficit causing many physiological and biochemical abnormalities that adversely affect plant growth (HAUSER and HORIE., 2010). The plants maintain a higher cytosolic K^+/Na^+ ratio via ion homeostasis but this ionic equilibrium is disturbed in salinity. The similar radius of Na^+ and K^+ makes transporting channels difficult to distinguish between these two ions. This creates an ionic imbalance as K^+ transporting channels take up toxic Na^+ ions which negatively affect plant growth. These elevated Na^+ concentrations in the plant inhibit K^+ absorption and thus affect important metabolic processes such as photosynthesis and protein synthesis by inhibiting the function of key enzymes involved in such processes (HAUSER and HORIE., 2010). The salinity affects water and air movement, water holding capacity, plant root penetration, seedling emergence and tillage operations.

Salt stress leads to severe osmotic disbalance, causing detrimental changes at physiological and molecular levels in cellular components (VINOCUR and ALTMAN., 2005). Plants exposed to salt stress adapt all their biological processes to tolerate changes in soil and water. Therefore, the plant produces reactive oxygen species known as free radicals, Hydroxyl radicals (OH), and Hydrogen Peroxide (H_2O_2) or Superoxide $O2^-$ (ROS). These destroy organelles of living cells such as mitochondria and chloroplasts by degrading the cytoskeleton. ROS affect plant growth and production through damaging cell membranes (MISHRA and CHOUDHARI., 1999) or inhibition of Calvin cycle enzymes (FATMA et al., 2014).

Proteins regulate these processes under normal and salt stress conditions (IQBAL et al., 2019), which are responsible for regulating cellular metabolism, organic and inorganic solute transport, water transport, osmoregulation, redox balance, sensing and signalling, hormonal balance, cell division, cell enlargement, and growth and development (ZHANG et al., 2012a). Changes in protein expression under salt stress are mainly associated with several biological functions and posttranscriptional and post-translational changes (CHICONATO et al., 2021; JHA., 2022). These proteins are directly involved in determining new phenotypes that can adapt to salt-stressed environments by contributing to vital metabolic processes. Thus, the contribution of specific proteins is more important for salt tolerance mechanisms than protein quantity.

Maintenance of ion homeostasis is a key component of salt tolerance, regulated by several membrane proteins responsible for ion transport, including K^+ and Na^+ channels and pumps (HUANG et al., 2020), and transport-facilitating cytosolic proteins involved in intra-cellular communication, such as CIPK6 (ROY et al., 2013). Such proteins increase when plants are exposed to salt stress (QUN et al., 2017; LUO et al., 2018).

Numerous studies have confirmed the effectiveness of the utilization of *in vitro* salt stress in the mass production of plant secondary metabolites (GUPTA et al., 2016). Accordingly, *in vitro,* salt stress can be considered a powerful approach to improve the biosynthesis of special phytoconstituents and pharmaceuticals.

1.4.1. Exploring plant's salt stress defence mechanisms

The level of reduction in growth and tolerance to the stress condition varies among different plant species. On this basis, plants can be categorized as glycophytes and halophytes. Halophytes, also called salt-loving plants, are plants that can withstand salinity stress and possess salt-responsive genes and proteins to counter the adverse effects of salinity (ASKARI et al., 2006), whereas glycophytes, also referred to as salinity-sensitive plants, cannot tolerate the high salinity. *Calotropis procera* is a xerophytic perennial shrub or shallow tree that grows in many arid and semi-arid countries (LOTTERMOSER., 2011). It is a C_3 plant that can survive drought, salinity, extreme temperatures, high vapour pressure deficit, and high photosynthetic active radiations (FROSI et al., 2013; RIVAS et al., 2020).

Salinity levels in the growth medium can reduce the levels of endogenous hormones that encourage growth in tissues exposed. These are necessary for multiplication, elongation, growth, and survival under salt stress (KAYA et al., 2009). The high osmotic pressure, and presence of NaCl, in the growing substrate changes some plant tissue's behaviours. This forces the plant to direct the greater part of energy in different paths. This energy available for metabolic processes is directed towards building an osmotic potential inside the cell to encounter high osmotic pressure in the substrate. This occurred at the expense of the building processes necessary to perpetuate processes involved in growth cell division and expansion (SMITH et al., 1992).

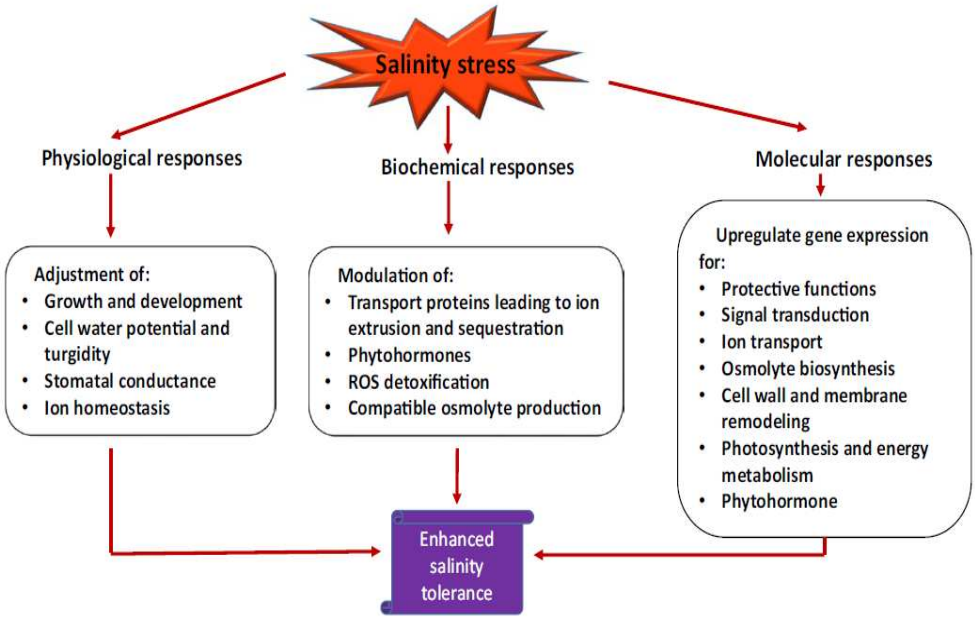


Figure 4- Mechanisms for crop salinity tolerance in response to saline conditions (MANSOUR et al., 2021).

The culture of plant cells in suspension offers a simplified model system for the study of cellular and molecular processes. Its predominant advantage is that a relatively homogenous, single-cell population allows a rapid and uniform response to external stimuli, thereby avoiding the complications of multicellular types at the whole plant level (MUSTAFA et al., 2011). Based on the above advantages, suspension cell cultures have been widely used in investigating the physiological and molecular mechanisms involved in plant responses to salt stress. The culture of salt-tolerant suspension cells is a useful tool for clarifying biological processes and primary transporters associated with Na^+ accumulation and compartmentation under salt stress in halophytes (VERA-ESTRELLA et al., 1999).

Many salt-tolerant related candidate genes and gene products involved in salt uptake and transport (GUAN et al., 2014), the elimination of reactive oxygen species (SUZUKI et al., 2012; PENG et al., 2014), the accumulation of organic compounds (ASHRAF and FOOLAD., 2007), the regulation of hormones (OSAKABE et al., 2014), and other processes have been conclusively

identified. Unfortunately, however, salt-tolerant crops using some of these genes have not been developed due to the complexity of the mechanisms of salt tolerance (WITZEL et al., 2010; SHABALA and MUNNS., 2012).

1.5. Elicitors: Exploring their nature and key role in plant responses

Different strategies to improve the production of phytochemicals have been studied extensively. One of them is by introducing elicitor, a compound that is known to induce stress in a plant that triggers the production of phytochemicals. Methyl jasmonate, salicylic acid, and melatonin are elicitors that have been described to be successfully enhancing the production of anthocyanin, a secondary metabolite that is beneficial in pharmaceutical industries (AKULA and RAVISHANKAR., 2011). The application of elicitors in plant tissue culture mostly focused on inducing plant-derived compounds, an important source of active pharmaceuticals. While elicitation is one of the most effective biotechnological strategies for augmenting the biosynthesis of bioactive compounds in cell or tissue cultures, its corresponding effect on culture biomass is rather diverse. During the past decade, various strategies have been formulated for the enhancement of major secondary metabolites in plant cell cultures (AKULA and RAVISHANKAR., 2011).

Both Jasmonic acid (JA) and Salicylic acid (SA) when provided exogenously, trigger responses like pathogen exposure or other external stimuli (GIRI and ZAHEER., 2016). They initiate signal transduction pathways that lead to the transcription of various genes thereby triggering the accumulation of molecules such as polyphenols, alkaloids, terpenoids, and steroids involved in the defence and resistance of plants (PIETERSE and VAN LOON., 1999; GIRI and ZAHEER., 2016). These phytoconstituents are known to have a range of pharmacological activities such as anticancer, antidiabetic, antiasthma, antimalarial, antimicrobial, and antiviral. They have been used in medicine either directly or as the starting point for drug discovery (AWUCHI., 2020).

SA and methyl jasmonate (MeJA) have been reported to have positive or negative effects on callus growth depending on the concentrations used in the cultures. It is important to determine the optimal concentration of elicitors to prevent hypersensitivity that leads to cell death (NAIK and AL-KHAYRI., 2016). Cai et al. (2017) reported that higher concentrations of elicitors including

MeJA and SA suppressed cell growth of the cell suspension cultures of *Changium smyrnioides*. A similar decrease in biomass in response to SA elicitation has been reported in the cell cultures of *S. miltiorrhiza* (DONG et al., 2010). Nonetheless, low concentrations of JA and SA have been shown to improve the growth of callus in normal or stressful conditions (AL-QATRANI et al., 2021).

1.5.1. Different types of elicitors and their functionalities

Elicitors can be divided into two types including abiotic and biotic (NAMDEO., 2007; NARAYANI and SRIVASTAVA., 2017). Abiotic elicitors with non-biological origin, are grouped into chemical, physical and hormonal factors (NARAYANI and SRIVASTAVA., 2017). Among abiotic elicitors, chemical elicitors have been widely applied in recent years as an effective technique to enhance the production of different plant-derived compounds with valuable pharmacological properties (NARAYANI and SRIVASTAVA., 2017). Chemical elicitors are grouped into different types as heavy metals and hormones (NARAYANI and SRIVASTAVA., 2017).

Abiotic elicitors are a group of major elicitors for the biosynthesis and production of novel pharmaceutically active metabolites. So far, there are limited reports available in the field of abiotic elicitors, but now abiotic elicitation is one of the emerging and effective approaches to enhance the production of bioactive compounds through *in vitro* culture conditions (KUMARI et al., 2020). Biotic elicitors are derived from plants and microorganisms such as fungal, bacterial, and yeast. Further, they are either of defined composition such as a combination of yeast extract and homogenized fungus, inactivated enzymes, chitosan, curdlan polysaccharides such as Pectin, cellulose, and chitin (VASCONSUELO and BOLAND., 2007). Phytohormones and biopolymers, such as methyl jasmonate, salicylic acid and chitosan, respectively, are some common and well-explored biotic elicitors (MAHENDRAN et al., 2022; NARAYANI and SRIVASTAVA., 2017). Elicitors regulate plant metabolic processes by stimulating physiological cascades. Elicitors as signalling molecules regulate a complex array of oxidative defence machinery in plant cells by initially increasing ROS production, elevating ionic currents across the plasma membrane,

activation of downstream transcription factors that ultimately leads to activating defence-related genes, and the synthesis and accumulation of phytoalexins (ZHAO et al., 2005; WANG and WELLER., 2006). However, sometimes and with overstimulation, it led to disruption of ROS homeostasis and subsequent explosion of oxidative stress. Elicitation typically involves the activation of signalling pathways within the plant that ultimately result in the production of a particular compound in a better quantity (EDER and COSIO., 1994). Researchers are interested in understanding the mechanisms underlying elicitation to develop new strategies for crop sustainability, drug discovery, and other applications. For this purpose, several protocols are being used worldwide, and an enormous number of research studies are being conducted on the use of elicitation (PATEL and KRISHNAMURTHY., 2013). For this purpose, *in vitro,* callus cultures (CCs) provide a useful tool for studying the production of bioactive compounds in plants and developing new strategies for their sustainable and enhanced production. They offer a controlled and scalable system for elicitation studies and can be used to explore the potential of various plant species to produce metabolic compounds (RUIZ-GARCÍA and GÓMEZ-PLAZA., 2013).

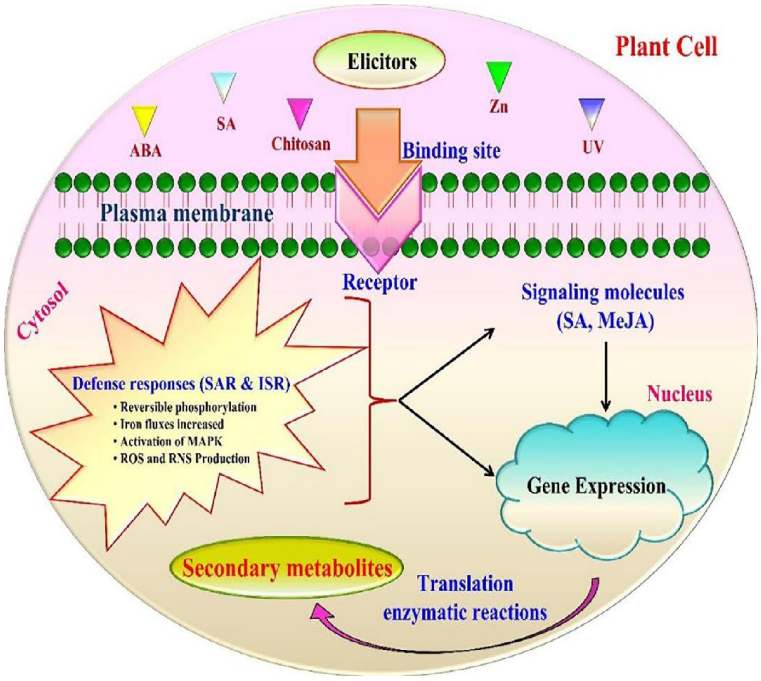


Figure 5- Secondary metabolites production by abiotic/biotic elicitors (JEYASRI et al., 2023).

1.5.2. Salicylic acid (SA): Exploring its significance in plant vitality and defence

Salicylic acid (SA), or orthohydroxy benzoic acid, is ubiquitously distributed in the whole plant kingdom (RASKIN et al., 1990). Its name is derived from the word *Salix,* the scientific name for the willow tree. Salicin, the glucoside of salicylic alcohol, was first isolated from willow bark in 1828, while the synthetic derivate of SA, acetyl SA, which is one of the best-known "antistress compounds" used by human beings was registered as "aspirin" in 1899 (RAINSFORD., 1984). SA is generally present in plants in quantities of few mg/g fresh weight or less (RASKIN et al., 1990), either in a free state or in the form of glycosylated, methylated, glucose-ester, or amino acid conjugates (LEE et al., 1995). SA can be detected in the largest quantities in thermogenic flowers at flowering or after pathogenic infection (RASKIN., 1992a).

In plants, SA can be synthesized via two distinct and compartmentalized enzymatic pathways, both requiring the primary metabolite chorismate. L-phenylalanine, derived from chorismate, can be converted into SA via the precursors free benzoic acid (BA), benzoyl glucose or *ortho*-coumaric acid (*ortho*-hydroxy-cinnamic acid: *o*HCA), depending on the plant species, but chorismate can also be converted into SA via isochorismate (CATINOT et al., 2008; DEMPSEY et al., 2011).

Although the therapeutic effect of SA in humans has been well studied for about 200 years, its role in plants has only been recognized in the last three decades and the full picture is still not clear. It was thought for a long time that phenolic compounds were non-essential for all organisms and were therefore called "secondary metabolites" (DEKOCK et al., 1974). Since then, several studies have focused on the role of both endogenous and exogenous SA in plant growth and development and have proved that this hormone regulates processes such as seed germination, vegetative growth, uptake and loss of water, nutrient uptake, nitrogen metabolism, photosynthesis, respiration, and the activity of the enzymes involved in these processes. Seed production and especially seed quality parameters are also affected by SA, which may also induce senescence and a type of cell death that is not associated with the hypersensitive response (HAYAT et al., 2010; RIVAS-SAN VICENTE and PLASENCIA., 2011).

SA could contribute to maintaining cellular redox homeostasis, through the regulation of antioxidant enzyme activity (SLAYMAKER et al., 2002) and the induction of the alternative respiratory pathway (MOORE et al., 2002), and to regulate gene expression by inducing an RNA-

dependent RNA polymerase, which is important for post-transcriptional gene silencing (XIE et al., 2001). Interactions between SA and other phytohormones, especially ABA, jasmonic acid and ethylene have been much studied, but have not yet been clarified (SINGH et al., 2011). SA treatment can influence pigment and protein contents, lignin biosynthesis and photosynthesis (JANDA et al., 2012). Both endogenous and exogenous SA have been shown to have various effects (RASKIN., 1992b), but these cannot always be generalised, as the studies were carried out on various plant species in various systems (from the whole plant to cell suspensions). The analysis of plants responding to microbial pathogen infection revealed another function of SA. As a key signal, it has an important role in the activation of disease resistance. The role of SA in the signal transduction processes of biotic stress tolerance has already been widely studied. SA is involved in the development of the hypersensitive reaction (HR): in tobacco leaves infected with tobacco mosaic virus, there is an increase in the level of endogenous SA (known as the salicylate burst) both in the necrotic lesion and in surrounding tissues (ENYEDI et al., 1992).

The external application of SA induces the expression of pathogenesis-related (PR) proteins in tobacco (MALAMY et al., 1990; YALPANI et al., 1991) and rice (RAKWAL et al., 2001). A large body of evidence indicates that SA is also required for the development of systemic acquired resistance (SAR). The level of endogenous SA increased in cucumber plants when acquired resistance developed (MÉTRAUX et al., 1990). Nevertheless, SA does not appear to be the signal molecule transported from the site of infection to more distant tissues, though the accumulation of SA in the given tissues is essential if SAR is to develop (VERNOOIJ et al., 1994). There is an increasing body of evidence suggesting that SA is involved not only in biotic stress but also in abiotic stress, as the endogenous SA content changes during abiotic stresses and the protective effect of exogenously applied SA has also been demonstrated (HORVÁTH et al., 2007; HAYAT et al., 2010).

Yang et al. (2004) divided plants into SA-sensitive and SA-insensitive species and suggested that in SA-insensitive plants such as rice, although SA may play an important role in modulating the redox balance and protecting plants from the oxidative damage caused by various biotic and abiotic factors, it is unable to act as an effective secondary signal for the activation of defence genes and induced resistance. The role of salicylic acid in various stress signal transduction processes is also supported by the fact that various abiotic stress factors induce the accumulation

of SA. The SA content increased in several plant species during treatment with various heavy metals (YANG et al., 2003; ZAWOZNIK et al., 2007).

SA treatment has been shown to improve callus traits in *in vitro* cultures (NAZIR et al., 2021). SA can induce various physiological and biochemical fluctuations in plants, including an increased synthesis of phytochemicals and antioxidants, enhanced defence responses, and improved tolerance to stresses. Various research studies have shown that the application of SA to *in vitro* callus cultures can lead to an increase in fresh and dry weights and chlorophyll content (GOLKAR et al., 2019). SA can also induce the production of molecules that have important medicinal and commercial value. In addition, SA can enhance the activity of antioxidant enzymes and can improve the stress tolerance and longevity of the callus culture (NECHAEVA et al., 2020). SA can also induce changes in gene expression and signal transduction pathways, which can lead to the activation of various stress-responsive genes and the synthesis of stress-related proteins. These changes can improve the resilience of the callus culture to external stressors, such as salt, drought, or pathogen attacks (MICLEA et al., 2020).

SA has been shown to enhance the activity of phenylalanine ammonia-lyase (*PAL*), which is an enzyme involved in the production of phenolic compounds. SA can also enhance the activity of chalcone synthase (*CHS*), an enzyme involved in the biosynthesis of flavonoids (KHATTAB et al., 2022). SA application to barley induced a pre-adaptive response to salt stress, promoted protective reactions to the photosynthetic pigments, and maintained membrane integrity, leading to improvement of plant growth (EL TAYEB., 2005). The application of SA to tomatoes via root drenching protected against NaCl stress and increased photosynthetic rates under salt stress (STEVENS et al., 2006; POÓR et al., 2011).

Coordinated expression of PR gene regulons is thought to be mediated, in part, by the accumulation of the small diffusible signalling molecules salicylic acid (SA) and jasmonic acid (JA) (ENYEDI et al., 1992; SCHENK et al., 2000). The production of PR proteins is known to be induced through two major pathways, including, salicylic acid (SA) dependent, and jasmonic acid (JA) and/or ethylene-dependent pathways. However, there may be some common control points for the activation of both pathways, resulting in the accumulation of different PR proteins (THIBAUD et al., 2004; ALI et al., 2018). Several PR-proteins are involved in plant development and may produce signal molecules through their enzymatic processes that could function as

endogenous elicitors during morphogenesis (VAN LOON et al., 2006). Chitinases are among the most abundant PR-proteins, constitutively present in plants at low concentrations and specifically induced on encounters with certain biotic and abiotic elicitors (VAN LOON et al., 2006; KUMUDINI et al., 2018). An increase in the transcription levels of chitinases was reported in the cell suspension cultures and hypocotyls of beans in response to elicitation with fungal cell wall extract and the fungus *Colletotrichum lindemuthianum*, respectively. The transcripts were found to be a maximum of two hours post-treatment (HEDRICK et al., 1988).

1.5.3. The impact of methyl jasmonate (MeJA) on plant growth and defence

Methyl jasmonate (MeJA) with chemical formula $C_{13}H_{20}O_3$ is a colourless liquid that as a plant hormone is effective in gene expression and metabolite pathways regulation, defensive responses induction and reproduction (SAISAVOEY et al., 2014). It also plays an important role in reducing free radicals by enhancing antioxidant enzymes, so that by maintaining high levels of antioxidant enzymes such as catalase and superoxide dismutase, prevents free radicals' effects from stress on the membrane (WANG., 1999). MeJA, a volatile methyl ester of jasmonic acid has been identified as a major signalling molecule in abiotic and biotic stresses (WANG et al., 2021). MeJA is used for plant defence gene expression and is commonly used as an elicitor in the production of secondary metabolite because of its ability to boost signal receptors of the plant defence system against external stimuli (XU et al., 2015). Methyl jasmonate is widely used in plants as a plant growth regulator and causes many reactions in plants (SCHALLER et al., 2004).

Jasmonic acid and methyl jasmonate belong to the group of jasmonates, these substances are directly and indirectly involved in plant reactions to adverse conditions. Internal jasmonates are hormonal substances that promote ageing, but external jasmonates act as a stress reliever. Methyl jasmonate increases plant resistance to physical and chemical damage (KOSHIOKA et al., 1998). Jasmonates play a regulatory role in growth and response to environmental stresses. In this regard, jasmonic acid is used as a gene encoder of inhibitory proteins such as threonine, smutin, hydroxyproline and proline proteins, as well as enzymes involved in flavonoid biosynthesis (CHAVAN., 2014). Moreover, studies on the molecular biology of laticifers show that latex

abundance may be induced by jasmonic acid, and therefore latex production may be enhanced under specific conditions. Since jasmonic acid is a well-known regulator involved in signalling in plant defence, this finding strengthens the value of laticifers and latex in facing enemies (CASTELBLANQUE et al., 2018).

Previous studies reported that MeJA stimulates a signal transduction process that regulates the defence response in plants and is effective in inducing the production of bioactive phytoconstituents (MENDOZA et al., 2018; RESTIANI et al., 2022). The use of methyl jasmonate in *in vitro* cultures has been shown to activate antioxidant enzymes and stimulate the expression of defence-related genes and the production of botanical compounds (HO et al., 2020).

MeJA also elicits beneficial effects on active components in plants. Takahashi and Hara (2014) sprayed MeJA on the leaves of several plants and found that MeJA promotes the accumulation of starch by up-regulating the expression genes of starch biosynthetic in *Arabidopsis thaliana*. Methyl jasmonate is known to regulate genes associated with pathogenesis, proteinase inhibitors, antifungal proteins, antioxidant enzymes, and photosynthesis in plants (YU et al., 2019). It has been reported in many plants that the defence mechanism is induced, and phytonutrients accumulation increases with the exogenous application of MeJA, which is naturally found in plants (TAKAHASHI and HARA., 2014).

Due to its volatile nature as well as the capacity to permeate via biological membranes, MeJA is regarded as a significant phytohormone that can facilitate intra-and inter-communications in plants, regulating defence responses, particularly antioxidant systems. Implementation of exogenous MeJA to plant *in vitro* cultures has been recognized as a novel method for the enhanced production of phytoconstituents, increased activity of antioxidant enzymes, and the expression of genes associated with defence (MURTHY et al., 2014).

The primitive stage of MeJA signalling includes many metabolic pathways to be activated. In general, the biosynthesis of polypeptides or the formation of free radicals indicates the introduction of biotic and abiotic stress. The activation of cell receptors, and protein molecules as well as over activity of transporters also represent the stressed condition. In response to pathogen attack, oligosaccharide signals were found in symplastic and apoplastic pathways, which indicates the plant is experiencing a stress reversal pathway. Moreover, the activation of phospholipases (PLAs) is essential for MeJA biosynthesis and signalling. PLAs produce linoleic acid from the

plasma membrane which serves as a precursor for MeJA. Several mutant and transgenic plants have been used to study the PLAs activity (YAN et al., 2013).

1.6. *In vitro* culture of plant tissues for enhanced growth and development

Plant tissue culture is a basic, fundamental science from the branch of plant biotechnology that helps in understanding the growth and development of plants at the cellular level. Tissue culture is defined as the growing or culturing of desired cells, tissues, or organs on a designed sterile synthetic medium under controlled conditions of temperature, light, and humidity (THORPE., 2007). Domestication of plants was one of the earliest developments in ancient agriculture (OBEIDAT et al., 2012). However, early breeding was primarily concerned with the selection of needed plants, such as a reduction in the shattering of seed heads in cereals. Today, crops are bred for traits such as greater resistance to pests and diseases, improved tolerance to abiotic stresses, salt stress, heat stress, improved yield, increased nutritional value, and the ability to be grown in a wider environmental range (HADDAD et al., 2020).

Plant tissue culture has been utilized for many years. During this time, many protocols have been developed for many plants (OSMAN et al., 2021). The use of plant cell culture techniques has several advantages over traditional cultivation, for example, control of production conditions, weather independence, and continuous production (SMETANSKA., 2008). Moreover, these methods allow amendments for enhanced production of specific bioactive compounds. Therefore, plant tissue provides an easy screening of plantlets for studying plant development and growth below a controlled atmosphere (HADDAD et al., 2020; SHHAB et al., 2021; SHATNAWI et al., 2021). *In vitro* methods seem to be perceived as a significant tool for plant development to increase elite collections and to progress appropriate cultivars in the least time (OSMAN et al., 2021; AL SHHAB et al., 2021). Tissue culture is used as a means for fast quantity propagation for a diverse variety of vegetation, in addition to genetic operation studies and the preservation of plant genetic resources to ensure the endurance of endangered plants.

The different techniques are used to multiply plants rapidly in a short time (HADDAD et al., 2020). Micropropagation involves growing plants in sterile, controlled conditions. Plants are

usually grown in a medium consisting of various macro and micronutrients, vitamins, amino acids, carbon sources, and plant growth regulators (HADDAD et al., 2020; OSMAN et al., 2021). Many parameters can affect the tissue culture of plants, particularly the whole plant regeneration. Among these variables, genotype, explant origin, culture medium, and plant hormones are the most effective (MINAEI CHENAR et al., 2016). By manipulating the growing conditions, it is possible to alter the growing responses of the explant. For example, this may involve changing the medium composition or altering environmental conditions, such as light and temperature.

Plant tissue culture system offers rapid propagation of elite plant varieties irrespective of climatic conditions and ecological-related factors. It also facilitates the implementation of different biotechnological approaches for the enhancement of therapeutically important plant-derived chemicals (MURTHY et al., 2014). Cell culture protocols are still very useful tools for the production and extraction of pharmacologically relevant compounds (MALIK et al., 2011). Plant cell and tissue cultures provide an excellent source of a continuous and uniform supply of raw material throughout the year without seasonal or geopolitical interference. Cell culture system is one such technique, generally utilized for plant propagation, hairy root culture, induction of somaclonal variations and biotransformation. In the present context, cell suspension cultures are more extensively used compared to other culture systems in elicitor treatment experiments (GIRI and ZAHEER., 2016).

The main areas in which plant tissue culture has gained major industrial importance are genetics, biochemistry, physiology, and pathology (ZHONG., 2001). Elicitation using distinct abiotic and biotic elicitors is the most powerful of these platforms (BAENAS et al., 2014).

1.6.1. Understanding plant biology using tissue culture methods

Cultures of *Calotropis procera* provide a unique system to study the cellular differentiation leading to laticifers formation (DHIR et al., 1984; DATTA and DE., 1986) and its regulation by growth regulators (SURI and RAMAWAT., 1995). One of the strategies that can be used is by adding the elicitor to the culture media.

Plant tissue culture can develop suitable conditions to study the stress of many plant species (GULZAR et al., 2020). Plant tissue culture has been used for different plant species to increase the mass production of plants under controlled environments (KIM et al., 2017). The role of plant tissue culture in different studies such as salinity stress is very important (THORPE., 2007).

In vitro modelling of stress is one of the promising avenues for plant breeding for resistance to negative environmental factors (MUNNS and TESTER., 2008; TERLETSKAYA et al., 2013). The benefits of *in vitro* selection include better control of the cultivation conditions and nutrient medium composition. The use of selective nutrient media (with increased levels of osmotically active substances, salts, herbicides, etc.) allows us to simulate natural stress conditions. This ensures the expression of resistance genes and helps us to select variants (individual cells, tissues, whole plants) with the desired characteristics (RAI et al., 2011), to preserve and propagate them *in vitro* in the short term.

The most common abiotic stress factors are known to be drought and salination. *In vitro* culture has been used extensively to study the impact of these factors on plants (KRUGLOVA et al., 2018). Increasing concentrations of sodium chloride (NaCl) in the culture medium allows us to model both salinity stress and osmotic stress facilitating the selection of salt-tolerant and drought-resistant forms (RAI et al., 2011). It was shown that salinity produces the greatest negative effect on plants (MUNNS and TESTER., 2008; KHUDOLIEIEVA and KUTSOKON., 2018). There have been some positive results on the selection of stress-tolerant plants through *in vitro* selection, but mainly for agricultural and fruit crops (KRUGLOVA et al., 2018; DASGUPTA et al., 2008).

1.6.2. Improving crops using callus culture techniques

By using the appropriate combination and concentration of auxin and cytokinin, shoots, roots, and embryos are produced from an undifferentiated mass of cells called callus, which was demonstrated for the first time in tobacco callus cultures (SKOOG and MILLER., 1957). Among all *in vitro* techniques, callus induction and plant regeneration techniques may enhance the potential for achieving this goal. They enable the production of many raw plant materials in small explants obtained from a mother seedling in a short time so that there could be alternative ways for the

conservation and rapid multiplication of many indigenous rare, threatened, and valuable medicinal plants (KARAKAS and TURKER., 2016).

Studies with halophytes and non-halophytes have shown that the extent of salt stress adaptation observed in the whole plant is also exhibited in callus tissue (ARZANI., 2008). *In vitro* cultures provide a feasible approach to studying the response of plants to stress conditions. Among *in vitro* techniques, callus culture, which can regenerate into a whole plant, is undoubtedly the most effective tool in the development of salt-tolerant plants. There are numerous reports of physiological alterations in calli under salinity (DOGAN., 2020; ZHANG et al., 2019).

The use of callus cultures to produce botanic drugs offers several advantages over traditional methods of plant cultivation, growth, and subsequent extraction of phytoconstituents. For example, callus cultures can be maintained in a controlled environment, allowing to production of high-quality, consistent plant material regardless of season or geographic location. Additionally, the use of callus cultures can reduce the need for wild harvesting of rare or endangered plant species alleviating biotic threats for plants to become extinct (HAKKIM et al., 2007).

In plant tissue culture, the effects of plant growth regulators (PGRs) have been extensively studied (KUMAR et al., 2016; ABIRI et al., 2017). Callus initiation and plant regeneration influenced by PGRs can swiftly produce a large number of plants (BINTE MOSTAFIZ and WAGIRAN., 2018). Using the right combination and concentration of PGRs in the medium can form callus with optimal quality. The combination of endogenous hormones with exogenous auxins and cytokinins plays an essential part in callus induction (SIL., 2021). The combination of different growth regulators generates superior outcomes compared to using either of them alone (ANTOGNONI et al., 2012; PARTHIBHAN et al., 2018).

It is well known that callus formation is a result of cell division and cell enlargement as well as auxins increase cell growth, while cytokinins stimulate cell division (GAMBORG and SHYLUK., 1982). Cytokinin growth regulators can stimulate callus formation when combined with auxin groups (HEMMATI et al., 2020). Auxins and cytokinins can induce callus by stimulating cell division and elongation through antagonistic, additive, and synergistic interaction, thus promoting cell growth (CASTRO et al., 2016). The process of callus formation begins during the swelling of the explant. The first indication of callus appearance is an enlargement of the callus, and then the callus forms on the explant's cut end (SHARMA et al., 2017). According to previous

reports, the success of callus induction and plantlet regeneration is determined by the types of explants, the media constituents, including PGRs, and the nature of the genotype (BENDERRADJI et al., 2012).

Callus is a potent source of phytoconstituents because laticiferous cell differentiation is minimal at this stage which is an interfering factor in plant-based extractions. Callus culture can be advantageous over various plant tissues for phyto-constituent extractions. Callus cells have the advantage that cells with or without laticiferous tissue can be differentiated *in vitro* (DATTA and DE., 1986). Recently, the hairy root and callus of the plant *Calotropis gigantea* have been shown to produce cardiac glycosides (SUN et al., 2012; TRIPATHI et al., 2013). To save natural resources and to maintain a uniform source and quality, an alternative protocol has been developed where *in vitro* explants of the germinating seeds of *C. procera* were grown in culture, and the callus and roots thus formed have been utilized to produce pharmacologically active proteins (TEIXEIRA et al., 2011).

1.7. Exploring plant pathogenesis-related proteins (PR proteins)

Plants, being immobile, are often subjected to different environmental stresses that lead to a decrease in plant growth and productivity (BHAT et al., 2019; ALI et al., 2018). They are exposed to infection by pathogens such as fungi, bacteria and viruses or are attacked by insects and other herbivorous along their life cycle (TIFFIN and MOELLER., 2006). However, to combat these external threats, plants have developed well-established defence mechanisms. For example, a small group of heterogeneous proteins called pathogenesis-related (PR) proteins are produced following the attack of disease pathogens, and these proteins play a critical role in inducing plants' potential to resist pathogen attack (MIR et al., 2020; ALI et al., 2017). Many studies have documented the accumulation and activation of these proteins under multiple abiotic stresses, and thus they are recognized as part of multiple defence systems. Up to now, many families of PR proteins have been characterized (MIR et al., 2020).

Pathogenesis-related (PR) proteins are members of a super large protein family in plants related to defence against abiotic and biotic stresses, and the over-expression of these proteins

renders plants tolerant to these stresses (KAUR and KUMAR., 2020). PR proteins are low molecular weight and are induced by phytopathogens as well as defence-related signalling molecules, like salicylic and jasmonic acid. Due to their mode of action in biotic and abiotic stress conditions, they are one of the most promising targets for engineering multiple stress-tolerant varieties (NAWROT., 2020; ALI et al., 2018). Based on the primary structure, amino acid composition, biochemical activities, and serological relationship, PR proteins have been classified into 17 families, i.e., β 1,3 glucanases (PR-2), chitinases (PR-3, 4, 8 and 11), thaumatin-like proteins (PR-5), ribosome-inactivating proteins (PR-10), defensins (PR-12), thionins (PR-13), and lipid transfer proteins (PR-14) (KAUR and KUMAR., 2020). Among these, pathogenesis-related protein family 5 (PR-5) in particular, is well-known for its role in osmoregulation, and plant development, and for its important antimicrobial activities (MANI et al., 2012). PR-5 members share high amino acid sequence similarity with the sweet-tasting protein thaumatin, isolated from the fruits of the West African shrub *Thaumatococcus daniellii* (VAN DER WEL and LOEVE., 1972). Therefore, members of the PR-5 family are also known as thaumatin-like proteins (TLPs).

Various PR-proteins are constitutive proteins that have their synthesis augmented in response to infection (VAN LOON., 1997). Osmotin and thaumatin-like proteins belong to family 5 of PR proteins. These proteins have been purified from banana, tomato, grape, barley, wheat, tobacco and sorghum, and exhibit remarkable activity against several fungi (PRESSEY., 1997; TATTERSALL et al., 1997). Thaumatin was first observed on *Thaumatococcus daniellii* and osmotin was first isolated from salt-adapted tobacco cell culture (CORNELISSEN et al., 1986; LAROSA et al., 1985). Osmotin-like proteins (OLPs) are proteins that have primary structures very close to osmotin. OLPs have been reported in latex fluids (FREITAS et al., 2011; LOOZE et al., 2009). Supplementary studies suggested the presence of OLPs in the latex of *Cryptostegia grandiflora* R.Br., *Plumeria rubra* L. and *Himatanthus drasticus* (Mart.) Plumel (FREITAS et al., 2015). Therefore, constitutive OLPs seem to be produced and stored by laticifers of different species. However, the involvement of these proteins in plant defence remains to be better clarified. In addition to their antifungal activities, OLPs have also been indicated in other developmental and physiological functions, including roles in flower formation and fruit ripening (NEALE et al., 1990; SALZMAN et al., 1998), protections against osmotic stress (ZHU et al., 1995) and antifreeze activities (HON et al., 1995).

The osmotin and osmotin-like proteins (OLPs) among PR-5 have been considered most important in provoking tolerance to several biotic and abiotic stresses in plants (CHOWDHURY et al., 2017; BASHIR et al., 2020). Since the last decade, several studies have been conducted to explore the functions of osmotin and OLPs in a diverse range of plants. The over-expression of these genes provides tolerance to drought, salinity, cold, high/low temperatures, plant development, and tolerance to plant diseases caused by fungi, bacteria, or viruses. Most of the research to date has been conducted on the functions and mechanisms of osmotin and OLPs.

1.7.1. Implications of Osmotins in plant defence and stress

Osmotins are plant proteins classified in family 5 of pathogenesis-related proteins (PR-5), involved in the response to biotic and abiotic stresses (BASHIR et al., 2020). Osmotin protein was first discovered in tobacco (Nicotiana tabacum var. Wisconsin 38) cells adapted to a low osmotic potential environment that exhibited salt stress tolerance (SINGH et al., 1987). Thus, it was named "osmotin" due to its function in lowering osmotic potential under stress (VIKTOROVA et al., 2012). Later, some homolog proteins were also identified throughout the plant kingdom involving various monocot and dicot plants (VIKTOROVA et al., 2019).

Many studies have demonstrated that the expression of OLPs can be activated by microbial infections and by a variety of abiotic stress factors (VELAZHAHAN et al., 1999). Although the biological functions of OLPs have not yet been fully established, some have been shown to act as antifungal proteins *in vitro.* For example, tobacco osmotin was reported to cause spore lyses and growth inhibition of *Phytophthora infestans* (ABAD et al., 1996). Grape osmotin exhibited inhibition of the hyphal growth of *Botrytis cinerea* (SALZMAN et al., 1998). Moreover, studies have demonstrated that over-expression of PR-5 proteins in transgenic plants conferred enhanced resistance to pathogens (VELAZHAHAN and MUTHUKRISHNAN., 2003). However, the molecular mechanism that accounts for the antifungal activity of PR-5 proteins is still not clear. A mechanism involving membrane permeabilization was proposed by Abad et al. (1996). Another study conducted by Yun et al. (1998) demonstrated that osmotin could subvert the target cell signal transduction pathway to increase its cytotoxic efficacy. A more recent study showed that tobacco

osmotin-induced apoptosis in *Saccharomyces cerevisiae,* was correlated with intracellular accumulation of reactive oxygen species and was mediated via the RAS2/cAMP pathway (NARASIMHAN et al., 2001).

Osmotin expression is modulated by salt and by other environmental factors such as drought and cold, and transgenic plants that overexpress osmotin genes are tolerant to these abiotic stresses (ZHU et al., 1996; GUO et al., 2004). For example, transgenic potato plants expressing the tobacco osmotin delayed the development of disease symptoms after inoculation with spore suspensions of *Phytophthora infestans,* which causes late blight disease in potatoes (LIU et al., 1994). Thus, osmotin genes have the potential to be exploited for the improvement of crops, providing higher tolerance or resistance to stresses that reduce crop viability and productivity.

CpOsm is a 22-kDa osmotin-like protein purified from the latex of the Sodom apple, *Calotropis procera* (Apocynaceae) that has antifungal activity against *Fusarium solani, Neurospora sp.* and *Colletotrichum gloeosporioides* (DE FREITAS et al., 2011a). The protein is relatively thermostable and retains its antifungal activity over a wide pH range (DE FREITAS et al., 2011b). *CpOsm* induces membrane permeabilization of spores and hyphae of *F. solani,* and although its mechanism of action is not yet fully understood, the antifungal activity of *CpOsm* is likely the result of its binding to the cell wall and/or a cell membrane receptor, as previously suggested (ABAD et al., 1996; YUN et al., 1997a). Based on these features, *CpOsm* may be useful in the development of new antifungal drugs or transgenic crops with enhanced resistance to phytopathogenic fungi. However, a better understanding of its mode of action is needed. Latex protein extracts containing *CpOsm* were shown to enhance an anti-inflammatory effect in a model of oral mucositis and to ameliorate infection-derived inflammatory processes (NASCIMENTO et al., 2016, RAMOS et al., 2020).

Osmotin (*OsOSM1*) gene over-expression in rice (*O. sativa* var. Xudao 3) increased resistance to sheath blight, the most destructive rice disease caused by *Rhizoctonia solani.* The study demonstrated the induction of jasmonic acid-responsive marker genes in response to the upregulation of jasmonic acid in transgenic rice plants. It suggests that the jasmonic acid signalling pathway activation may account for the increased resistance in transgenic lines (XUE et al., 2016). Overexpression of osmotin enhanced the tolerance to cold in tomatoes growing at higher altitudes (SARAD et al., 2004).

In the literature, there are only a few works that describe the purification, characterization, and biological activities of latex osmotins (FREITAS et al., 2011; LOOZE et al., 2009). Looking for the responsiveness of the latex osmotin gene, in salt-stressed cultured callus of *C. procera,* it was observed that increased content of mRNA corresponding to osmotin. However, the protein was not detected (SOUZA et al., 2017). It is thought that specific requirements for osmotin synthesis such as post-translational modifications and post-transcriptional events are not available on callus. Whether they are only found in laticifers remains undefined. When plants encounter high salinity levels, they activate various stress-responsive genes, including osmotin. Osmotin is involved in osmotic stress adaptation and contributes to the plant's ability to cope with challenging environmental conditions. Additionally, osmotin can also be stimulated by elicitors. Elicitors are molecules that trigger defence responses in plants. When elicitors bind to specific receptors on plant cell surfaces, they activate defence-related genes, including osmotin. Osmotin plays a multifaceted role in both salt stress responses and defence mechanisms triggered by elicitors.

1.7.2. The role of chitinase enzymes in plant stress responses

Chitinases are enzymes responsible for hydrolyzing β-1,4-glycosidic bonds of chitin, acting as defensive proteins against pathogenic fungi and insects. After proteases, the chitinases are the proteins most studied in latex fluids. In general, they present several isoforms that exhibit insecticidal and antifungal activities (FREITAS et al., 2016). Chitinases are ubiquitous in nature and are found in living organisms across different kingdoms of life (XU et al., 2016). The proteins are categorized into two glycosyl hydrolases (GH) families, GH18 & GH19, based on the presence of specific catalytic domains (CAO and TAN., 2019). Individual families are further classified into several classes, in which classes I, II, and IV correspond to GH19, and classes III and V correspond to GH18 (NEUHAUS et al., 1996). Most plant species express different classes of chitinases as part of tolerance mechanisms against various types of biotic or abiotic stress (KASPRZEWSKA., 2003; BRUNNER et al., 1998). A lack of chitin in the plant cell wall and other tissue parts makes chitinase an important component of the plant defence system (WANG et al., 2015).

Chitinase is an enzyme system that employs chitin as a substrate and hydrolyzes it to N-acetyl oligosaccharide and glucose. As a subgroup of pathogenesis-related (PR) proteins, chitinase is widely present in various organs of higher plants and can be rapidly produced and accumulated when plants are subjected to pathogen infection or abiotic stress sources, such as heavy metals and drought (BRAVO et al., 2003; WANG et al., 2015; LI et al., 2018). Backiyarani et al. (2015) identified 14 chitinase genes with complete open reading frame sequences in the Musa genome hub and analysed their gene structure and conserved domains, as well as their intron and exon regions, and their expression patterns.

Chitinase activity in plants is very low under normal circumstances, but when plants are affected by exogenous factors such as fungi, bacteria, viruses and other signalling factors such as elicitors, growth regulators (e.g. ethylene), chemicals (e.g. salicylic acid), heavy metals, or wounding (DING et al., 2002; YU et al., 2001), the level of chitinase activity increases markedly; in some cases, there is also a concomitant increase in β-1,3-glucanase activity, to significantly inhibit the growth of fungi (SELA-BUURLAGE et al., 1993). Studies have shown that plant chitinase genes play an important role in not only disease and insect pest resistance but also in plant development, symbiotic nitrogen fixation and freezing tolerance (STRESSMANN et al., 2004).

Chitinases identified from *C. procera* affected larval survival and weight, mean developmental time and emergence of adult insects (RAMOS et al., 2010). Marchant et al reported that expression of the chitinase transgene reduced the severity of black spot development by 13−43% in rose (MARCHANT et al., 1998). The mechanism of chitinase's action on insects is not well established. It is postulated that it may involve the hydrolysis of chitin present in their peritrophic membranes, which are responsible for protection against mechanical damage and invasion by microorganisms or parasites.

Chitinases, proteolytic enzymes and proteins involved in cellular anti-oxidative machinery were detected in the latex of *C. procera*. In additional studies, the proteins of the latex were shown to be deleterious to insects (RAMOS et al., 2007; RAMOS et al., 2010). Of the pathogenesis-related (PR) proteins, involved in the response mechanisms in plants, dedicated to battle pathogen attacks, plant chitinases (EC 3.2.1.14) were expressed as a common defence response to herbivores, fungal pathogens, and were also shown to be induced by abiotic stress (GROVER., 2012).

Chitinases are induced by various stress factors, i.e. drought, salinity, wounding, heavy metals in their environment, endogenous and exogenous elicitor treatment, and plant growth regulators (YU et al., 2001; XIE et al., 1999). Kumar et al. (2016) developed a transgenic tomato showing enhanced tolerance to salt and drought stress by the expression of Osmotin-like protein and *chi11* genes (KUMAR et al., 2016). During the pathogenic attack, the pathogenesis response proteins are released by plant chitinase as a self-defence whereas some of them are expressed after environmental stress such as increased salt concentration cold and drought (LILIANE et al., 1997; SEIDL et al., 2005; YAMAMOTO et al., 2000). Expression of Lt*chi7* gene encoding a class III endochitinase was also seen to be induced by drought stress, salt stress, hydrogen peroxide, and abscisic acid in the roots of *Lotus tenuis* and *Lotus japonicus* (TAPIA et al., 2011). Overexpression of Lc*CHI2* gene that encodes a class II chitinase from *Leymus chinensis* (false wheatgrass) increased chitinase activity in transgenic tobacco and maize, and enhanced pathogen resistance as well as salt stress tolerance (LIU et al., 2020).

1.7.3. Understanding how plants use proteases to respond to stress

Plant proteases are proteolytic enzymes that hydrolyse peptide bonds in proteins and are found in various plant tissues and organs (VAN DER HOORN., 2008; SHARMA and GAYEN., 2021). Their activities are tightly regulated through the transcriptional control of protease transcripts, post-translational modifications of their proenzymes, actions of endogenous protease inhibitors, and/or compartmentalization into organelles and cellular compartments to avoid random acts of protein degradation (VIERSTRA., 1996; DIAZ-MENDOZA et al., 2016). For instance, plant proteases are located in the cytosol, chloroplasts, vacuoles, nuclei, endoplasmic reticulum, proteasome, mitochondria, and cell walls (KIDRIC et al., 2014; DIAZ-MENDOZA et al., 2016), and also secreted into the extracellular matrix (NGCALA et al., 2020; GODSON and VAN DER HOORN., 2021). Each of these cellular compartments may possess specialised proteolytic pathways. For example, in the cytosol, protein degradation is mainly carried out by the highly selective ubiquitin-proteasome system (UPS), which consists of ubiquitin, the proteasome and associated components (HOPKINS and WILLIAM., 2008; XU and XUE., 2019).

Proteases are structurally and functionally diverse and are classified based on their catalytic activity, such as aspartic, cysteine, serine and threonine peptidases (CALLIS., 1995; SCHALLER., 2004). Alternatively, proteolytic enzymes are grouped into endo and exo-peptidases depending on the site of cleavage on the peptide chain (PALMA et al., 2002; SCHALLER., 2004).

Abiotic stresses such as drought and extreme temperatures, including biotic stress factors like phytopathogenic microorganisms trigger the production of extracellular and intracellular protease enzymes. Several studies have indicated that endogenous and exogenous secretion of protease enzymes in plant cell's cytoplasm is associated with their exposure to biotic and abiotic stress (ALI and BAEK., 2020; VAN DER HOORN and KLEMENCIC., 2021).

Proteases also play a key regulatory role in plant metabolism by maintaining effective protein quality controls, and eliminating nonfunctional proteins, and are used in systemic defence responses. Among these important biological roles, the biochemical degradation of cell proteins through hydrolysis of peptide bonds serves as the main primary function of proteolytic enzymes (MORRELL and SADANANDOM., 2019). However, these enzymes were also found to be associated with the occurrence of cell death (necrosis, excessive chlorosis, and programmed cell death) during senescence of tissues and organs, cell differentiation (SANTOS and FIGUEREDO., 2021), and additionally acting as critical regulators during embryogenesis, cuticle formation, chloroplast biogenesis and stomatal development (VAN DER HOORN and KLEMENCIC., 2021). According to D'ippolito et al. (2021), plant proteases were also found to be involved in signal transductions among phytohormones and the adjustment of stomatal apertures during the exposure of plants to drought stress.

Nevertheless, protease enzymes also induce the formation of reactive oxygen species (ROS) detected during plant exposure and response to abiotic stresses, especially water deficit stress (ALI and BAEK., 2020). Low and feverish temperatures were also reported to induce proteases that diminish plant productivity by causing a rapid burst of ROS in the chloroplasts (LUO and KIM., 2021). The dynamic changes in environmental conditions involving pathogen invasion also caused the expression of distinct digestive enzymes produced either by host plants or invading pathogen and herbivorous insect pests. Al-Ani et al. (2022) demonstrated a trypsin-serine-like protease activity of fungi and nematodes during plant parasitism and antibiosis. Most fungal and nematode species inject secretions into plant cells with trypsin-like or serine proteinase activity.

For instance, two serine protease enzymes were demonstrated in soybean cyst nematode (*Heterodera* spp. and *Globodera* spp.), and saprophytic fungi used to digest plant tissue proteins to favour the invading pathogenic metabolism and spreading of the infections (SILVA et al., 2018; RODRÍGUEZ-SIFUENTES et al., 2020).

When combined with environmental stress, proteolytic enzymes could be very debilitating to crops and plant life in general. Recombinant protease inhibitors have been expressed in various crop species to specifically confer resistance and protection against such various types of stress factors. Both proteases and their inhibitors may be harmful to pests, despite being essential for the maintenance and survival of plants during their acclimation to stressful habitat conditions, causing phenotypic and cellular disruptions when present in the cells in higher concentrations (SHARMA and GAYEN., 2021).

2. HYPOTHESIS

Osmotins, chitinases and peptidases are PR-proteins constitutively found in the latex of *Calotropis procera*. Previous studies indicated these proteins are involved in plant defence against predators and invaders. However, there is no evidence or consolidated data on whether these proteins are responsive to stress or plant elicitors. This question was approached in this study by treating cultured cells of *Calotropis procera* with Sodium chloride as an abiotic stressor agent and both, plant elicitors, Salicylic acid, and Methyl Jasmonate. The hypothesis evaluated proposes that these proteins are responsive to salt stress and can be increased in concentration when stimulated by both cited elicitors.

3. JUSTIFICATION

The current study introduces procedures for exploring numerous aspects of *in vitro* cultivation approaches and the biological functions performed by calluses produced from *Calotropis procera*. This strategy is utilized to estimate in what way salinity, salicylic acid, and

methyl jasmonate, influence the expression of osmotins, chitinases, and proteases. As was formerly documented, these proteins are considered pathogenesis-related (PR) proteins and are correlated to biological processes that comprise plant defence against biotic and abiotic stress. These proteins are intricately linked to the plant's defence processes against insect herbivores and microbial pathogens. This study engaged in three extremely significant areas. The primary objective was to develop controlled settings that would allow for an evaluation of the effects of salinity, salicylic acid, and methyl jasmonate at the cellular level. Subsequently, the investigation's second goal was to define the pathways triggered by salt stress and the signalling molecules. Lastly, the inquiry centred around the hypothesis that drove the investigation was that the expression levels of PR proteins would increase in response to salinity-induced stress and the presence of elicitors. The conclusion of this study aims to shed light on the responses of the callus proteins of the laticifer plant *"C. procera"* under study to salt stress, salicylic acid, and methyl jasmonate. Additionally, it seeks to develop strategies for overcoming these challenges by enhancing our understanding of how plant cells respond to saline stress and elicitation. Investigating the relationship between osmotin, chitinase, and protease as well as adaptive mechanisms sensitive to stressful conditions and signalling molecules is an additional critical factor to consider. This assessment builds the possibility of a connection between them and reducing the adverse effects of salt stress and elicitors. In this way, this work contributed to the understanding of the physiological role of the callus proteins in plant defence.

4. OBJECTIVES

4.1. General objective

- Evaluating the effects of salt stress, salicylic acid, and methyl jasmonate on *in vitro* callus cultures of *Calotropis procera* and analysing the expression of pathogenesis-related proteins (osmotin, chitinase, and protease) in response to salinity and signalling inducers.

4.2. Specific objectives

- Investigate the progression of *in vitro* callus cultures derived from the hypocotyls of *C. procera,* cultivated under varying NaCl concentrations (0, 40, 60, 80, and 100 mM), salicylic acid (0, 100, 200, and 400 µM), and methyl jasmonate (0, 50, 100, and 200 µM) aiming to understand their developmental responses.
- Examine the photosynthetic pigments level in the cultured *Calotropis procers* callus exposed to salt stress and elicitors.
- Utilize a zymogram and measure the proteolytic activity in cultured *Calotropis procera* callus exposed to salt stress and elicitors.
- Utilize electrophoresis to determine the expression of soluble proteins in the callus of *C. procera* cultivated under salt stress and signalling inducers.
- Assess the performance of enzymes (ascorbate peroxidase, catalase, guaiacol peroxidase, and superoxide dismutase) responsible for reactive oxygen species detoxification, in *Calotropis procera* callus grown under conditions of saline stress and elicitors.
- Examine the expression levels of osmotin, chitinase, and protease transcripts in the callus of *Calotropis procera* cultivated under different levels of salt stress and elicitors.
- Employ confocal microscopy to assess chlorophyll, lignin, and phenolic compounds in the callus of *C. procera* subjected to varied degrees of salt stress and elicitors.
- Utilize the TUNEL assay to assess DNA integrity and quantify cell numbers in *C. procera* callus subjected to diverse concentrations of salt stress and signalling molecules.

5. MATERIALS AND METHODS

5.1. Biological material

Mature fruits of *C. procera* (Ait) R. Br. were collected from plants found growing on the campus of the Federal University of Ceara, Brazil, situated at the geographical coordinates 3º75′29″S, 38º57′38″W. The fruits were carefully stored at the temperature range of 24–27 ºC until the seeds were removed. The seed's fibre was removed mechanically, and the seeds were visually examined for physical integrity. Only the healthy and undamaged seeds were used for the treatments. In a laminar flow cabinet, the seeds (n = 192) were disinfected with 1% (active chlorine) NaOCl solution for 3 min and rinsed five times with sterile distilled water for 2 min. Subsequently, the seeds were placed in Petri dishes lined with sterile filter paper. The seeds utilized in all experimental procedures were sourced from fruits gathered within a time frame of 1 to 5 days before their application.

5.2. Methods

5.2.1. Seed germination

Following the disinfestation process, a total of 192 seeds were introduced into sterile tubes containing 15 mL of ½ MS medium (as described by Murashige and Skoog., 1962), which had been previously autoclaved at 121 ºC for 15 minutes. The medium was supplemented with 100 mg/L myo-inositol, 2% (w/v) sucrose (pH 5.8) and solidified with 0.7% (w/v) agar (Sigma Chemical Co.) according to methods described by Teixeira et al. (2011). To initiate seed germination, the tubes holding the seeds were placed in a controlled environment with a temperature of 25 ± 2 °C and subjected to a photoperiod of 16 hours of light followed by 8 hours of darkness. The light source was provided by white, fluorescent light-emitting diodes (LED) with an intensity of 10–15 μmol/m^2/s.

5.2.2. Callus induction

After four weeks of sowing, the seedlings of the species under study were used as the primary source for callus induction. These seedlings had a size of approximately 4 cm. To generate the callus cultures, the hypocotyl segments were aseptically detached and subsequently longitudinally sliced into segments measuring 10 mm in length, all under aseptic conditions. For callus induction, five hypocotyl explants (n = 325) were inoculated in Petri dishes containing 25 mL of the same nutritive medium previously used for seed germination but supplemented with 3 µM 1- naphthyl acetic acid (NAA) and 4.6 µM kinetin (KIN). This procedure was similar to that described by Suri and Ramawat (1995) proposal with modifications. The cultivation of callus was conducted following a methodology like that of seed germination. After 28 days, calli were successfully generated and served as the biological materials for subsequent assays.

5.2.3. Effects of salt-induced stress in callus of *Calotropis procera*

Following the acquisition of *C. procera* callus, a fresh medium, resembling the one employed for callus growth, was prepared. Various concentrations of NaCl (0, 40, 60, 80, and 100 mM) were added to this medium, and 25 mL of each mixture was distributed onto Petri dishes. Calli that were 28 days old and viable were then carefully transplanted into the newly prepared saline media. A total of 125 calluses were allocated across five conditions (125/5 = 25). These 25 calluses were then distributed among five petri dishes (five replicates), each with five calluses present in each petri dish for every concentration, including the control. The Petri dishes were subsequently kept under the same experimental conditions as previously established for callus induction, with an additional duration of 28 days for maintenance. At the end of the salt stress treatment, the calli were removed and subjected to analysis of physiological parameters (fresh weight, dry weight, growth rate, water content, protein content, hydrogen peroxide, proteolytic activity, photosynthetic pigments, and the activity of anti-oxidative enzymes). These measurements were conducted with the primary objective of verifying the successful induction of salt stress before proceeding with the analysis of protease, chitinase, and osmotin transcript profiles.

5.2.4. Growth rate and water content of salt-induced stress callus of *Calotropis procera*

A total of five calluses from each petri dish (n = 5 plates) from every concentration, including control, were weighed using an analytical balance on the initial day of salt treatment (28 days old), and the average weight was recorded. The same calli were weighed again 28 days later, at the end of the salt treatment. These measurements were used to calculate the growth rate, and the second weight measurement served as the initial measurement for water content. Following the second weighing, the calli were freeze-dried and weighed again for the final water content estimation. The growth rate (GR) was calculated by taking the initial weight of the callus at the time of transference to the medium containing NaCl (Wi), and the weight after 28 days of treatment with NaCl (Wa). The mean was calculated by: $[(Wa-Wi)/Wi] \times 100$. The percent of tissue water content (TWC %) was calculated with the weight values obtained from fresh (FW) and dry (DW) callus, applying the following equation: $TWC\ (\%) = [(FW-DW)/FW] \times 100$.

5.2.5. Effects of signalling inducers in callus of *Calotropis procera*

Following the acquisition of *C. procera* callus, a fresh medium, resembling the one employed for callus growth, was prepared. Various concentrations of salicylic acid (0, 100, 200, and 400 µM), and methyl jasmonate (0, 50, 100, and 200 µM) were added to this medium, and 25 mL of each mixture was distributed onto petri dishes. Viable calli aged 28 days were transplanted into the newly prepared media with varying concentrations of salicylic acid and methyl jasmonate. A total of 200 calluses were evenly distributed across treatment groups for both compounds. Each treatment group contained 100 calluses, divided into four conditions, resulting in 25 calluses per condition. These 25 calluses were then allocated among five petri dishes, each representing a replicate, with five calluses per dish for every concentration, including the control, in both salicylic acid and methyl jasmonate treatments. The Petri dishes were subsequently kept under the same experimental conditions as previously established for callus induction, with an additional duration of one week for salicylic acid and methyl jasmonate treatment. At the end of treatment, the calli were removed and subjected to analysis of physiological parameters (fresh weight, dry weight, growth rate, water content, protein content, proteolytic activity, photosynthetic pigments, and the

activity of anti-oxidative enzymes). These measurements were carried out with the main objective of verifying the effect of salicylic acid and methyl jasmonate induction on callus growth before proceeding with the analysis of the transcript profiles of protease, chitinase, and osmotin.

5.2.6. Growth rate and water content of signalling inducers in callus of *Calotropis procera*

A total of five calluses from each petri dish (n = 5 plates) from every concentration, including control, were weighed using an analytical balance on the initial day of salicylic acid and methyl jasmonate treatment (aged 28 days), and the average weight was recorded. The same calli were weighed again after one week of the treatment. These measurements were used to calculate the growth rate, and the second weight measurement served as the initial measurement for water content. Following the second weighing, the calli were freeze-dried and weighed again for the final water content estimation. The growth rate (GR) was calculated by taking the initial weight of the callus at the time of transference to the medium containing salicylic acid, and methyl jasmonate (Wi), and the weight after one week of treatment with salicylic acid, and methyl jasmonate (Wa). The mean was calculated by: $[(Wa-Wi)/Wi] \times 100$. The percent of tissue water content (TWC %) was calculated with the weight values obtained from fresh (FW) and dry (DW) callus, applying the following equation: $TWC\ (\%) = [(FW-DW)/FW] \times 100$.

5.2.7. Protein extraction

After the treatment of salt (28 days), salicylic acid and methyl jasmonate (one week) the calli from the control MS medium and the salt treatments at 40, 60, 80, and 100 mM NaCl concentrations, salicylic acid at 100, 200, and 400 µM concentrations, and methyl jasmonate at 50, 100, and 200 µM concentrations were collected and subjected to lyophilization. Following the lyophilization process, the calli of *C. procera* were thoroughly macerated in a mortar using liquid nitrogen for further analysis. A protein extract was obtained from 500 mg of both the control group and the calli subjected to salt, salicylic acid, and methyl jasmonate treatments. After that, 2 mL of

100 mM potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl, 3% PEG, 1 mM PMSF, 2 mM EDTA and 1% PVPP was added. The mixture was macerated for 10 min in a mortar under an ice bath. The homogenate was centrifuged (10,000×*g*, 4 ºC, 10 min). The soluble phase was carefully retrieved and subsequently dialyzed against the extraction buffer over 24 hours at a temperature of 4 ºC. The total soluble protein concentration was quantified using the Bradford method (BRADFORD., 1976). These protein extracts served as the foundation for exploring various enzymatic activities. Notably, all enzymatic assays were conducted upon completion of the 24-hour dialysis period. The protein extract for carrying out electrophoresis and zymogram was obtained using the same methodology mentioned above.

5.2.8. Enzymatic activities in *Calotropis procera* callus under salt-induced stress and signalling inducers

The dialyzed extracts obtained from *Calotropis procera* hypocotyl callus, were exposed to NaCl concentrations (0, 40, 60, 80, and 100 mM) for 28 days, as well as salicylic acid (0, 100, 200, and 400 µM) and methyl jasmonate (0, 50, 100, and 200 µM) for one week, were utilized to evaluate enzymatic activities.

The activity of guaiacol peroxidase enzymes (POX, EC 1.11.1.7) was determined followed by the method described by Urbanek et al. (1991). Guaiacol and H_2O_2 were used as substrates. Aliquots of 0.8 mL of 50 mM sodium acetate buffer (pH 5.2) were added to 0.5 mL of 60 mM H_2O_2 and 0.5 mL of 20 mM guaiacol. This reaction mixture was left in a water bath at 30 °C for 10 min. The addition of 0.2 mL of dialyzed extract started the reaction and the formation of a coloured compound was measured every 20 s, at 480 nm in a spectrophotometer (Novaspec III Pharmacia). One activity unit (AU) was established as the change in absorbance per minute and expressed as AU/mg of protein (AU/mgP).

The catalase (CAT, EC. 1.11.1.6) activity was measured as described by Havir and McHale (1987) and Peixoto et al. (1999). In a 0.8 mL aliquot of 75 mM potassium phosphate buffer, pH 7.0, 0.1 mL of the dialyzed extract was added, and this reaction mixture was left in a water bath at 30 °C for 10 min. After that, 0.1 mL of H_2O_2 112.5 mM was added to the reaction mixture and the

enzyme activity was measured at 240 nm based on the decrease in absorbance at 20 s intervals. The AU was established as the change in absorbance per minute and expressed as AU/mgP.

The ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined according to Koshiba (1993) and Peixoto et al. (1999), with modification. Initially, 0.1 mL of dialyzed extract was added to 0.8 mL of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5 mM ascorbate. The mixture was left in a water bath at 30 °C for 10 min. APX activity was initiated by the addition of 2 mM H_2O_2. The measurement was performed with a spectrophotometer at 290 nm, at 30 s intervals. The activity unit (AU) was established as the change in absorbance per minute and expressed as AU/mgP.

The superoxide dismutase (SOD, EC 1.15.1.1) was evaluated by the ability of the enzyme to inhibit the photoreduction of nitro blue tetrazolium (NBT) in a reaction medium containing 0.05M potassium phosphate (pH 7.8), 19.5 mM methionine, EDTA 0.1 mM, NBT 750 µM and riboflavin 10 µM (BEYER and FRIDOVICH., 1987). The tubes with the reaction medium and the samples were illuminated for 15 min under two 20-W fluorescent tubes at 25 ºC. As a control, the same reaction medium without the sample was illuminated under the same conditions, while the blank solution was kept in the dark. Readings were taken at 560 nm in a spectrophotometer, where one unit of SOD was considered as the amount of enzyme able to inhibit by 50% the photoreduction of NBT under the experimental conditions. The SOD activity was expressed in AU/mgP.

These assessments were conducted in triplicate for each of the three independent treatments to ensure accuracy and reliability. Mean values were calculated independently for each treatment group.

5.2.9. Proteolytic activity

The total proteolytic activity was tested through the colorimetric reaction, which uses azocasein as a non-specific substrate. This protein, upon undergoing degradation by proteases, releases a compound called azo that functions as a chromophore, being detected at a wavelength of 420 nm. To carry out this test, an aliquot of 100 µL of the sample of protein extracts from the callus of *C. procera* which was subjected to 28 days of treatment in the presence of different

concentrations of NaCl (0, 40, 60, 80, and 100 mM), and one-week treatment of salicylic acid (0, 100, 200, and 400 µM), and methyl jasmonate (0, 50, 100, and 200 µM). These samples were added to the reaction with 200 µL of 1% azocasein and the volume was adjusted to 500 µL with 50 mM sodium acetate buffer, pH 5.0. After 60 minutes at 37 ºC, the reaction was stopped with the addition of 300 µL of 10% trichloroacetic acid (TCA). The tubes were centrifuged at 5,000 x *g*, for 10 minutes, at 25 °C and 400 µL of the supernatant was mixed with 400 µL of sodium hydroxide (NaOH) 2 M. Colour development was measured by spectrophotometer at 420 nm. One unit of activity was defined as the amount of protein capable of increasing absorbance by 0.01 in one hour (XAVIER-FILHO et al., 1989). The results were presented in AU μg^{-1} of protein. At least three independent measurements were performed for each treatment.

5.2.10. Content of photosynthetic pigments

The photosynthetic pigments, chlorophylls *a* and *b* as well as total carotenoids *x* + *c*, were extracted with 80% acetone and then centrifuged at 15,000×*g* for 30 min, using the redetermined extinction coefficients and equations established by Lichtenthaler (1987). The samples were treated with NaCl (0, 40, 60, 80, and 100 mM) for 28 days, and one week with salicylic acid (0, 100, 200, and 400 µM), and methyl jasmonate (0, 50, 100, and 200 µM) were stored in a light-protected environment to prevent light exposure and were later subjected to absorbance readings at 665, 649 and 480 nm. Pigment contents were determined using the equations: Chlorophyll *a* =12.47 (A_{665}) – 3.62 (A_{649}); Chlorophyll *b* = 25.06 (A_{649}) – 6.50 (A_{665}); Chlorophyll (*a* + *b*) =7.15 (A_{665}) –18.71 (A_{649}); Carotenoids = [1000 (A_{480}) – 1.29 (chlorophyll *a*) – 53.78 (chlorophyll *b*)]/220.

5.2.11. Analysis of H_2O_2 content in *Calotropis procera* callus under salt-induced stress

The assessment of H_2O_2 content was carried out immediately after subjecting *Calotropis procera* calluses to various concentrations of NaCl treatment (0, 40, 60, 80, and 100 mM) for 28 days. The H_2O_2 was extracted and quantified according to Gay et al. (1999). The calluses were

macerated in the presence of borax-borate 50 mM buffer (pH 8.4) in a 1: 5 (w/v) ratio. The homogenate was centrifuged (12,000×*g*, 4 °C, 20 min) and the supernatant was collected. In the assay, 0.2 mL of the supernatant was added to 1 mL of xylenol orange solution, and the mixture was incubated for 30 min (25 °C) in the dark. The xylenol orange solution was formed by 0.1 mL of solution A (iron sulfate 25 mM, ammonium sulfate 25 mM, sulfuric acid 2.5 M and ultrapure water) with 10 mL of solution B (xylenol orange 125 µM, sorbitol 100 mM and ultrapure water). The samples were subjected to absorbance measurements at 560 nm in triplicate. The H_2O_2 content was quantified and expressed as nmol H_2O_2 per gram of fresh weight (nmol H_2O_2/gfw). The reported results represent the mean of three separate measurements.

5.2.12. Protein profile analysis by SDS-PAGE

Electrophoresis of proteins was performed to visualise the proteins from *C. procera* callus treated with NaCl (0, 40, 60, 80, and 100 mM) for 28 days, and with salicylic acid (0, 100, 200, and 400 µM) and methyl jasmonate (0, 50, 100, and 200 µM) for one week.

Electrophoresis was proceeded according to Laemmli (1970). Stacking gels of 5% polyacrylamide were used with 15% polyacrylamide resolving gels. To estimate molecular mass, the following markers were used: phosphorylase β (97.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

The runs were executed in a constant current of 30 mA per gel and tension of 110 V per gel, in an average duration of 2 hours at 25 ºC. In each lane, 30 µL of each sample diluted to 1 mg/mL in distilled water was applied. Protein bands were visualized after colouring with Coomassie Brilliant Blue R-250 in a solution of methanol, acetic acid, and water (4:1:5; v:v:v). The revealing was done using the same solution without the Coomassie dye.

5.2.13. Gelatin-based zymography

The proteolytic activity of the *C. procera* callus treated with NaCl (0, 40, 60, 80, and 100 mM) for 28 days, and with salicylic acid (0, 100, 200, and 400 µM) and methyl jasmonate (0, 50, 100, and 200 µM) for one week was also evaluated by zymography containing 0.1% gelatin as substrate according to Toth and Fridman (2001). The fractions were separated by electrophoresis in 15% SDS-PAGE, as described before, containing 0.1% gelatin at 25 ºC. Afterwards, the gel was incubated in a renaturing solution containing 2.5% Triton X-100, and gently shaken for 30 minutes to remove SDS and allow protein rearrangement. The gels were then washed with distilled water and incubated in 50 mM PBS (pH 7.0) containing 3 mM DTT for 24 h at 37 ºC before being stained with 0.2% Coomassie Brilliant Blue R-350. Enzymatic activity was detected as clear bands in a dark background. Bovine serum albumin was used as a negative control for proteolytic activity.

5.2.14. Analysis of chlorophyll, lignin, and phenol compounds in *Calotropis procera* callus under salt-induced stress using confocal microscopy

To analyse the chlorophyll, lignin, and phenol compounds, fresh calluses of *Calotropis procera* treated with various concentrations of NaCl (0, 40, 60, 80, and 100 mM) were collected after 28 days of treatment. These calluses were delicately sectioned into thin slices and then immersed in a solution of 90% glycerine in PBS for 15 minutes according to Baldacci-cresp et al. (2020) and Hutzler et al. (1998). Subsequently, the sections were carefully mounted onto slides, ensuring complete coverage to eliminate any gaps between the slides. The prepared slides were then examined under an Ar laser (λ = 488 nm) by confocal microscopy. Excitation signals were collected between 500 and 580 nm.

Fluorescent images were captured using a Carl Zeiss laser scanning microscope (LSM 710) with a 20x magnification objective lens (Carl Zeiss, Jena, Germany). For each treatment group, a minimum of five independent analyses were conducted.

5.2.15. TUNEL assay analysis of *Calotropis procera* callus under salt-induced stress

To assess DNA damage, fresh calluses of *C. procera* treated with various concentrations of NaCl (0, 40, 60, 80, and 100 mM) were collected after 28 days of treatment. The TUNEL fluorometric assay (TdT-mediated dUTP Nick-End Labeling) was conducted following the manufacturer's instructions. After treatment the calluses were collected, sectioned into thin slices, and fixed in 4% formaldehyde in PBS for 15 minutes according to Shamsi et al. (2008). Excess formaldehyde was removed through washing, and the TUNEL protocol was subsequently executed.

The prepared sections were mounted on slides and examined using confocal microscopy. Cells with damaged DNA were identified by the incorporation of fluorescein-12-dUTP, emitting at 520 nm. Moreover, the 647 nm wavelength was employed to visualize propidium iodide, highlighting individual cells. Fluorescent images were captured using a Carl Zeiss laser scanning microscope (LSM 710) equipped with a 20x magnification objective lens (Carl Zeiss, Jena, Germany). A minimum of five independent analyses were performed for each treatment group.

5.2.16. Cell count analysis in *Calotropis procera* callus under salt-induced stress

Fresh calluses of *Calotropis procera* subjected to different NaCl concentrations (0, 40, 60, 80, and 100 mM) were collected after 28 days of treatment. Cell counts from callus sections on slides were obtained according to Klais et al. (2003) with modifications, utilizing confocal microscopy software in both manual and automated modes. The defined area was marked using a drawing tool by confocal microscope software and the cells were manually counted by observing DNA-stained nuclei with propidium iodide in the TUNEL assay within the bounded area. Results were based on cell density and presented as the number of cells/mm^2.

5.2.17. Total RNA extraction and transcriptomic analyses

Callus tissues obtained from hypocotyls of *C. procera* were used for RNA extraction. These calluses were subjected to saline stress (MS added with 80 mM NaCl), salicylic acid treatment (MS added with 400 µM of salicylic acid), methyl jasmonate treatment (MS added with 200 µM of methyl jasmonate), and control conditions (MS medium). For the salt treatment, different collection times were 0, 2, 12, 24 and 48 hours and 4, 7, 14 and 28 days of contact with the culture medium while for the salicylic acid and methyl jasmonate treatment, different collection times were 0, 2, 12, 24, and 48 hours and 4 and 7 days of contact with the culture medium.

For the extraction procedure, 100 mg of callus was used. This material was obtained through a sample composed of callus from different replications. The calluses were macerated in liquid nitrogen, using a mortar and pestle. From the plant material macerated, total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

In microtubes, 700 µL of RLT buffer and 10 µL of β-mercaptoethanol to the macerated callus samples, and the mixture was homogenized using vortex, and the obtained solution was transferred by pipetting to a mini-column from the kit (QIAshedder spin column), coupled to a 2 mL collection tube and centrifuged at 12,000 x g, at 25 °C for 2 minutes. The supernatant was recovered and transferred to a new microtube, where 400 µL of 95% ethanol was added and the solution obtained was mixed by inversion.

The previously obtained solution was transferred to a new column in the kit. (RNeasy mini spin column), also attached to a 2 mL collection tube and centrifuged at 25 °C for 15 seconds at 8,000 x g. The eluate was discarded and 400 µL of RW1 buffer was added to the column, centrifuging at 8000 x g, temperature environment for 15 seconds. After washing with RW1, the column was transferred to a new collection tube and 500 µL of RPE buffer was added to the column, which was subjected to new centrifugation at 8000 x g, at 25 °C for 1 minute. The eluate was discarded and another 500 µL of RPE buffer was added to the column. The sample was subjected to a new centrifugation at 8000 x g, at 25 °C for 2 minutes, to eliminate any ethanol residue that could interfere with subsequent reactions.

The eluate and collection tubes were discarded, and the column was transferred to a new 1.5 mL collection tube. Then, 30 µL of RNase-free water was added directly to the column and kept for 3 minutes at 25 °C and then subjected to the new centrifugation to 8000 x g, for 1 minute to RNA elution. Then again 20 µL of RNase-free water was added directly to the column,

subjecting the new centrifugation to 8000 x g, for 1 minute to RNA elution. The eluted RNA was stored at -20 °C for later quantification, electrophoretic analyses, reverse transcription (RT) reactions and RT-PCR assays.

The RNA concentration was estimated based on the absorbance readings of the samples at a wavelength of 260 nm, using a GeneQuant spectrophotometer (Pharmacia). The degree of purity of this RNA was also evaluated in this equipment using the ratio of absorbances 260/280 nm and the ratio of 260/230 nm. The samples were quantified in triplicate and then an average was taken from the reading of the absorbance.

The integrity of total RNA and verification of contamination with genomic DNA was examined using 0.5 µg of total RNA. For this, electrophoresis was carried out in 1% agarose gel. Agarose gels for electrophoretic migration were prepared according to the protocol described by Sambrook (1989). To produce the gel, agarose was melted in TAE buffer, pH 8.0 (0.04 M Tris-acetate and 0.001 M EDTA) containing 0.5 µg of ethidium bromide. Soon after, this mixture was placed in a horizontal vat suitable for inserting combs, enabling the formation of wells.

After polymerization of the gel, a volume of 5 µL containing the RNA samples. To prepare these, 500 ng of total RNA was used (with varied volumes), 1.5 µL of sample buffer (phenol bromine blue) and deionized water autoclaved to complete the final volume of 5 µL.

Electrophoretic runs were carried out in a horizontal tank filled with TAE buffer (pH 8). The parameters used were a constant amperage of 35 mA and a voltage of 70 V. The duration of the race was approximately 30 minutes and was carried out at 25 ºC. Ribosomal RNA bands (18S and 28S) in the agarose gel were visualized by exposure to UV light, on a Vilber Lourmat transilluminator (Marne-la-Vallée, France), and the image was captured by a photo documentation system coupled to the equipment.

After quantifying the RNA samples, a run was performed electrophoretic analysis to identify whether the RNA samples were truly homogeneous and complete. Once the homogeneity of the RNA samples was confirmed, the reverse transcription was performed.

Total RNA was used for the synthesis of complementary DNA (cDNA), using the IMpromII^{TM} Reverse Transcriptase Kit (Promega, United States), according to the manufacturer's instructions.

For the reaction to occur, 1μg of the RNA sample was incubated with 500 ng of Oligo $(dT)_{18}$ initiator, for 5 minutes, at a temperature of 70 °C and then at 4 °C, for another 5 minutes. Soon after, a reaction mixture was added containing: 4 μL of buffer reverse transcription 5 times concentrate, 2.4 μL of 0.025 M $MgCl_2$, 1 μL of 0.01 M dNTP and 1 μL of reverse transcriptase enzyme at 1 AU/ μL. The complementary strand synthesis reaction occurred at 25 °C for 5 minutes, 42 °C for 1 hour, followed by 75 °C for 15 minutes (inactivation of the enzyme). The cDNA obtained was stored at -20 °C for subsequent PCR reactions.

To carry out the RT-PCR, four oligonucleotide primers were used degenerates (Primers): *EF1α*, *CpOsm*, *CpChit*, and *CpProt*. The degenerate *CpOsm* oligonucleotides were designed by Souza. (2015) and produced based on the N-terminal sequence obtained from osmotin from *C. procera.*

Other oligonucleotides used were *EF1α* (synthesis elongation factor protein in eukaryotes), this being a gene with constitutive expression. The objective of its use was to normalize the data obtained through its stable expression. These oligonucleotides were described by some researchers (COSTA et al., 2010; GONDIM et al., 2012). Oligonucleotides were designed from the 3' untranslated region (3'UTR). The nucleotide sequences of the primer oligonucleotides employed in the experiment are as follows:
Sequence of oligonucleotides used.

Name	Sequence
EF1α F	5' AGRTTYGAGAARGARGCTGC 3'
EF1α R	5' CCACGCTTSAGATCCTTVAC 3'
CpOsm F	5' CCGGCCACNTTYACNATHCGNAACAAYTGYCC 3'
CpOsm R	5' CCGGGRCARAANAYAACYCTRTARTTDGT 3'
CpChit F	5' CAAGATGTCGGTTCAATCGT 3'
CpChit R	5' AGTATTCCACAGTAACGCCTA 3'
CpProt F	5' CCTATGGATACTTCGTTAAGT 3'
CpProt R	5' GATGCTGATATGTCCTTCGTC 3'

Table 1- Primers utilized for transcriptome-level assessment of target protein-encoding genes.

To verify the most appropriate temperature for the annealing of the *EF1α*, *CpOsm*, *CpChit*, and *CpProt* primer oligonucleotides, a gradient of temperature. For this, a pool of cDNA samples was collected. This procedure was carried out in a Mastercycler realplex 4S thermocycler (Eppendorf®). The temperatures used were (50.8; 52; 53; 54; 55; 56; 57.1; and 59.6 °C).

This step was carried out to verify the best number of cycles to amplify the gene product of the *EF1α*, *CpOsm*, *CpChit*, and *CpProt* primers. For this, a pool of *C. procera* cDNA samples and tested with 24 cycles, 27 cycles, 30 cycles, 33 cycles, 35 cycles, 37 cycles, and 39 amplification cycles. Next, the best number of cycles to occur was determined in polymerase chain reaction. The reaction was carried out in a Mastercycler thermocycler, realplex 4S (Eppendorf®), with an annealing temperature of 57 °C.

After standardizing the temperatures and number of cycles of the *EF1α*, *CpOsm*, *CpChit*, and *CpProt* RT-PCR was performed to evaluate the expression level of osmotin, chitinase, protease and the gene *EF1α* in *C. procera* calli subjected to saline stress, salicylic acid, and methyl jasmonate at different exposure times.

The products of the RT reactions (single-stranded cDNA) were subjected to PCR reactions using degenerate primers for the osmotin gene (CpOsmF and CpOsmR), for chitinase (CpChitF and CpChitR), for protease (CpProtF and CpProtR) and the *EF1α* elongation factor gene (EF1αF and EF1αR). The number of cycles used for the different genes was different: 33 cycles were used for osmotin, 30 cycles for chitinase, 37 cycles for protease and 24 cycles for *EF1α*. The procedure used for obtaining the PCR reaction was as indicated by the supplier. The kit for performing the PCR was GoTaq® DNA Polymerase from Promega.

For the reaction, 1 µL of cDNA obtained by reverse transcription was pipetted into 0.2 mL microtubes. This sample was incubated in a reaction mixture consisting of 0.1 µL (0.5 U) GoTaq DNA Polymerase 5 U/µL, 5 µL 5x colourless reaction buffer, 1 µL of a 0.001 M dNTP mixture, 1 µL of each primer oligonucleotide in concentration of 5 µM (sense and antisense) and sterile deionized water. In addition to samples tested in the PCR reaction, a negative control containing all components of the reaction except DNA was always used. The reaction cycles consisted of an initial stage of denaturation at 95 °C for 3 min and a series of 33 cycles (for osmotin primers), 30 cycles (for chitinase primers), 37 cycles (for protease primers) and 24 cycles (for *EF1α* primers) at the following temperatures: 94 °C (denaturation step), 57.5 °C (primer annealing step) and 72 °C

(strand extension), and all these steps had lasted 1 minute. After these cycles, there was a finalization stage, which consisted of 4 minutes at 72 °C. This reaction was carried out in the Mastercycler realplex 4S thermocycler (Eppendorf®).

The products of the PCR reactions were stored at -20 °C until further use. To analyse the reaction products, an 8 µL aliquot was subjected to gel electrophoresis of 1% agarose.

5.2.18. Statistical analysis

The average obtained from the calli cultivated in the MS media with different concentrations of salt 40, 60, 80, and 100 mM, salicylic acid 100, 200, and 400 µM, and methyl jasmonate 50, 100, and 200 µM were compared between them. Statistical analysis was performed using the R-software. Data normality was tested. For data from a normal distribution, ANOVA was considered and subsequently, the Tukey test, otherwise the non-parametric tests, Kruskal-Wallis and Wilcoxon were considered. A significance level of 5% was considered.

6. RESULTS AND DISCUSSION

6.1. Salt-induced stress in *C. procera* callus

6.1.1. Cultivation of *C. procera* calluses

In the initial phase of the experiment, protocols were employed to cultivate calluses from *C. procera*. These calluses were developed as a model to explore the physiological and biochemical responses in these species under saline stress.

The substrate utilized for seed germination successfully facilitated the growth of *C. procera*. Twenty-eight days after sowing, viable seedlings, measuring approximately 4 cm in size, were obtained, serving as explants for callus generation. Hypocotyl segments from these seedlings were selected as the primary source of explants for callus formation.

The calluses that emerged from the hypocotyls displayed a greenish colouration. Callus formation began about two weeks after the explants were introduced to the nutrient medium. By the fourth week, the calluses had significantly developed. Notably, better callus formation, averaging 1 gram per callus, was observed in the hypocotyl tissues. These calluses were subsequently utilized to progress the experiment, as indicated in (Figure 6).

Healthy and viable calli, with an average weight of 1 gram and free from contamination, were transplanted to a fresh MS culture medium. This new medium contained increasing concentrations of NaCl, aimed at inducing salt stress. The calli were subjected to the salt medium for 28 days. After this period, specific criteria were assessed to understand their development and response mechanisms to salt-induced stress.

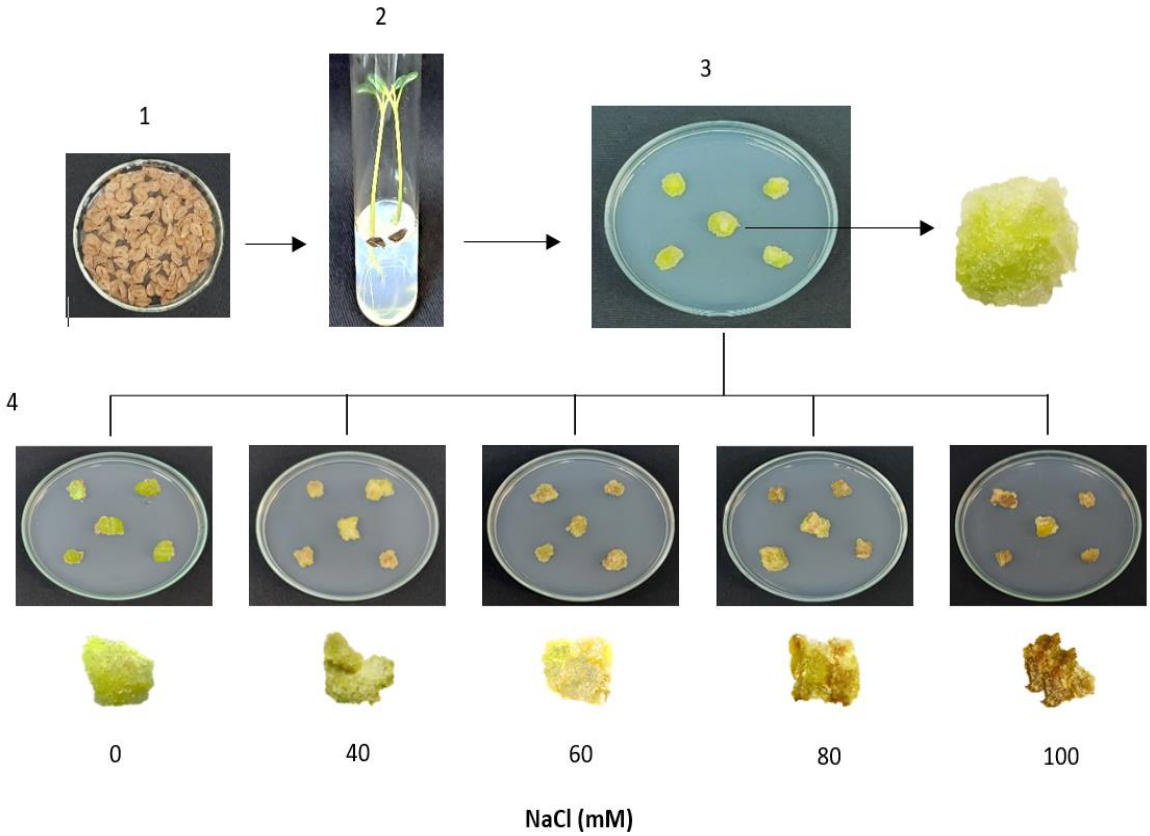


Figure 6- Seeds of *C. procera* were grown in 1/2 MS media for 28 days (2). Calluses were produced from the hypocotyl segments in the same nutritive medium used for seed germination but supplemented with 3 µM 1-naphthylacetic acid (NAA) and 4.6 µM kinetin (KIN) (3). After 28 days the calluses were transferred into the newly prepared saline media (0, 40, 60, 80 and 100 mM) for a further 28 days (4).

6.1.2. Salinity impact on callus physiology

The saline environment induces various detrimental effects on plant cells, resulting in a significant reduction in water content, growth, productivity, and both fresh and dry weight as a final physiological response. To evaluate the impact of heightened salinity on the culture medium, we analysed the relative growth rate (RGR) of calli exposed to a 28-day cultivation period in a NaCl-infused medium. In this study, the RGR, derived from the total fresh mass of callus before and after exposure to stress, exhibited susceptibility to high NaCl concentrations.

Our findings revealed a progressive reduction in the growth rate of calli as the NaCl concentration in the medium increased. The lowest relative growth rate was observed in 80 and 100 mM of salt-treated calluses. Consequently, the water content percentage decreased in calluses treated with higher levels of NaCl compared to the control. Water content is the same for concentrations of 0, 40 and 80 mM. However, a statistically significant reduction was confirmed only in calli treated with 100 mM NaCl (Figure 7).

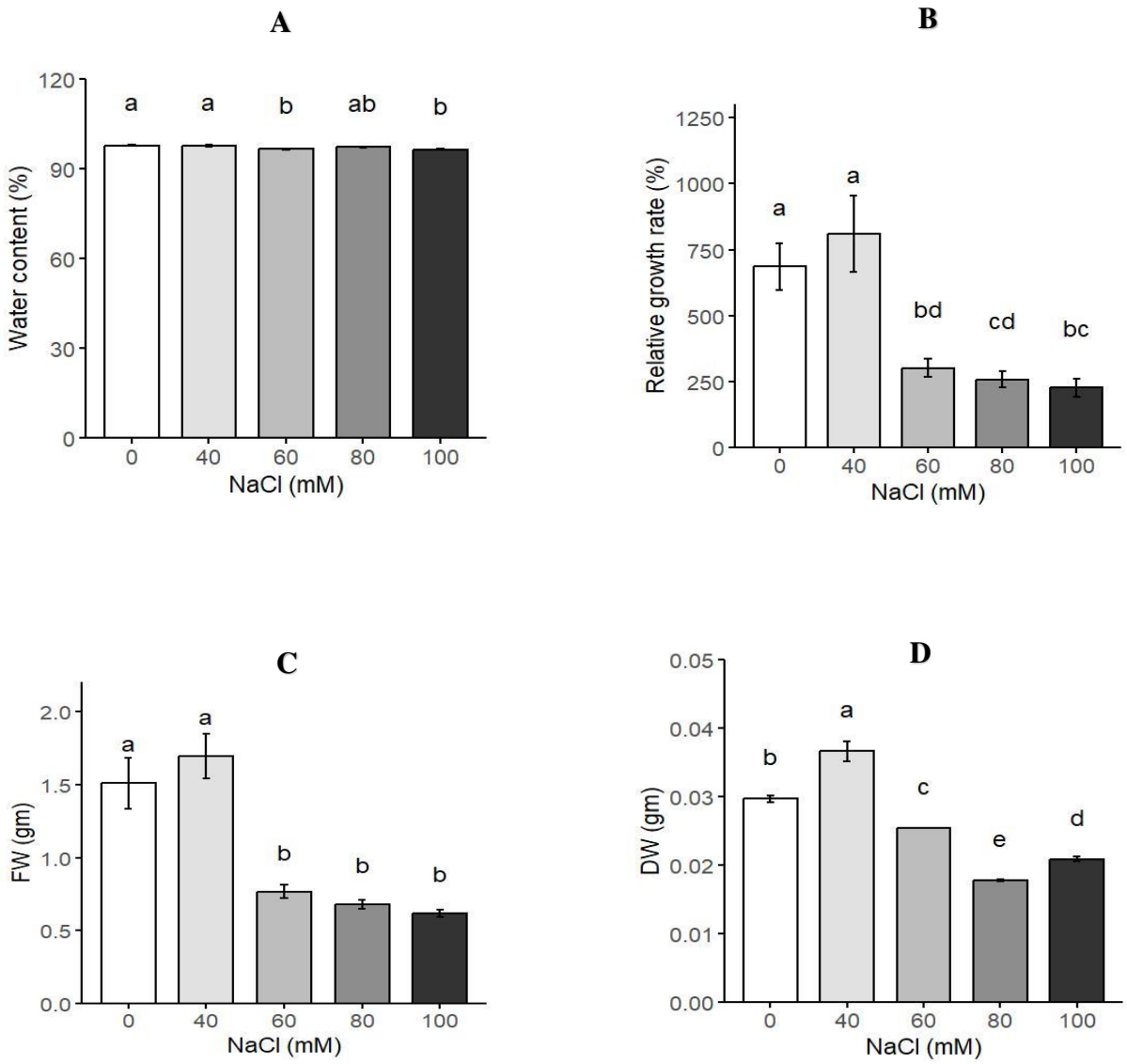


Figure 7- Performance of callus of *C. procera* in terms of water content (A), relative growth rate (B), fresh weight (C), and dry weight (D) when cultivated in increasing concentrations of NaCl. Data are mean ± SMD of five independent measurements. Columns labelled with different letters are significantly different at $P < 0.05$ based on one-way ANOVA with Tukey multiple range test using R-software. Kruskal-Wallis was used for Relative growth rate (B), and Dry weight (D).

The observed decline in growth under saline stress conditions aligns with findings from various studies involving callus from diverse species, including rice, sugarcane, wheat, and niger (REDDY and VAIDYANATH., 1986; BARAKAT and ABDEL-LATIF., 1996; PATADE et al., 2008; GHANE et al., 2014). Reddy and Vaidyanath (1986) attribute this reduction in callus growth

to osmotic and ionic stress induced by the high salt concentration in the culture medium. Additionally, cells cultured under stress exhibit reduced growth compared to those in a non-stressful medium, as energy resources are diverted towards pathways that confer resistance, to stress, thereby inhibiting normal growth (CROUGHAN et al., 1981; CUSHMAN et al., 1990).

Patade et al. (2008) proposed an alternative explanation for the observed decline in callus growth in a culture medium containing NaCl. According to their findings, the presence of Na^+ and Cl^- ions introduce a nutritional imbalance by competing with essential nutrients crucial for the metabolic processes essential to callus growth within the culture medium.

In this study, the observed decrease in callus growth in the species under study is likely attributed to two potential factors: the presence of toxic levels of ions and/or a nutritional imbalance induced by high NaCl concentrations. This hypothesis gains support from the identification of ion accumulation in calluses subjected to stress. Additionally, cells cultivated in a saline medium incur higher metabolic expenditures, raising the production of compatible solutes and consequently slowing down their growth.

After 28 days of exposure to a culture medium containing NaCl, the calli exposed to high NaCl specifically 100 mM showed lower water content compared to the control (Figure 7), aligning with values observed in calluses of halophyte species exposed to similar stress conditions (SHARMA and RAMAWAT., 2014). Consequently, the water content percentage decreased in calluses treated with higher levels of NaCl compared to the control. The sensitivity observed in the calluses of *C. procera* was similar to that recorded in the *niger* callus under NaCl-induced stress (GHANE et al., 2014).

6.1.3. Salt-induced changes in callus proteolysis and biochemical parameters

In our investigation to understand the impact of various salt stress concentrations in comparison to control conditions, we assessed the proteolytic activity in our selected plant species. Our findings revealed a notable increase in proteolytic activity at higher concentrations of NaCl, specifically at 80 and 100 mM, when compared to the control conditions. This observation

underscores a concentration-dependent response in proteolytic activity under salt stress, providing valuable insights into the plant's adaptive mechanisms in challenging environmental conditions (Figure 8). Athar et al. (2022) and Ahmad et al. (2016) have also reported similar findings in their studies on salt stress proteins in plants. Athar et al. (2022) provided an overview of salt-stress proteins in plants and their role in regulating growth and development under salt stress. Ahmad et al. (2016) demonstrated that nitric oxide mitigates salt stress by regulating levels of osmolytes and antioxidant enzymes in chickpeas. These studies provide additional support for our findings and suggest that proteolytic activity is an important mechanism for plants to adapt to salt stress.

To assess the impact of salinity, we conducted a comprehensive examination of hydrogen peroxide (H_2O_2) activity in the calluses of *C. procera* under both control and saline conditions. Our observations disclosed a concentration-dependent effect, indicating that hydrogen peroxide activity increased proportionally with higher salt concentrations. Particularly noteworthy were the highest levels of hydrogen peroxide activity observed in samples subjected to 100 mM NaCl. This observed indication establishes a clear correlation between salt concentration and heightened hydrogen peroxide activity, offering a quantitative foundation for understanding the oxidative stress response in the context of salinity (Figure 8). This finding is consistent with the work of Wang et al. (2013), who conducted a study on the callus of *Malus domestica L.* Their results similarly showed an elevation in the level of H_2O_2 corresponding to an increase in NaCl concentration. The consistency between our study and the work of Wang et al. reinforces the conception that the modulation of hydrogen peroxide is a shared response across different plant species, providing additional support for the proposed quantitative relationship between salt concentration and hydrogen peroxide activity. This collective evidence contributes to a more comprehensive understanding of the oxidative stress dynamics in plants exposed to salinity-induced conditions.

In our analysis to determine the soluble protein content in our studied species and evaluate the impact of varying NaCl concentrations on protein levels compared to control conditions, we utilized a robust methodology. Our findings demonstrated a notable increase in soluble protein content at high NaCl concentrations, particularly at 80 and 100 mM of NaCl, in contrast to the control conditions (Figure 8). Athar et al. (2022) reported similar trends in protein content modulation under saline conditions in a related species.

In our investigation aimed at elucidating the impact of salinity on photosynthetic pigments relative to control conditions in the calluses of our selected plant species, we conducted a photosynthetic pigments assay. The results revealed distinct patterns in the levels of chlorophyll *a*, *b*, and carotenoids under different salinity concentrations. Specifically, chlorophyll *a* exhibited elevated levels in the presence of 80 and 100 mM NaCl. Conversely, chlorophyll *b* levels were notably higher at 80 mM NaCl compared to the control, while carotenoid levels exhibited a similar increase at 80 mM NaCl (Figure 8). For instance, a study on *Salvinia auriculata* found that higher chlorophyll *a* content was observed in plants treated with 50 mmol L-1 NaCl, decreasing in higher NaCl concentrations, while chlorophyll *b* content decreased with increasing NaCl concentrations (GOMES et al., 2017). This is somewhat consistent with our observation of elevated chlorophyll *a* level at 80 and 100 mM NaCl and chlorophyll *b* levels at 80 mM NaCl. In terms of carotenoid levels, a decrease in carotenoid levels in plants under salinity stress has been reported in many species (UARROTA et al., 2018). However, another study found that excessive salinity resulted in a higher overall content of pigments (chlorophyll, carotenoids) but also a higher variation of concentration of these compounds during cultivation (VENCKUS et al., 2021). This explains the increase in carotenoid levels we observed at 80 mM NaCl. It is important to note that the response of photosynthetic pigments to salinity can vary significantly among different plant species and even among different varieties of the same species (UARROTA et al., 2018). Therefore, the patterns we observed in our selected plant species might not be generalizable to all plants.

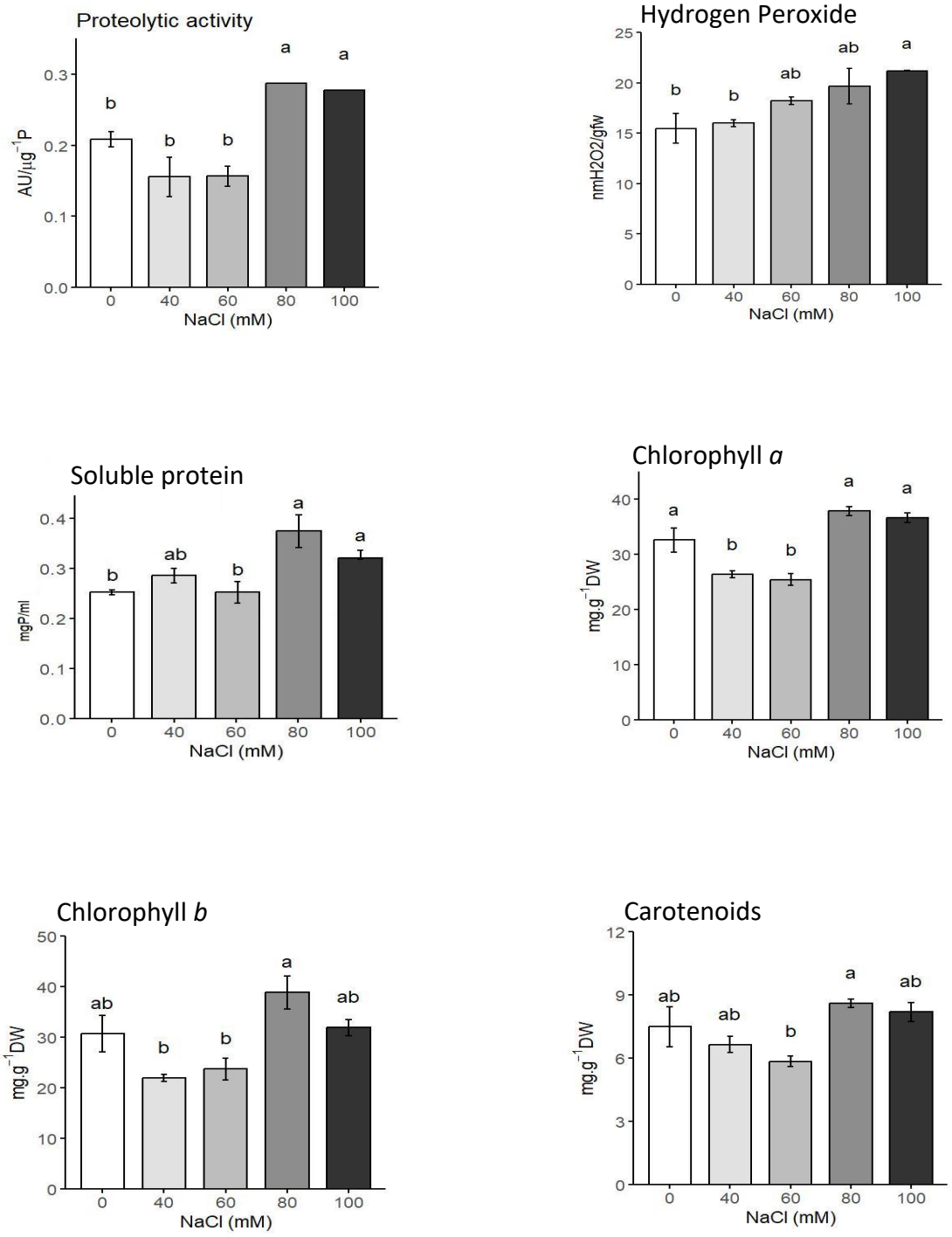


Figure 8- Performance of callus of *C. procera* in terms of Proteolytic activity, Hydrogen Peroxide, Soluble protein, and Photosynthetic pigments, when cultivated in increasing concentrations of NaCl. Data are mean ± SMD of five independent measurements. Columns labelled with different letters are significantly different at $P < 0.05$ based on one-way ANOVA with Tukey multiple range test using R-software.

6.1.4. Protein profiling in salt-stressed callus

The SDS-PAGE allowed us to separate proteins based on their molecular weights, providing a visual representation of the protein composition. In parallel, the zymogram was employed to assess the enzymatic activity of proteins, specifically highlighting enzymes involved in digesting substrates incorporated into the gel. Upon analysis, our observations revealed distinctive patterns. In the SDS-PAGE, certain polypeptides exhibited intensified staining, suggesting alterations in protein abundance or modifications in response to varying salt concentrations. Additionally, novel polypeptides were detected, appearing more heavily stained and prominent, particularly in the 100 mM salt-tolerant calli. Interestingly, under control conditions, these polypeptides were visibly lighter. These findings indicate that the proteomic profile of *C. procera* calli responds dynamically to salt stress, leading to changes in protein abundance and enzymatic activity. Further analysis of these identified proteins can provide insights into the molecular mechanisms governing salt tolerance in *C. procera* calli (Figure 9). Yen et al. (1997) reported similar results in their SDS-PAGE analysis. Their study on the callus of *Mesembryanthemum crystallinum* revealed that elevated salt levels correlated with increased protein content. This parallel observation across studies underscores the consistency of the relationship between high salt concentrations and enhanced protein abundance in plant callus systems. The convergence of outcomes further strengthens the credibility of the association between salinity and protein content, as established through SDS-PAGE analyses in distinct plant species. In a parallel study by Tyburski and Mucha (2023), consistent results were observed on the Zymogram, demonstrating heightened enzymatic activity in response to high NaCl concentrations in the callus of Red Beet (*Beta vulgaris*). The consistent results from both studies highlight the robustness of the association between elevated salt levels and heightened enzymatic activity, demonstrating its uniformity across diverse plant species.

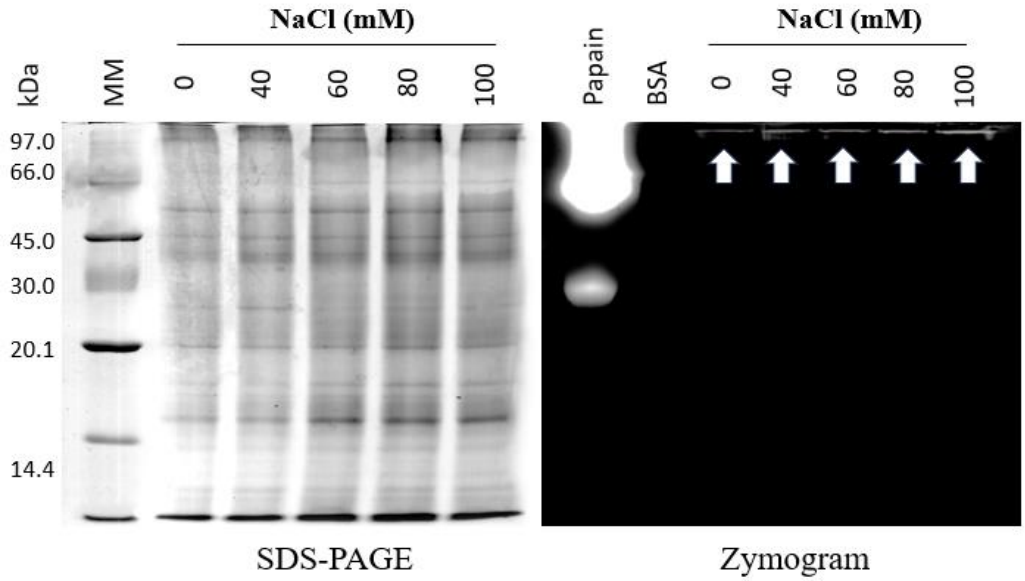


Figure 9- SDS-PAGE and zymogram of soluble proteins. In comparison to control callus, in gels of salt tolerant calli (40, 60, 80 and 100 mM of salt). Some polypeptides appeared heavily stained, and some new polypeptides were also detected, which appeared heavier stained in 100 mM of salt-tolerant calli. An equal amount of proteins were loaded on the gels. In SDS-PAGE molecular markers (MM) loaded in the 1st row, C in the 2nd while increasing concentrations of salt loaded in the rest of the rows. In the zymogram, Papain loaded in the 1st row, BSA in the 2nd, and C in the 3rd while increasing concentrations of salt loaded in the remaining rows. MM: molecular markers; 97.0 kDa: phosphorylase β; 66.0 kDa: albumin; 45.0 kDa: ovalbumin; 30.0 kDa: carbonic anhydrase; 20.1 kDa: trypsin inhibitor and α- lactalbumin 14.4 kDa.

6.1.5. Exploring enzyme responses in salt-treated callus

Salt stress induces intricate mechanisms involving both increased and decreased enzymatic activity. To assess the dynamics of oxidative stress enzymes, we quantified the activity levels of catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX), and superoxide dismutase (SOD).

To investigate catalase (CAT) enzyme levels in the callus of *C. procera* by examining their responses to different salt concentrations. The findings revealed clear patterns in catalase (CAT) enzyme activity, providing insights into how this important antioxidant responds to salt-induced stress. Our findings demonstrated that the highest levels of CAT enzymes were observed in the control group and at 40 mM NaCl, indicative of optimal enzymatic activity under normal and low salinity conditions. This suggests that CAT plays a pivotal role in mitigating oxidative stress under

baseline and mildly stressful conditions. The observed elevation in CAT activity at 40 mM NaCl aligns with its adaptive response to cope with the initial impact of salt stress. In contrast, CAT enzyme levels were lowest with 80 mM NaCl, hinting at a possible slowdown in catalase activity at this salt level (Figure 10). This suggests that excessive salt might hinder the enzyme's function, possibly due to complex changes in the cell environment, such as shifts in available substances or alterations in the enzyme's shape. In a related study, Lokhande et al. (2010) investigated CAT activity in callus cultures of *Sesuvium portulacastrum L.* and reported low catalase activity in response to a high concentration of NaCl (400 mM). The parallel findings between our study and Lokhande et al.'s research underscore the conserved nature of CAT activity modulation in response to high salt concentrations across different plant species. This collective knowledge enhances our comprehension of the versatile and species-specific responses of catalase to salt-induced stress, contributing to the broader understanding of plant stress physiology.

Our study noted an elevation in ascorbate peroxidase (APX) activity in the calluses subjected to NaCl treatment. This observation suggests a responsive modulation of APX enzymatic activity under both normal and salt-stress conditions, highlighting the adaptive nature of the enzyme in the cellular environment. This finding contributes to a deeper understanding of the regulation process governing APX activity and its potential implications in stress response pathways (Figure 10). These outcomes align with related research findings. For instance, Yasar et al. (2008) reported a similar phenomenon, noting an elevation in APX activity in response to salt stress in the leaves of the halophyte *Suaeda salsa.* This consistency across studies reinforces the idea that the modulation of APX activity is a conserved response mechanism across different plant species facing salt-induced stress, contributing to the broader understanding of the enzyme's role in stress adaptation.

Likewise, peroxidase (POX) activity exhibited variations across different salt concentrations. Specifically, higher levels of POX activity were observed in the presence of 40 and 80 mM NaCl, whereas lower activity levels were noted at 60 mM NaCl. This nuanced response underscores the sensitivity of POX activity to varying salt conditions and provides valuable insights into the regulatory dynamics of this enzyme in the context of salt-induced stress (Figure 10). Our findings align with research conducted by Kumar et al. (2008), who reported enhanced POX activity in callus cultures of *Jatropha curcas* under high concentrations of NaCl. This consistent

trend in POX activity across different plant species and experimental conditions suggests a conserved response mechanism. The observed elevation in POX activity in the presence of higher salt concentrations may be associated with the enzyme's role in mitigating oxidative stress, acting as a crucial component of the plant's defence system. Moreover, the lower POX activity observed at 60 mM NaCl in our study prompts interesting inquiries about the threshold impact of salt concentrations on POX regulation. This may reflect a complex interplay of signalling pathways and cellular responses under moderate salt stress. Exploring these nuances contributes to a more comprehensive understanding of POX dynamics and its role in coordinating adaptive responses to salt-induced stress.

The investigation into superoxide dismutase (SOD) activity in the callus of *C. procera* revealed distinctive trends among different salt treatments. The highest SOD activity was observed in the calluses exposed to 60 mM NaCl, indicating a notable response to moderate salt stress. Conversely, the lowest SOD activity levels were recorded in calluses treated with 100 mM NaCl, suggesting a potential inhibitory effect on SOD activity at higher salt concentrations. This observation contributes valuable insights into the nuanced modulation of SOD activity under varying salt stress conditions, emphasizing the importance of considering specific salt concentrations in understanding enzymatic responses. The inhibition of SOD activity under high salt stress conditions could be attributed to complex cellular responses, including potential feedback mechanisms or alterations in the cellular redox status (Figure 10). The insights gained from our study emphasize the significance of considering specific salt concentrations when interpreting enzymatic responses. The nuanced modulation of SOD activity at different salt levels highlights the complexities of plant stress responses, requiring a careful examination of the concentration-dependent effects on enzyme activities. This observed phenomenon is consistent with the work of Cherian et al. (2003), who reported low SOD activity in callus cultures of *Suaeda nudiflora Moq* under high concentrations of NaCl. The parallel findings between our study and Cherian et al.'s research across different plant species further strengthen the notion that SOD activity is intricately regulated in response to varying salt stress conditions. This collective knowledge contributes to a more comprehensive understanding of the adaptive strategies employed by plants in coping with salinity-induced oxidative stress.

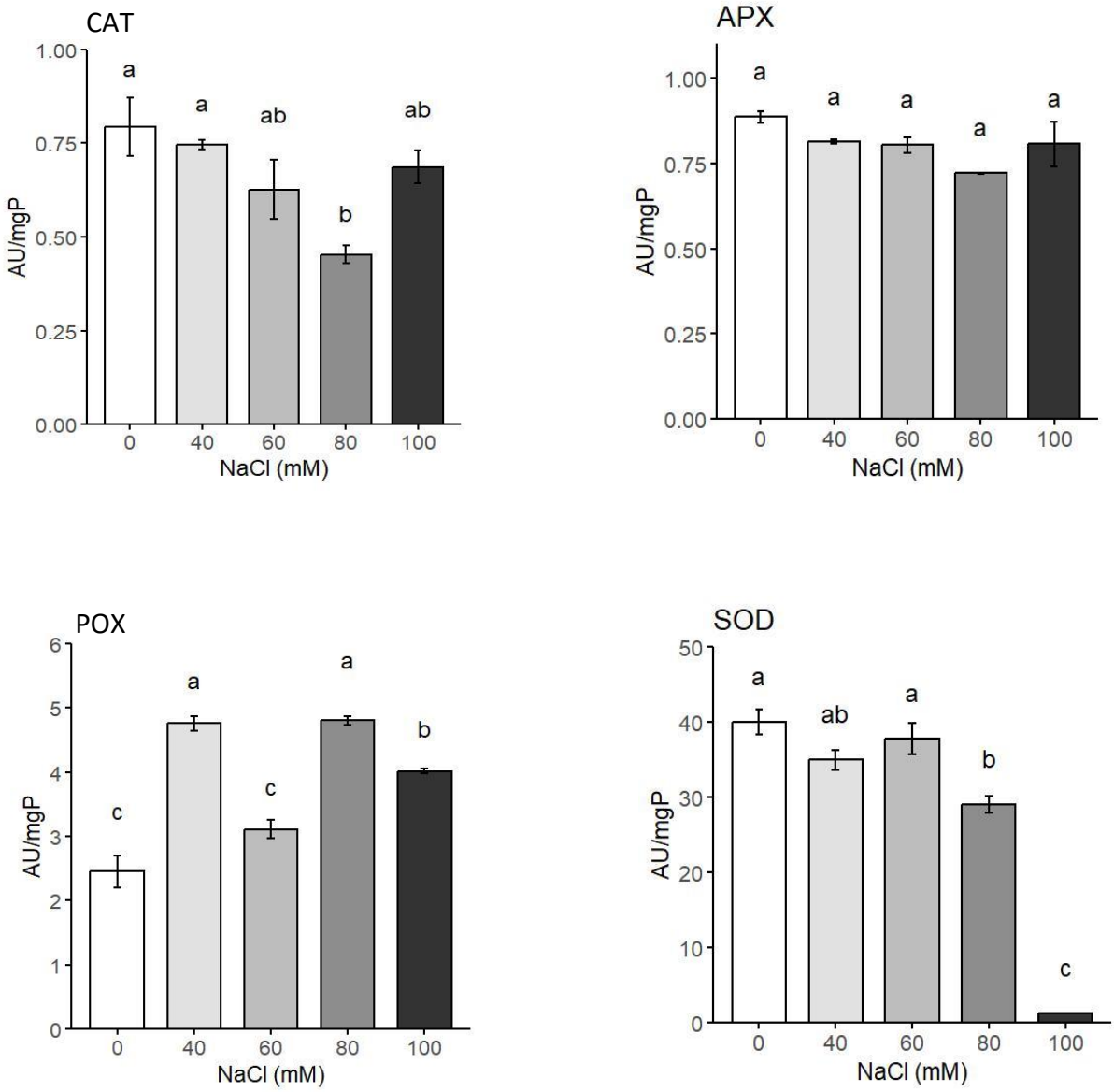


Figure 10- Performance of callus of *C. procera* in terms of CAT, APX, POX, and SOD when cultivated in increasing concentrations of NaCl. Data are mean ± SMD of five independent measurements. Columns labelled with different letters are significantly different at $P < 0.05$ based on one-way ANOVA with Tukey multiple range test using R-software. Kruskal-Wallis was used for APX.

Plants employ a range of antioxidant enzymes as a protective mechanism to detoxify reactive oxygen species (ROS), as highlighted by Mittler (2002). Under salinity conditions, the

activity of these enzymes is commonly influenced, exhibiting an overall increasing trend. This observation underscores the adaptive response of antioxidant enzymes in plants to mitigate the impact of salinity-induced oxidative stress, emphasizing the significance of these enzymatic processes in plant defence mechanisms.

6.1.6. Chlorophyll, phenols, and lignin under salt stress: Confocal analysis

To evaluate the impact of various NaCl concentrations on chlorophyll content in *C. procera* calli in contrast to control conditions, confocal microscopy analyses were conducted. The results exposed distinct variations in chlorophyll percentages across different salt treatments. Specifically, the highest chlorophyll percentage was observed in calli treated with 40 mM NaCl, showing an increase in chlorophyll content compared to the control conditions. On the other hand, the lowest chlorophyll percentage was identified in calli subjected to a high NaCl concentration of 100 mM. This concentration-dependent response indicates a potential inhibitory effect on chlorophyll synthesis or stability under high salt stress conditions. The observed variation suggests a complex interplay between salt stress and chlorophyll metabolism, which can have significant implications for the plant's photosynthetic capacity and overall physiological responses (Figure 11). In the study conducted by Yasar et al. (2008), a reduced level of chlorophyll content was also documented in response to high concentrations of NaCl.

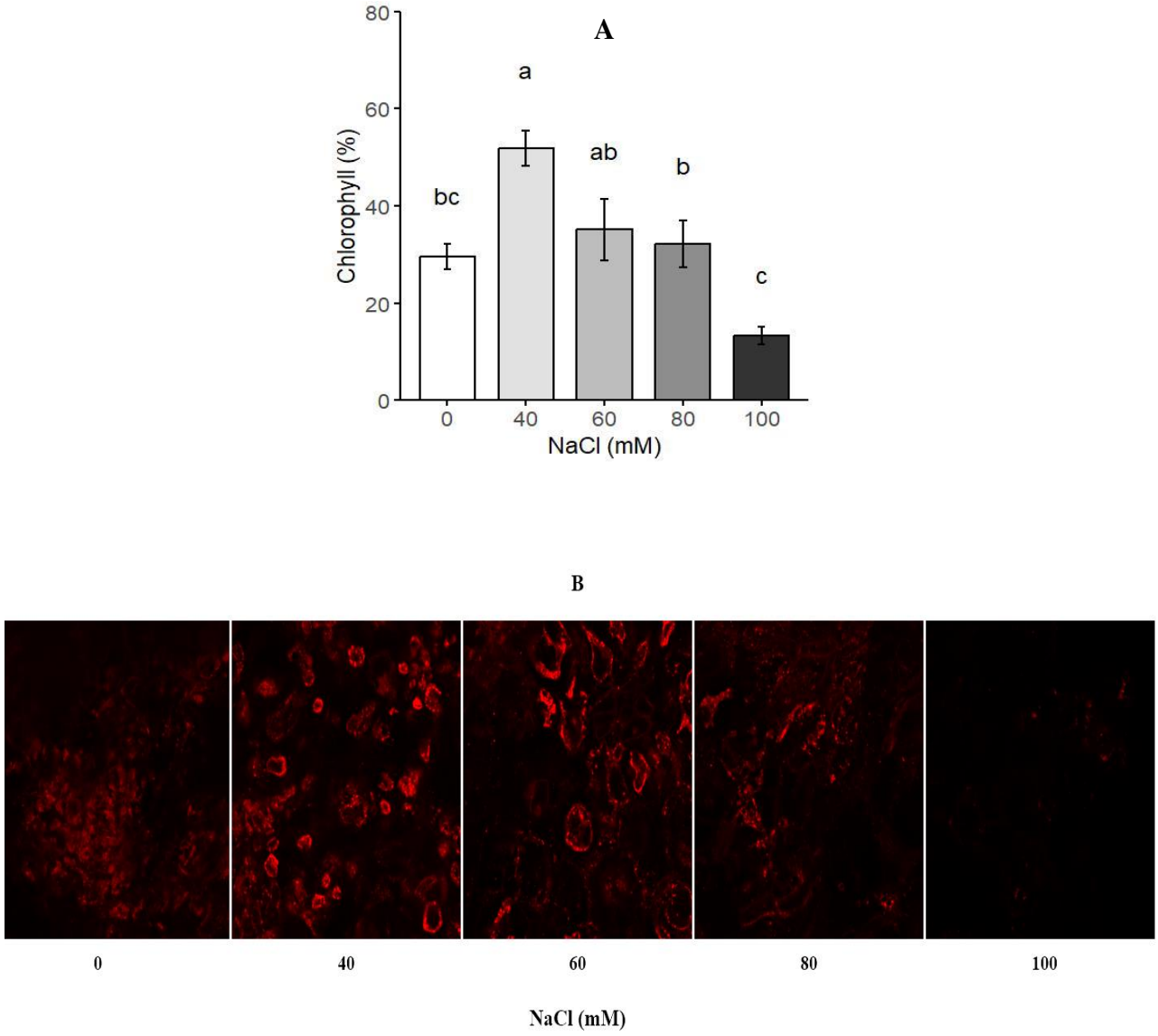


Figure 11- Performance of callus of *C. procera* in terms of chlorophyll % (A), and chlorophyll fluorescence detected by confocal microscopy (λ = 488 nm) (B) when cultivated in increasing concentrations of NaCl. Data are mean ± SMD of five independent measurements. Columns labelled with different letters are significantly different at $P < 0.05$ based on one-way ANOVA with Tukey multiple range test using R-software.

To measure the impact of varying NaCl concentrations compared to the control group on phenol content in *C. procera* callus, confocal microscopy analyses were conducted. The results revealed distinct variations in phenol percentages across different levels of salt treatments. In

comparison to the NaCl treatment samples, the control group exhibited a higher percentage of phenols. Among the NaCl treatment samples, the highest phenol percentage was observed in the 100 mM NaCl treatment, while the lowest quantity was recorded in the 80 mM NaCl treatment (Figure 12). These findings suggest a concentration-dependent response of phenol accumulation in *C. procera* callus under salt stress conditions. Notably, the observed increase in phenol content at higher NaCl concentrations aligns with the findings of Valifard et al. (2014), who reported an elevation in total phenolic content in *Salvia mirzayanii* under increased NaCl concentrations. The intricate variation in phenol content observed in *C. procera* callus under different NaCl concentrations points out the dynamic nature of phytochemical production in response to salt stress. Conducting further investigations focused on identifying specific phenolic compounds and understanding their potential roles in salt adaptation mechanisms will contribute to a more comprehensive understanding of the plant's responses to stress.

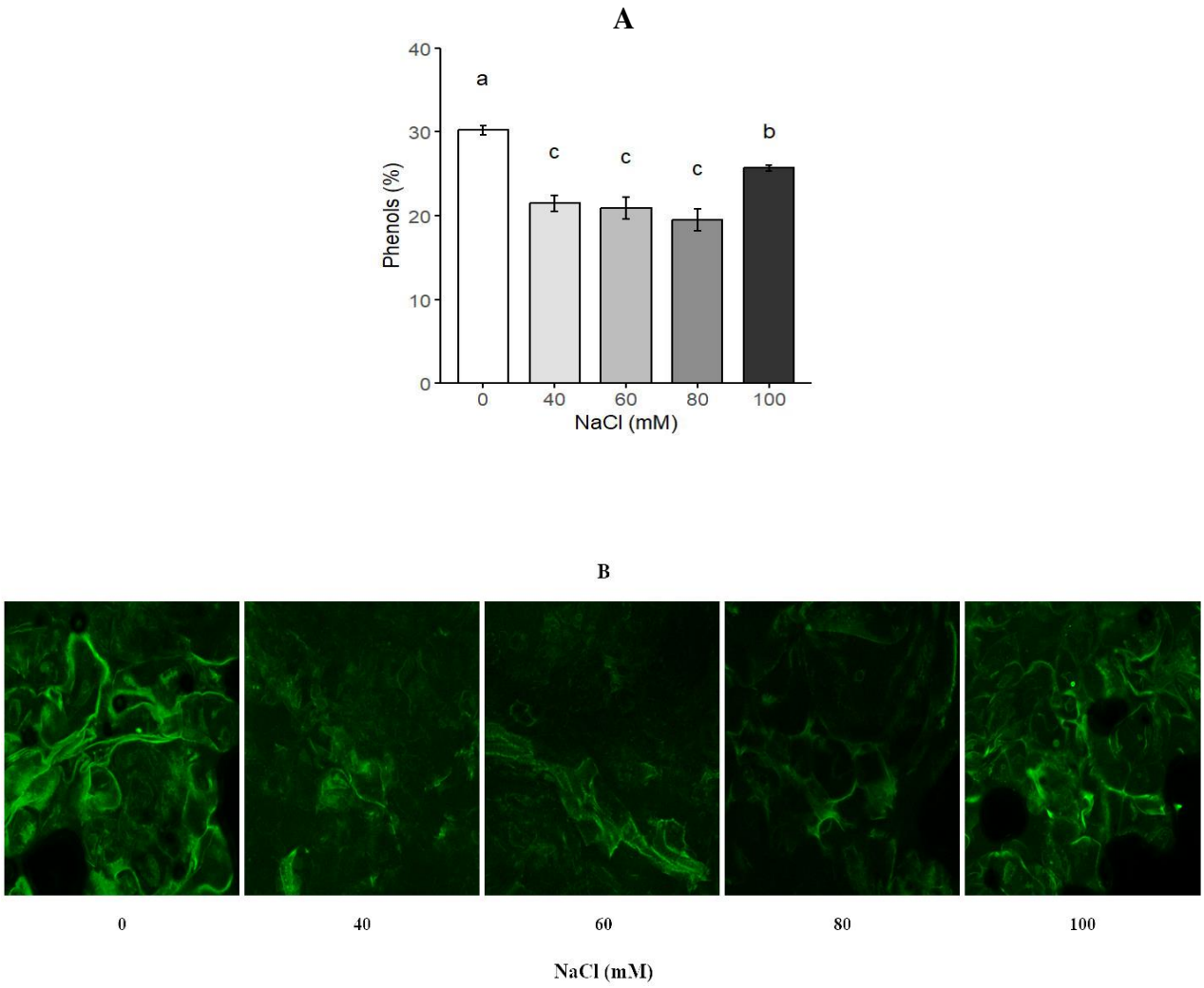


Figure 12- Performance of callus of *C. procera* in terms of phenols % (A), and phenols fluorescence detected by confocal microscopy (λ = 488 nm) (B) when cultivated in increasing concentrations of NaCl. Data are mean ± SMD of five independent measurements. Columns labelled with different letters are significantly different at $P < 0.05$ based on one-way ANOVA with Tukey multiple range test using R-software.

The investigation into lignin content in *C. procera* callus, under varying NaCl concentrations compared to control conditions, revealed distinctive patterns. Notably, the lignin percentage exhibited an increase at 100 mM NaCl, reaching a higher level compared to the control conditions. On the contrary, the minimum lignin content was observed at 40 mM NaCl treatment (Figure 13). This concentration-dependent response in lignin biosynthesis under salt stress

conditions suggests a regulatory mechanism influenced by NaCl levels. The observed elevation in lignin content lines up with the findings of Kelij et al. (2015), who reported increased lignin biosynthesis in *Aeluropus littoralis* under high concentrations of NaCl treatment. The significant correlation between NaCl concentration and lignin accumulation in *C. procera* callus provides valuable insights into the plant's response to salt stress at the cellular level. Lignin, as a key component of the plant cell wall, plays a crucial role in structural integrity and defence mechanisms. The findings highlight the adaptability of lignin biosynthesis in response to environmental stressors, contributing to the plant's flexibility under hard environments. Further exploration of the specific lignin subunits involved, and the underlying molecular mechanisms would enhance our understanding of lignin biosynthesis in *C. procera* under salt stress, contributing to broader insights into plant adaptation strategies.

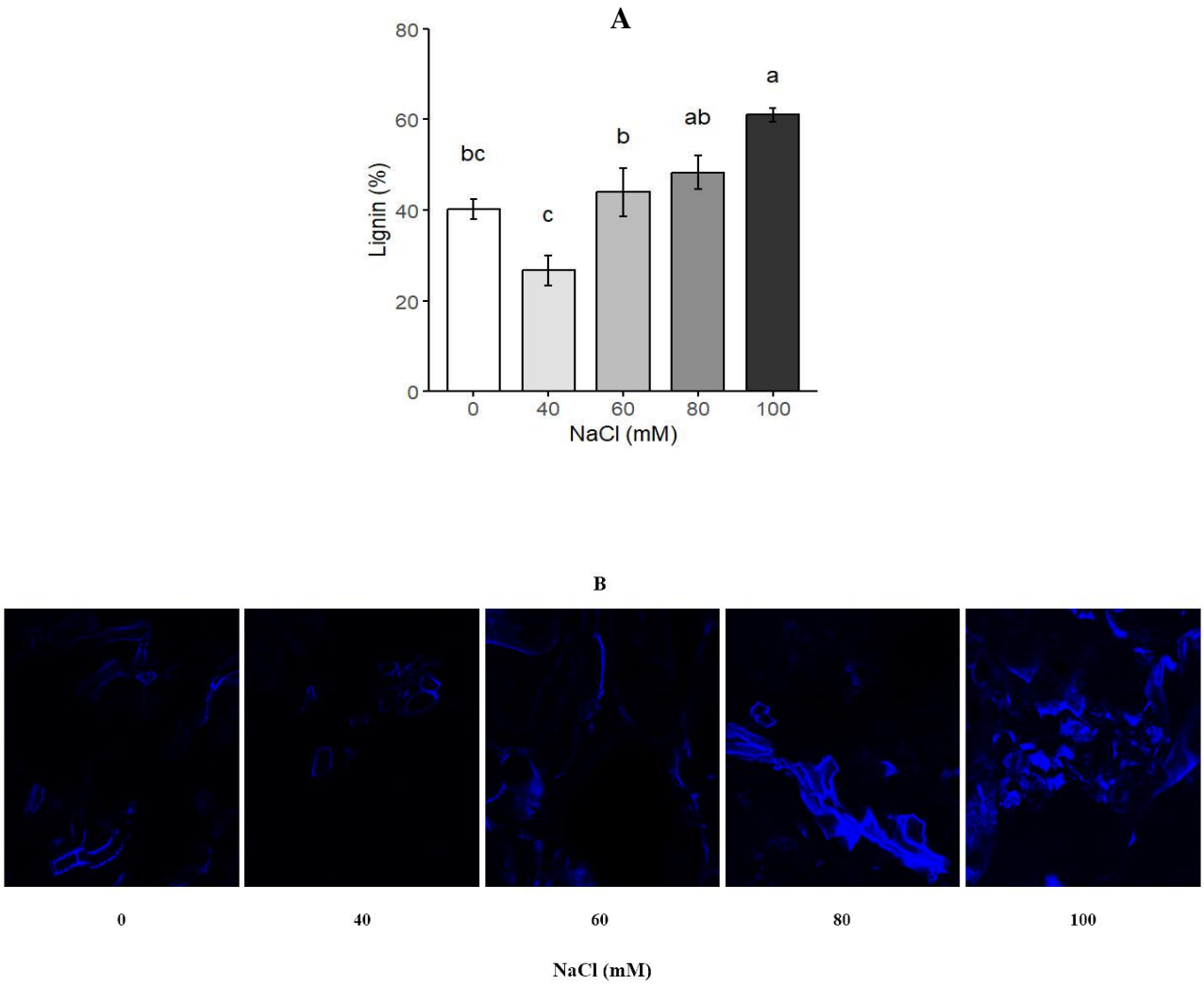


Figure 13- Performance of callus of *C. procera* in terms of lignin % (A), and lignin fluorescence detected by confocal microscopy (λ = 488 nm) (B) when cultivated in increasing concentrations of NaCl. Data are mean ± SMD of five independent measurements. Columns labelled with different letters are significantly different at $P < 0.05$ based on one-way ANOVA with Tukey multiple range test using R-software.

6.1.7. Analysing DNA integrity in salt-treated callus

The investigation into DNA integrity in *C. procera* calli, under both control conditions and various NaCl treatments, employed the TUNEL fluorometric assay (TdT-mediated dUTP Nick-End Labeling). This assay is a sensitive method for detecting DNA fragmentation, providing insights into the impact of different concentrations of NaCl compared to control conditions. Calli

were sectioned, and the control and NaCl treatment groups were separately examined under confocal microscopy. The assessment involved the incorporation of fluorescein-12-dUTP with emission at 520 nm, to identify damaged cells. The results were visualized through fluorescein staining (Figure 14b). Notably, the quantified green fluorescence was observed to be high in the calli treated with 100 mM of NaCl in comparison to the control samples. This observation implies that, under the conditions of this experiment, the treatment with 100 mM NaCl did not induce significant DNA damage or fragmentation in the calli. The TUNEL assay, coupled with confocal microscopy and fluorescein staining, provided a detailed and quantitative assessment of DNA integrity in response to different NaCl concentrations. These findings contribute to our understanding of the molecular responses of *C. procera* calli to salt stress and emphasize the importance of preserving DNA integrity, a fundamental aspect of cellular function and viability under challenging environmental conditions.

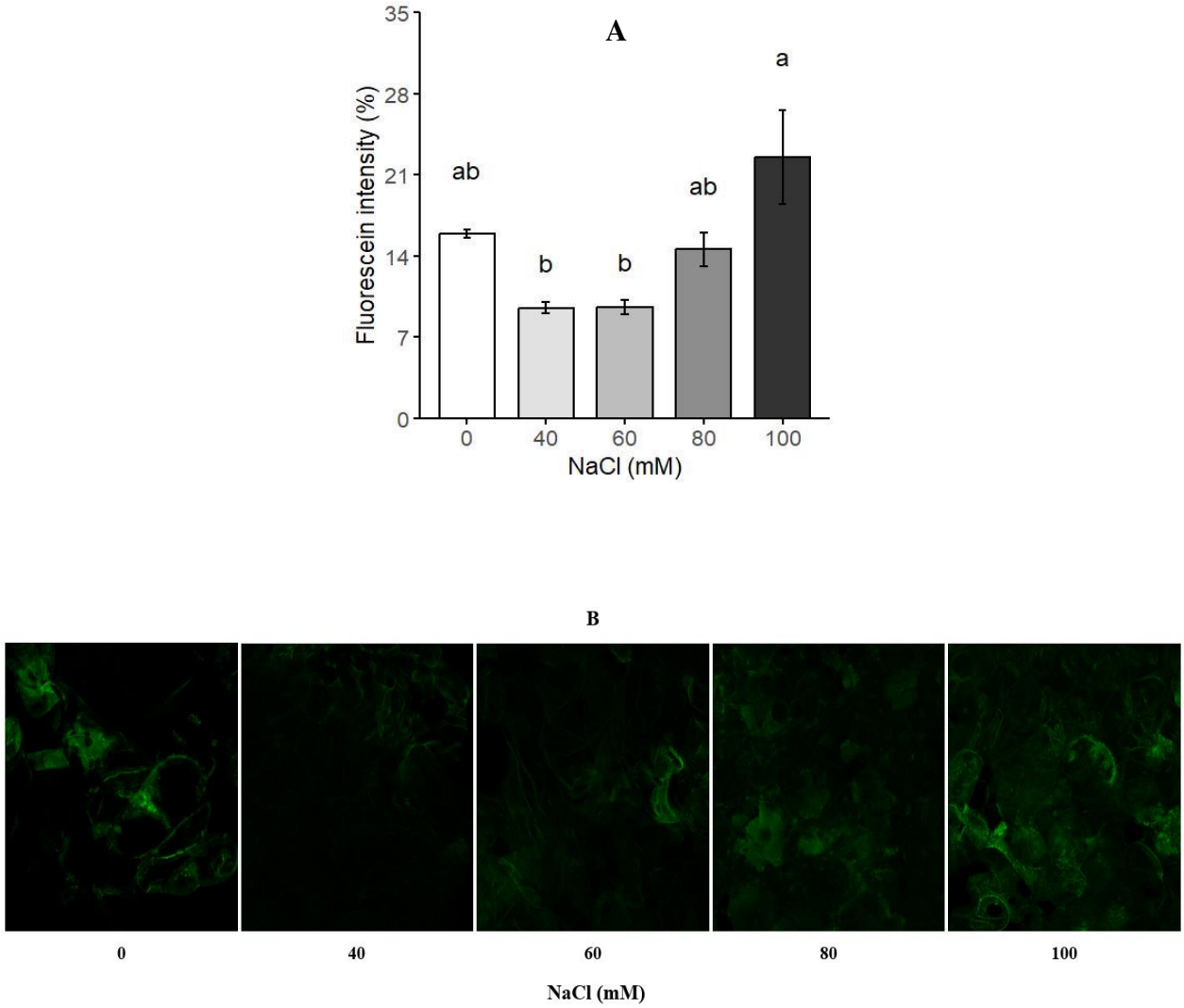


Figure 14- Analysis of DNA integrity by TUNEL assay in the calli of *C. procera* in terms of fluorescein intensity % (A), Tissue section visualized by fluorescein fluorescence detected by confocal microscopy (scale bars: 50 µm) (B) when cultivated in increasing concentrations of NaCl. Data are mean ± SMD of five independent measurements. Columns labelled with different letters are significantly different at $P < 0.05$ based on one-way ANOVA with Tukey multiple range test using R-software.

To further explore the DNA integrity, confocal microscopy utilized the emission of 647 nm to detect propidium iodide, which stained individual cells in the TUNEL assay. This allowed for the evaluation of cell quantity in both control and NaCl treatment calli. The total number of cells

within a defined area (0.03 mm²) was determined in each section by counting DNA stained with propidium iodide. The images presented in (Figure 15b) reveal that the estimated cell count in calli treated with 40 and 60 mM of NaCl was statistically higher than in the control group. This suggests that an optimal level of NaCl exposure likely stimulated faster cell multiplication, acting as a mitogen-like inducer under the experimental conditions. On the contrary, high NaCl exposure appeared to have a suppressive effect on cell multiplication, resulting in a reduced cell count in the calli. These findings underscore the intricate relationship between NaCl concentration and cell proliferation in *C. procera* calli. The observed increase in cell count at moderate NaCl levels suggests a potential positive impact on cell multiplication, while excessive NaCl levels may induce adverse effects, leading to a decline in cell numbers. These detailed observations emphasize the importance of fine-tuning NaCl concentrations for optimal plant growth and cellular processes. The application of propidium iodide as a DNA stain, in combination with confocal microscopy, offered a quantitative approach to evaluate cell numbers and, indirectly, cell proliferation. These insights contribute to our understanding of the dynamic cellular responses to different NaCl concentrations and shed light on the delicate balance between stimulatory and inhibitory effects on cell multiplication under salt stress conditions.

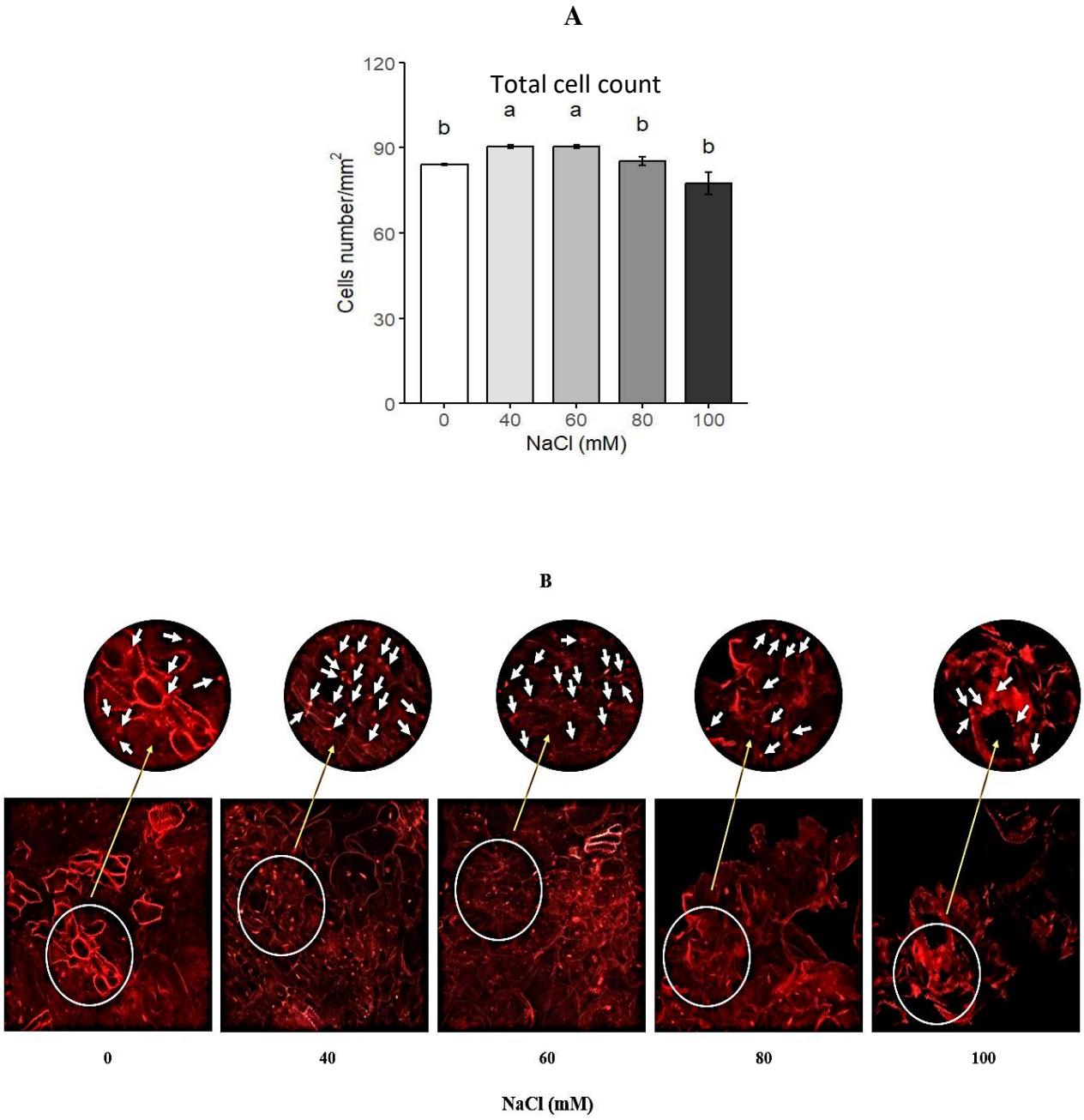


Figure 15- Analysis of cell count by confocal software on nuclear DNA stained in slides of *C. procera* calli in terms of Total cell count (A), Selected area of the calli of *C. procera* (scale bars: 50 µm) (B) when cultivated in increasing concentrations of NaCl. White arrows indicate stained nuclear DNA. Data are mean ± SMD of five independent measurements. Columns labelled with different letters are significantly different at $P < 0.05$ based on one-way ANOVA with Tukey multiple range test using R-software.

6.1.8. RNA quality in salt-treated callus

To evaluate the impact of NaCl (80 mM) compared to the control condition NaCl (0 mM) on gene expression in the callus of *C. procera,* comprehensive transcriptomic analyses were conducted. The process of RNA extraction was initiated to make sure we have good-quality material for further analysis. The quality of the extracted RNA was thoroughly evaluated to ensure its integrity and absence of contamination with carbohydrates and proteins. Spectrophotometric readings were employed to assess RNA quality. Both the control condition NaCl (0 mM) and salt-treated NaCl (80 mM) samples demonstrated non-degraded RNA, with a 260/280 ratio exceeding 2. This suggests the effective removal of proteins during the extraction process. The 260/230 ratio, which varied from 1.07 to 2.3 among treatments, indicated slight variability possibly attributed to the presence of secondary compounds and other composites in the callus. Although some samples had a less-than-ideal 260/230 ratio, the successful gene amplification in PCR analyses provides evidence of the generally high-quality RNA suitable for gene expression studies in the callus of *C. procera* (Table 2).

Callus of *C. procera*	**ng/µL**	**260/280**	**260/230**			
Before treatment (0h)	127.75	2.17	1.3			
Time	**NaCl (0 mM) ng/µL**	**260/280**	**260/230**	**NaCl (80 mM) ng/µL**	**260/280**	**260/230**
2h	139.39	2.15	1.15	216.52	2.15	1.41
12h	243.5	2.16	2.02	276.25	2.15	2.16
24h	235.86	2.18	1.15	328.58	2.16	1.93
48h	330.35	2.14	2.18	328.12	2.16	2.01
4 days	303.6	2.12	2.05	301.59	2.14	1.96
7 days	451.78	2.11	1.07	573.83	2.19	2.13
14 days	356.59	2.14	2.3	404.7	2.13	1.74
28 days	172.65	2.18	1.84	202.09	2.18	1.49

Table 2- Average concentration values and absorbance ratios of RNAs extracted from the callus of *C. procera* cultivated for 4 weeks in MS medium under control and NaCl (80 mM) of treatment.

The high-quality RNA is evident through the clear visualization of distinct rRNA bands (28s and 18s) in agarose gel electrophoresis. The integrity of total RNA is indicated by the intactness of the rRNA bands and the intensity ratio of 28s to 18s rRNA. The gel electrophoresis exposed distinctive bands corresponding to 28s and 18s rRNA, and notably, the intensity of the 28s band was approximately double that of the 18s band. This difference implies that there is minimal degradation of RNA. Furthermore, the absence of DNA bands alongside the presence of well-defined 18s and 28s bands confirms the lack of DNA contamination. The 18s and 28s bands represent distinct components of the ribosome, a cellular organelle crucial for protein synthesis. Given that rRNA constitutes 80% of total cellular RNA, its appearance and integrity serve as reliable indicators for overall RNA quality. Since mRNA, which constitutes only 5% of total RNA, is not visible in the gel, the integrity of rRNA serves as an alternate measure, suggesting that if rRNA is of good quality, mRNA is likely of good quality as well (Figure 16).

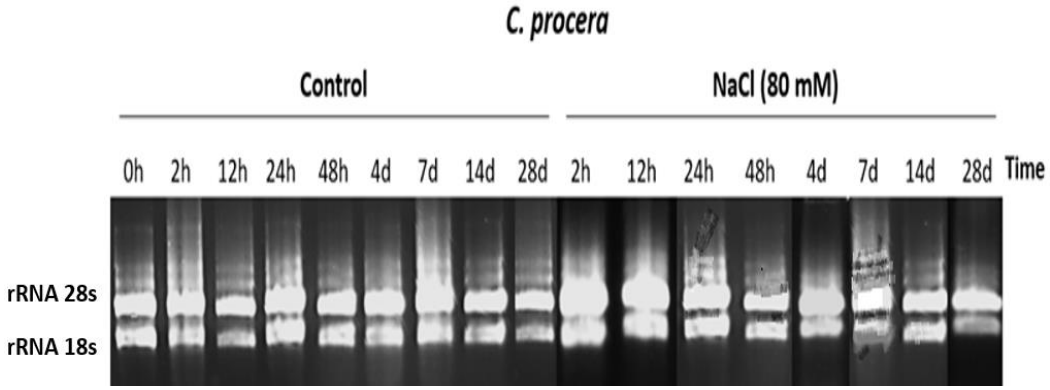


Figure 16- Agarose gel electrophoresis (1%), showing the 18s and 28s ribosomal RNA bands. RNA samples were extracted from the calli of *C. procera* at different time points and exposure duration to control MS medium (bands 1−9) and MS medium with 80 mM NaCl (bands 10−17).

6.1.9. RT-PCR optimization for stress-responsive genes in salt-stressed callus

To standardize the Reverse Transcription Polymerase Chain Reaction (RT-PCR) procedure, optimal annealing temperatures were determined by conducting a temperature gradient

RT-PCR. The selected annealing temperatures were 57.1 °C for Osmotin, 59.6 °C for Chitinase, 50.8 °C for Protease, and 57.1 °C for *EF1α*.

The chosen number of cycles for each primer pair varied: 33 cycles for Osmotin, 30 cycles for Chitinase, 37 cycles for Protease, and 24 cycles for the *EF1α* coding sequence. These standardized conditions ensure robust and specific amplification for each target gene during RT-PCR analysis.

To assess the integrity, quality, and homogeneity of synthesized cDNAs, an initial amplification step was conducted using degenerate primers targeting the elongation factor *EF1α* gene. This amplification was performed across all cDNA samples to ensure a uniform expression pattern. *EF1α* was chosen as a constitutive control due to its high expression across diverse plant tissues, classifying it as a "Housekeeping" gene. The band corresponding to the *EF1α* gene amplicon, utilized in the reactions, was consistently observed in the various samples analysed. This observation indicates the good quality and homogeneity of the produced cDNA samples, validating their suitability for downstream analyses (SARAIVA., 2013).

To elucidate the impact of salt stress on the transcriptional regulation of osmotin, chitinase, and protease in *C. procera* calli, transcriptional analysis was conducted. The results revealed that exposure to the MS culture medium induced the expression of osmotin, chitinase, and protease genes; however, this induction was more prominent and sustained when the calli were subjected to an MS medium supplemented with NaCl (Figure 17).

For osmotin, transcripts with lower intensity bands were clear at 24 hours of exposure to 80 mM NaCl, followed by higher intensity bands at 48 hours. Transcript accumulation continued until the 7^{th} day under salt stress. In the control MS medium, a lower intensity band appeared at 24 hours and on the 14^{th} day.

Chitinase transcripts were observed from 12 hours to the 14^{th} day under 80 mM NaCl exposure. In the control MS medium, transcripts started at 0 hours, with a very low-intensity band at 2 hours, reaching higher intensity bands at 12 hours until the 28^{th} day. This suggests a more sustained and robust response to both control and salt stress in chitinase expression.

Protease transcripts, like chitinase, show an early response to salt stress, characterized by lower intensity bands at 2 hours and higher intensity bands from 24 hours to the 7^{th} day under 80

mM NaCl stress. In the control, a very low-intensity band emerged at 12 hours, followed by higher intensity bands on days 4 and 7, concluding with a decreased intensity band on the 14th and 28th day. This varied pattern suggests a complex regulatory mechanism for protease under salt stress, possibly involving feedback loops or post-transcriptional modifications.

Similarly, Subramanyam et al. (2011) demonstrated an increase in salt tolerance in transgenic chilli pepper *(Capsicum annum L.)* by overexpressing the osmotin gene. Wan et al. (2017) also highlighted the pivotal role of osmotin and osmotin-like proteins in enhancing plant salt tolerance, aligning with our findings. The studies by Zhou et al. (2020), Liu et al. (2020), and Zheng et al. (2018) further contribute to our understanding of stress response mechanisms. Zhou et al. (2020) observed the overexpression of chitinase genes in *Zizania latifolia* in response to abiotic stress, while Liu et al. (2020) demonstrated improved pathogen resistance and saline-alkali stress tolerance in transgenic tobacco and maize through the chitinase gene. Zheng et al. (2018) reported that overexpression of the cysteine protease gene from *Salix matsudana* enhances salt tolerance in transgenic Arabidopsis.

Additionally, Leandro et al. (2021) documented the essential role of protease in salt stress tolerance in the plant growth-promoting bacterium *Gluconacetobacter diazotrophicus* PAL5. These collective studies provide intense support to our results, emphasizing a consistent theme, the crucial involvement of osmotin, chitinase, and protease-related mechanisms in plant's responses to salt stress. The convergence of findings across diverse plant species and experimental setups strengthens the credibility of our observations and aligns with the broader understanding of stress adaptation mechanisms in plants.

The transcriptional analysis of osmotin, chitinase, and protease in *C. procera* calli under salt stress provides valuable insights into the molecular responses of these genes to environmental challenges. The induction of osmotin, chitinase, and protease expression in response to the MS culture medium suggests a baseline activation of these genes under normal conditions. However, this induction becomes more pronounced and sustained when the calli were exposed to MS medium supplemented with NaCl, showing an intensified response to salt stress. Overall, these results underscore the specificity and sensitivity of osmotin, chitinase, and protease genes in responding to salt stress. Further investigations into the precise molecular pathways and functional

implications of these genes under salt stress will provide a more comprehensive understanding of their roles in plant stress responses.

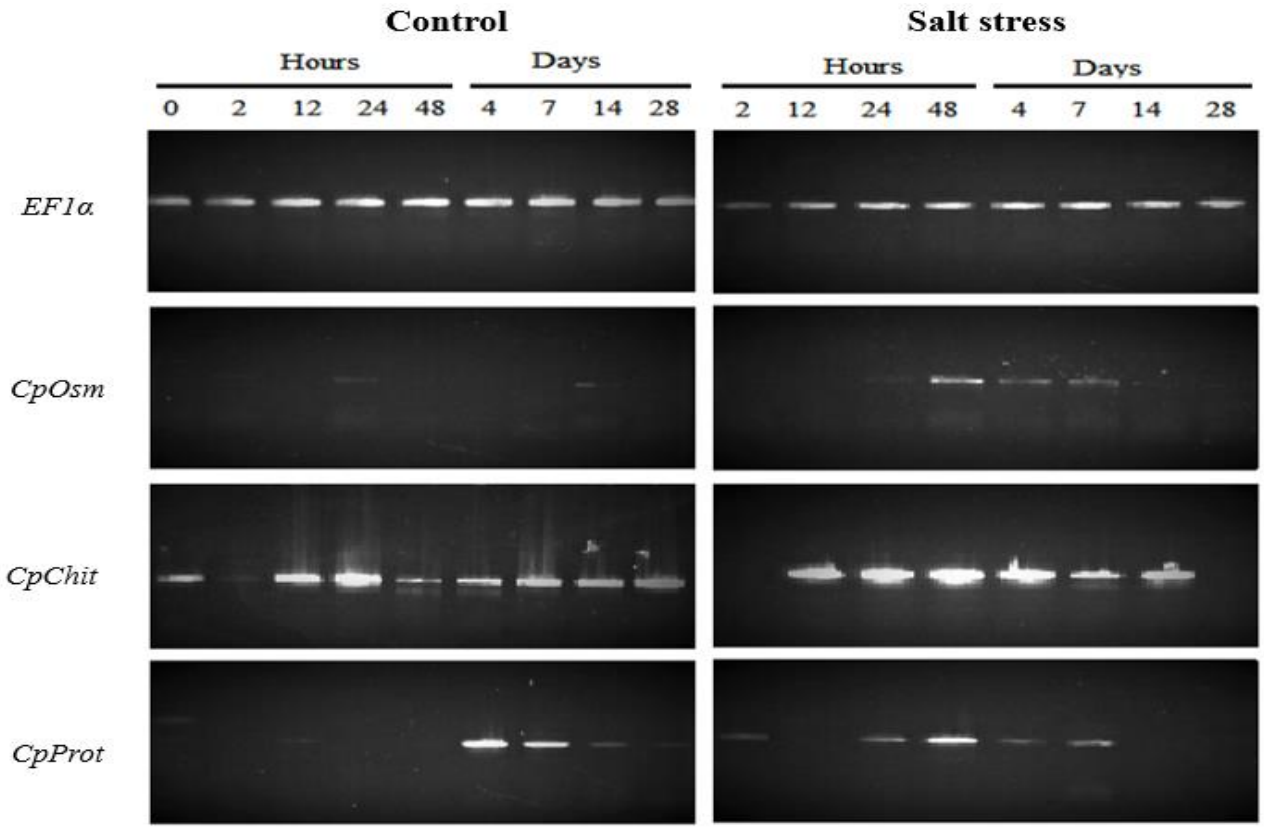


Figure 17- Agarose gel electrophoresis (1%) showing time-course transcript analysis of *EF1α*, Osmotin (*CpOsm*), Chitinase (*CpChit*), and Protease (*CpProt*) genes in the calli of *C. procera* under control and Salt stress (80 mM).

6.2. Signalling inducers impact on *C. procera* callus

6.2.1. Calluses development in *C. procera*

Calluses were induced from hypocotyl segments of *C. procera* plants following established protocols to investigate physiological and biochemical responses under signalling molecules, specifically Salicylic acid (SA) and Methyl jasmonate (MeJA). Upon achieving healthy greenish calli growth after four weeks, approximately 1 gram of viable and uncontaminated calli were selected for further experimentation with SA and MeJA. The calli were then transferred to a fresh MS culture medium with increasing concentrations of SA and MeJA, to induce the effects of these signalling molecules. The calli were exposed to this medium for one week (7 days). Following this period, specific criteria were evaluated to comprehend their development and response mechanisms to SA and MeJA (Figure 18).

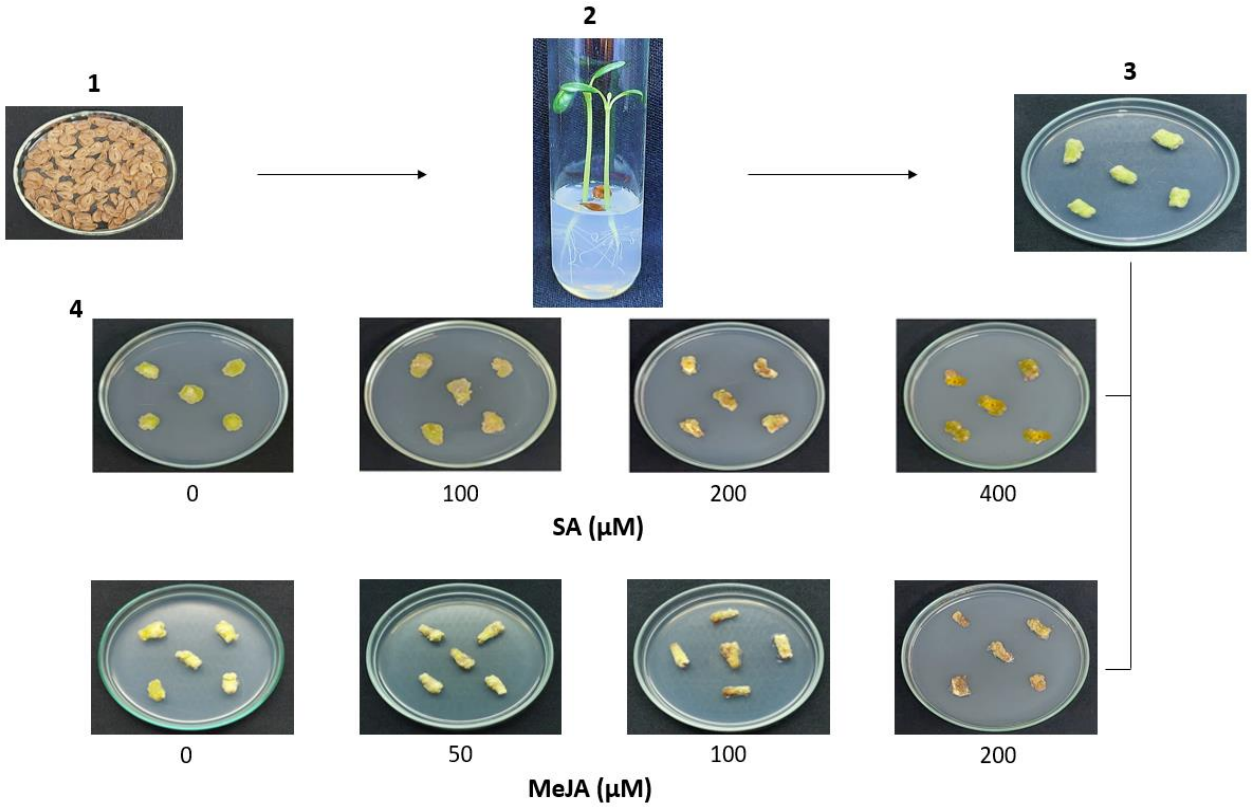


Figure 18- Seeds of *C. procera* were grown in $^{1}/_{2}$ MS media for 28 days (2). Calluses were produced from the hypocotyl segments in the same nutritive medium used for seed germination but supplemented with 3 µM 1-naphthylacetic acid (NAA) and 4.6 µM kinetin (KIN) (3). After 28 days the calluses were transferred into the newly prepared MS media with increasing concentrations of SA and MeJA for a further 7 days (4).

6.2.2. Impact of signalling inducers on callus physiology

In this study, we aimed to assess the impact of increasing concentrations of Salicylic acid (SA) and Methyl jasmonate (MeJA) on the physiological responses of *C. procera* calli. Our study focused on water content percentage and relative growth rate percentage (RGR) after subjecting the calli to a 7-day cultivation period in a medium designed to induce signalling pathways. The RGR is calculated by evaluating the total fresh mass both before and after exposure to the signalling inducers. Remarkably, our results revealed no obvious progressive alterations in water content (%)

and relative growth rate (%) in the calli as the concentrations of SA and MeJA increased within the medium (Figure 19).

The absence of a clear dose-dependent relationship between SA and MeJA concentrations and the physiological parameters of interest suggests that there might be an optimal range for inducing measurable changes.

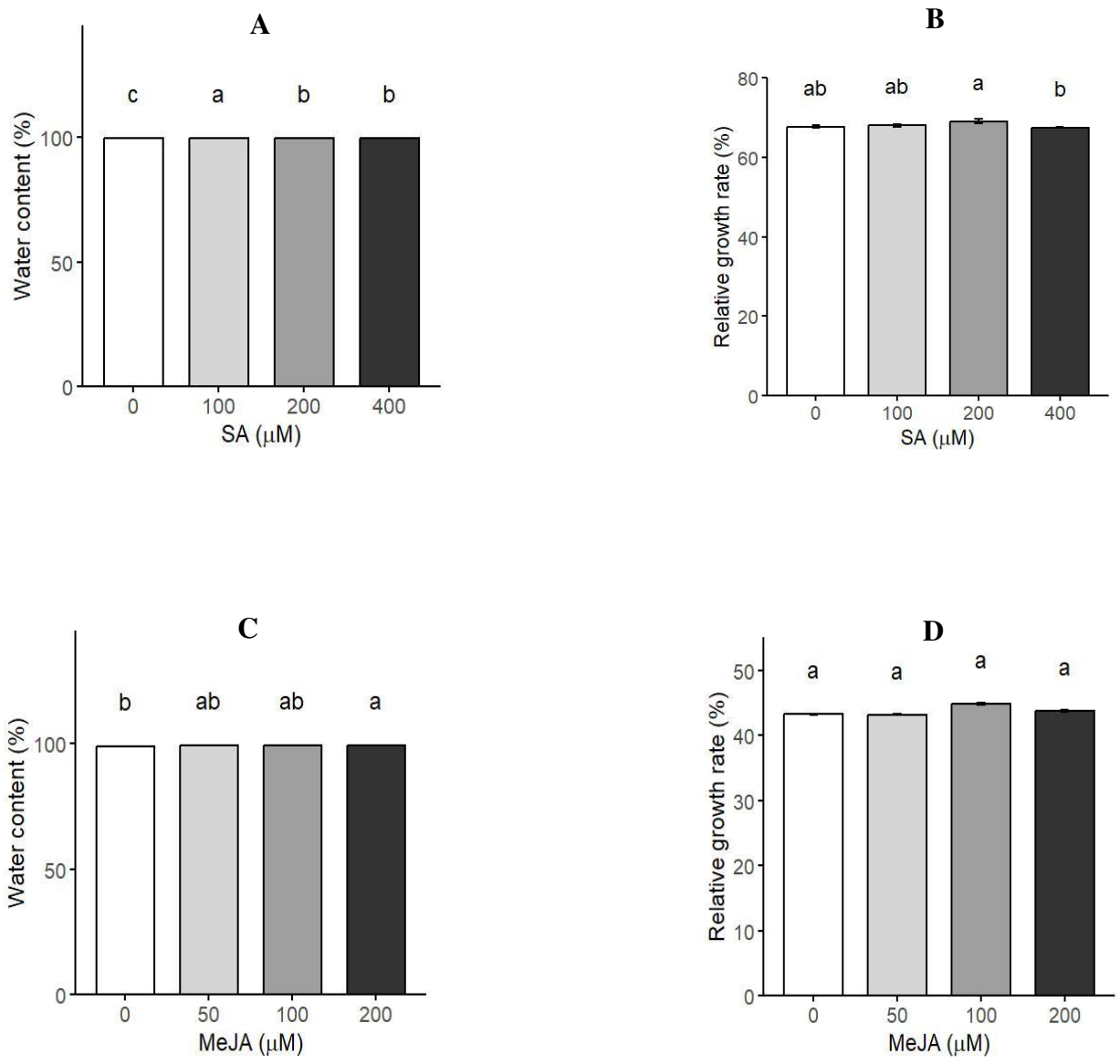


Figure 19- Performance of callus of *C. procera* in increasing concentrations of SA (A) and (B); MeJA (C) and (D) in terms of water content and relative growth rate. Data are mean ± SMD of five independent measurements. Columns labelled with different letters are significantly different at $P < 0.05$ based on one-way ANOVA with Tukey multiple range test using R-software. Kruskal-Wallis was used for the Relative growth rate (D) in MeJA treatment.

The fresh weight (FW) of calli showed no significant difference with increasing SA concentration. However, in terms of dry weight (DW), 100 µM of SA resulted in a decrease

compared to the control. In MeJA treatment, FW increased with higher concentrations, but DW decreased as MeJA concentration rose (Figure 20).

The observed trends in fresh and dry weights highlight the differential effects of SA and MeJA on *C. procera* callus growth. The decrease in dry weight at 100 µM SA suggests a potential inhibitory effect on biomass accumulation. Conversely, MeJA appears to enhance fresh weight at higher concentrations but may adversely impact dry weight, indicative of altered biomass allocation or metabolic shifts.

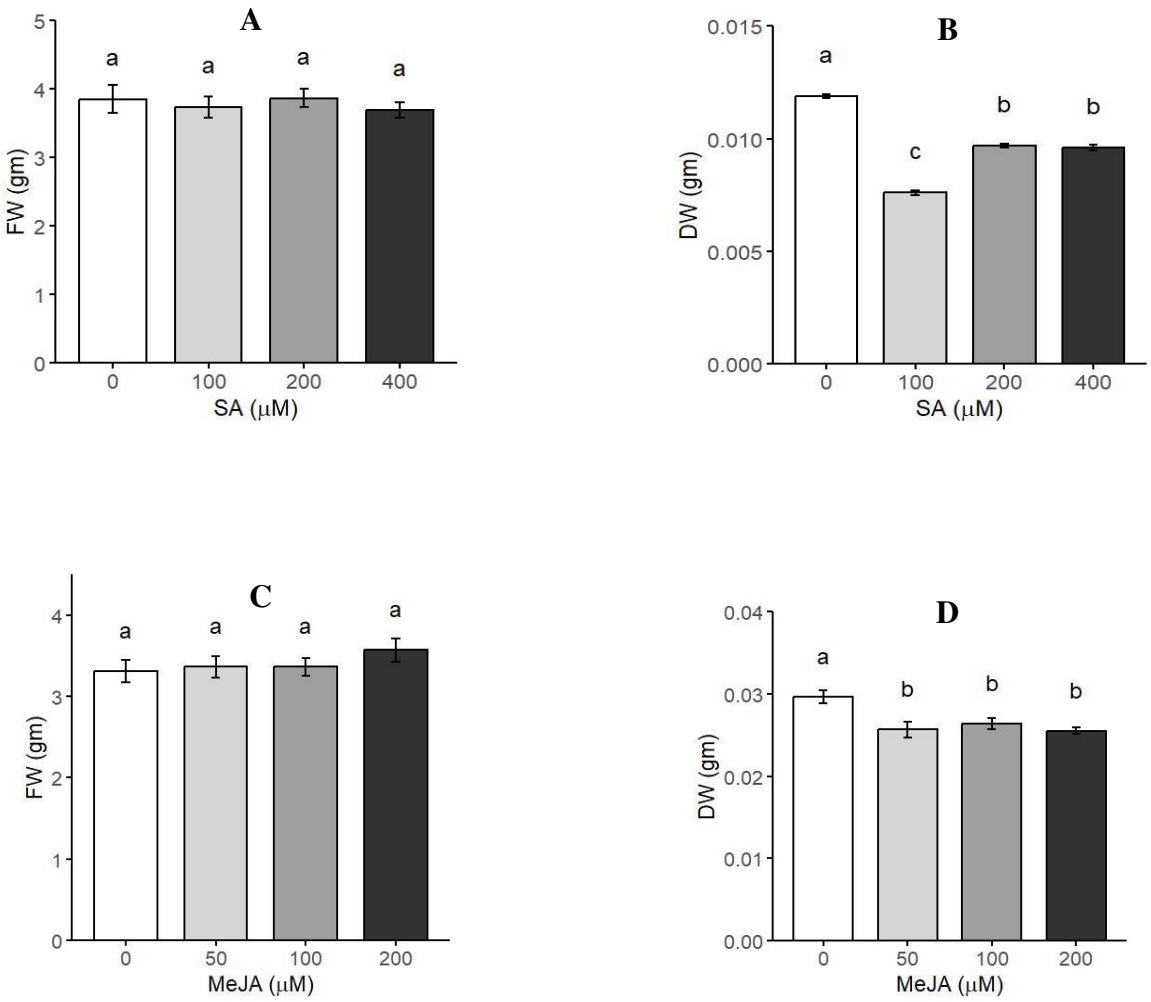


Figure 20- Performance of callus of *C. procera* in increasing concentrations of SA (A) and (B); MeJA (C) and (D) in terms of fresh weight (FW) and dry weight (DW). Data are mean ± SMD of five independent measurements. Columns labelled with different letters are significantly different at $P < 0.05$ based on one-way ANOVA with Tukey multiple range test using R-software.

6.2.3. Impact of signalling inducers on callus proteolysis and biochemical parameters

To evaluate the proteolytic activity and soluble protein content in SA and MeJA-treated calli compared to control conditions, our observations reveal distinctive patterns. The high SA concentration (400 µM) exhibited lower proteolytic activity than control conditions, while the highest soluble protein content was noted in calli treated with 200 µM SA compared to control. In contrast, the MeJA treatment group displayed extremely low proteolytic activity in the 50 µM

MeJA-treated calli compared to the control, and the soluble protein content was also significantly lower in the 50 µM MeJA-treated calli compared to control conditions (Figure 21). These results align with the findings of Baghizadeh et al. (2014), who reported increased protein content in response to high SA concentration in *Brassica napus* L. (Canola). However, the specific responses may vary between plant species due to their distinct physiological characteristics. The decrease in proteolytic activity at a high concentration of SA (400 µM) may be linked to the regulatory role of SA in inhibiting proteolytic enzymes. SA is known to modulate various defence responses in plants, and its higher concentration might suppress protease activity as part of a stress response strategy. The observed variations in proteolytic activity and soluble protein content suggest distinct responses of *C. procera* calli to SA and MeJA treatments, emphasizing the intricate regulatory mechanisms involved in these signalling pathways.

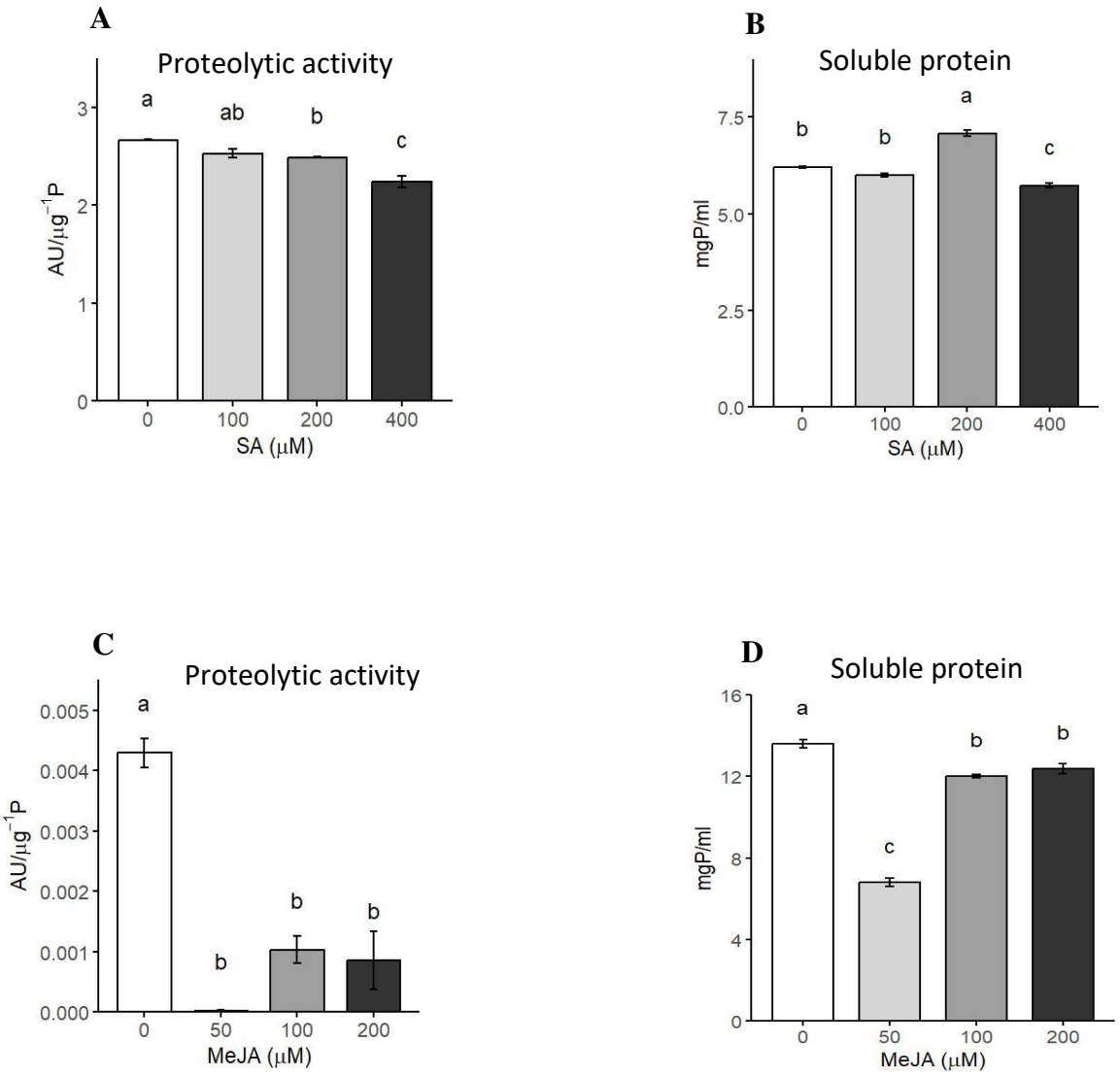


Figure 21- Performance of callus of *C. procera* in increasing concentrations of SA (A) and (B); MeJA (C) and (D) in terms of proteolytic activity and soluble protein. Data are mean ± SMD of five independent measurements. Columns labelled with different letters are significantly different at $P < 0.05$ based on one-way ANOVA with Tukey multiple range test using R-software.

In our investigation aimed at interpreting the influence of salicylic acid (SA) and methyl jasmonate (MeJA) on photosynthetic pigments in *C. procera* calli relative to control conditions, we conducted a comprehensive photosynthetic pigments assay. The outcomes unveiled distinctive patterns in the concentrations of chlorophyll *a*, *b*, and carotenoids under increasing concentrations of SA and MeJA. In the SA treatment group, 400 µM of SA exhibited the highest levels of

chlorophyll *a*, *b*, compared to the control, with a contemporary elevation in carotenoid content noted in calli treated with 200 µM of SA. Contrarily, in the MeJA treatment group, chlorophyll *a* content was reduced at 50 and 200 µM MeJA compared to the control, while chlorophyll *b* remained consistent across all treatment groups and control conditions. Notably, carotenoid levels were heightened only in the 50 µM MeJA treatment group compared to the others (Figure 22). Zahraa and Faiq (2019) reported elevated content of chlorophyll *a*, *b*, and carotene in *Petunia sp.* in the presence of SA. In contrast, a study by Kalariya et al. (2023) demonstrated a decrease in chlorophyll *a*, *b*, and carotenoid content in *Andrographis paniculata* with increasing MeJA concentrations compared to the control.

The notable elevation in chlorophyll *a*, *b*, levels at 400 µM SA implies a positive regulatory impact on photosynthetic pigments. SA, recognized for its involvement in defence mechanisms, likely contributes to heightened chlorophyll synthesis, indicative of its multifaceted roles in plant physiology while the heightened carotenoid levels at 200 µM SA suggest a potential role of SA in reinforcing photoprotection. The increased carotenoids play a vital role in photoprotection and antioxidative defence, indicating a responsive mechanism.

In the MeJA the drop in chlorophyll *a* at 50 and 200 µM suggests that MeJA might slow down chlorophyll production. MeJA, connected to defence, could be shifting resources from photosynthesis to defence mechanisms, causing this decline. The stable chlorophyll *b* levels in both MeJA-treated groups and the control condition imply that MeJA might specifically influence chlorophyll *a* biosynthesis without notably affecting chlorophyll *b*. The MeJA may selectively affect the production of chlorophyll *a* over chlorophyll *b* due to specific plant processes it influences. MeJA can modify gene activity and plant chemistry, and its impact on the pathways for making chlorophyll may vary between the genes linked to chlorophyll *a*, *b*. Also, since chlorophyll *a*, *b*, serve different roles in photosynthesis, they might respond differently to external factors like MeJA. This response is likely due to the complex molecular processes governing chlorophyll production and the plant's ability to adapt to MeJA. The specific increase in carotenoid content at 50 µM MeJA may be attributed to the optimal concentration triggering the plant's response mechanisms. Different concentrations of MeJA can elicit varied responses, and at 50 µM, the conditions might have favoured carotenoid accumulation.

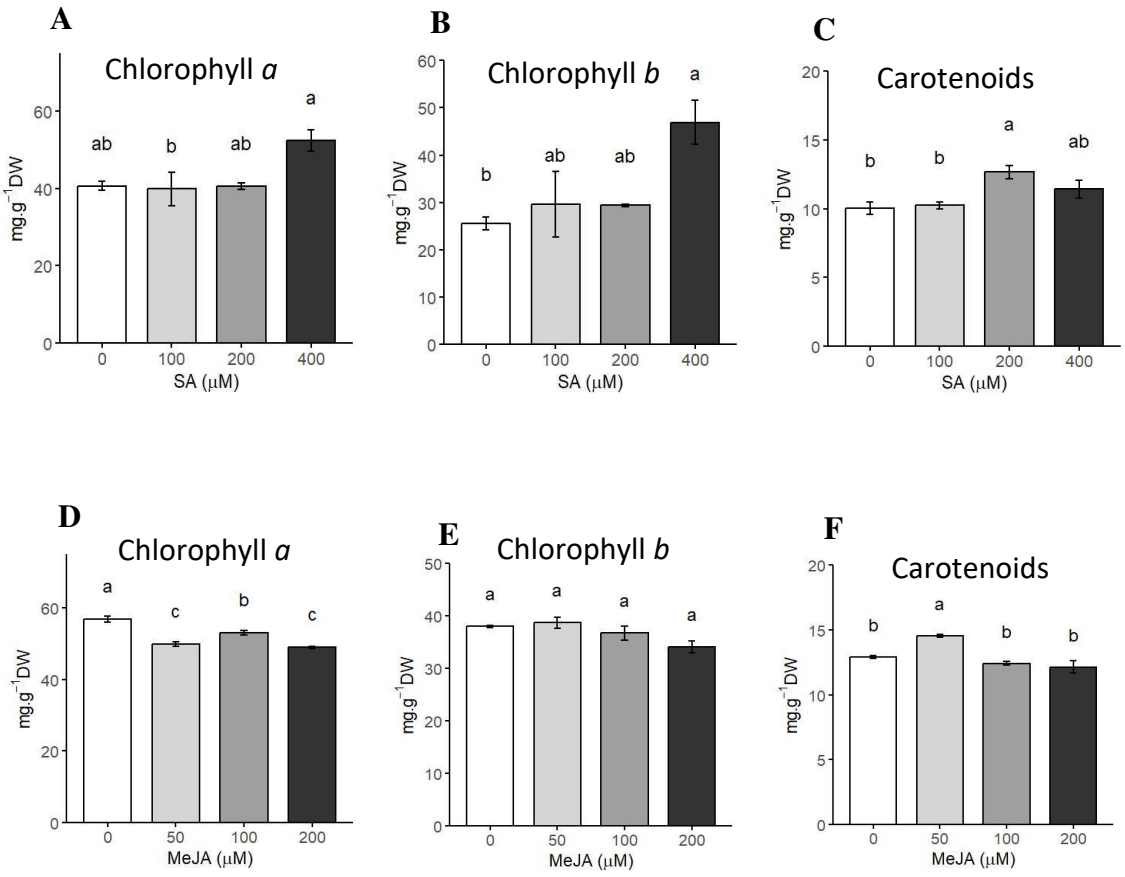


Figure 22- Performance of callus of *C. procera* in increasing concentrations of SA (A), (B) and (C); MeJA (D), (E), and (F) in terms of Photosynthetic pigments. Data are mean ± SMD of five independent measurements. Columns labelled with different letters are significantly different at $P < 0.05$ based on one-way ANOVA with Tukey multiple range test using R-software.

6.2.4. The impact of signalling inducers on callus protein profiles

To investigate the impact of varying concentrations of salicylic acid (SA) and methyl jasmonate (MeJA) on proteins in *C. procera* calli, particularly under control conditions, and to assess the enzymatic activity of these proteins, two complementary techniques were employed: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and zymogram. Upon analysis, distinctive patterns emerged. In the SA treatment, SDS-PAGE revealed novel polypeptides more heavily stained and prominent at 400 µM compared to the control, a pattern consistent with the zymogram at 400 µM. In MeJA treatment, the 200 µM concentration exhibited

heavy bands in both SDS-PAGE and its corresponding zymogram compared to the control conditions (Figure 23).

The enhanced detection of novel polypeptides in SDS-PAGE and their increased prominence at 400 µM SA compared to the control suggests that higher concentrations of salicylic acid may induce the synthesis or activation of specific proteins in *C. procera* calli. This could be attributed to the signalling role of SA in plant defence responses, leading to the production of proteins involved in stress-related pathways. Similarly, the heavy bands observed at 200 µM MeJA in both SDS-PAGE and the corresponding zymogram indicate that methyl jasmonate at this concentration might trigger the expression or activation of specific proteins associated with MeJA signalling pathways. These findings align with the established roles of SA and MeJA as signalling molecules in plant stress responses, influencing the proteomic profile of the calli. Prachi et al. (2002) demonstrated salicylic acid-induced insensitivity to *Fusarium oxysporum* f.sp. *zingiberi* culture filtrate in *Zingiber officinale* Roscoe calli. Additionally, Sabater-jara et al. (2014) reported the induction of extracellular defence-related proteins in suspension-cultured cells of *Daucus carota* elicited with cyclodextrins and methyl jasmonate in a similar study.

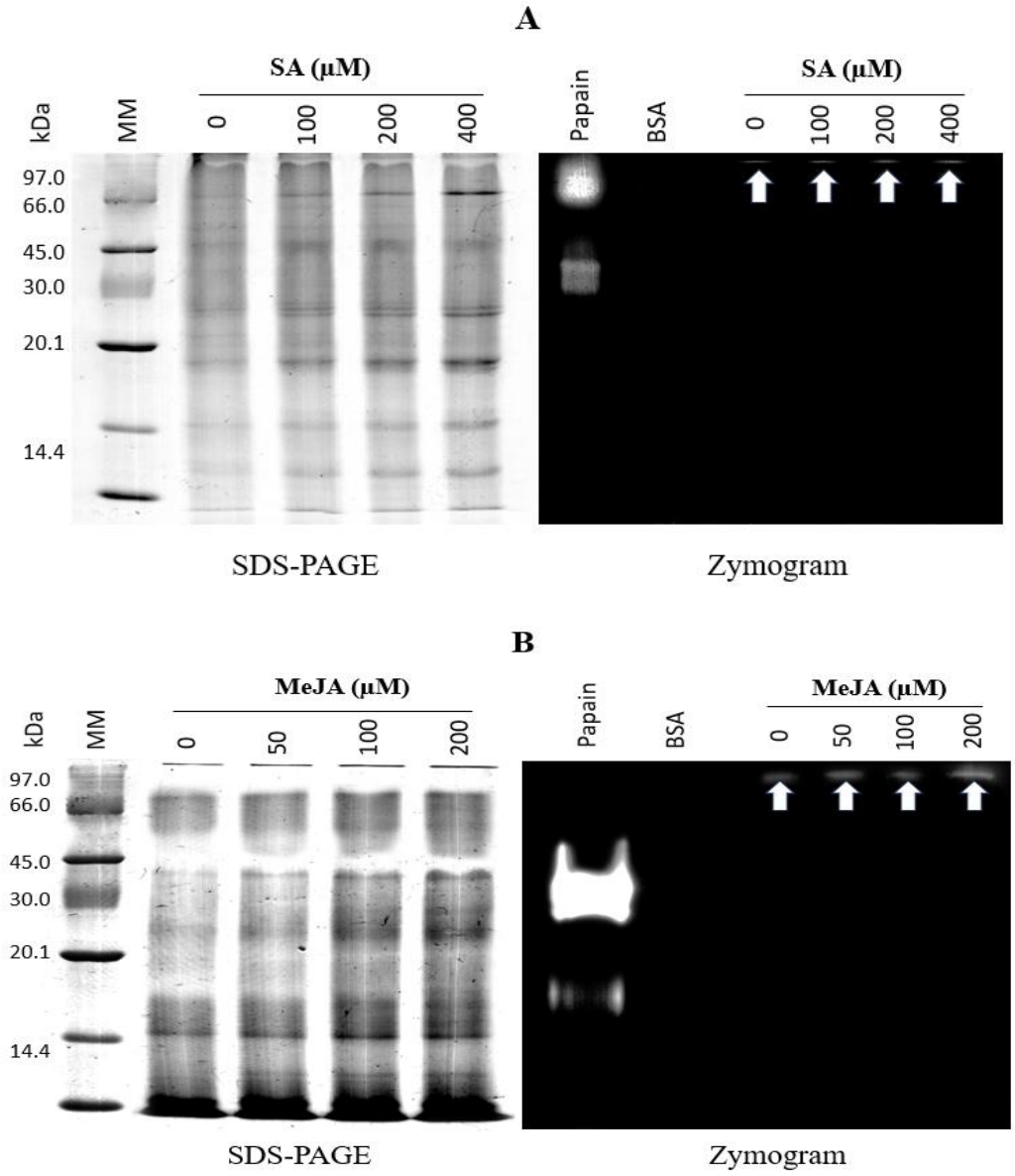


Figure 23- Analysis of soluble proteins through SDS-PAGE and zymogram of *C. procera* calli treated with varying concentrations of SA (A) and MeJA (B) in comparison to control. Some polypeptides appeared heavily stained, and some new polypeptides were also detected, which appeared heavier stained in 400 µM of SA and 200 µM of MeJA-treated calli. An equal amount of proteins were loaded on the gels. In SDS-PAGE molecular markers (MM) loaded in the 1st row, C in the 2nd while increasing concentrations of SA and MeJA loaded in the rest of the rows. In the zymogram, Papain loaded in the 1st row, BSA in the 2nd, and C in the 3rd while increasing concentrations of SA and MeJA loaded in the remaining rows. MM: molecular markers; 97.0 kDa: phosphorylase β; 66.0 kDa: albumin; 45.0 kDa: ovalbumin; 30.0 kDa: carbonic anhydrase; 20.1 kDa: trypsin inhibitor and α- lactalbumin 14.4 kDa.

6.2.5. Signalling inducers impact on enzyme responses in callus

The impact of salicylic acid (SA) and methyl jasmonate (MeJA) on enzymatic activity reveals complex interactions, with both enhancing and inhibitory effects. The activities of catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX), and superoxide dismutase (SOD) were quantified to elucidate these dynamics.

At 400 µM of SA, CAT activity exhibited a concentration-dependent decrease compared to the control, paralleled by a similar trend in APX activity, which also showed lower levels compared to the control. In MeJA the CAT and APX activities were observed to be lower in the control conditions in comparison to the MeJA treatment group (Figure 24). The reduction in CAT activity at 400 µM of SA compared to the control group may be indicative of a concentration-dependent response. While lower concentrations of SA might serve as signalling molecules triggering stress responses, higher concentrations could potentially act in a regulatory manner, leading to a downregulation of CAT activity. The simultaneous decrease in APX activity with increasing SA concentrations suggests a coordinated response. SA is known to influence the redox status of cells and regulate antioxidant enzyme activities. The lower CAT and APX activities observed in the control conditions compared to the MeJA-treated group suggest a potential priming effect of MeJA on antioxidant enzyme activities. In a study conducted by Asghari et al. (2015), it was observed that the activity of the catalase enzyme decreased after treatment with methyl jasmonate. However, the inhibitory effect of methyl jasmonate on catalase activity was reduced by the presence of salicylic acid. The observed differences in CAT and APX activities between SA and MeJA treatments indicate the complex interplay between signalling molecules. SA and MeJA may activate different signalling pathways, leading to varied responses in antioxidant enzyme activities.

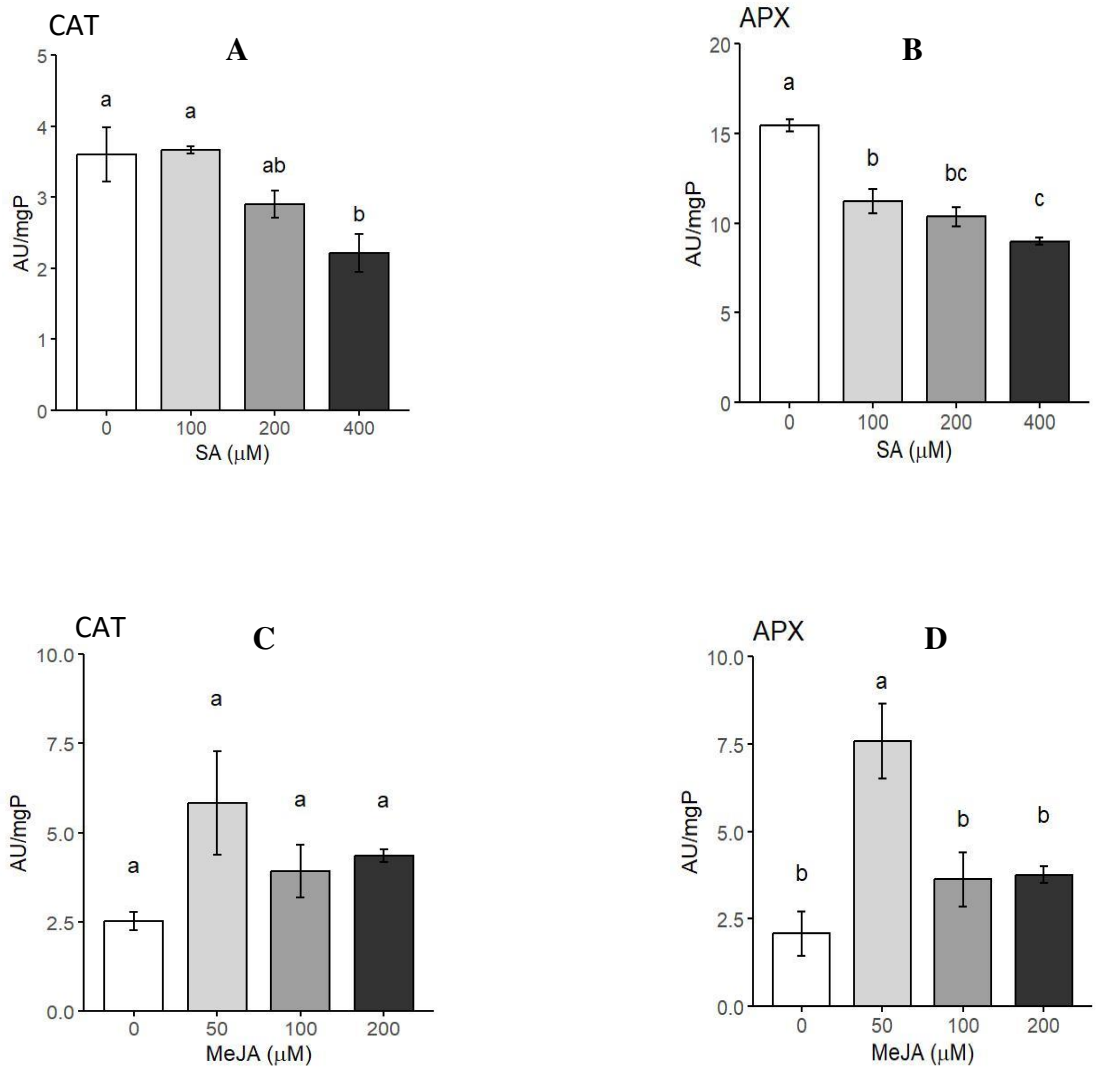


Figure 24- Performance of callus of *C. procera* in increasing concentrations of SA (A) and (B); MeJA (C) and (D) in terms of CAT and APX. Data are mean ± SMD of five independent measurements. Columns labelled with different letters are significantly different at $P < 0.05$ based on one-way ANOVA with Tukey multiple range test using R-software.

In our investigation of the impact of increasing concentrations of salicylic acid (SA) and methyl jasmonate (MeJA) on the enzymatic activity of peroxidase (POX) and superoxide dismutase (SOD) in *C. procera* calli, we observed elevated POX activity in the high SA treatment group (400 µM) compared to the control, and a similar trend was observed for SOD activity. In the MeJA

treatment group, the highest POX activity was noted at 50 µM of MeJA compared to the control, and a similar pattern was observed for SOD activity (Figure 25).

The results align with findings by Rao et al. (1997), who reported increased POX and SOD activity in response to high SA treatment compared to the control. Similarly, Abdelgawad et al. (2014) observed elevated POX and SOD activity in the MeJA treatment group compared to the control conditions.

The enhanced POX and SOD activities in response to higher concentrations of SA and MeJA suggest a potential involvement of these signalling molecules in triggering antioxidant defence mechanisms. POX plays a crucial role in scavenging reactive oxygen species (ROS), while SOD is involved in detoxifying superoxide radicals.

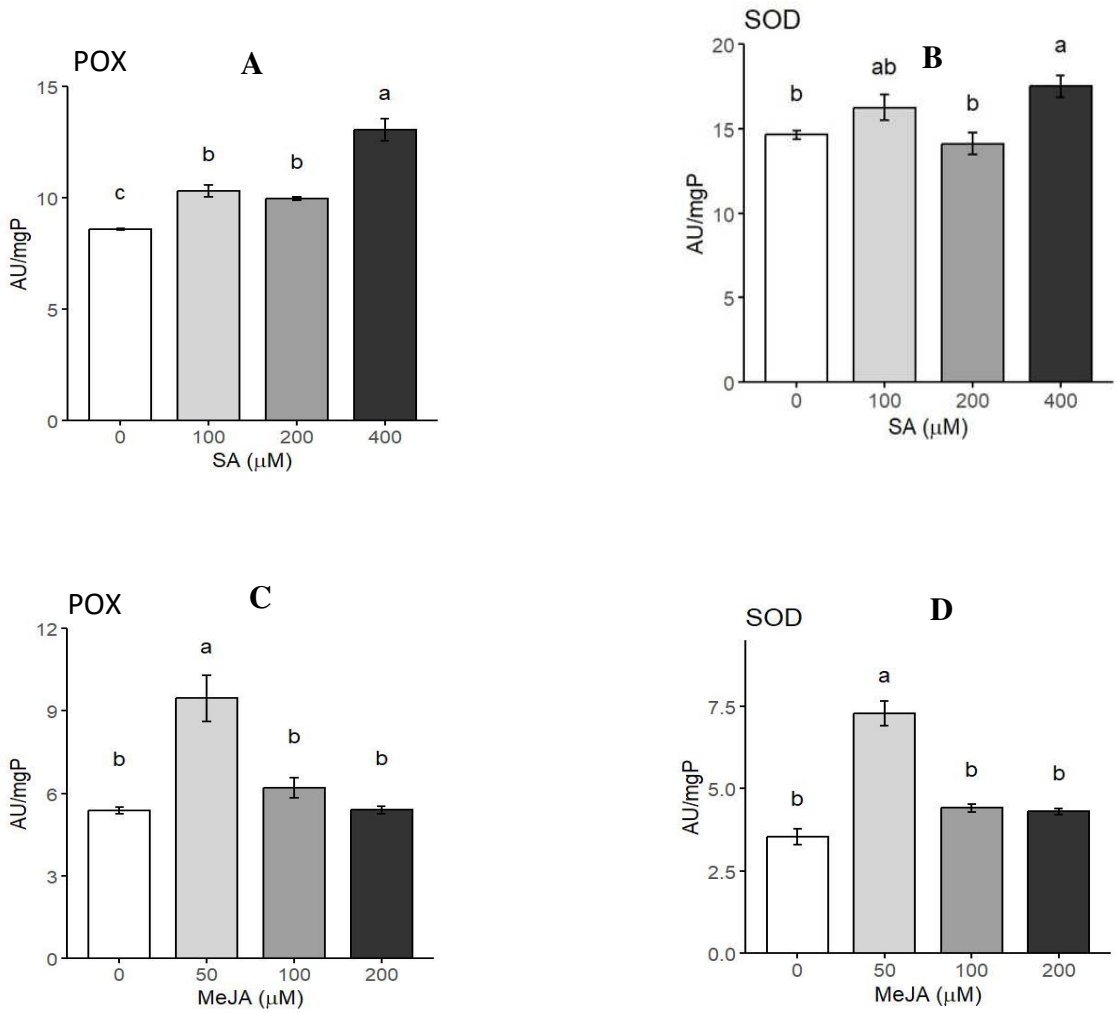


Figure 25- Performance of callus of *C. procera* in increasing concentrations of SA (A) and (B); MeJA (C) and (D) in terms of POX and SOD. Data are mean ± SMD of five independent measurements. Columns labelled with different letters are significantly different at $P < 0.05$ based on one-way ANOVA with Tukey multiple range test using R-software.

6.2.6. RNA quality in signalling inducer-treated callus

In assessing the impact of SA (400 µM) and MeJA (200 µM) in comparison to control conditions on gene expression in *C. procera* callus, comprehensive transcriptomic analyses were executed. The RNA extraction process was thoroughly initiated to ensure the acquisition of high-quality material for subsequent analysis. Comprehensive assessments were carried out to confirm the integrity of the extracted RNA and ensure it was free from contamination with carbohydrates and proteins. Spectrophotometric readings were employed for a thorough assessment of RNA

quality. The control, SA-treated (400 µM), and MeJA-treated (200 µM) samples exhibited non-degraded RNA, with a 260/280 ratio exceeding 2, indicating effective protein removal during extraction. The 260/230 ratio, varying from 1.07 to 2.3 among treatments, revealed some variability in MeJA-treated calli at 12 and 24 hours, presenting lower 260/230 ratios (0.62 and 0.53, respectively). This suggests the presence of residual polysaccharides and slight variability, possibly attributed to secondary compounds and other composites in the callus. However, successful gene amplification in PCR analyses provides evidence of generally high-quality RNA suitable for gene expression studies in *C. procera* callus (Table 3).

Callus of *C. procera*	ng/µL	260/280	260/230						
Before treatment (0h)	127.75	2.17	1.3						
Time	Control ng/µL	260/280	260/230	SA (400 µM) ng/µL	260/280	260/230	MeJA (200 µM) ng/µL	260/280	260/230
2h	139.39	2.15	1.15	171.48	2.16	1.51	159.56	2.14	1.08
12h	243.5	2.16	2.02	273.7	2.15	2.05	137.98	2.15	0.62
24h	235.86	2.18	1.15	304.61	2.17	1.33	151.53	2.15	0.53
48h	330.35	2.14	2.18	396.51	2.13	2.14	406.93	2.13	1.43
4 days	303.6	2.12	2.05	414.14	2.13	1.56	221.15	2.16	1.75
7 days	451.78	2.11	1.07	419.69	2.13	2.02	224.56	2.16	2.17

Table 3- Average concentration values and absorbance ratios of RNAs extracted from the callus of *C. procera* cultivated for 1 week in MS medium under control, SA (400 µM), and MeJA (200 µM) of treatment.

6.2.7. Optimizing RT-PCR for stress-responsive genes in signalling-induced callus

To ensure reliable and specific amplification during RT-PCR, optimal annealing temperatures and cycle numbers were determined for each target gene. The chosen temperatures (57.1 °C for Osmotin, 59.6 °C for Chitinase, 50.8 °C for Protease, and 57.1 °C for *EF1α*) and cycles

(33 for Osmotin, 30 for Chitinase, 37 for Protease, and 24 for *EF1α*) establish a foundation for robust and consistent gene amplification. This standardization guarantees the accuracy of subsequent analyses.

An initial amplification step using degenerate primers targeting the *EF1α* gene ensured the integrity and homogeneity of synthesized cDNAs. *EF1α*, chosen as a "Housekeeping" gene, exhibited consistent expression across samples, confirming the quality of cDNA. The uniformity in *EF1α* expression validated the suitability of the synthesized cDNAs for downstream analyses.

Transcriptional analysis revealed differential expression of osmotin, chitinase, and protease genes in *C. procera* calli under SA (400 µM) and MeJA (200 µM) treatments compared to the control. Exposure to MS culture medium induced gene expression, but this induction was more pronounced and sustained with SA and MeJA supplementation (Figure 26).

For Osmotin, transcripts exhibited elevated intensity bands at 12 hours of exposure to 400 µM SA, and this accumulation persisted until the 7th day under SA treatment. In MeJA 200 µM treatment, higher intensity bands emerged at 24 hours and persisted until the 7th day, while in the control MS medium, a considerably lower intensity band appeared at 24 hours.

Chitinase transcripts were detected from 2 hours until the 7th day of SA 400 µM treatment. In MeJA treatment, clear bands were observed throughout the entire period (from 2 hours to the 7th day). In the control MS medium, transcripts initiated at 0 hours with a very low-intensity band at 2 hours, reaching higher intensity bands at 12 hours and continuing until the 7th day.

Protease transcripts revealed a faint band at 24 and 48 hours, concluding with a prominent high-intensity band on day 4 in the SA treatment. In MeJA treatment, bands were visible throughout the entire period from 2 hours to the 7th day. In the control, bands were only observed on days 4 and 7.

The findings in our study align with related research, providing a broader context for understanding the transcriptional dynamics observed in *C. procera* calli under SA and MeJA treatments. Kim et al. (2002) demonstrated that osmotin transcript levels increased in response to octadecanoid pathway intermediates, aspirin, and salicylic acid. Their results highlighted the developmental regulation of osmotin, emphasizing its involvement in wound stress signal transduction. Similarly, Zhu et al. (1995) observed a moderate increase in osmotin-like protein

genes in potatoes in response to SA, indicating a role in osmotic stress and fungal infection. The regulatory influence of SA and MeJA on chitinase gene expression is consistent with studies on pine by Davis et al. (2002). This suggests a shared regulatory mechanism across plant species in response to these signalling molecules. Kovács et al. (2016) reported SA-induced cysteine protease activity during programmed cell death in tomato plants, supporting our observation of protease transcript dynamics under SA treatment. Interestingly, Zinovieva et al. (2021) noted a significant decrease in nematode invasion after SA or JA (jasmonic acid) treatment, emphasizing the potential role of the studied proteinase inhibitors in resistance induced by signalling molecules. Pluskota et al. (2019) highlighted the involvement of JA and ethylene in the accumulation of osmotin in germinating tomato seeds, adding another layer to the interconnected regulatory network. The dual involvement of the chitinase gene in both the SA and jasmonate/ethylene signalling pathways, as reported by Zhang et al. (2012), reinforces the intricate crosstalk between these pathways, confirming the complex dynamics we observed in our study. Finally, Farmer et al. (1992) elucidated the regulation of proteinase inhibitor genes by MeJA and JA, aligning with our findings on the role of MeJA in the expression of protease transcripts in *C. procera* calli. These findings align with the broader literature, reinforcing the role of SA and MeJA in regulating gene expression associated with stress responses and plant defence mechanisms. The observed gene expression patterns provide valuable insights into the complex interplay of signalling molecules in *C. procera* calli.

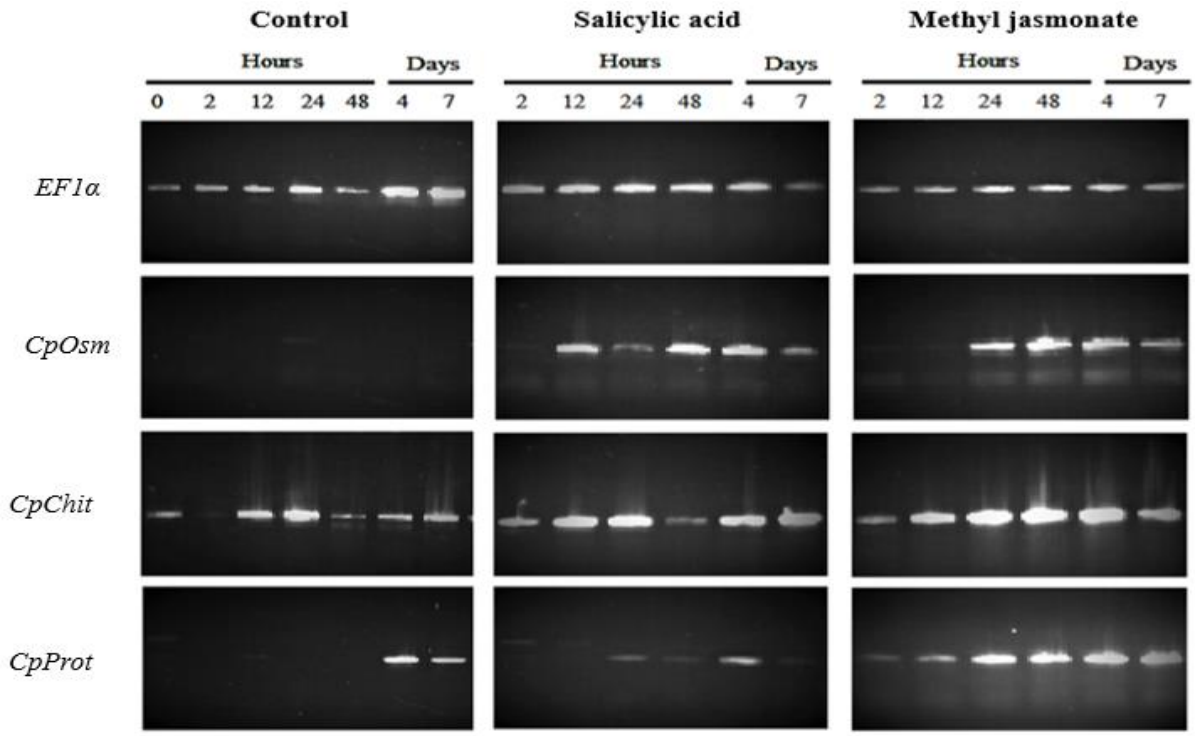


Figure 26- Agarose gel electrophoresis (1%) showing time-course transcript analysis of *EF1α*, Osmotin (*CpOsm*), Chitinase (*CpChit*), and Protease (*CpProt*) genes in the calli of *C. procera* under control, SA (400 µM), and MeJA (200 µM) of treatment.

7. CONCLUSION

In the context of *C. procera* calli, the exposure to salinity and signalling inducers resulted in distinct physiological and biochemical responses compared to control conditions. Notably, the expression of osmotin, chitinase, and protease genes was heightened under salt stress and signalling inducer treatments. This upregulation indicates a defensive role for these genes in response to unfavourable conditions, signifying their potential importance in stress adaptation. DNA integrity remained intact in calli subjected to salt stress, suggesting a preservation of cellular stability. Preliminary evidence suggests that salt stress may trigger cellular metabolic activation, potentially influencing cellular proliferation. Osmotin, chitinase, and protease, identified as highly responsive genes, hold promise for gene cloning research, presenting opportunities for genetic modifications in agriculturally significant plant species. This research provides valuable insights into the biological effects of salinity and signalling molecules in *C. procera* callus culture. Such understanding not only contributes to plant defence strategies but also opens avenues for novel applications in agriculture, fostering advancements in technology and market prospects.

REFERENCES

ABAD, Laura R. et al. Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. **Plant Science**, v. 118, n. 1, p. 11-23, 1996.

ABDELGAWAD, Z. A. et al. Impact of methyl jasmonate on antioxidant activity and some biochemical aspects of maize plant grown under water stress condition. **Agricultural Sciences**, v. 5, n. 12, p. 1077, 2014.

ABDELKHALEK, Ahmed; AL-ASKAR, Abdulaziz A. Green synthesized ZnO nanoparticles mediated by Mentha spicata extract induce plant systemic resistance against Tobacco mosaic virus. **Applied Sciences**, v. 10, n. 15, p. 5054, 2020.

ABDULHAFIZ, Ferid et al. Plant cell culture technologies: A promising alternatives to produce high-value secondary metabolites. **Arabian Journal of Chemistry**, v. 15, n. 11, p. 104161, 2022.

ABIRI, R. et al. Enhancing somatic embryogenesis of Malaysian rice cultivar MR219 using adjuvant materials in a high-efficiency protocol. **International Journal of Environmental Science and Technology**, v. 14, p. 1091-1108, 2017.

ACHUO, E. A.; PRINSEN, E.; HÖFTE, Monica. Influence of drought, salt stress and abscisic acid on the resistance of tomatoes to Botrytis cinerea and Oidium neolycopersici. **Plant pathology**, v. 55, no. 2, p. 178-186, 2006.

AERTS, Rob J. et al. Signalling molecules and the synthesis of alkaloids in Catharanthus roseus seedlings. **Phytochemistry**, v. 42, n. 2, p. 417-422, 1996.

AGRAWAL, Anurag A. et al. Toxic cardenolides: chemical ecology and coevolution of specialized plant–herbivore interactions. **New Phytologist**, v. 194, n. 1, p. 28-45, 2012.

AGRAWAL, Anurag A.; KONNO, Kotaro. Latex: a model for understanding mechanisms, ecology, and evolution of plant defense against herbivory. **Annu. Rev. Ecol. Evol. Syst.**, v. 40, p. 311-331, 2009.

AHMAD, M. et al. Improvement for biotic and abiotic stress tolerance in crop plants. **Biological and Clinical Sciences Research Journal**, v. 2021, n. 1, 2021.

AHMAD, Parvaiz et al. Mitigation of sodium chloride toxicity in Solanum lycopersicum L. by supplementation of jasmonic acid and nitric oxide. **Journal of plant interactions**, v. 13, n. 1, p. 64-72, 2018.

AHMAD, Parvaiz et al. Nitric oxide mitigates salt stress by regulating levels of osmolytes and antioxidant enzymes in chickpea. **Frontiers in plant science**, v. 7, p. 347, 2016.

AHMADI MOUSAVI, E.; MANOCHEHRI KALANTARI, K.; JAFARI, S. R. Change of some osmolytes accumulation in water-stressed colza (Brassica napus L.) as affected by 24-epibrassinolide. **Iranian Journal of Science and Technology (Sciences)**, v. 33, n. 1, p. 1-11, 2009.

AHMADI, F. I.; KARIMI, K.; STRUIK, P. C. Effect of exogenous application of methyl jasmonate on physiological and biochemical characteristics of Brassica napus L. cv. Talaye under salinity stress. **South African Journal of Botany**, v. 115, p. 5-11, 2018.

AHMED, Nasar Uddin et al. Identification and expression analysis of chitinase genes related to biotic stress resistance in Brassica. **Molecular biology reports**, v. 39, p. 3649-3657, 2012.

AKTE, J. et al. In vitro screening of rice genotypes using polyethylene glycol under drought stress. **Progressive agriculture**, v. 27, n. 2, p. 128-135, 2016.

AKULA, Ramakrishna; RAVISHANKAR, Gokare Aswathanarayana. Influence of abiotic stress signals on secondary metabolites in plants. **Plant signalling & behaviour**, v. 6, n. 11, p. 1720-1731, 2011.

AL SULAIBI, Mazen AM; THIEMANN, Carolin; THIEMANN, Thies. Chemical constituents and uses of Calotropis procera and Calotropis gigantea–a review (Part I–the plants as material and energy resources). **Open Chemistry Journal**, v. 7, n. 1, 2020.

AL-ANI, Laith Khalil Tawfeeq et al. Strategy of nematophagous fungi in determining the activity of plant parasitic nematodes and their prospective role in sustainable agriculture. **Frontiers in Fungal Biology**, v. 3, p. 863198, 2022.

ALENCAR, Nylane Maria Nunes et al. Pro-and anti-inflammatory activities of the latex from Calotropis procera (Ait.) R. Br. are triggered by compounds fractionated by dialysis. **Inflammation Research**, v. 55, p. 559-564, 2006.

ALI, Md Sarafat; BAEK, Kwang-Hyun. Protective roles of cytosolic and plastidal proteasomes on abiotic stress and pathogen invasion. **Plants**, v. 9, n. 7, p. 832, 2020.

ALI, Sajad et al. Identification and comparative analysis of Brassica juncea pathogenesis-related genes in response to hormonal, biotic and abiotic stresses. **Acta physiologiae plantarum**, v. 39, p. 1-15, 2017.

ALI, Sajad et al. Pathogenesis-related proteins and peptides as promising tools for engineering plants with multiple stress tolerance. **Microbiological research**, v. 212, p. 29-37, 2018.

ALI, Zahraa H.; ALI, Faiq H. Effect of Salicylic Acid on Chlorophyll A, B and Carotene Content of Petunia sp. **seeds**, v. 1, p. 3, 2019.

AL-ISSA, Ruba Muhammad Soud et al. The impact of NaCl on different genotypes of tomato (Solanum Lycopersicon Mil) on germination, some physiology characteristics and gene expression. **Eurasian Journal of Biosciences**, v. 14, n. 2, p. 4467-4470, 2020.

ALORI, Elizabeth T.; GLICK, Bernard R.; BABALOLA, Olubukola O. Microbial phosphorus solubilization and its potential for use in sustainable agriculture. **Frontiers in microbiology**, v. 8, p. 971, 2017.

AL-QATRANI, Muna KJ; AL KHALIFA, Aqeel AS; OBAID, Nada Ab. Effect of jasmonic acid on stimulating the growth and development of date palm callus (Phoenix dactylifera L.) cultivar shukar in vitro under salt stress conditions. In: **IOP Conference Series Earth and Environmental Science**. IOP Publishing, p. 012017, 2021.

AL-QURA'N, S. Ethnopharmacological survey of wild medicinal plants in Showbak, Jordan. **Journal of Ethnopharmacology**, v. 123, n. 1, p. 45-50, 2009.

AL-QUWAIE, Diana AH. Bacterial community dynamics with rhizosphere of Calotropis procera and Senna alexandrina desert plants in Saudi Arabia. **Bioinformation**, v. 16, n. 8, p. 567, 2020.

AL-ROWAILY, Saud L. et al. Essential oil of Calotropis procera: Comparative chemical profiles, antimicrobial activity, and allelopathic potential on weeds. **Molecules**, v. 25, n. 21, p. 5203, 2020.

AL-SALT, Jordan. Antimicrobial activity of crude extracts of some plant leaves. **Res J Microbiol**, v. 7, p. 59-67, 2012.

ALTPETER, Fredy et al. Advancing crop transformation in the era of genome editing. **The Plant Cell**, v. 28, n. 7, p. 1510-1520, 2016.

AMINI, Mohammad Humayoon et al. Important insights from the antimicrobial activity of Calotropis procera. **Arabian Journal of Chemistry**, v. 14, n. 7, p. 103181, 2021.

ANSARI, S. H. et al. New oleanene triterpenes from root bark of Calotropis procera. **Journal of Medicinal and Aromatic Plant Sciences**, v. 21, n. 4, p. 978-981, 1999.

ANTOGNONI, Fabiana et al. Elicited Teucrium chamaedrys cell cultures produce high amounts of teucrioside, but not the hepatotoxic neo-clerodane diterpenoids. **Phytochemistry**, v. 81, p. 50-59, 2012.

ARZANI, A.; ASHRAF, M. Smart engineering of genetic resources for enhanced salinity tolerance in crop plants. **Critical Reviews in Plant Sciences**, v. 35, n. 3, p. 146-189, 2016.

ARZANI, Ahmad. Improving salinity tolerance in crop plants: a biotechnological view. **In Vitro Cellular & Developmental Biology-Plant**, v. 44, p. 373-383, 2008.

ASGHARI, Mohammadreza; HASANLOOE, Ali Rashid. Interaction effects of salicylic acid and methyl jasmonate on total antioxidant content, catalase and peroxidase enzymes activity in "Sabrosa" strawberry fruit during storage. **Scientia Horticulturae**, v. 197, p. 490-495, 2015.

ASHRAF, M. F. M. R.; FOOLAD, Majid R. Roles of glycine betaine and proline in improving plant abiotic stress resistance. **Environmental and experimental botany**, v. 59, n. 2, p. 206-216, 2007.

ASHRAF, M. P. J. C.; HARRIS, P. J. C. Potential biochemical indicators of salinity tolerance in plants. **Plant science**, v. 166, n. 1, p. 3-16, 2004.

ASHRAF, M.; MUNNS, R. Evolution of approaches to increase the salt tolerance of crops. **Critical Reviews in Plant Sciences**, v. 41, n. 2, p. 128-160, 2022.

ASHRAF, Muhammad; FOOLAD, Majid R. Crop breeding for salt tolerance in the era of molecular markers and marker-assisted selection. **Plant Breeding**, v. 132, n. 1, p. 10-20, 2013.

ASKARI, Hossein et al. Effects of salinity levels on proteome of Suaeda aegyptiaca leaves. **Proteomics**, v. 6, n. 8, p. 2542-2554, 2006.

ATHAR, Habib-ur-Rehman et al. Salt stress proteins in plants: An overview. **Frontiers in Plant Science**, vol. 13, p. 999058, 2022.

ATKINSON, Nicky J.; URWIN, Peter E. The interaction of plant biotic and abiotic stresses: from genes to the field. **Journal of experimental botany**, v. 63, n. 10, p. 3523-3543, 2012.

AWORH, OC; NAKAI, S. Separation of sodom apple proteinases by gel filtration. **International Journal of Food Science & Technology** , vol. 23, no. 4, p. 419-423, 1988.

AWUCHI, Chinaza Godswill. The biochemistry, toxicology, and Uses of the acologically active phytochemicals: Alkaloids, terpenes, polyphenols, and glycosides. **Merit Research Journals**, v. 5, n. 1, p. 6-21, 2020.

BACKIYARANI, S. et al. Genome-wide analysis and differential expression of chitinases in banana against root lesion nematode (Pratylenchus coffeae) and eumusa leaf spot (Mycosphaerella eumusae) pathogens. **Applied biochemistry and biotechnology**, v. 175, p. 3585-3598, 2015.

BAENAS, Nieves; GARCÍA-VIGUERA, Cristina; MORENO, Diego A. Elicitation: a tool for enriching the bioactive composition of foods. **Molecules**, v. 19, n. 9, p. 13541-13563, 2014.

BAGHIZADEH, A. et al. Effects of Salicylic acid on some physiological and biochemical parameters of Brassica napus L. (Canola) under salt stress. **Int. J. Agric. Sci** , vol. 4, no. 2, p. 147-152, 2014.

BALDACCI-CRESP, Fabien et al. A rapid and quantitative safranin-based fluorescent microscopy method to evaluate cell wall lignification. **The Plant Journal**, v. 102, n. 5, p. 1074-1089, 2020.

BARAKAT, M. N.; ABDEL-LATIF, T. H. In vitro selection of wheat callus tolerant to high levels of salt and plant regeneration. **Euphytica**, v. 91, p. 127-140, 1996.

BASHIR, Muhammad Ajmal et al. Osmotin: a cationic protein leads to improve biotic and abiotic stress tolerance in plants. **Plants**, v. 9, n. 8, p. 992, 2020.

BATOOL, Hina et al. A review on Calotropis procera its phytochemistry and traditional uses. **Big Data Agric**, v. 2, n. 11, p. 29-31, 2020.

BEBAWI, Faiz F.; CAMPBELL, Shane D.; MAYER, Robert J. Seed bank longevity and age to reproductive maturity of Calotropis procera (Aiton) WT Aiton in the dry tropics of northern Queensland. **The Rangeland Journal**, vol. 37, no. 3, p. 239-247, 2015.

BENDERRADJI, Laid et al. Callus induction, proliferation, and plantlets regeneration of two bread wheat (Triticum aestivum L.) genotypes under saline and heat stress conditions. **International Scholarly Research Notices**, v. 2012, 2012.

BEYER JR, Wayne F.; FRIDOVICH, Irwin. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. **Analytical biochemistry**, v. 161, n. 2, p. 559-566, 1987.

BHAGYASHRI, A. Chavan et al. Plant latex: an inherent spring of pharmaceuticals. **World Journal of Pharmacy and Pharmaceutical Sciences (WJPPS)**, v. 4, n. 4, p. 1781-1796, 2015.

BHARTI, S.; WAHANE, V. D.; KUMAR, V. L. Protective effect of Calotropis procera latex extracts on experimentally induced gastric ulcers in rat. **Journal of Ethnopharmacology**, v. 127, n. 2, p. 440-444, 2010.

BHAT, Javaid Akhter et al. Role of silicon in mitigation of heavy metal stresses in crop plants. **Plants**, v. 8, n. 3, p. 71, 2019.

BINTE MOSTAFIZ, Suraiya; WAGIRAN, Alina. Efficient callus induction and regeneration in selected indica rice. **Agronomy**, v. 8, n. 5, p. 77, 2018.

BORASTON, Alisdair B. et al. Carbohydrate-binding modules: fine-tuning polysaccharide recognition. **Biochemical journal**, v. 382, n. 3, p. 769-781, 2004.

BORIBOONKASET, Thanaphol et al. Regulation of some carbohydrate metabolism-related genes, starch and soluble sugar contents, photosynthetic activities and yield attributes of two contrasting rice genotypes subjected to salt stress. **Protoplasma**, v. 250, p. 1157-1167, 2013.

BORSANI, Omar; VALPUESTA, Victoriano; BOTELLA, Miguel A. Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in Arabidopsis seedlings. **Plant physiology**, v. 126, n. 3, p. 1024-1030, 2001.

BOUIC, P. J.; LAMPRECHT, Johan H. Plant sterols and sterolins: a review of their immune-modulating properties. **Altern Med Rev**, v. 4, n. 3, p. 170-7, 1999.

BRADFORD, Marion M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical biochemistry**, v. 72, n. 1-2, p. 248-254, 1976.

BRAVO, Juan Manuel et al. Fungus-and wound-induced accumulation of mRNA containing a class II chitinase of the pathogenesis-related protein 4 (PR-4) family of maize. **Plant molecular biology**, v. 52, p. 745-759, 2003.

BRUNNER, Frédéric et al. Substrate specificities of tobacco chitinases. **The Plant Journal**, v. 14, n. 2, p. 225-234, 1998.

CAI, Jing et al. Elicitation of furanocoumarins in Changium smyrnioides suspension cells. **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 130, p. 1-12, 2017.

CAI, Xia; LI, Wei; YIN, Lingfang. Ultrastructure and cytochemical localization of acid phosphatase of laticifers in Euphorbia kansui Liou. **Protoplasma**, v. 238, n. 1-4, p. 3-10, 2009.

CAI, Yizhong et al. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. **Life sciences**, v. 74, n. 17, p. 2157-2184, 2004.

CALLIS, Judy. Regulation of protein degradation. **The plant cell**, v. 7, n. 7, p. 845, 1995.

CAO, Jun; TAN, Xiaona. Comprehensive analysis of the chitinase family genes in tomato (Solanum lycopersicum). **Plants**, v. 8, n. 3, p. 52, 2019.

CARRILLO, Laura et al. Expression of a barley cystatin gene in maize enhances resistance against phytophagous mites by altering their cysteine-proteases. **Plant cell reports**, v. 30, p. 101-112, 2011.

CASTELBLANQUE, Lourdes et al. LOL 2 and LOL 5 loci control latex production by laticifer cells in Euphorbia lathyris. **New Phytologist**, v. 219, n. 4, p. 1467-1479, 2018.

CASTRO, Ana Hortência Fonsêca et al. Callus induction and bioactive phenolic compounds production from Byrsonima verbascifolia (L.) DC. (Malpighiaceae). **Revista Ciência Agronômica**, v. 47, p. 143-151, 2016.

CATINOT, Jérémy et al. Salicylic acid production in response to biotic and abiotic stress depends on isochorismate in Nicotiana benthamiana. **FEBS letters**, v. 582, n. 4, p. 473-478, 2008.

CHANDRAN, Hema et al. Plant tissue culture as a perpetual source for production of industrially important bioactive compounds. **Biotechnology reports**, v. 26, p. e00450, 2020.

CHARTRES, Colin J.; NOBLE, Andrew. Sustainable intensification: overcoming land and water constraints on food production. **Food security**, v. 7, p. 235-245, 2015.

CHAVAN, Suchitra Arun. **Expression profiling and analysis of resistance to Ustilago maydis in maize and teosinte**. Tese de Doutorado, University of Georgia, 2014.

CHEESEMAN, John M. Mechanisms of salinity tolerance in plants. **Plant physiology**, v. 87, n. 3, p. 547-550, 1988.

CHENAR, Hamzeh MINAEI et al. Callus induction is affected by explant type and plant growth regulators in chickpea (Cicer arietinum L.). **Biharean Biologist**, v. 10, n. 1, p. 28-32, 2016.

CHENG, Tielong et al. Physiological and proteomic analyses of leaves from the halophyte Tangut Nitraria reveals diverse response pathways critical for high salinity tolerance. **Frontiers in Plant Science**, v. 6, p. 30, 2015.

CHERIAN, S.; REDDY, M. P. Evaluation of NaCl tolerance in the callus cultures of Suaeda nudiflora Moq. **Biologia Plantarum**, v. 46, p. 193-198, 2003.

CHICONATO, Denise A. et al. Proteomic analysis of young sugarcane plants with contrasting salt tolerance. **Functional Plant Biology**, v. 48, n. 6, p. 588-596, 2021.

CHOWDHURY, Supriyo; BASU, Arpita; KUNDU, Surekha. Overexpression of a new osmotin-like protein gene (SindOLP) confers tolerance against biotic and abiotic stresses in sesame. **Frontiers in Plant Science**, v. 8, p. 410, 2017.

CLEMENTE, Marina et al. Plant serine protease inhibitors: biotechnology application in agriculture and molecular farming. **International journal of molecular sciences**, v. 20, n. 6, p. 1345, 2019.

COÊLHO, Maria RV et al. Salt tolerance of Calotropis procera begins with immediate regulation of aquaporin activity in the root system. **Physiology and Molecular Biology of Plants**, v. 27, p. 457-468, 2021.

CORNELISSEN, Ben JC; HOOFT VAN HUIJSDUIJNEN, Rob AM; BOL, John F. A tobacco mosaic virus-induced tobacco protein is homologous to the sweet-tasting protein thaumatin. **Nature**, v. 321, n. 6069, p. 531-532, 1986.

COSTA, Jose Helio et al. Stress-induced co-expression of two alternative oxidase (VuAox1 and 2b) genes in Vigna unguiculata. **Journal of plant physiology**, v. 167, n. 7, p. 561-570, 2010.

COURT, William A.; HENDEL, John G.; ELMI, Jama. Reversed-phase high-performance liquid chromatography determination of ginsenosides of Panax quinquefolium. **Journal of chromatography A**, v. 755, n. 1, p. 11-17, 1996.

CROUGHAN, T. P.; STAVAREK, S. J.; RAINS, D. W. In vitro development of salt resistant plants. **Environmental and Experimental Botany**, v. 21, n. 3-4, p. 317-324, 1981.

CUSHMAN, John C. Gene expression during adaptation to salt stress. **Environmental Injury to Plants.**, p. 137-171, 1990.

DANA, María de las Mercedes; PINTOR-TORO, José A.; CUBERO, Beatriz. Transgenic tobacco plants overexpressing chitinases of fungal origin show enhanced resistance to biotic and abiotic stress agents. **Plant Physiology**, v. 142, n. 2, p. 722-730, 2006.

DANG, Zhen-hua et al. Transcriptomic profiling of the salt-stress response in the wild recretohalophyte Reaumuria trigyna. **BMC genomics**, v. 14, n. 1, p. 1-18, 2013.

DANTON, Ombeline et al. Ethnopharmacological survey of plants used in the traditional treatment of pain conditions in Mali. **Journal of Herbal Medicine**, v. 17, p. 100271, 2019.

DASGUPTA, M. et al. Evaluation of orange-fleshed sweet potato (Ipomoea batatas L.) genotypes for salt tolerance through shoot apex culture under in vitro NaCl mediated salinity stress conditions. **Plant Cell, Tissue and Organ Culture**, v. 94, p. 161-170, 2008.

DASS, Suchismita; RAMAWAT, K. G. Studies on somatic cell variability in Commiphora wightii (Arnott.). **Bhandari for guggulsterone production,** 2009.

DATTA, S. K.; SIBAPRASAD, D. E. Laticifer differentiation of Calotropis gigantea R. Br. ex Ait. in cultures. **Annals of botany**, v. 57, n. 3, p. 403-406, 1986.

DAVIS, John M. et al. Pathogen challenge, salicylic acid, and jasmonic acid regulate expression of chitinase gene homologs in pine. **Molecular plant-microbe interactions**, v. 15, n. 4, p. 380-387, 2002.

DE ALENCAR, Nylane Maria Nunes et al. Side-effects of Irinotecan (CPT-11), the clinically used drug for colon cancer therapy, are eliminated in experimental animals treated with latex proteins from Calotropis procera (Apocynaceae). **Phytotherapy Research**, v. 31, n. 2, p. 312-320, 2017.

DE FREITAS, Cleverson Diniz Teixeira et al. Osmotin from Calotropis procera latex: new insights into structure and antifungal properties. **Biochimica et Biophysica Acta (BBA)-Biomembranes**, v. 1808, n. 10, p. 2501-2507, 2011.

DE FREITAS, Cleverson Diniz Teixeira et al. Osmotin purified from the latex of Calotropis procera: biochemical characterization, biological activity and role in plant defense. **Plant physiology and biochemistry**, v. 49, n. 7, p. 738-743, 2011.

DE OLIVEIRA NASCIMENTO, Danielle Cristina et al. Latex protein extracts from Calotropis procera with immunomodulatory properties protect against experimental infections with Listeria monocytogenes. **Phytomedicine**, v. 23, n. 7, p. 745-753, 2016.

DE OLIVEIRA, Jefferson Soares et al. In vitro cytotoxicity against different human cancer cell lines of laticifer proteins of Calotropis procera (Ait.) R. Br. **Toxicology in vitro**, v. 21, n. 8, p. 1563-1573, 2007.

DE OLIVEIRA, Keciany Alves et al. Phytomodulatory proteins isolated from Calotropis procera latex promote glycemic control by improving hepatic mitochondrial function in HepG2 cells. **Saudi Pharmaceutical Journal**, v. 29, n. 9, p. 1061-1069, 2021.

DEINLEIN, Ulrich et al. Plant salt-tolerance mechanisms. **Trends in plant science**, v. 19, n. 6, p. 371-379, 2014.

DEKOCK, P. C.; GRABOWSKA, FLORA B.; INNES, ALISON M. The effect of salicylic acid on the growth of Lemna gibba. **Annals of Botany**, v. 38, n. 4, p. 903-908, 1974.

DEMARCO, Diego; DE MORAES CASTRO, Marilia; ASCENSÃO, Lia. Two laticifer systems in Sapium haematospermum—new records for Euphorbiaceae. **Botany**, v. 91, n. 8, p. 545-554, 2013.

DEMARCO, Diego; CASTRO, Marilia de Moraes. Anastomosing articulated laticifers in species of Asclepiadeae (Asclepiadoideae, Apocynaceae) and their ecological implications. **Brazilian Journal of Botany**, v. 31, p. 701-713, 2008.

DEMPSEY, D.'Maris Amick et al. Salicylic acid biosynthesis and metabolism. **The Arabidopsis book/American Society of Plant Biologists**, v. 9, 2011.

DGHIM, Fairouz et al. Laticifers identification and natural rubber characterization from the latex of Periploca angustifolia Labill. (Apocynaceae). **Flora-Morphology, Distribution, Functional Ecology of Plants**, v. 217, p. 90-98, 2015.

DHILEEPAN, K. Prospects for the classical biological control of Calotropis procera (Apocynaceae) using coevolved insects. **Biocontrol Science and Technology**, v. 24, n. 9, p. 977-998, 2014.

DHIR, S. K. et al. Development of laticifer cells in callus cultures of Calotropis procera (Ait.) R. Br. **Plant cell reports**, v. 3, p. 206-209, 1984.

DIAZ-MENDOZA, Mercedes et al. Plant senescence and proteolysis: two processes with one destiny. **Genetics and molecular biology**, v. 39, p. 329-338, 2016.

DICKE, Marcel; VAN LOON, Joop JA. Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. **Entomologia experimentalis et applicata**, v. 97, n. 3, p. 237-249, 2000.

DING, Chang-Kui et al. Jasmonate and salicylate induce the expression of pathogenesis-related-protein genes and increase resistance to chilling injury in tomato fruit. **Planta**, v. 214, p. 895-901, 2002.

D'IPPÓLITO, Sebastián et al. Role of proteases in the response of plants to drought. **Plant Physiology and Biochemistry**, v. 168, p. 1-9, 2021.

DISSANAYAKE, Bhagya M. et al. Distinct salinity-induced changes in wheat metabolic machinery in different root tissue types. **Journal of Proteomics**, v. 256, p. 104502, 2022.

DOGAN, Muhammet. Effect of salt stress on in vitro organogenesis from nodal explant of Limnophila aromatica (Lamk.) Merr. and Bacopa monnieri (L.) Wettst. and their physio-morphological and biochemical responses. **Physiology and molecular biology of plants**, v. 26, p. 803-816, 2020.

DONG, Juane et al. Investigation on ultrasound-assisted extraction of salvianolic acid B from Salvia miltiorrhiza root. **Ultrasonics Sonochemistry**, v. 17, n. 1, p. 61-65, 2010.

DURÁN, Mateo David León; ZABALA, Mario Evelio Arias; LONDOÑO, Guillermo Antonio Correa. Optimization of flavonoid production in plant cell culture of Thevetia peruviana elicited with methyl jasmonate and salicylic acid. **Brazilian Archives of Biology and Technology**, v. 64, p. e21210022, 2021.

EDER, Jürgen; COSIO, Eric G. Elicitors of plant defense responses. In: **International review of cytology**. Academic Press, p. 1-36, 1994.

ELSAHOOKIE, M. Some Environmental. **Data Concerns Crop Productivity in Baghdad Dept. of Field Crop, Coll. of Agric., Univ. of Baghdad**, p. 13, 2013.

EL-TAYEB, M. A. Response of barley grains to the interactive e. ect of salinity and salicylic acid. **Plant growth regulation**, v. 45, p. 215-224, 2005.

ENYEDI, Alexander J. et al. Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. **Proceedings of the National Academy of Sciences**, v. 89, n. 6, p. 2480-2484, 1992.

ENYEDI, Alexander J. et al. Signal molecules in systemic plant resistance to pathogens and pests. **Cell**, v. 70, n. 6, p. 879-886, 1992.

ESPINOSA-LEAL, Claudia A.; PUENTE-GARZA, César A.; GARCÍA-LARA, Silverio. In vitro plant tissue culture: means for production of biological active compounds. **Planta**, v. 248, p. 1-18, 2018.

ETESAMI, Hassan; FATEMI, Hamideh; RIZWAN, Muhammad. Interactions of nanoparticles and salinity stress at physiological, biochemical and molecular levels in plants: A review. **Ecotoxicology and Environmental Safety**, v. 225, p. 112769, 2021.

EVERT, Ray F. **Esau's plant anatomy**: meristems, cells, and tissues of the plant body: their structure, function, and development. John Wiley & Sons, 2006.

FAILLACE, Giulia Ramos et al. Genome-wide analysis and evolution of plant thaumatin-like proteins: a focus on the origin and diversification of osmotins. **Molecular Genetics and Genomics**, v. 294, p. 1137-1157, 2019.

FAN, Linlin et al. Amelioration of postharvest chilling injury in cowpea (Vigna sinensis) by methyl jasmonate (MeJA) treatments. **Scientia horticulturae**, v. 203, p. 95-101, 2016.

FARMER, Edward E.; JOHNSON, Russell R.; RYAN, Clarence A. Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. **Plant Physiology**, v. 98, n. 3, p. 995-1002, 1992.

FARRELL, Brian D.; DUSSOURD, David E.; MITTER, Charles. Escalation of plant defense: do latex and resin canals spur plant diversification? **The American Naturalist**, v. 138, n. 4, p. 881-900, 1991.

FATMA, Mehar et al. Excess sulfur supplementation improves photosynthesis and growth in mustard under salt stress through increased production of glutathione. **Environmental and Experimental Botany**, v. 107, p. 55-63, 2014.

FISCHER, Joern et al. Reframing the food–biodiversity challenge. **Trends in Ecology & Evolution**, v. 32, n. 5, p. 335-345, 2017.

FISCHER, R. A.; BYERLEE, Derek R.; EDMEADES, Gregory O. **Can technology deliver on the yield challenge to 2050?** 2009.

FLOWERS, T. J. Improving crop salt tolerance. **Journal of Experimental botany**, v. 55, n. 396, p. 307-319, 2004.

FLOWERS, Timothy J. et al. Salt sensitivity in chickpea. **Plant, cell & environment**, v. 33, n. 4, p. 490-509, 2010.

FLOWERS, Timothy J.; COLMER, Timothy D. Salinity tolerance in halophytes. **New phytologist**, p. 945-963, 2008.

FLOWERS, Timothy J.; GLENN, Edward P.; VOLKOV, Vadim. Could vesicular transport of Na+ and Cl–be a feature of salt tolerance in halophytes? **Annals of botany**, v. 123, n. 1, p. 1-18, 2019.

FREITAS, Cleverson Diniz T. et al. Enzymatic activities and protein profile of latex from Calotropis procera. **Plant Physiology and Biochemistry**, v. 45, n. 10-11, p. 781-789, 2007.

FREITAS, Cleverson DT et al. Insights into milk-clotting activity of latex peptidases from Calotropis procera and Cryptostegia grandiflora. **Food Research International**, v. 87, p. 50-59, 2016.

FREITAS, Cleverson DT et al. New constitutive latex osmotin-like proteins lacking antifungal activity. **Plant Physiology and Biochemistry**, v. 96, p. 45-52, 2015.

FROSI, Gabriella et al. Ecophysiological performance of Calotropis procera: an exotic and evergreen species in Caatinga, Brazilian semi-arid. **Acta Physiologiae Plantarum**, v. 35, p. 335-344, 2013.

FUJITA, Miki et al. Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. **Current opinion in plant biology**, v. 9, n. 4, p. 436-442, 2006.

GAJ, Malgorzata D. Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to Arabidopsis thaliana (L.) Heynh. **Plant Growth Regulation**, v. 43, p. 27-47, 2004.

GAMBORG, Oluf L.; SHYLUK, Jerry P. Nutrition, media and characteristics of plant cell and tissue cultures. 1982.

GARG, ROHINI et al. Deep transcriptome sequencing of wild halophyte rice, Porteresia coarctata, provides novel insights into the salinity and submergence tolerance factors. **DNA research**, v. 21, n. 1, p. 69-84, 2014.

GAXIOLA, Roberto; REGMI, Kamesh; HIRSCHI, Kendal D. Moving on Up. **Trends in Biotechnology**, 2016.

GAY, Craig; COLLINS, James; GEBICKI, Janusz M. Hydroperoxide assay with the ferric–xylenol orange complex. **Analytical biochemistry**, v. 273, n. 2, p. 149-155, 1999.

GHAFIYEHSANJ, E. et al. The effects of salicylic acid on some of biochemical characteristics of wheat (Triticum aestivum L.) under salinity stress. **Annals of Biological Research**, v. 4, n. 6, p. 242-248, 2013.

GHANE, Savaliram Goga; LOKHANDE, Vinayak Haribhau; NIKAM, Tukaram Dayaram. Growth, physiological, and biochemical responses in relation to salinity tolerance for In Vitro selection in oil seed crop Guizotia abyssinica Cass. **Journal of Crop Science and Biotechnology**, v. 17, p. 11-20, 2014.

GIRI, Charu Chandra; ZAHEER, Mohd. Chemical elicitors versus secondary metabolite production in vitro using plant cell, tissue and organ cultures: recent trends and a sky eye view appraisal. **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 126, p. 1-18, 2016.

GIRI, Lalit et al. In vitro production of phenolic compounds and antioxidant activity in callus suspension cultures of Habenaria edgeworthii: A rare Himalayan medicinal orchid. **Industrial crops and products**, v. 39, p. 1-6, 2012.

GODSON, Alice; VAN DER HOORN, Renier AL. The front line of defence: a meta-analysis of apoplastic proteases in plant immunity. **Journal of Experimental Botany**, v. 72, n. 9, p. 3381-3394, 2021.

GOŁĘBIOWSKA-PIKANIA, Gabriela et al. Changes in phenolic acid abundance involved in low temperature and Microdochium nivale (Samuels and Hallett) cross-tolerance in winter triticale (x Triticosecale Wittmack). **Acta Physiologiae Plantarum**, v. 41, p. 1-14, 2019.

GOLKAR, Pooran; TAGHIZADEH, Marzieh; YOUSEFIAN, Zeinab. The effects of chitosan and salicylic acid on elicitation of secondary metabolites and antioxidant activity of safflower under in vitro salinity stress. **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 137, p. 575-585, 2019.

GOMES, Maria Angélica da Conceição et al. Salinity effects on photosynthetic pigments, proline, biomass and nitric oxide in Salvinia auriculata Aubl. **Acta Limnologica Brasiliensia**, v. 29, 2017.

GONDIM, Franklin Aragão et al. Catalase plays a key role in salt stress acclimation induced by hydrogen peroxide pretreatment in maize. **Plant Physiology and Biochemistry**, v. 56, p. 62-71, 2012.

GROVER, Anita. Plant chitinases: genetic diversity and physiological roles. **Critical reviews in plant sciences**, v. 31, n. 1, p. 57-73, 2012.

GUAN, Rongxia et al. Salinity tolerance in soybean is modulated by natural variation in G m SALT 3. **The Plant Journal**, v. 80, n. 6, p. 937-950, 2014.

GULL, Audil; LONE, Ajaz Ahmad; WANI, Noor Ul Islam. Biotic and abiotic stresses in plants. **Abiotic and biotic stress in plants**, p. 1-19, 2019.

GULZAR, Basit et al. Plant tissue culture: agriculture and industrial applications. In: **Transgenic technology-based value addition in plant biotechnology**. Academic Press, p. 25-49, 2020.

GUNDLACH, Heidrun et al. Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. **Proceedings of the National Academy of Sciences**, v. 89, n. 6, p. 2389-2393, 1992.

GUO, Ze-Jian et al. Overexpression of the AP2/EREBP transcription factor OPBP1 enhances disease resistance and salt tolerance in tobacco. **Plant Molecular Biology**, v. 55, p. 607-618, 2004.

GUPTA, Pratibha; SHARMA, Satyawati; SAXENA, Sanjay. Effect of abiotic stress on growth parameters and steviol glycoside content in Stevia rebaudiana (Bertoni) raised in vitro. **Journal of Applied Research on Medicinal and Aromatic Plants**, v. 3, n. 4, p. 160-167, 2016.

GUPTA, Sneha et al. Alleviation of salinity stress in plants by endophytic plant-fungal symbiosis: Current knowledge, perspectives and future directions. **Plant and Soil**, v. 461, p. 219-244, 2021.

GURIB-FAKIM, Ameenah. Medicinal plants: traditions of yesterday and drugs of tomorrow. **Molecular aspects of Medicine**, v. 27, n. 1, p. 1-93, 2006.

HADDAD, Moawiya A. et al. Herbs and medicinal plants in Jordan. **Journal of AOAC International**, v. 103, n. 4, p. 925-929, 2020.

HADI, M. R.; KHAVARINEJAD, R.; GHOSHKOLGH-SIMA, N. A. NaCl Effects on Accumulation of Minerals (Na+, K+, Cl^-) and Proline in Triticum turgidum L. **Asian Journal of Plant Sciences**, 2007.

HAGEL, Jillian M.; YEUNG, Edward C.; FACCHINI, Peter J. Got milk? The secret life of laticifers. **Trends in plant science**, v. 13, n. 12, p. 631-639, 2008.

HAKKIM, F. Lukmanul; SHANKAR, C. Gowri; GIRIJA, S. Chemical composition and antioxidant property of holy basil (Ocimum sanctum L.) leaves, stems, and inflorescence and their in vitro callus cultures. **Journal of agricultural and food chemistry**, v. 55, n. 22, p. 9109-9117, 2007.

HASEGAWA, P. M. et al. Tissue culture in the improvement of salt tolerance in plants. In: **Soil mineral stresses: Approaches to crop improvement**. Berlin, Heidelberg: Springer Berlin Heidelberg, p. 83-125, 1994.

HASHEMI, Seyed Mohammad et al. Effects of abiotic elicitors on expression and accumulation of three candidate benzophenanthridine alkaloids in cultured greater celandine cells. **Molecules**, v. 26, n. 5, p. 1395, 2021.

HASSAN, Loutfy M. et al. The biology of Calotropis procera (Aiton) WT. **Trees**, v. 29, p. 311-320, 2015.

HAUSER, Felix; HORIE, Tomoaki. A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K+/Na+ ratio in leaves during salinity stress. **Plant, cell & environment**, v. 33, n. 4, p. 552-565, 2010.

HAVIR, Evelyn A.; MCHALE, Neil A. Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. **Plant physiology**, v. 84, n. 2, p. 450-455, 1987.

HAYAT, Qaiser et al. Effect of exogenous salicylic acid under changing environment: a review. **Environmental and experimental botany**, v. 68, n. 1, p. 14-25, 2010.

HEDRICK, Susan A. et al. Chitinase cDNA cloning and mRNA induction by fungal elicitor, wounding, and infection. **Plant Physiology**, v. 86, n. 1, p. 182-186, 1988.

HEMMATI, Niloofar; CHENIANY, Monireh; GANJEALI, Ali. Effect of plant growth regulators and explants on callus induction and study of antioxidant potentials and phenolic metabolites in Salvia tebesana Bunge. **Botanica serbica**, v. 44, n. 2, p. 163-173, 2020.

HERNANDEZ, Jose A. et al. Salt-induced oxidative stress mediated by activated oxygen species in pea leaf mitochondria. **Physiologia Plantarum**, v. 89, no. 1, p. 103-110, 1993.

HERWADE, Abhijeet P. et al. Characterization of a Bowman–Birk type trypsin inhibitor purified from seeds of Solanum surattense. **Scientific reports**, v. 11, n. 1, p. 8648, 2021.

HICKEY, Lee T. et al. Breeding crops to feed 10 billion. **Nature biotechnology**, v. 37, n. 7, p. 744-754, 2019.

HILLEL, Daniel. **Salinity management for sustainable irrigation: integrating science, environment, and economics**. World Bank Publications, 2000.

HINKSON, Izumi V.; ELIAS, Joshua E. The dynamic state of protein turnover: It's about time. **Trends in cell biology**, v. 21, no. 5, p. 293-303, 2011.

HINOJOSA, Leonardo et al. Quinoa abiotic stress responses: A review. **Plants**, v. 7, n. 4, p. 106, 2018.

HIRANO, Shigehiro; HAYASHI, Masahiko; OKUNO, Seiji. Soybean seeds surface-coated with depolymerised chitins: chitinase activity as a predictive index for the harvest of beans in field culture. **Journal of the Science of Food and Agriculture**, v. 81, n. 2, p. 205-209, 2001.

HIRAOKA, Noboru et al. Alkaloid production by somatic embryo cultures of Corydalis ambigua. **Plant Biotechnology**, v. 21, n. 5, p. 361-366, 2004.

HO, Thanh-Tam; MURTHY, Hosakatte Niranjana; PARK, So-Young. Methyl jasmonate induced oxidative stress and accumulation of secondary metabolites in plant cell and organ cultures. **International journal of molecular sciences**, v. 21, n. 3, p. 716, 2020.

HON, Wai-Ching et al. Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. **Plant physiology**, v. 109, n. 3, p. 879-889, 1995.

HOPKINS, William G. **Introduction to plant physiology**. John Wiley & Sons, 2008.

HORVÁTH, E. et al. Exogenous 4-hydroxybenzoic acid and salicylic acid modulate the effect of short-term drought and freezing stress on wheat plants. **Biologia plantarum**, v. 51, p. 480-487, 2007.

HOSHAN, Muhsen N. Review of Reclamation of salinity affected soils by leaching and their effect on soil properties and plant growth. **Tikrit Journal for Agricultural Sciences** v. 22, n. 1, p. 149-168, 2022.

HUANG, Lu et al. The HKT transporter HvHKT1; 5 negatively regulates salt tolerance. **Plant Physiology**, v. 182, n. 1, p. 584-596, 2020.

HUTZLER, Peter et al. Tissue localization of phenolic compounds in plants by confocal laser scanning microscopy. **Journal of Experimental Botany**, v. 49, n. 323, p. 953-965, 1998.

IDM'HAND, Elhassan; MSANDA, Fouad; CHERIFI, Khalil. Ethnobotanical study and biodiversity of medicinal plants used in the Tarfaya Province, Morocco. **Acta Ecologica Sinica**, v. 40, n. 2, p. 134-144, 2020.

IQBAL, Zafar et al. Anthelmintic activity of Calotropis procera (Ait.) Ait. F. flowers in sheep. **Journal of ethnopharmacology**, v. 102, n. 2, p. 256-261, 2005.

ISAH, Tasiu et al. Secondary metabolism of pharmaceuticals in the plant in vitro cultures: strategies, approaches, and limitations to achieving higher yield. **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 132, p. 239-265, 2018.

ISAYENKOV, S. V. Physiological and molecular aspects of salt stress in plants. **Cytology and Genetics**, v. 46, n. 5, p. 302-318, 2012.

JACOMINI, Débora et al. Lipid profile and antiproliferative activity of callus cultures of Cereus peruvianus Mill. **Industrial Crops and Products**, v. 69, p. 408-414, 2015.

JAFARINIA, M.; SHARIATI, M. Effects of salt stress on photosystem II of canola plant (Barassica napus, L.) probing by chlorophyll a fluorescence measurement. **Iranian Journal of Science**, v. 36, n. 1, p. 71-76, 2012.

JANARDHAN REDDY, P.; VAIDYANATH, K. In vitro characterization of salt stress effects and the selection of salt tolerant plants in rice (Oryza sativa L.). **Theoretical and applied genetics**, v. 71, p. 757-760, 1986.

JANDA, Katalin et al. Salicylic acid may indirectly influence the photosynthetic electron transport. **Journal of Plant Physiology**, v. 169, n. 10, p. 971-978, 2012.

JAVID, Majid Ghorbani et al. The role of phytohormones in alleviating salt stress in crop plants. **Australian Journal of Crop Science**, v. 5, n. 6, p. 726-734, 2011.

JESUS, Ana et al. A morphometric approach to track opium poppy domestication. **Scientific reports**, v. 11, n. 1, p. 9778, 2021.

JEYASRI, Rajendran et al. Methyl jasmonate and salicylic acid as powerful elicitors for enhancing the production of secondary metabolites in medicinal plants: an updated review. **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 153, n. 3, p. 447-458, 2023.

JHA, Shweta. Proteome responses of pearl millet genotypes under salinity. **Plant Gene**, v. 29, p. 100347, 2022.

JIANG, Jingjing et al. WRKY transcription factors in plant responses to stresses. **Journal of integrative plant biology**, v. 59, n. 2, p. 86-101, 2017.

JUNCKER, Tom et al. UNBS1450 from Calotropis procera as a regulator of signaling pathways involved in proliferation and cell death. **Biochemical Pharmacology**, v. 78, n. 1, p. 1-10, 2009.

KAINAMA, Healthy et al. The relationship of free radical scavenging and total phenolic and flavonoid contents of Garcinia lasoar PAM. **Pharmaceutical Chemistry Journal**, v. 53, p. 1151-1157, 2020.

KALARIYA, Kuldeepsingh A. et al. Effect of genotypes and foliar spray of methyl jasmonate and salicylic acid on andrographolide yield in Andrographis paniculata (Burm. f.) Wall. ex Nees. under semi-arid climate. **Bulletin of the National Research Centre**, v. 47, n. 1, p. 1-13, 2023.

KANDHOL, Nidhi; JAIN, Mukesh; TRIPATHI, Durgesh Kumar. Nanoparticles as potential hallmarks of drought stress tolerance in plants. **Physiologia Plantarum**, v. 174, n. 2, p. e13665, 2022.

KARAKAS, Fatma Pehlivan; TURKER, Arzu Ucar. Improvement of shoot proliferation and comparison of secondary metabolites in shoot and callus cultures of Phlomis armeniaca by LC-ESI-MS/MS analysis. **In Vitro Cellular & Developmental Biology-Plant**, v. 52, p. 608-618, 2016.

KARAR, Mohamed Gamaleldin Elsadig; KUHNERT, Nikolai. Herbal drugs from Sudan: Traditional uses and phytoconstituents. **Pharmacognosy reviews**, v. 11, n. 22, p. 83, 2017.

KASPRZEWSKA, A. N. N. A. Plant chitinases-regulation and function. **Cellular and Molecular Biology Letters**, v. 8, n. 3, p. 809-824, 2003.

KAUR, Amanpreet; KUMAR, Anil. Cloning and characterization of Osmotin (OsmSt) alleles from potato cultivar ‘Kufri Chipsona 1’. **bioRxiv**, p. 2020.01. 03.894030, 2020.

KAUR, Amarpreet et al. An overview of the characteristics and potential of Calotropis procera from botanical, ecological, and economic perspectives. **Frontiers in Plant Science**, v. 12, p. 690806, 2021.

KAYA, C.; TUNA, A. L.; YOKAŞ, I. The role of plant hormones in plants under salinity stress. **Salinity and water stress improving crop efficiency**, p. 45-50, 2009.

KELIJ, Sedigheh et al. Activation of lignin biosynthetic enzymes during internodal development of Aeluropus littoralis exposed to NaCl. **Journal of Genetic Resources**, v. 1, n. 1, p. 19-24, 2015.

KETCHUM, Raymond EB et al. The kinetics of taxoid accumulation in cell suspension cultures of Taxus following elicitation with methyl jasmonate. **Biotechnology and bioengineering**, v. 62, n. 1, p. 97-105, 1999.

KHALID, Noreen et al. Vehicle pollution toxicity induced changes in physiology, defence system and biochemical characteristics of Calotropis procera L. **Chemistry and Ecology**, v. 34, n. 6, p. 565-581, 2018.

KHAN, Faheema; HAKEEM, Khalid Rehman. Cell signaling during drought and salt stress. **Plant signaling understanding the molecular crosstalk**, p. 227-239, 2014.

KHAN, M. Iqbal R. et al. Salicylic acid-induced abiotic stress tolerance and underlying mechanisms in plants. **Frontiers in plant science**, v. 6, p. 462, 2015.

KHATTAB, Salah; YAP, Yun-Kiam; EL SHERIF, Fadia. Salicylic Acid Foliar Spray Enhanced Silybum marianum Growth and Yield, as Well as Its Chemical Constituents and Chalcone Synthase Gene Activity. **Horticulturae**, v. 8, n. 6, p. 556, 2022.

KHUDOLIEIEVA, Lidiya; KUTSOKON, Nataliya. In vitro evaluation of salt tolerance of poplars and willows. 2018.

KHURSHID, Razia et al. Biosynthesis of precious metabolites in callus cultures of Eclipta alba. **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 135, p. 287-298, 2018.

KIDRIČ, Marjetka et al. Proteases and their endogenous inhibitors in the plant response to abiotic stress. **Botanica serbica**, v. 38, n. 1, p. 139-158, 2014.

KIDWAI, Maria; AHMAD, Iffat Zareen; CHAKRABARTY, Debasis. Class III peroxidase: An indispensable enzyme for biotic/abiotic stress tolerance and a potent candidate for crop improvement. **Plant cell reports**, v. 39, p. 1381-1393, 2020.

KIM, Doo Hwan; GOPAL, Judy; SIVANESAN, Iyyakkannu. Nanomaterials in plant tissue culture: the disclosed and undisclosed. **RSC advances**, v. 7, n. 58, p. 36492-36505, 2017.

KIM, Hoyeun et al. Molecular cloning and characterization of the gene encoding osmotin protein in Petunia hybrida. **Plant Science**, v. 162, n. 5, p. 745-752, 2002.

KLAIS, Christina; BÜHREN, Jens; KOHNEN, Thomas. Comparison of endothelial cell count using confocal and contact specular microscopy. **Ophthalmologica**, v. 217, n. 2, p. 99-103, 2003.

KONNO, Kotaro. Plant latex and other exudates as plant defense systems: roles of various defense chemicals and proteins contained therein. **Phytochemistry**, v. 72, n. 13, p. 1510-1530, 2011.

KOSHIBA, Tomokazu. Cytosolic ascorbate peroxidase in seedlings and leaves of maize (Zea mays). **Plant and Cell Physiology**, v. 34, n. 5, p. 713-721, 1993.

KOSHIOKA, M. et al. Identification of endogenous gibberellins in stems and leaves in vegetative growth stage of Gentiana triflora. **Journal of plant physiology**, v. 153, n. 1-2, p. 230-232, 1998.

KOSOVÁ, Klára et al. Plant proteome responses to salinity stress–comparison of glycophytes and halophytes. **Functional Plant Biology**, v. 40, n. 9, p. 775-786, 2013.

KOUTOUA, Ayolié et al. **NaCl stress induced growth, water and ions contents changes on in vitro selection of salt tolerant and salt sensitive callus of wheat**. 2011.

KOVÁCS, Judit et al. Salicylic acid induced cysteine protease activity during programmed cell death in tomato plants. **Acta Biologica Hungarica**, v. 67, n. 2, p. 148-158, 2016.

KRUGLOVA, N. N.; SELDIMIROVA, O. A.; ZINATULLINA, A. E. In vitro callus as a model system for the study of plant stress-resistance to abiotic factors (on the example of cereals). **Biology Bulletin Reviews**, v. 8, p. 518-526, 2018.

KSOURI, Riadh et al. Salinity effects on polyphenol content and antioxidant activities in leaves of the halophyte Cakile maritima. **Plant Physiology and Biochemistry**, v. 45, n. 3-4, p. 244-249, 2007.

KUMAR, Nitish et al. Effects of NaCl on growth, ion accumulation, protein, proline contents and antioxidant enzymes activity in callus cultures of Jatropha curcas. **Biologia**, v. 63, n. 3, p. 378-382, 2008.

KUMAR, S. Anil et al. Beyond just being foot soldiers–osmotin like protein (OLP) and chitinase (Chi11) genes act as sentinels to confront salt, drought, and fungal stress tolerance in tomato. **Environmental and Experimental Botany**, v. 132, p. 53-65, 2016.

KUMAR, Suresh et al. Anti-diarrhoeal activity of the latex of Calotropis procera. **Journal of Ethnopharmacology**, v. 76, n. 1, p. 115-118, 2001.

KUMAR, SURESH et al. Recent advances in understanding the role of growth regulators in plant growth and development in vitro-I: conventional growth regulators. **Indian Forester**, v. 142, n. 5, p. 459-470, 2016.

KUMAR, V. L.; ARYA, S. Medicinal uses and pharmacological properties of Calotropis procera. **Recent progress in medicinal plants**, v. 11, p. 373-388, 2006.

KUMARI, Nisha et al. Biotic elicitation: A tool for producing bioactive compounds. **Plant Arch**, v. 20, p. 3385-3391, 2020.

KUMUDINI, Belur S. et al. Primary plant metabolism during plant–pathogen interactions and its role in defense. **Plant metabolites and regulation under environmental stress**, p. 215-229, 2018.

KUPCHAK, Brian R. et al. Dissecting the regulation of yeast genes by the osmotin receptor. **Biochemical and Biophysical Research Communications**, v. 374, n. 2, p. 210-213, 2008.

LAEMMLI, Ulrich Karl. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **nature**, v. 227, n. 5259, p. 680-685, 1970.

LAN, Ping et al. iTRAQ protein profile analysis of Arabidopsis roots reveals new aspects critical for iron homeostasis. **Plant physiology**, v. 155, n. 2, p. 821-834, 2011.

LAROSA, P. Christopher et al. Abscisic acid accelerates adaptation of cultured tobacco cells to salt. **Plant Physiology**, v. 79, n. 1, p. 138-142, 1985.

LATA, Charu; PRASAD, Manoj. Role of DREBs in regulation of abiotic stress responses in plants. **Journal of experimental botany**, v. 62, n. 14, p. 4731-4748, 2011.

LEAL, Laura C. et al. Germination responses of the invasive Calotropis procera (Ait.) R. Br.(Apocynaceae): comparisons with seeds from two ecosystems in northeastern Brazil. **Anais da Academia Brasileira de Ciências**, v. 85, p. 1025-1034, 2013.

LEANDRO, Mariana Ramos et al. DegP protease is essential for tolerance to salt stress in the plant growth-promoting bacterium Gluconacetobacter diazotrophicus PAL5. **Microbiological Research**, v. 243, p. 126654, 2021.

LEE, Hyung-Il; LEON, Jose; RASKIN, Ilya. Biosynthesis and metabolism of salicylic acid. **Proceedings of the national academy of sciences**, v. 92, n. 10, p. 4076-4079, 1995.

LI, Ping et al. Drought stress impact on leaf proteome variations of faba bean (Vicia faba L.) in the Qinghai–Tibet Plateau of China. **3 Biotech**, v. 8, p. 1-12, 2018.

LIANG, Jin-gang et al. Expression profiles of Cry1Ab protein and its insecticidal efficacy against the invasive fall armyworm for Chinese domestic GM maize DBN9936. **Journal of Integrative Agriculture**, v. 20, n. 3, p. 792-803, 2021.

LICHTENTHALER, Hartmut K. [34] Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. In: **Methods in enzymology**. Academic Press, p. 350-382, 1987.

LILA, Mary Ann. 24 Valuable Secondary Products from In Vitro Culture. **Plant development and biotechnology**, p. 285, 2004.

LILIANE, B. de A. et al. Arabidopsis thaliana class IV chitinase is early induced during the interaction with Xanthomonas campestris. **Febs Letters**, v. 419, n. 1, p. 69-75, 1997.

LIU, D. O. N. G. et al. Osmotin overexpression in potato delays development of disease symptoms. **Proceedings of the National Academy of Sciences**, v. 91, n. 5, p. 1888-1892, 1994.

LIU, Xiangguo et al. A Na2CO3-responsive chitinase gene from Leymus chinensis improve pathogen resistance and saline-alkali stress tolerance in transgenic tobacco and maize. **Frontiers in plant science**, v. 11, p. 504, 2020.

LOIZOU, Stella et al. β-Sitosterol exhibits anti-inflammatory activity in human aortic endothelial cells. **Molecular nutrition & food research**, v. 54, n. 4, p. 551-558, 2010.

LOKHANDE, Vinayak Haribhau; NIKAM, Tukaram Dayaram; PENNA, Suprasanna. Biochemical, physiological and growth changes in response to salinity in callus cultures of Sesuvium portulacastrum L. **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 102, p. 17-25, 2010.

LOOZE, Yvan et al. Purification and characterization of a wound-inducible thaumatin-like protein from the latex of Carica papaya. **Phytochemistry**, v. 70, n. 8, p. 970-978, 2009.

LOSVIK, Aleksandra et al. Overexpression of the aphid-induced serine protease inhibitor CI2c gene in barley affects the generalist green peach aphid, not the specialist bird cherry-oat aphid. **PLoS One**, v. 13, n. 3, p. e0193816, 2018.

LOTTERMOSER, Bernd G. Colonisation of the rehabilitated Mary Kathleen uranium mine site (Australia) by Calotropis procera: toxicity risk to grazing animals. **Journal of Geochemical Exploration**, v. 111, n. 1-2, p. 39-46, 2011.

LUO, Meijie et al. Comparative proteomics of contrasting maize genotypes provides insights into salt-stress tolerance mechanisms. **Journal of proteome research**, v. 17, n. 1, p. 141-153, 2018.

LUO, Shengji; KIM, Chanhong. Current understanding of temperature stress-responsive chloroplast FtsH metalloproteases. **International Journal of Molecular Sciences**, v. 22, n. 22, p. 12106, 2021.

MAHENDRAN, Ganesan et al. Elicitation enhances swerchirin and 1, 2, 5, 6-tetrahydroxyxanthone production in hairy root cultures of Swertia chirayita (Roxb.) H. Karst. **Industrial Crops and Products**, v. 177, p. 114488, 2022.

MALAMY, Jocelyn et al. Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. **Science**, v. 250, n. 4983, p. 1002-1004, 1990.

MALIK, Sonia et al. Production of the anticancer drug taxol in Taxus baccata suspension cultures: a review. **Process Biochemistry**, v. 46, n. 1, p. 23-34, 2011.

MANI, Tomson; SIVAKUMAR, K. C.; MANJULA, S. Expression and functional analysis of two osmotin (PR5) isoforms with differential antifungal activity from Piper colubrinum: prediction of structure–function relationship by bioinformatics approach. **Molecular biotechnology**, v. 52, p. 251-261, 2012.

MANSOUR, Mohamed Magdy Fahim et al. Sorghum under saline conditions: Responses, tolerance mechanisms, and management strategies. **Planta**, v. 254, p. 1-38, 2021.

MARIANI, Luigi; FERRANTE, Antonio. Agronomic management for enhancing plant tolerance to abiotic stresses—drought, salinity, hypoxia, and lodging. **Horticulturae**, v. 3, n. 4, p. 52, 2017.

MARINHO, Cristina Ribeiro et al. Laticifer distribution in fig inflorescence and its potential role in the fig-fig wasp mutualism. **Acta Oecologica**, v. 90, p. 160-167, 2018.

MATHUR, Rajani et al. Anti-tumor studies with extracts of Calotropis procera (Ait.) R. Br. root employing Hep2 cells and their possible mechanism of action. **Indian J Exp Biol**, 47(5):343-8, 2009.

MCCOY, V. E.; WAPPLER, T.; LABANDEIRA, C. C. Exceptional fossilization of ecological interactions: Plant defenses during the four major expansions of arthropod herbivory in the fossil record. Fossilization: **The Material Nature of Ancient Plants and Animals in the Paleontological Record**, p. 187-220, 2021.

MCCOY, Victoria E. et al. Oldest fossil evidence of latex sabotaging behavior by herbivorous insects. **Review of Palaeobotany and Palynology**, v. 300, p. 104631, 2022.

MENDOZA, Dary et al. Effect of salicylic acid and methyl jasmonate in the production of phenolic compounds in plant cell suspension cultures of Thevetia peruviana. **Biotechnology reports**, v. 19, p. e00273, 2018.

MENGE, Enock O. et al. Density-dependent reproduction and pollen limitation in an invasive milkweed, Calotropis procera (Ait.) R. Br. (Apocynaceae). **Austral Ecology**, v. 42, n. 1, p. 61-71, 2017.

MENGE, EO; BELLAIRS, SM; LAWES, MJ Seed-germination responses of Calotropis procera (Asclepiadaceae) to temperature and water stress in northern Australia. **Australian Journal of Botany**, vol. 64, no. 5, p. 441-450, 2016.

METCALFE, Charles R. Distribution of latex in the plant kingdom. **Economic Botany**, v. 21, p. 115-127, 1967.

MÉTRAUX, J. P. et al. Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. **Science**, v. 250, n. 4983, p. 1004-1006, 1990.

MICLEA, Ileana et al. Effect of jasmonic acid and salicylic acid on growth and biochemical composition of in-vitro-propagated Lavandula angustifolia Mill. **Agronomy**, v. 10, n. 11, p. 1722, 2020.

MIR, Mohammad Yaseen et al. Sneak peek of Hypericum perforatum L.: phytochemistry, phytochemical efficacy and biotechnological interventions. **Journal of Plant Biochemistry and Biotechnology**, v. 28, p. 357-373, 2019.

MIR, Zahoor Ahmad et al. Genome-wide identification and characterization of Chitinase gene family in Brassica juncea and Camelina sativa in response to Alternaria brassicae. **Genomics**, v. 112, n. 1, p. 749-763, 2020.

MISHRA, A.; CHOUDHURI, M. A. Effects of salicylic acid on heavy metal-induced membrane deterioration mediated by lipoxygenase in rice. **Biologia Plantarum**, v. 42, p. 409-415, 1999.

MITTLER, Ron. Oxidative stress, antioxidants and stress tolerance. **Trends in plant science**, v. 7, n. 9, p. 405-410, 2002.

MIZUKAMI, Hajime; TABIRA, Yukari; ELLIS, Brian E. Methyl jasmonate-induced rosmarinic acid biosynthesis in Lithospermum erythrorhizon cell suspension cultures. **Plant Cell Reports**, v. 12, p. 706-709, 1993.

MOHAMED, Nadia H. et al. Cytotoxic cardenolides from the latex of Calotropis procera. **Bioorganic & medicinal chemistry letters**, v. 25, n. 20, p. 4615-4620, 2015.

MOORE, Anthony L. et al. Function of the alternative oxidase: is it still a scavenger? **Trends in Plant Science**, v. 7, n. 11, p. 478-481, 2002.

MORRELL, Rebecca; SADANANDOM, Ari. Dealing with stress: a review of plant SUMO proteases. **Frontiers in plant science**, v. 10, p. 1122, 2019.

MOSOLOV, V. V.; VALUEVA, T. A. Inhibitors of proteolytic enzymes under abiotic stresses in plants. **Applied Biochemistry and Microbiology**, v. 47, p. 453-459, 2011.

MUHAMMAD IQBAL, Muhammad Iqbal et al. Leaf proteome analysis signified that photosynthesis and antioxidants are key indicators of salinity tolerance in canola (Brassica napus L.). **Pak. J. Bot.** 1955–1968, 2019.

MUJEEB-KAZI, Abdul et al. Breeding strategies for structuring salinity tolerance in wheat. **Advances in agronomy**, v. 155, p. 121-187, 2019.

MUKHOPADHYAY, Raj et al. Soil salinity under climate change: Challenges for sustainable agriculture and food security. **Journal of Environmental Management**, v. 280, p. 111736, 2021.

MUNNS, Rana et al. Energy costs of salt tolerance in crop plants. **New Phytologist**, v. 225, n. 3, p. 1072-1090, 2020.

MUNNS, Rana et al. Osmotic adjustment and energy limitations to plant growth in saline soil. **New Phytologist**, v. 225, n. 3, p. 1091-1096, 2020.

MUNNS, Rana. Comparative physiology of salt and water stress. **Plant, cell & environment**, v. 25, n. 2, p. 239-250, 2002.

MUNNS, Rana; GILLIHAM, Matthew. Salinity tolerance of crops–what is the cost? **New phytologist**, v. 208, n. 3, p. 668-673, 2015.

MUNNS, Rana; TESTER, Mark. Mechanisms of salinity tolerance. **Annu. Rev. Plant Biol.**, v. 59, p. 651-681, 2008.

MURASHIGE, Toshio; SKOOG, Folke. A revised medium for rapid growth and bio assays with tobacco tissue cultures. **Physiologia plantarum**, v. 15, n. 3, p. 473-497, 1962.

MURTHY, Hosakatte Niranjana; LEE, Eun-Jung; PAEK, Kee-Yoeup. Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 118, p. 1-16, 2014.

MURTI, Y.; YOGI, B.; PATHAK, D. Pharmacognostic standardization of leaves of Calotropis procera (Ait.) R. Br. (Asclepiadaceae). **International journal of Ayurveda research**, v. 1, n. 1, p. 14, 2010.

MUSTAFA, Hina et al. Biosynthesis and characterization of titanium dioxide nanoparticles and its effects along with calcium phosphate on physicochemical attributes of wheat under drought stress. **Ecotoxicology and Environmental Safety**, v. 223, p. 112519, 2021.

MUSTAFA, Natali R. et al. Initiation, growth and cryopreservation of plant cell suspension cultures. **Nature protocols**, v. 6, n. 6, p. 715-742, 2011.

MUTWAKIL, Mohammed Z. et al. Transcriptomic and metabolic responses of Calotropis procera to salt and drought stress. **BMC plant biology**, v. 17, p. 1-11, 2017.

NADEEM, Muhammad et al. Calotropis procera: UHPLC-QTOF-MS/MS based profiling of bioactives, antioxidant and anti-diabetic potential of leaf extracts and an insight into molecular docking. **Journal of Food Measurement and Characterization**, v. 13, p. 3206-3220, 2019.

NADIR, Dhuha S.; IBRAHEM, M. M.; AHMAD, Talfan A. Effect of methyl jasmonate on the production of furanocoumarins in callus cultures of Ruta graveolens L. **Indian Journal of Ecology**, v. 48, n. 3, p. 950-954, 2021.

NAIK, Poornananda M.; AL-KHAYRI, Jameel M. Abiotic and biotic elicitors-role in secondary metabolites production through in vitro culture of medicinal plants. **Abiotic and biotic stress in plants—recent advances and future perspectives. Rijeka: InTech**, p. 247-277, 2016.

NAMDEO, A. G. et al. Plant cell elicitation for production of secondary metabolites: a review. **Pharmacogn Rev**, v. 1, n. 1, p. 69-79, 2007.

NARASIMHAN, Meena L. et al. A plant defense response effector induces microbial apoptosis. **Molecular Cell**, v. 8, n. 4, p. 921-930, 2001.

NARAYANI, M.; SRIVASTAVA, Smita. Elicitation: a stimulation of stress in in vitro plant cell/tissue cultures for enhancement of secondary metabolite production. **Phytochemistry reviews**, v. 16, p. 1227-1252, 2017.

NASCIMENTO, T. L. et al. Biodiversity of endophytic fungi in different leaf ages of Calotropis procera and their antimicrobial activity. **Fungal ecology**, v. 14, p. 79-86, 2015.

NAWROT, Robert. **Latex, laticifers and their molecular components: from functions to possible applications**. Academic Press, 2020.

NAZAR, Rahat; UMAR, Shahid; KHAN, Nafees A. Exogenous salicylic acid improves photosynthesis and growth through increase in ascorbate-glutathione metabolism and S assimilation in mustard under salt stress. **Plant signaling & behavior**, v. 10, n. 3, p. e1003751, 2015.

NAZIR, Saher et al. Callus culture of Thai basil is an effective biological system for the production of antioxidants. **Molecules**, v. 25, n. 20, p. 4859, 2020.

NAZIR, Saher et al. Synergistic effects of salicylic acid and light stress on bioactive metabolites in basil callus cultures. **Biocatalysis and agricultural biotechnology**, v. 37, p. 102176, 2021.

NEALE, Alan D. et al. Chitinase, beta-1, 3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. **The Plant Cell**, v. 2, n. 7, p. 673-684, 1990.

NECHAEVA, T. L.; NIKOLAEVA, T. N.; ZAGOSKINA, N. V. Salicylic and hydroxybenzoic acids affect the accumulation of phenolic compounds in tea-plant cultures in vitro. **Biology Bulletin**, v. 47, p. 374-380, 2020.

NEUHAUS, J. -M. et al. A revised nomenclature for chitinase genes. **Plant Molecular Biology Reporter**, v. 14, p. 102-104, 1996.

NGCALA, Mamosa G. et al. Heat stress triggers differential protein accumulation in the extracellular matrix of sorghum cell suspension cultures. **Proteomes**, v. 8, n. 4, p. 29, 2020.

OBATA, Toshihiro; FERNIE, Alisdair R. The use of metabolomics to dissect plant responses to abiotic stresses. **Cellular and Molecular Life Sciences**, v. 69, p. 3225-3243, 2012.

ORAIBI, Amjad Ibraim; HAMAD, Maha N. Phytochemical investigation of flavanoid of Calotropis Procera in Iraq, Isolation and identification of rutin, quercitin and kampferol. **Journal of Pharmaceutical Sciences and Research**, v. 10, n. 9, p. 2407-2411, 2018.

OSAKABE, Yuriko et al. ABA control of plant macroelement membrane transport systems in response to water deficit and high salinity. **New Phytologist**, v. 202, n. 1, p. 35-49, 2014.

OSMAN, Nahid Abd Elhamid et al. Salts induced salinity and in vitro multiplication of Paronychia argentea. **Ecological Engineering & Environmental Technology**, v. 22, 2021.

PALMA, José M. et al. Plant proteases, protein degradation, and oxidative stress: role of peroxisomes. **Plant Physiology and Biochemistry**, v. 40, n. 6-8, p. 521-530, 2002.

PANDEY, Prachi et al. Impact of combined abiotic and biotic stresses on plant growth and avenues for crop improvement by exploiting physio-morphological traits. **Frontiers in plant science**, v. 8, p. 537, 2017.

PANDOLFI, Camilla; MANCUSO, Stefano; SHABALA, Sergey. Physiology of acclimation to salinity stress in pea (Pisum sativum). **Environmental and Experimental Botany**, v. 84, p. 44-51, 2012.

PARIDA, Asish Kumar; JHA, Bhavanath. Salt tolerance mechanisms in mangroves: a review. **Trees**, v. 24, n. 2, p. 199-217, 2010.

PARROTTA, John A. et al. **Healing plants of peninsular India**. CABI publishing, 2001.

PARTHIBHAN, S. et al. Somatic embryogenesis from stem thin cell layers of Dendrobium aqueum. **Biologia plantarum**, v. 62, p. 439-450, 2018.

PASSIOURA, J. B.; ANGUS, J. F. Improving productivity of crops in water-limited environments. **Advances in agronomy**, v. 106, p. 37-75, 2010.

PATADE, Vikas Yadav; SUPRASANNA, Penna; BAPAT, Vishwas Anant. Effects of salt stress in relation to osmotic adjustment on sugarcane (Saccharum officinarum L.) callus cultures. **Plant Growth Regulation**, v. 55, p. 169-173, 2008.

PATEL, Heena; KRISHNAMURTHY, Ramar. Elicitors in plant tissue culture. **Journal of Pharmacognosy and Phytochemistry**, v. 2, n. 2, p. 60-65, 2013.

PATHANIA, Shelly et al. Genus Calotropis: a hub of medicinally active phytoconstituents. **Current Traditional Medicine**, vol. 6, no. 4, p. 312-331, 2020.

PATTNAIK, Pratap Keshari et al. Chemometric profile & antimicrobial activities of leaf extract of Calotropis procera and Calotropis gigantea. **Natural product research**, v. 31, n. 16, p. 1954-1957, 2017.

PEIXOTO, Paulo Henrique Pereira et al. Aluminum effects on lipid peroxidation and on the activities of enzymes of oxidative metabolism in sorghum. **Revista Brasileira de Fisiologia Vegetal**, v. 11, n. 3, p. 137-143, 1999.

PENG, Jinying et al. Salt-induced stabilization of EIN3/EIL1 confers salinity tolerance by deterring ROS accumulation in Arabidopsis. **PLoS Genetics**, v. 10, n. 10, p. e1004664, 2014.

PHOO, Zan Win Moh Moh et al. Evaluation of Indian milkweed (Calotropis gigantea) seed oil as alternative feedstock for biodiesel. **Industrial Crops and Products**, v. 54, p. 226-232, 2014.

PIETERSE, Corné MJ; VAN LOON, Leendert C. Salicylic acid-independent plant defence pathways. **Trends in plant science**, v. 4, n. 2, p. 52-58, 1999.

PLUSKOTA, Wioletta E. et al. Jasmonic acid and ethylene are involved in the accumulation of osmotin in germinating tomato seeds. **Journal of plant physiology**, v. 232, p. 74-81, 2019.

POLYA, Gideon M. Protein and non-protein protease inhibitors from plants. **Studies in natural products chemistry**, v. 29, p. 567-641, 2003.

POMPELLI, Marcelo F. et al. Mesophyll thickness and sclerophylly among Calotropis procera morphotypes reveal water-saved adaptation to environments. **Journal of Arid Land**, v. 11, p. 795-810, 2019.

POÓR, P. et al. Salicylic acid treatment via the rooting medium interferes with stomatal response, CO2 fixation rate and carbohydrate metabolism in tomato, and decreases harmful effects of subsequent salt stress. **Plant Biology**, v. 13, n. 1, p. 105-114, 2011.

POULEV, Alexander et al. Elicitation, a new window into plant chemodiversity and phytochemical drug discovery. **Journal of Medicinal Chemistry**, v. 46, n. 12, p. 2542-2547, 2003.

PRACHI; SHARMA, Tilak R.; SINGH, Brij M. Salicylic Acid Induced Insensitivity to Culture Filtrate of Fusarium oxysporum f. sp. zingiberi in the Calli of Zingiber officinale Roscoe. **European Journal of Plant Pathology**, v. 108, p. 31-39, 2002.

PRADO, Erika; DEMARCO, Diego. Laticifers and secretory ducts: similarities and differences. In: **Ecosystem services and global ecology**. IntechOpen, 2018.

PRESSEY, Russell. Two isoforms of NP24: a thaumatin-like protein in tomato fruit. **Phytochemistry**, v. 44, n. 7, p. 1241-1245, 1997.

RAI, Manoj K. et al. The role of abscisic acid in plant tissue culture: a review of recent progress. **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 106, p. 179-190, 2011.

RAINSFORD, Kim D. **Aspirin and the Salicylates**. Elsevier, 1984.

RAKWAL, Randeep; AGRAWAL, Ganesh K.; AGRAWAL, Vishwanath P. Jasmonate, salicylate, protein phosphatase 2A inhibitors and kinetin up-regulate OsPR5 expression in cut-responsive rice (Oryza sativa). **Journal of plant physiology**, v. 158, n. 10, p. 1357-1362, 2001.

RAMADAN, Ahmed et al. Metabolomic response of Calotropis procera growing in the desert to changes in water availability. **PLoS One**, v. 9, n. 2, p. e87895, 2014.

RAMAWAT, K. G. & Merillon, J. M. **Biotechnology, secondary metabolites**. Second edition, **Published by CRC Press**. PP, 586, 2019.

RAMOS, Márcio V. et al. Anti-inflammatory latex proteins of the medicinal plant Calotropis procera: a promising alternative for oral mucositis treatment. **Inflammation Research**, v. 69, p. 951-966, 2020.

RAMOS, Marcio V. et al. Crystal structure of an antifungal osmotin-like protein from Calotropis procera and its effects on Fusarium solani spores, as revealed by atomic force microscopy: Insights into the mechanism of action. **Phytochemistry**, v. 119, p. 5-18, 2015.

RAMOS, Márcio V. et al. Performance of distinct crop pests reared on diets enriched with latex proteins from Calotropis procera: role of laticifer proteins in plant defense. **Plant Science**, v. 173, n. 3, p. 349-357, 2007.

RAMOS, Márcio V. et al. The defensive role of latex in plants: detrimental effects on insects. **Arthropod-plant interactions**, v. 4, p. 57-67, 2010.

RAMOS, Márcio Viana et al. Laticifers, latex, and their role in plant defense. **Trends in plant science**, vol. 24, no. 6, p. 553-567, 2019.

RANI, R. et al. Antibacterial activity of twenty different endophytic fungi isolated from Calotropis procera and time kill assay. **Clin Microbiol**, v. 6, n. 3, p. 280, 2017.

RANI, Reena et al. Phytochemical analysis, antibacterial and antioxidant activity of Calotropis procera and Calotropis gigantea. **The natural products journal**, v. 9, n. 1, p. 47-60, 2019.

RAO, Mulpuri V. et al. Influence of salicylic acid on H2O2 production, oxidative stress, and H2O2-metabolizing enzymes (salicylic acid-mediated oxidative damage requires H2O2). **Plant Physiology**, v. 115, n. 1, p. 137-149, 1997.

RAO, S. Ramachandra; RAVISHANKAR, G. A. Plant cell cultures: chemical factories of secondary metabolites. **Biotechnology advances**, v. 20, n. 2, p. 101-153, 2002.

RASKIN, ILYA et al. Salicylic acid levels in thermogenic and non-thermogenic plants. **Annals of Botany**, v. 66, n. 4, p. 369-373, 1990.

RASKIN, Ilya. Role of salicylic acid in plants. **Annual review of plant biology**, v. 43, n. 1, p. 439-463, 1992.

RASKIN, Ilya. Salicylate, a new plant hormone. **Plant physiology**, v. 99, n. 3, p. 799, 1992.

RATHORE, Mala; MEENA, R. K. Potential of utilizing Calotropis procera flower biomass as a renewable source of energy. **Journal of Phytology**, v. 2, n. 1, 2010.

RAVEE, Rishiesvari et al. Protease activity is maintained in Nepenthes ampullaria digestive fluids depleted of endogenous proteins with compositional changes. **Physiologia plantarum**, v. 173, n. 4, p. 1967-1978, 2021.

RENGASAMY, Pichu. Soil salinization. In: **Oxford Research Encyclopedia of Environmental Science**. 2016.

RESTIANI, Ratih; BARLIN, Novianti. Dwi Aditiyarini. **Effect of Methyl Jasmonate on Biomass and Saponin Content in Javanese Ginseng**, p. 154-158.2022.

RIVAS, Rebeca et al. Ecophysiological traits of invasive C3 species Calotropis procera to maintain high photosynthetic performance under high VPD and low soil water balance in semi-arid and seacoast zones. **Frontiers in Plant Science**, v. 11, p. 717, 2020.

RIVAS, Rebeca et al. Photosynthetic limitation and mechanisms of photoprotection under drought and recovery of Calotropis procera, an evergreen C3 from arid regions. **Plant physiology and biochemistry**, v. 118, p. 589-599, 2017.

RIVAS-SAN VICENTE, Mariana; PLASENCIA, Javier. Salicylic acid beyond defence: its role in plant growth and development. **Journal of experimental botany**, v. 62, n. 10, p. 3321-3338, 2011.

RODRÍGUEZ-SIFUENTES, Lucio et al. Legumes protease inhibitors as biopesticides and their defense mechanisms against biotic factors. **International Journal of Molecular Sciences**, v. 21, n. 9, p. 3322, 2020.

ROY, S. et al. Antioxidant and protective effect of latex of Calotropis procera against alloxan-induced diabetes in rats. **Journal of Ethnopharmacology**, v. 102, n. 3, p. 470-473, 2005.

ROY, S. J. et al. A novel protein kinase involved in Na+ exclusion revealed from positional cloning. **Plant, cell & environment**, v. 36, n. 3, p. 553-568, 2013.

RUAN, Cheng-Jiang; TEIXEIRA DA SILVA, Jaime A. Metabolomics: creating new potentials for unraveling the mechanisms in response to salt and drought stress and for the biotechnological improvement of xero-halophytes. **Critical Reviews in Biotechnology**, v. 31, n. 2, p. 153-169, 2011.

RUBINSTEIN, Ian et al. The 220 MHz NMR spectra of phytosterols. **Phytochemistry**, v. 15, n. 1, p. 195-200, 1976.

RUGINI, E. et al. Kiwifruit transgenics for osmotin gene and inoculation tests with Botrytis cinerea and Cadophora luteo-Olivacea. In: **VII International Symposium on Kiwifruit 913**. p. 197-203, 2010.

RUIZ-GARCÍA, Yolanda; GÓMEZ-PLAZA, Encarna. Elicitors: A tool for improving fruit phenolic content. **Agriculture**, v. 3, n. 1, p. 33-52, 2013.

SABATER-JARA, Ana B.; ALMAGRO, Lorena; PEDREÑO, María A. Induction of extracellular defense-related proteins in suspension cultured-cells of Daucus carota elicited with cyclodextrins and methyl jasmonate. **Plant physiology and biochemistry**, v. 77, p. 133-139, 2014.

SADAT-HOSSEINI, Mohammad et al. Ethnopharmacological studies of indigenous medicinal plants in the south of Kerman, Iran. **Journal of Ethnopharmacology**, v. 199, p. 194-204, 2017.

SÁENZ-CARBONELL, Luis; LOYOLA-VARGAS, Víctor M. Datura stramonium hairy roots tropane alkaloid content as a response to changes in Gamborg's B 5 medium. **Applied biochemistry and biotechnology**, v. 61, p. 321-337, 1997.

SAHAI, O.; KNUTH, M. Commercializing plant tissue culture processes: economics, problems and prospects. **Biotechnology progress**, v. 1, n. 1, p. 1-9, 1985.

SAISAVOEY, Tanatorn et al. Effect of methyl jasmonate on isoflavonoid accumulation and antioxidant enzymes in Pueraria mirifica cell suspension culture. **J Med Plants Res**, v. 8, n. 9, p. 401-407, 2014.

SALZMAN, Ron A. et al. Coordinate accumulation of antifungal proteins and hexoses constitutes a developmentally controlled defense response during fruit ripening in grape. **Plant physiology**, v. 117, n. 2, p. 465-472, 1998.

SAMADI, Soghra; GHASEMNAJAD, Azim; ALIZADEH, Mahdi. Investigation on phenylalanine ammonia-lyase activity of artichoke (Cynara scolymus L.) affected by methyl jasmonate and salicylic acid in in-vitro conditions. **Journal of Plant Production Research**, v. 21, n. 4, p. 135-148, 2015.

SAMBROOK, Joseph et al. **Molecular cloning a laboratory manual**. Cold spring harbor laboratory press, 1989.

SANTOS, Rita B.; FIGUEIREDO, Andreia. Two sides of the same story in grapevine–pathogen interactions. **Journal of Experimental Botany**, v. 72, n. 9, p. 3367-3380, 2021.

SARAD, Nidhi et al. Genetically engineered tomatoes: new vista for sustainable agriculture in high altitude regions. In: **Proceedings of the Fourth international crop science congress Brisbane, Australia**. 2004.

SARAIVA, Katia Daniella da Cruz. Characterization, phylogenetic analysis and expression profile of the soybean [Glycine max (L.) MERR.] elongation factor 1 alpha (EF1α) multigene family: detection of normalizing genes for qPCR. 2013.

SAYED, Alaa El-Din H. et al. Antioxidant and antiapoptotic activities of Calotropis procera latex on Catfish (Clarias gariepinus) exposed to toxic 4-nonylphenol. **Ecotoxicology and environmental safety**, v. 128, p. 189-194, 2016.

SCHALLER, Andreas. A cut above the rest: the regulatory function of plant proteases. **Planta**, v. 220, p. 183-197, 2004.

SCHALLER, Florian; SCHALLER, Andreas; STINTZI, Annick. Biosynthesis and metabolism of jasmonates. **Journal of Plant Growth Regulation**, v. 23, p. 179-199, 2004.

SCHEBEN, Armin; YUAN, Yuxuan; EDWARDS, David. Advances in genomics for adapting crops to climate change. **Current Plant Biology**, v. 6, p. 2-10, 2016.

SCHENK, Peer M. et al. Coordinated plant defence responses in Arabidopsis revealed by microarray analysis. **Proceedings of the National Academy of Sciences**, v. 97, n. 21, p. 11655-11660, 2000.

SEIDL, Verena et al. A complete survey of Trichoderma chitinases reveals three distinct subgroups of family 18 chitinases. **The FEBS journal**, v. 272, n. 22, p. 5923-5939, 2005.

SELA-BUURLAGE, Marianne B. et al. Only specific tobacco (Nicotiana tabacum) chitinases and β-1, 3-glucanases exhibit antifungal activity. **Plant physiology**, v. 101, n. 3, p. 857-863, 1993.

SELEIMAN, Mahmoud F. et al. Drought stress impacts on plants and different approaches to alleviate its adverse effects. **Plants**, v. 10, n. 2, p. 259, 2021.

SENTHILKUMAR, Rajendran; CHENG, Chiu-Ping; YEH, Kai-Wun. Genetically pyramiding protease-inhibitor genes for dual broad-spectrum resistance against insect and phytopathogens in transgenic tobacco. **Plant biotechnology journal**, v. 8, n. 1, p. 65-75, 2010.

SHABALA, S., and Munns, R. **Plant Stress Physiology**. Oxford shire: CAB International. 2012.

SHAH, Drashti et al. Pharmacognostical and Phytochemical Evaluation of Arka Taila Gel-External dermal application. **The Journal of Phytopharmacology**, vol. 8, no. 2, p. 63-66, 2019.

SHAMSI, Anas; BANO, Bilqees. Journey of cystatins from being mere thiol protease inhibitors to at heart of many pathological conditions. **International journal of biological macromolecules**, v. 102, p. 674-693, 2017.

SHAMSI, M. B.; KUMAR, R.; DADA, R. Evaluation of nuclear DNA damage in human spermatozoa in men opting for assisted reproduction. **Indian Journal of Medical Research**, v. 127, n. 2, p. 115-123, 2008.

SHANAZARI, Mahdi; GOLKAR, Pooran; MIRMOHAMMADY MAIBODY, Ali Mohammad. Effects of drought stress on some agronomic and bio-physiological traits of Trititicum aestivum, Triticale, and Tritipyrum genotypes. **Archives of Agronomy and Soil Science**, v. 64, n. 14, p. 2005-2018, 2018.

SHARMA, Avinash; LAL, Dalpat; SUTRADHAR, Monoj. Effect of growth regulators on callus morphology of Rice anther culturec. **Journal of Applied Biology and Biotechnology**, v. 5, n. 3, p. 068-071, 2017.

SHARMA, N. et al. Role of chitinase in plant defense. **Asian Journal of Biochemistry**, v. 6, n. 1, p. 29-37, 2011.

SHARMA, P.; SHARMA, J. D. In-vitro schizonticidal screening of Calotropis procera. **Fitoterapia**, v. 71, n. 1, p. 77-79, 2000.

SHARMA, Punam; GAYEN, Dipak. Plant protease as regulator and signaling molecule for enhancing environmental stress-tolerance. **Plant Cell Reports**, v. 40, n. 11, p. 2081-2095, 2021.

SHARMA, Varsha; RAMAWAT, Kishan Gopal. Salt stress enhanced antioxidant response in callus of three halophytes (Salsola baryosma, Trianthema triquetra, Zygophyllum simplex) of Thar Desert. **Biologia**, v. 69, p. 178-185, 2014.

SHATNAWI, Mohamad A. et al. In vitro propagation and cryopreservation of Prunus avium using vitrification and encapsulation dehydration methods. **Journal of Food Agriculture and Environment**, v. 5, n. 2, p. 204, 2007.

SHATNAWI, Mohamad et al. Antimicrobial activity and micropropagation of some rare and endangered plants of Jordan. **Journal of Ecological Engineering**, v. 22, n. 6, 2021.

SHEN, Xinjie et al. PacMYBA, a sweet cherry R2R3-MYB transcription factor, is a positive regulator of salt stress tolerance and pathogen resistance. **Plant Physiology and Biochemistry**, v. 112, p. 302-311, 2017.

SHHAB, Mohammad Al et al. Micropropagation and in vitro conservation of Ruta graveolens plants. **Research on Crops**, v. 22, n. 2, p. 398-409, 2021.

SHIBLI, Rida A. et al. Regeneration via somatic embryogenesis of the endangered wild arum (Arum palaestinum). **In Vitro Cellular & Developmental Biology-Plant**, v. 48, p. 335-340, 2012.

SIL, Sudipta Kumar. Influence of auxin and cytokinin on callus induction of mulberry. **Annals of the Romanian Society for Cell Biology**, p. 2310–2318-2310–2318, 2021.

SILVA, Katchen Julliany P. et al. NPR1 as a transgenic crop protection strategy in horticultural species. **Horticulture research**, v. 5, 2018.

SILVA, Márcia Calheiros Chaves et al. Therapeutic and biological activities of Calotropis procera (Ait.) R. Br. **Asian Pacific Journal of Tropical Medicine**, v. 3, n. 4, p. 332-336, 2010.

SINGH, Narendra K. et al. Characterization of osmotin: a thaumatin-like protein associated with osmotic adaptation in plant cells. **Plant physiology**, v. 85, n. 2, p. 529-536, 1987.

SINGH, Narendra K. et al. Proteins associated with adaptation of cultured tobacco cells to NaCl. **Plant physiology**, v. 79, n. 1, p. 126-137, 1985.

SINGH, Pramod Kumar; CHATURVEDI, Varun Kumar; SINGH, Harikesh Bahadur. Cross talk signalling: an emerging defense strategy in plants. **Current Science**, v. 100, n. 3, p. 288-289, 2011.

SKOOG, Folke. Chemical regulation of growth and organ formation in plant tissue cultured in vitro. In: **Symp. Soc. Exp. Biol.** p. 118-131, 1957.

SLAYMAKER, David H. et al. The tobacco salicylic acid-binding protein 3 (SABP3) is the chloroplast carbonic anhydrase, which exhibits antioxidant activity and plays a role in the hypersensitive defense response. **Proceedings of the National Academy of Sciences**, v. 99, n. 18, p. 11640-11645, 2002.

SMETANSKA, Iryna. Production of secondary metabolites using plant cell cultures. **Food biotechnology**, p. 187-228, 2008.

SMITH, M. A. L. et al. Effects of NaCl salinity on miniature dwarf tomato 'Micro-Tom': II. Shoot and root growth responses, fruit production, and osmotic adjustment. **Journal of plant nutrition**, v. 15, n. 11, p. 2329-2341, 1992.

SOARES, Paula Matias et al. Antinociceptive activity of Calotropis procera latex in mice. **Journal of ethnopharmacology**, v. 99, n. 1, p. 125-129, 2005.

SOUZA, Isabel CC et al. The osmotin of Calotropis procera latex is not expressed in laticifer-free cultivated callus and under salt stress. **Plant Physiology and Biochemistry**, v. 119, p. 312-318, 2017.

SOUZA, Isabel Cristina da Cósta. Biochemical characterization of callus from dairy species in response to saline stress and analysis of osmotin transcription. 2015.

STARCK, Zofia; KOZIŃSKA, Maria. Effect of phytohormones on absorption and distribution of ions in salt-stressed bean plants. **Acta Societatis Botanicorum Poloniae**, v. 49, n. 1-2, p. 111-125, 1980.

STEVENS, Jason; SENARATNA, Tissa; SIVASITHAMPARAM, Krishnapillai. Salicylic acid induces salinity tolerance in tomato (Lycopersicon esculentum cv. Roma): associated changes in gas exchange, water relations and membrane stabilisation. **Plant Growth Regulation**, v. 49, p. 77-83, 2006.

STRESSMANN, Maja et al. Calcium interacts with antifreeze proteins and chitinase from cold-acclimated winter rye. **Plant Physiology**, v. 135, n. 1, p. 364-376, 2004.

SUBRAMANYAM, Kondeti et al. Ectopic expression of an osmotin gene leads to enhanced salt tolerance in transgenic chilli pepper (Capsicum annum L.). **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 105, p. 181-192, 2011.

SUN, Jian et al. Improved cardenolide production in Calotropis gigantea hairy roots using mechanical wounding and elicitation. **Biotechnology letters**, v. 34, p. 563-569, 2012.

SUN, Yan-Lin; HONG, Soon-Kwan. Effect of chitinase on resistance to fungal pathogens in sea buckthorn, Hippophae rhamnoides, and cloning of Class I and III chitinase genes. **Biochemical genetics**, v. 50, p. 600-615, 2012.

SUPRASANNA, P. et al. Genomic roadmaps for augmenting salinity stress tolerance in crop plants. **Salinity Responses and Tolerance in Plants, Volume 2: Exploring RNAi, Genome Editing and Systems Biology**, p. 189-216, 2018.

SURI, S. S.; RAMAWAT, K. G. In vitro hormonal regulation of laticifer differentiation in Calotropis procera. **Annals of botany**, v. 75, n. 5, p. 477-480, 1995.

SUZUKI, Nobuhiro et al. ROS and redox signalling in the response of plants to abiotic stress. **Plant, cell & environment**, v. 35, n. 2, p. 259-270, 2012.

SWEIDAN, Nuha I.; ABU ZARGA, Musa H. Two novel cardenolides from Calotropis procera. **Journal of Asian natural products research**, v. 17, n. 9, p. 900-907, 2015.

TAKAHASHI, Ikuo; HARA, Masakazu. Enhancement of starch accumulation in plants by exogenously applied methyl jasmonate. **Plant biotechnology reports**, v. 8, p. 143-149, 2014.

TALEBI, S.; KALAT, SM Nabavi; DARBAN, AL Sohani. The study effects of heavy metals on germination characteristics and proline content of Triticale (Triticoseale Wittmack). **International Journal of Farming and Allied Sciences**, v. 3, n. 10, p. 1080-1087, 2014.

TAPIA, G. et al. Molecular characterization of Ltchi7, a gene encoding a Class III endochitinase induced by drought stress in Lotus spp. **Plant Biology**, vol. 13, no. 1, p. 69-77, 2011.

TATTERSALL, David Bruce; VAN HEESWIJCK, Robyn; HOJ, P. Bordier. Identification and characterization of a fruit-specific, thaumatin-like protein that accumulates to very high levels in conjunction with the onset of sugar accumulation and berry softening in grapes. **Plant physiology**, vol. 114, no. 3, p. 759-769, 1997.

TEIXEIRA, Fabiano M. et al. In vitro tissue culture of the medicinal shrub Calotropis procera to produce pharmacologically active proteins from plant latex. **Process Biochemistry**, v. 46, n. 5, p. 1118-1124, 2011.

TERLETSKAYA, Nina; KHAILENKO, Nina; ZHAMBAKIN, Kabl. Stability of cereal crops to drought and saline stress in vivo and in vitro. **Journal of Life Sciences**, v. 7, n. 2, p. 135, 2013.

TESTER, Mark; DAVENPORT, Romola. Na+ tolerance and Na+ transport in higher plants. **Annals of botany**, v. 91, n. 5, p. 503-527, 2003.

THAKUR, Menaka et al. Improving production of plant secondary metabolites through biotic and abiotic elicitation. **Journal of Applied Research on Medicinal and Aromatic Plants**, v. 12, p. 1-12, 2019.

THIBAUD, Marie-Christine et al. Sucrose increases pathogenesis-related PR-2 gene expression in Arabidopsis thaliana through an SA-dependent but NPR1-independent signalling pathway. **Plant Physiology and Biochemistry**, v. 42, n. 1, p. 81-88, 2004.

THORPE, Trevor A. History of plant tissue culture. **Molecular biotechnology**, v. 37, p. 169-180, 2007.

TIFFIN, Peter; MOELLER, David A. Molecular evolution of plant immune system genes. **Trends in genetics**, vol. 22, no. 12, p. 662-670, 2006.

TOHIDFAR, Masoud; KHOSRAVI, Solmaz. Transgenic crops with an improved resistance to biotic stresses. A review. **Base**, 2015.

TOSCANO, Stefania et al. Physiological and biochemical responses in two ornamental shrubs to drought stress. **Frontiers in plant science**, v. 7, p. 645, 2016.

TOTH, Marta; FRIDMAN, Rafael. Assessment of gelatinases (MMP-2 and MMP-9 by gelatin zymography. **Metastasis Research Protocols: Volume I: Analysis of Cells and Tissues**, p. 163-174, 2001.

TRAORE, M. S. et al. Ethnobotanical survey on medicinal plants used by Guinean traditional healers in the treatment of malaria. **Journal of ethnopharmacology**, v. 150, n. 3, p. 1145-1153, 2013.

TRIPATHI, Pankaj K. et al. Callus culture and in vitro biosynthesis of cardiac glycosides from Calotropis gigantea (L.) Ait. **In Vitro Cellular & Developmental Biology-Plant**, v. 49, p. 455-460, 2013.

TRIVEDI, V. R.; CHORAWALA, M. R.; SHAH, G. B. Antiatherosclerotic activity of osmotin, an adiponectin agonist in atherogenic diet induced hypertriglyceridemia and hypercholesterolemia in wistar rats. **Adv. Res. Pharmaceuticals Biol**, v. 2, p. 196-207, 2012.

TRIVEDI, Vandit R.; CHORAWALA, Mehul R.; SHAH, Gaurang B. Osmotin: a new adiponectin agonist, in type-II diabetes and obesity. **Int. J. Pharm. Sci. Rev. Res**, v. 16, p. 70-74, 2012.

TWAIJ, Baan Munim; HASAN, Md Nazmul. Bioactive secondary metabolites from plant sources: Types, synthesis, and their therapeutic uses. **International Journal of Plant Biology**, v. 13, n. 1, p. 4-14, 2022.

TYBURSKI, Jarosław; MUCHA, Natalia. Antioxidant Response in the Salt-Acclimated Red Beet (Beta vulgaris) Callus. **Agronomy**, v. 13, n. 9, p. 2284, 2023.

UARROTA, Virgílio Gavicho et al. Revisiting carotenoids and their role in plant stress responses: from biosynthesis to plant signaling mechanisms during stress. **Antioxidants and antioxidant enzymes in higher plants**, p. 207-232, 2018.

ULLAH, Rahim; MUHAMMAD, Said. Heavy metals contamination in soils and plants along with the mafic–ultramafic complex (Ophiolites), Baluchistan, Pakistan: Evaluation for the risk and phytoremediation potential. **Environmental Technology & Innovation**, v. 19, p. 100931, 2020.

URBANEK, H.; KUZNIAK-GEBAROWSKA, E.; HERKA, K. Elicitation of defence responses in bean leaves by Botrytis cinerea polygalacturonase. **Acta Physiologiae Plantarum**, v. 13, n. 1, 1991.

VALERIO, M. S. et al. β-Sitosterol modulates TLR4 receptor expression and intracellular MyD88-dependent pathway activation in J774A. 1 murine macrophage. **Cellular immunology**, v. 285, n. 1-2, p. 76-83, 2013.

VALERIO, Michael; AWAD, Atif B. β-Sitosterol down-regulates some pro-inflammatory signal transduction pathways by increasing the activity of tyrosine phosphatase SHP-1 in J774A. 1 murine macrophage. **International immunopharmacology**, v. 11, n. 8, p. 1012-1017, 2011.

VALIFARD, M. et al. Effects of salt stress on volatile compounds, total phenolic content and antioxidant activities of Salvia mirzayanii. **South African Journal of Botany**, v. 93, p. 92-97, 2014.

VAN DER HOORN, Renier AL. Plant proteases: from phenotypes to molecular mechanisms. **Annu. Rev. Plant Biol.**, v. 59, p. 191-223, 2008.

VAN DER HOORN, Renier AL; KLEMENČIČ, Marina. Plant proteases: from molecular mechanisms to functions in development and immunity. **Journal of Experimental Botany**, v. 72, n. 9, p. 3337-3339, 2021.

VAN DER WEL, Henrik; LOEVE, Kees. Isolation and characterization of thaumatin I and II, the sweet-tasting proteins from Thaumatococcus daniellii Benth. **European Journal of Biochemistry**, v. 31, n. 2, p. 221-225, 1972.

VAN LOON, L. C. Induced resistance in plants and the role of pathogenesis-related proteins. **European journal of plant pathology**, v. 103, p. 753-765, 1997.

VAN LOON, L. C.; VAN KAMMEN, A. Polyacrylamide disc electrophoresis of the soluble leaf proteins from Nicotiana tabacum var.'Samsun'and 'Samsun NN': II. Changes in protein constitution after infection with tobacco mosaic virus. **Virology**, v. 40, n. 2, p. 199-211, 1970.

VAN LOON, Leendert C.; REP, Martijn; PIETERSE, Corné MJ. Significance of inducible defense-related proteins in infected plants. **Annu. Rev. Phytopathol.**, v. 44, p. 135-162, 2006.

VAN WIJK, Klaas J. Protein maturation and proteolysis in plant plastids, mitochondria, and peroxisomes. **Annual review of plant biology**, v. 66, p. 75-111, 2015.

VAN ZELM, Eva; ZHANG, Yanxia; TESTERINK, Christa. Salt tolerance mechanisms of plants. **Annual review of plant biology**, v. 71, p. 403-433, 2020.

VASCONSUELO, Andrea; BOLAND, Ricardo. Molecular aspects of the early stages of elicitation of secondary metabolites in plants. **Plant science**, v. 172, n. 5, p. 861-875, 2007.

VEGA, Andrea S.; CASTRO, Maria A.; ANDERSON, William R. Occurrence and phylogenetic significance of latex in the Malpighiaceae. **American Journal of Botany**, v. 89, n. 11, p. 1725-1729, 2002.

VELAZHAHAN, R.; MUTHUKRISHNAN, S. Transgenic tobacco plants constitutively overexpressing a rice thaumatin-like protein (PR-5) show enhanced resistance to Alternaria alternata. **Biologia Plantarum**, v. 47, p. 347-354, 2003.

VELAZHAHAN, Rethinasamy; DATTA, Swapan K.; MUTHUKRISHNAN, Subbaratnam. The PR-5 family: thaumatin-like proteins. **Pathogenesis-related proteins in plants**, p. 107-129, 1999.

VENCKUS, Petras; CICCHI, Bernardo; CHINI ZITTELLI, Graziella. Effects of medium salinity on growth and biochemical composition of the green microalga Tetraselmis suecica. **Journal of Applied Phycology**, v. 33, p. 3555-3563, 2021.

VERA-ESTRELLA, Rosario et al. Salt stress in Mesembryanthemum crystallinum L. cell suspensions activate adaptive mechanisms similar to those observed in the whole plant. **Planta**, v. 207, p. 426-435, 1999.

VERNOOIJ, Bernard et al. Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. **The Plant Cell**, v. 6, n. 7, p. 959-965, 1994.

VERONESE, Paola et al. In defense against pathogens. Both plant sentinels and foot soldiers need to know the enemy. **Plant Physiology**, v. 131, n. 4, p. 1580-1590, 2003.

VIANA, Carolina Araújo et al. Cytotoxicity against tumor cell lines and anti-inflammatory properties of chitinases from Calotropis procera latex. **Naunyn-Schmiedeberg's archives of pharmacology**, v. 390, p. 1005-1013, 2017.

VIERSTRA, Richard D. Proteolysis in plants: mechanisms and functions. **Post-Transcriptional Control of Gene Expression in Plants**, p. 275-302, 1996.

VIKTOROVA, J. et al. Osmotin, a pathogenesis-related protein. **Current Protein and Peptide Science**, v. 13, n. 7, p. 672-681, 2012.

VIKTOROVA, Jitka et al. Recombinant expression of osmotin in barley improves stress resistance and food safety during adverse growing conditions. **PLoS One**, v. 14, n. 5, p. e0212718, 2019.

VILLARET, Joran et al. Adaptation of a microbead assay for the easy evaluation of traditional anti-sickling medicines: application to DREPANOSTAT and FACA. **Pharmaceutical biology**, vol. 56, no. 1, p. 385-392, 2018.

VINOCUR, Basia; ALTMAN, Arie. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. **Current opinion in biotechnology**, v. 16, n. 2, p. 123-132, 2005.

WAN, Qun et al. Salinity tolerance mechanism of osmotin and osmotin-like proteins: a promising candidate for enhancing plant salt tolerance. **Current genomics**, v. 18, n. 6, p. 553-556, 2017.

WANG, Jing et al. Enhanced production of flavonoids by methyl jasmonate elicitation in cell suspension culture of Hypericum perforatum. **Bioresources and Bioprocessing**, v. 2, p. 1-9, 2015.

WANG, Juncheng et al. Physiological and proteomic analyses of salt stress response in the halophyte H alogeton glomeratus. **Plant, Cell & Environment**, v. 38, n. 4, p. 655-669, 2015.

WANG, Kai et al. Influence of salt stress on growth and antioxidant responses of two malus species at callus and plantlet stages. **Pak. J. Bot**, v. 45, n. 2, p. 375-381, 2013.

WANG, Lijun; WELLER, Curtis L. Recent advances in extraction of nutraceuticals from plants. **Trends in Food Science & Technology**, v. 17, n. 6, p. 300-312, 2006.

WANG, Li-Ying et al. Molecular cloning of class III chitinase gene from Avicennia marina and its expression analysis in response to cadmium and lead stress. **Ecotoxicology**, v. 24, p. 1697-1704, 2015.

WANG, S. Y. Methyl jasmonate reduces water stress in strawberry. **Journal of plant growth regulation**, v. 18, p. 127-134, 1999.

WANG, Xuchu et al. Comparative proteomics of Thellungiella halophila leaves from plants subjected to salinity reveals the importance of chloroplastic starch and soluble sugars in halophyte salt tolerance. **Molecular & Cellular Proteomics**, v. 12, n. 8, p. 2174-2195, 2013.

WANG, Yun et al. Function and mechanism of jasmonic acid in plant responses to abiotic and biotic stresses. **International Journal of Molecular Sciences**, v. 22, n. 16, p. 8568, 2021.

WINTER, Jonathan M. et al. Representing water scarcity in future agricultural assessments. **Anthropocene**, v. 18, p. 15-26, 2017.

WITZEL, Katja et al. Comparative analysis of the grain proteome fraction in barley genotypes with contrasting salinity tolerance during germination. **Plant, Cell & Environment**, vol. 33, no. 2, p. 211-222, 2010.

XAVIER-FILHO, J. et al. Poor correlation between the levels of proteinase inhibitors found in seeds of different cultivars of cowpea (Vigna unguiculata) and the resistance/susceptibility to predation by Callosobruchus maculatus. **Journal of Agricultural and Food Chemistry**, v. 37, n. 4, p. 1139-1143, 1989.

XIE, Zhixin et al. An important role of an inducible RNA-dependent RNA polymerase in plant antiviral defense. **Proceedings of the National Academy of Sciences**, v. 98, n. 11, p. 6516-6521, 2001.

XIE, Zhi-Ping et al. Symbiosis-stimulated chitinase isoenzymes of soybean (Glycine max (L.) Merr.). **Journal of Experimental Botany**, v. 50, n. 332, p. 327-333, 1999.

XING, Bingcong et al. Phenolic acid production is more effectively enhanced than tanshinone production by methyl jasmonate in Salvia miltiorrhiza hairy roots. **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 134, p. 119-129, 2018.

XU, Ai; ZHAN, Ji-Cheng; HUANG, Wei-Dong. Effects of ultraviolet C, methyl jasmonate and salicylic acid, alone or in combination, on stilbene biosynthesis in cell suspension cultures of Vitis vinifera L. cv. Cabernet Sauvignon. **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 122, p. 197-211, 2015.

XU, Fa-Qing; XUE, Hong-Wei. The ubiquitin-proteasome system in plant responses to environments. **Plant, cell & environment**, v. 42, n. 10, p. 2931-2944, 2019.

XU, Jun et al. Discovery and identification of candidate genes from the chitinase gene family for Verticillium dahliae resistance in cotton. **Scientific reports**, v. 6, n. 1, p. 29022, 2016.

XUE, X. et al. Overexpression of OsOSM1 enhances resistance to rice sheath blight. **Plant Disease**, v. 100, n. 8, p. 1634-1642, 2016.

YALPANI, Nasser et al. Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. **The Plant Cell**, v. 3, n. 8, p. 809-818, 1991.

YAMAMOTO, T. et al. Transgenic grapevine plants expressing a rice chitinase with enhanced resistance to fungal pathogens. **Plant Cell Reports**, v. 19, p. 639-646, 2000.

YAN, Liuhua et al. Role of tomato lipoxygenase D in wound-induced jasmonate biosynthesis and plant immunity to insect herbivores. **PLoS Genetics**, v. 9, n. 12, p. e1003964, 2013.

YANG, Ya-jun et al. Effects of inhibitors on the protease profiles and degradation of activated Cry toxins in larval midgut juices of Cnaphalocrocis medinalis (Lepidoptera: Pyralidae). **Journal of Integrative Agriculture**, v. 20, n. 8, p. 2195-2203, 2021.

YANG, Yingli et al. Salinity-induced Physiological Modification in the Callus from Halophyte Nitraria tangutorum Bobr. **Journal of plant growth regulation**, v. 29, p. 465-476, 2010.

YANG, Yinong; QI, Min; MEI, Chuansheng. Endogenous salicylic acid protects rice plants from oxidative damage caused by aging as well as biotic and abiotic stress. **The Plant Journal**, v. 40, n. 6, p. 909-919, 2004.

YANG, Zhi-Min et al. Salicylic acid-induced aluminum tolerance by modulation of citrate efflux from roots of Cassia tora L. **Planta**, v. 217, p. 168-174, 2003.

YASAR, Fikret; ELLIALTIOGLU, Sebnem; YILDIZ, Kenan. Effect of salt stress on antioxidant defense systems, lipid peroxidation, and chlorophyll content in green bean. **Russian journal of plant physiology**, v. 55, p. 782-786, 2008.

YEBOUK, Cheikh et al. Ethnobotanical study of medicinal plants in the Adrar Province, Mauritania. **Journal of ethnopharmacology**, v. 246, p. 112217, 2020.

YEN, H. Emilie et al. Salt-induced changes in protein composition in light-grown callus of Mesembryanthemum crystallinum. **Physiologia Plantarum**, v. 101, n. 3, p. 526-532, 1997.

YIN, Heng; DU, Yuguang; DONG, Zhongmin. Chitin oligosaccharide and chitosan oligosaccharide: two similar but different plant elicitors. **Frontiers in Plant Science**, v. 7, p. 522, 2016.

YOUSEFIAN, Shirin et al. Effect of methyl jasmonate on phenolic acids accumulation and the expression profile of their biosynthesis-related genes in Mentha spicata hairy root cultures. **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 142, n. 2, p. 285-297, 2020.

YU XIAXIA, Yu XiaXia et al. The roles of methyl jasmonate to stress in plants. 2019.

YU, Xiao-Ming; GRIFFITH, Marilyn; WISEMAN, Steven B. Ethylene induces antifreeze activity in winter rye leaves. **Plant physiology**, v. 126, n. 3, p. 1232-1240, 2001.

YUN, Dae-Jin et al. Osmotin, a plant antifungal protein, subverts signal transduction to enhance fungal cell susceptibility. **Molecular Cell**, v. 1, n. 6, p. 807-817, 1998.

YUN, Dae-Jin; BRESSAN, Ray A.; HASEGAWA, Paul M. Plant antifungal proteins. **Plant breeding reviews**, v. 14, p. 39-88, 1997.

ZAFAR, Shamaila et al. Calotropis procera (root) escalates functions rehabilitation and attenuates oxidative stress in a mouse model of peripheral nerve injury. **Pakistan Journal of Pharmaceutical Sciences**, v. 33, 2020.

ZAFAR, Zafar Ullah et al. Strategies to improve crop salt and drought tolerance: Success and limitations. **Agrobios (India)**, v. 11, p. 265-298, 2017.

ZAHID, Noosheen et al. Regulation of inducible enzymes and suppression of anthracnose using submicron chitosan dispersions. **Scientia Horticulturae**, v. 193, p. 381-388, 2015.

ZAID, Abbu; MOHAMMAD, Firoz. Methyl jasmonate and nitrogen interact to alleviate cadmium stress in Mentha arvensis by regulating physio-biochemical damages and ROS detoxification. **Journal of Plant Growth Regulation**, v. 37, n. 4, p. 1331-1348, 2018.

ZAKARIA, A. L. et al. Callus induction, plant regeneration, and growth on barley (Hordeum vulgare L.). **Southwest. J. Hortic. Biol Environ**, v. 3, p. 25-39, 2012.

ZAWOZNIK, Myriam S. et al. Endogenous salicylic acid potentiates cadmium-induced oxidative stress in Arabidopsis thaliana. **Plant Science**, v. 173, n. 2, p. 190-197, 2007.

ZHANG, Fuli et al. Overexpression of a chitinase gene from Trichoderma asperellum increases disease resistance in transgenic soybean. **Applied biochemistry and biotechnology**, v. 180, p. 1542-1558, 2016.

ZHANG, Heng et al. Mechanisms of plant salt response: insights from proteomics. **Journal of proteome research**, v. 11, n. 1, p. 49-67, 2012.

ZHANG, Ji-Yu et al. Identification and Molecular Characterization of a Class I Chitinase Gene (Mhchit 1) from Malus hupehensis. **Plant molecular biology reporter**, v. 30, p. 760-767, 2012.

ZHANG, Yongxue et al. NaCl-responsive ROS scavenging and energy supply in alkaligrass callus revealed from proteomic analysis. **BMC genomics**, v. 20, p. 1-16, 2019.

ZHAO, Jian; DAVIS, Lawrence C.; VERPOORTE, Robert. Elicitor signal transduction leading to production of plant secondary metabolites. **Biotechnology advances**, v. 23, n. 4, p. 283-333, 2005.

ZHAO, Jiang-Lin; ZHOU, Li-Gang; WU, Jian-Yong. Effects of biotic and abiotic elicitors on cell growth and tanshinone accumulation in Salvia miltiorrhiza cell cultures. **Applied Microbiology and Biotechnology**, v. 87, p. 137-144, 2010.

ZHENG, Liu et al. Overexpression of cysteine protease gene from Salix matsudana enhances salt tolerance in transgenic Arabidopsis. **Environmental and Experimental Botany**, v. 147, p. 53-62, 2018.

ZHONG, Jian-Jiang. Biochemical engineering of the production of plant-specific secondary metabolites by cell suspension cultures. **Plant cells**, p. 1-26, 2001.

ZHOU, Niannian et al. Identification and expression analysis of chitinase genes in Zizania latifolia in response to abiotic stress. **Scientia Horticulturae**, v. 261, p. 108952, 2020.

ZHOU, Ya-Fu; LIU, Wen-Zhe. Laticiferous canal formation in fruits of Decaisnea fargesii: a programmed cell death process? **Protoplasma**, v. 248, p. 683-694, 2011.

ZHU, Baolong; CHEN, Tony HH; LI, Paul H. Analysis of late-blight disease resistance and freezing tolerance in transgenic potato plants expressing sense and antisense genes for an osmotin-like protein. **Planta**, v. 198, p. 70-77, 1996.

ZHU, Baolong; CHEN, Tony HH; LI, Paul H. Expression of three osmotin-like protein genes in response to osmotic stress and fungal infection in potato. **Plant molecular biology**, v. 28, p. 17-26, 1995.

ZHU, Jian-Kang. Plant salt tolerance. **Trends in plant science**, v. 6, n. 2, p. 66-71, 2001.

ZINOVIEVA, S. V. et al. Gene expression of protease inhibitors in tomato plants with invasion by root-knot nematode Meloidogyne incognita and modulation of their activity with salicylic and jasmonic acids. **Biology Bulletin**, v. 48, p. 130-139, 2021.

ZULFIQAR, Faisal; ASHRAF, Muhammad. Nanoparticles potentially mediate salt stress tolerance in plants. **Plant Physiology and Biochemistry**, v. 160, p. 257-268, 2021.

ZUO, Guowei et al. Total saponins of Panax ginseng induces K562 cell differentiation by promoting internalization of the erythropoietin receptor. **The American journal of Chinese medicine**, v. 37, n. 04, p. 747-757, 2009.