UNDERSTANDING THE ROLE OF ABIOTIC STRESS IN AZADIRACHTIN ACCUMULATION IN CALLUS CULTURES OF NEEM (*Azadirachta indica*) THROUGH PROTEOMICS APPROACH

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FACULTY OF SCIENCE
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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES
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Field of Study: **BIOLOGY AND BIOCHEMISTRY**

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UNDERSTANDING THE ROLE OF ABIOTIC STRESS IN AZADIRACHTIN ACCUMULATION IN CALLUS CULTURES OF NEEM (*Azadirachta indica*) THROUGH PROTEOMICS APPROACH

ABSTRACT

Abiotic stress such as salinity and jasmonic acid have the potential to impact plant growth and synthesis of secondary compounds. Abiotic stresses might also act as elicitors which may stimulate the production of secondary metabolites as a part of defense system. In this research, *in vitro* callus culture of neem, a highly valuable medicinal tree was developed and induced with salinity and jasmonic acid stress to explore their effects on the production of azadirachtin, an essential neem secondary metabolite. Further investigation was carried out to evaluate the impact of these stresses on the expression and regulation of neem proteins via comparative proteomics. In this study, callus cultures were subjected to various concentrations of salinity stress (0.05 - 1.5% of NaCl) and jasmonic acid (1 - 6 mg/L) for 4 weeks. The outcomes showed that the callus was able to survive at increasing NaCl and jasmonic acid concentrations. However, overall fresh weight reduction was observed as the stress concentration increased. Remarkably, callus grown under salt and jasmonic stress showed an increase in the level of azadirachtin production compared to control. The highest total azadirachtin contents were recorded from callus supplemented with 6 mg/L jasmonic acid and 1.5% NaCl. In order to acquire some molecular insights of stress adaptations undergone by the callus cultures, the samples were subjected to shotgun proteomics analysis. From the analysis, a total of 129 (36 upregulated and 93 downregulated) proteins were differentially regulated in salinity stress; meanwhile, 320 (34 upregulated and 286 downregulated) proteins were associated with jasmonic acid elicitation. Overall, altered proteins in salinity stress were related to several functional groups such as cellular process, metabolic process, response to stimulus (stress, abiotic stimulus, and chemical), biological regulation, and biological

process involved in interspecies interaction between organisms. Meanwhile, in jasmonic stress, differentially abundant proteins were related to several functional groups, for instance, cellular process, metabolite process, response to stimulus, localization, and development process involved in reproduction. The findings suggest that elicitation with jasmonic acid and salinity stress significantly improved the content of azadirachtin in the sample compared to control media. Thus, this information on stress-induced improvement of secondary metabolites synthesis can be used as a tool in manipulation of biosynthesis pathways for large scale production to produce natural product-like compounds under controlled conditions.

Keywords: *Azadirachta indica*, azadirachtin, abiotic stress, growth, proteomics

MEMAHAMI PERANAN TEKANAN ABIOTIK DALAM PENGUMPULAN AZADIRACHTIN DALAM KULTUR KALUS SEMAMBU (*Azadirachta indica*) MELALUI PENDEKATAN PROTEOMIK

ABSTRAK

Tekanan abiotik seperti kemasinan dan asid jasmonik mempunyai potensi untuk memberi kesan kepada pertumbuhan tumbuhan dan sintesis sebatian sekunder. Tekanan abiotik juga mungkin bertindak sebagai elisitor yang boleh merangsang pengeluaran metabolik sekunder sebagai sebahagian daripada sistem pertahanan. Dalam penyelidikan ini, kultur kalus *in vitro* semambu, pokok ubatan yang sangat berharga telah dibangunkan dan diinduksi dengan kemasinan dan tekanan asid jasmonik yang berbeza untuk meneroka kesannya ke atas penghasilan azadirachtin, metabolik sekunder semambu yang penting. Penyiasatan lanjut telah dijalankan untuk menilai kesan tekanan ini terhadap ekspresi dan pengawalan protein semambu melalui proteomik perbandingan. Dalam kajian ini, kultur kalus tertakluk kepada pelbagai tahap kepekatan kemasinan (0.05 - 1.5% NaCl) dan asid jasmonik (1 - 6 mg/L) selama 4 minggu. Hasil kajian menunjukkan bahawa kalus dapat bertahan dengan peningkatan kepekatan NaCl dan asid jasmonik. Walau bagaimanapun, pengurangan berat keseluruhan telah diperhatikan apabila kepekatan tekanan meningkat. Kalus yang tumbuh di bawah tekanan garam dan jasmonik menunjukkan peningkatan tahap pengeluaran azadirachtin berbanding kawalan. Jumlah kandungan azadiracthin tertinggi direkodkan pada kalus yang ditambah dengan 6 mg/L asid jasmonik dan 1.5% NaCl. Untuk memperolehi beberapa pandangan molekul tentang penyesuaian tekanan yang dialami oleh kultur kalus, sampel telah tertakluk kepada analisis proteomik ‘shotgun’. Daripada analisis ini, sejumlah 129 (36 pengawalaturan menaik dan 93 pengawalaturan menurun) protein dikawal secara berbeza dalam tekanan kemasinan; Sementara itu, 320 (34 pengawalaturan menaik dan 286 pengawalaturan menurun) protein dikaitkan dengan elisitasi asid jasmonik. Secara keseluruhannya, protein

menunjukkan perubahan dalam tekanan kemasinan adalah berkaitan dengan beberapa kumpulan yang berfungsi seperti proses selular, proses metabolik, tindak balas kepada rangsangan (tekanan, rangsangan abiotik, dan kimia), statut biologi, dan proses biologi yang terlibat dalam interaksi interspesies antara organisma. Sementara itu, dalam tekanan jasmonik, protein yang variasi secara berbeza dikaitkan dengan beberapa kumpulan yang berfungsi, contohnya, proses selular, proses metabolik, tindak balas kepada rangsangan, penyetempatan, dan proses perkembangan yang terlibat dalam pembiakan. Penemuan menunjukkan bahawa elisitasi dengan asid jasmonik dan tekanan kemasinan secara signifikan meningkatkan kandungan azadirachtin dalam sampel berbanding dengan media kawalan. Oleh itu, maklumat mengenai penambahbaikan akibat tekanan sintesis metabolik sekunder ini boleh digunakan sebagai alat dalam manipulasi laluan biosintesis untuk pengeluaran skala besar untuk menghasilkan sebatian seperti produk semula jadi di bawah keadaan terkawal.

Kata kunci: *Azadirachta indica*, azadirachtin, tekanan abiotik, pertumbuhan, proteomik

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LIST OF SYMBOLS AND ABBREVIATIONS

=	:	Equals to
≈	:	Almost equal to
<	:	Less than
>	:	More than
≤	:	Less than or equal to
%	:	Percentage
°C	:	Degree Celsius
µg	:	Microgram
µL	:	Microlitre
µM	:	Micromolar
1-D	:	1-Dimension
2-D	:	2-Dimension
2,4-D	:	2,4-Dichlorophenoxyacetic acid
AA	:	Amino acid
ACN	:	Acetonitrile
ANOVA	:	Analysis of Variance
ATP	:	Adenosine triphosphate
BA	:	Benzyladenine
BAP	:	6-benzylaminopurine
BSA	:	Bovine serum albumin
CAT	:	Catalase
Cl-	:	Chloride ion
CHAPS	:	3-[(3-cholamidopropyl) dimethylammonium]-1-propanesulfonate
CID	:	collision-induced

Da	:	Dalton
DAPs	:	Differentially abundant proteins
DCM	:	Dichloromethane
dH2O	:	Distilled water
DMRT	:	Duncan's multiple range test
DW	:	Dry weight
DTT	:	Dithiothreitol
EASY-nLC	:	EASY-nano liquid chromatography
FA	:	Formic acid
FC	:	Fold changes
FW	:	Fresh weight
G	:	Gram
$g\ mol^{-1}$	:	Gram per molar
g/L	:	Gram per litre
GAPDH	:	Glyceraldehyde-3-phosphate dehydrogenase
GI	:	Growth index
GO	:	Gene Ontology
GPX	:	Glutathione peroxidase
H+	:	Hydrogen ion
H_2O_2	:	Hydrogen peroxide
HCl	:	Hydrochloric acid
HCD	:	high-energy collision-induced
HPLC	:	High performance liquid chromatography
Hsp	:	Heat shock protein
JAS	:	Jasmonic acid
K+	:	Potassium ion

kDa	:	Kilodalton
kPa	:	Kilopascal
LCMS/MS	:	Liquid Chromatography Mass Spectrometry/ Mass Spectrometry
mg	:	Milligram
mg/g DW	:	Milligram per gram of dry weight
mg/mL	:	Milligram per milliliter
mg/L	:	Milligram per liter
min	:	Minute
mL	:	Milliliters
mm	:	Milli meter
mM	:	Milli molar
MeOH	:	Methanol
MeJa	:	Methyl jasmonate
MDAR	:	monodehydroascorbate reductase
MS	:	Murashige and Skoog media
MW	:	Molar weight
m/z	:	Mass divided by charge number
Na+	:	Sodium ion
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
nL/min	:	Normal air liter per minute
Nano-LC	:	Nano- Liquid Chromatography
Nm	:	Newton-meter
OCM	:	Optimized callus induction media
OEE		Oxygen evolving enhancer protein
PCA	:	Principal Component Analysis

PGR	:	Plant growth regulators
pI	:	Isoelectric point
POD	:	Peroxidase
Ppm	:	Parts per million
PS II	:	Photosystem II
PVPP	:	Polyvinylpyrrolidone
Rubisco	:	Ribulose bisphosphate carboxylase
ROS	:	Reactive oxygen species
Rpm	:	Revolutions per minute
SE	:	Standard error
SOD	:	Superoxide dismutase
SPSS	:	Statistical Package for the Social Sciences
TCA	:	Trichloroacetic acid
TCAP	:	Trichloroacetic acid -acetone precipitate with PVPP
TDZ	:	Thidiazuron
TRX	:	Thiredoxin
v/v	:	Volume per volume
w/v	:	Weight per volume

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 General introduction

Since the beginning of human civilization, various parts of plants have been extracted and employed for medicinal purposes (Khan, 2014). They are used in traditional and modern medicines, as food supplements and pharmaceutical drugs (Yuan et al., 2016). Initially, the term 'herbs' was used to refer to non-woody plants, including shrubs and trees. Herbs refer to any part of a plant-like seed, stem, bark, root, flower, leaf, and non-woody plant (Shaheen et al., 2019). A medicinal plant is a plant that produces chemical compounds that possess biological functions such as biopesticides, defense against fungi, antioxidant properties, etc. (Subapriya & Nagini, 2005).

To adapt to their changing environments, plants produce chemical compounds having medicinal activities (Dias et al., 2012). Plants undergo evolution where they have evolved various mechanisms for defense and produce a broad range of bioactive compounds. Plant secondary metabolites such as flavonoids act as antioxidants and are essential for human health (Kumar & Pandey, 2013). Plants are eaten as food; thus, boosting the amount of these important secondary metabolites in certain food crops is crucial for human health and may help lessen the reliance on synthetically-produced dietary supplements (Bhatti et al., 2022).

Biotic and abiotic stressors are recognized to stimulate enhanced yields of secondary metabolites in various plant cell cultures in short periods (DiCosmo & Misawa, 1985). In response to abiotic challenges, including high salinity, dehydration, and severe osmotic stress, plants have developed several adaptation strategies (Wang et al., 1999). Morphological, developmental, physiological, and biochemical alterations are all examples of adaptive processes (Han et al., 2016). Among them, the buildup of compatible solutes in accordance with metabolic reactions has received much attention (Saeedipour, 2013). To date, many studies have used cultured cells as a model system to

investigate the responses of cells to a variety of abiotic stressors. Some of these studies have even attempted to differentiate between short-term and long-term adaptations, including changes in physiological and biochemical processes (Fallon & Phillips, 1989; Leone et al., 1994; Wang et al., 1999).

In recent years, the focus has led to the improvement of multipurpose trees such as neem (*Azadirachta indica*) (Adhikari et al., 2020), which has diverse uses in the production of timber and fuelwood, fertilizer, pesticides, medicine, and natural insecticide (George et al., 2007). Traditionally, neem leaf extracts have been used to treat illnesses such as leprosy, eye disorder, bloody nose, intestinal worms, stomach upset, fever, diabetes, and liver problems (Alzohairy, 2016; CABI, 2019; Campos et al., 2016; S. Kumar et al., 2015; Kumar & Navaratnam, 2013). Its vast agricultural and pharmacological benefits have rendered it highly important and should be further researched. Since ancient times, the extracts from neem trees have been widely utilized in agriculture and as bio-pesticides throughout Asia, Africa, and other developing countries (Ezin & Chabi, 2023). Secondary metabolites from neem, especially azadirachtin, a tetraterpenoid, is the active ingredient in neem's insecticidal properties, which can be extracted from leaves, fruit, and seed kernel and has the ability to manage agricultural and domestic pests as well as illness (Ashrafuzzaman et al., 2008; Ezin & Chabi, 2023). Because of its significant economic value, this tree has attracted increased attention from scientists throughout the globe (Akula et al., 2003).

In this study, *in vitro* cultures of neem were used with the addition of abiotic stress factors to investigate the possible scale-up of bioactive compound production. Studies were conducted on the buildup of secondary metabolites in neem callus in response to jasmonic acid (a plant chemical related to abiotic stress signaling) and salt stress treatment. Proteomics studies were carried out on the callus culture that had been treated with different stressors. The protein profiles of *Azadirachta indica* callus exposed to

jasmonic acid and salt stress were then analyzed using a proteomic approach consisting of LCMS and mass spectrometry techniques. Utilizing a proteomics technique allows for a better knowledge of the biosynthesis pathways that have been altered, allowing the possibility of manipulating these processes in the future to produce secondary metabolites of interest.

1.2 Problem Statement

However, owing to the lack of sufficient natural resources to fulfil the market's present expectations, the commercialization of neem products is still in progress (Ashokhan et al., 2020). Neem is conventionally propagated through seeds, but the heterogeneity of the seeds due to its diverse geographical, climate and habitat regions affects the production of its bioactive compounds (Kumar & Roy, 2011). Therefore, to overcome this difficulty, scientists have introduced plant tissue culture technique as an alternative method. This study uses this technique to ensure uniform product production while serving as the ideal system for studies on secondary metabolite production.

1.3 Research Questions

Many studies have been done on protein extraction of vegetables, fruits, and woody plants. However, only a few number of proteomics-based research have been undertaken to investigate the effect of abiotic stress on *in vitro* callus cultures of *Azadirachta indica*.

The research answered the following research questions:

1. Do abiotic stresses (salinity and jasmonic acid) cause an increase in the production of azadirachtin in *Azadirachta indica*?
2. How will abiotic stresses (salinity and jasmonic acid) affect the protein expression and regulation in stressed-induced callus?

1.4 Research Objectives

In this research, the neem plant was utilized to examine the influence of various abiotic factors on the accumulation of azadirachtin and the protein expression profile of *in vitro* neem callus using proteomics. The following are the objective of the current study:

1. To evaluate the effect of salinity and jasmonic acid elicitation on the azadirachtin production in elicited and non-elicited *Azadirachta indica* callus culture.
2. To identify the proteins associated with abiotic stresses (salinity and jasmonic acid) on the regulation of proteins in the callus cultures of *Azadirachta indica* via comparative proteomics.

1.5 Significance of Study

This study improves the growth of neem by employing the technique of plant tissue culture. It has used jasmonic acid and salt treatment in the media culture as abiotic elicitors to initiate and promote the accumulation of azadirachtin in the callus of *Azadirachta indica*. The accumulation of this bioactive molecule is predicted, and its synthesis is elevated in periods of stress. Besides, the stress-tolerance mechanism system can be understood by analyzing the differential abundance and identification of proteins involved in treated neem callus.

CHAPTER 2: LITERATURE REVIEW

2.1 Origin and Taxonomy (*Azadirachta indica*)

Neem (*Azadirachta indica*) is one of the most valuable medicinal plants that belong to the family Meliaceace (Table 2.1). Meliaceae is a woody plant family cultivated mainly in the Indian subcontinent (Kumar & Navaratnam, 2013; Tiwari et al., 2014). However, the precise native origin of this plant is still unknown, and it was thought to be indigenous to South Asia, where it grows in natural forests in dry areas in Afghanistan, Pakistan, India, Sri Lanka, Bangladesh, and Myanmar (Akula et al., 2003; Jhariya et al., 2013). It has been widely cultivated in subtropical, tropical, and arid areas. It can grow well in many dried tropical and subtropical areas with rainfall between 400-1200mm per annum. According to Champion et al. (1965) and Pankaj et al. (2011), neem trees are now commonly found in Pakistan, Africa, and India, where the trees can be grown in dry deciduous and thorn forests and also grow in the mixed forest with other plant species such as Acacia species (World Agroforestry Centre, 2002). Neem tree is tolerant of a broad range of climatic conditions except for extreme cold temperature and flooding (Petruzzello, 2023).

Table 2.1: The taxonomy classification of *Azadirachta indica.* Adapted from Singh and Tiwari (2021) and Tripathi and Singh (2020)

Kingdom	Plantae
Division	Magnoliophyta
Class	Dipsacales
Order	Rutales
Suborder	Rutinae
Family	Meliaceae
Subfamily	Melioideae
Tribe	Melieae
Genus	Azadirachta
Species	*Azadirachta Indica*

Currently, neem trees have been introduced and are growing in about 72 countries worldwide in Asia, Africa, Australia, North, Central, South America, and Southeast Asia (Kumar & Navaratnam, 2013; Ogbuewu et al., 2011; Tinghui et al., 2001). Neem trees were also introduced in Pan Zhihua, Sichuan province, China (Zhang et al., 2007). While in 1995, neem trees were also introduced in Yunnan by a research institute named Luxi Biological Resources Development Institute (Tinghui et al., 2001). Nowadays, it is the most extensive area where neem is artificially planted, with over 400 000 neem trees. Neem trees can also be found all over in Malaysia, such as in Kedah, Penang, Langkawi, and Perlis (Kumar & Navaratnam, 2013). This shows that they have started to change their distribution and expand in other regions as well (Benelli et al., 2017; Kumar & Navaratnam, 2013; Patel et al., 2016).

The latin term of *Azadirachta indica* is derived from the Persian name of the tree where the meaning of Azad is “free,” dirakt stands for “tree,” and I for Hind, which belongs to “Indian Origin” (Kumar & Navaratnam, 2013; Tiwari et al., 2014). Hence, it means “the tree of India,” which also has been declared as the “Tree of the 21st century” by the United Nations (Ogbuewu et al., 2011). The neem tree is also called “arista” in Sanskrit, which means perfect, complete, and imperishable (Jhariya et al., 2013). Mondal and Chakraborty (2016) claimed that in Indian Ayurvedic tradition, this tree is also known as the “Divine Tree," “Heal All," “Village Pharmacy,” and “Panacea for all disease” (Maithani et al., 2011). While in East Africa, it is known as Muarubaini (Swahili), which means the “Tree of the 40” because it is believed to be able to cure 40 different diseases (Mondal & Chakraborty, 2016).

2.1.1 Botanical description of *Azadirachta indica*

Neem is a fast-growing tree species with small to medium size that can reach a height between 15 to 20 meters, though it occasionally reaches 30 meters (Mridha & Al, 2014; Tinghui et al., 2001). It is evergreen because this tree will retain green leaves throughout the year. However, it may shed most of its leaves in dry areas or during a severe drought (Girish & Shankara, 2008; Pandey et al., 2014). The tree branches are wide and spread, forming a large round crown that may reach a diameter of 20 to 25 meters (Hashmat et al., 2012). The color of the bark varies depending on the part of the plant and its age. The mature bark on an old tree has thick, straight, deeply fissured, and flaking with a grey to the greyish-black color of outer bark, while the younger branch has a lighter shade of bark (Figure 2.2) (Maithani et al., 2011). The inner bark is fibrous and reddish in color. The sap of neem is sticky, unpleasant smelling, and lacks any discernible color.

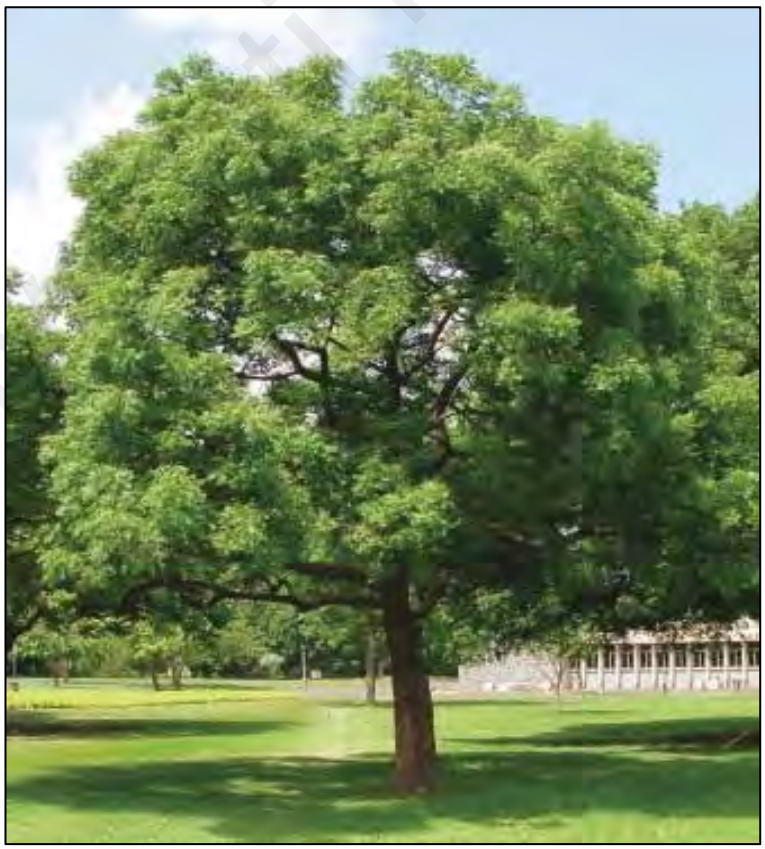

Figure 2.1: A neem tree. Sources from Satdive et al. (2006)

Species in the *Azadirachta* genus sometimes have been confused with the species in the genus *Melia* because of their similar appearance. However, it can be distinguished by observing the leaf and ovary morphology between these two genera. *Azadirachta* spp has simple pinnate leaves with circular glands, elongated glands at the base, and an ovary with three cells, while *Melia* spp. possess bipinnate leaves with one pair of globular glands and a 4- to 8-locular ovary (Lemmens et al., 1995; Rojas-Sandoval & Acevedo-Rodriguez, 2014). The neem leaves (20-40 cm long) are alternate, clustered near the branch tips, petiolate, with 20 to 31 leaflets about 3 to 8 centimeters long, and the leaf stalks are short (Puri, 2003). The upper side of the leaf has a darker green color, while the underside is lighter in color. The leaves show a distinct toothed margin and smooth surface, but it reveals the presence of a resin-secreting gland near the shoot apex with a closer observation of the young leaves.

The flower buds are tiny and arranged in axillary panicles which are up to 25 cm long (Figure 2.2) (Maithani et al., 2011). Flower bud are usually open in the evening and has a slightly honey-like fragrance that becomes more scented at night. An individual flower consists of 3 to 5 wide, rounded calyx; 5 white oblong, oval in the bud corolla; 10 stamens combined into a long tube; and a carpel with a rounded ovary and slender style (Puri, 2003). The flowers can cross-pollinate because they have both male and female sexes in the same flower, which can also be called a hermaphrodite flower (Jhariya et al., 2013).

The fruits are olive-shaped drupes with a smooth surface that varies in shape from elongate oval to nearly roundish and are 1 to 2 cm seeds long (Figure 2.2) (Parrotta & Chaturvedi, 1994). The fruit skin (exocarp) is thin when young and green with milky white juice, then turns yellow when ripe (Alzohairy, 2016). The bitter-sweet pulp (mesocarp) is yellowish-white, very fibrous, and 0.3 to 0.5 cm thick. The fruit's firm inner shell or endocarp encloses the seed with a brown seed coat (kernel). The fruit will get darker in color and wrinkled upon maturity.

Based on studies by Raj and Toppo (2015), the neem tree starts fruiting at the age of 3 to 5 years old and will achieve total production of the economic yield of fruit at approximately 10 to 12 years old (CABI, 2019; Raj & Toppo, 2015; Tinghui et al., 2001). Fruit ripening corresponds to the rainy season and under natural conditions. When the fruit starts to ripen, it will fall on the ground, and within 15 days, it will begin germinating. Besides, neem seeds also can be dispersed by birds and bats as the neem fruits are also a part of their diet. The flowering and ripening of the fruits depend on the climatic condition, location, and habitat. However, the seed loses their viability within a few weeks after ripening, thus serving as a significant problem for the propagation of this species.

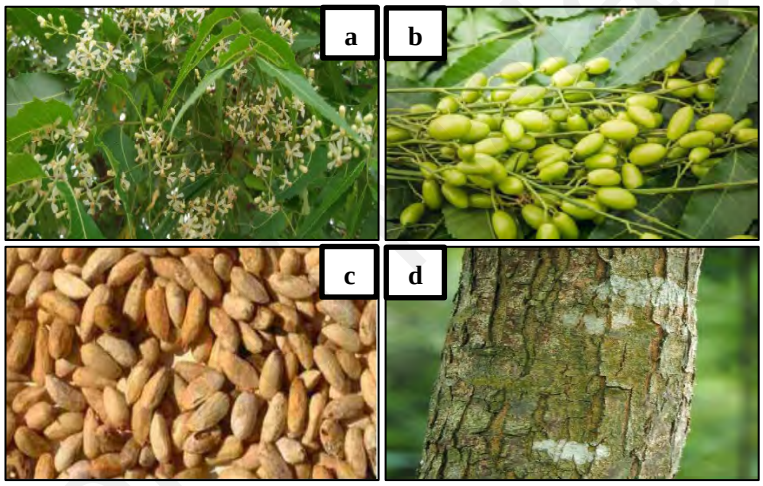

Figure 2.2: Several parts of *Azadirachta indica* a) flowers, b) fruits, c) seeds, d) bark. Adapted from Haque et al. (2016) and Mbembo et al. (2021)

2.2 *Azadirachta indica* and its uses

Azadirachta indica is a multifunction timber tree producing various economically valuable products that have been extracted for use as insecticides, fertilizer, timber industry, pesticides, and medicine (Ashrafuzzaman et al., 2008). The wood from this tree is hard and resistant to shock, boner, termites, and fungi. Therefore, the neem tree is suitable for general-purpose plywood and plywood for the block board. It also has good nail-holding properties, and it is not difficult to cut, which is easy to work by hand or using a machine (Parrotta & Chaturvedi, 1994). Due to its insect-repellent properties, it has been used for making doors and windows, ship and boat buildings, and furniture production (Gehlot et al., 2017; Tiwari et al., 2014). However, it is not preferred to be used for furniture manufacturing because it has a rough grain and does not polish well (CABI, 2019). However, neem wood provides good fuel; in some countries, for example, Africa (Parrotta & Chaturvedi, 1994), it has become one of the sources of fuelwood and makes good charcoal.

Besides, neem trees have been used for environmental protection where the trees help to encounter desertification, and soil erosion, for soil conservation, and to lessen global warming impact (Ogbuewu et al., 2011). It is also a drought-tolerant plant that can improve soil fertility. It has high water holding capacity due to its distinctive characteristic of calcium mining, which transform the acidic soil into natural soil (Gehlot et al., 2017). Neem trees are grown to support a sustainable land-use system, thus increasing the demand for its continuous propagation (Kavithra et al, 2010). When neem trees mature, they will start to lose their leaves quickly, and when the leaves fall off after a few weeks, they restore a lot of nutrients to the soil, which in turn helps to replenish the soil (Orakpo, 2012).

Many studies have been conducted concerning the medicinal properties of neem plants. *Azadirachta indica* is an essential medicinal plant, and the extract from this plant

is widely utilized as a stimulant, tonic, and treatment against various illnesses (Dhawan & Patnaik, 1993). Traditionally, neem or margosa is the first medicinal plant that has been mentioned in ancient Tamil Literature (Kumar & Navaratnam, 2013). Various parts of neem have been used in the traditional system of medicine (Ayurvedic) (Uchegbu et al., 2011) for the treatment of illnesses such as malaria, ulcers, cardiovascular disease, skin problem, burns, wounds, sprains, headaches, sore throat and bruises (Gupta et al., 2017; Kumar & Navaratnam, 2013). For example, the bark and leaves have been used to heal skin diseases, while the flower has been consumed as a tonic to relieve stomachache. The leaf extract is further taken with salt to cure intestinal worms and honey to treat skin disorders and jaundice (CABI, 2019).

Azadirachta indica has a medicinal function in preventing and treating several ailments owing to its high antioxidants and other beneficial active compounds (Alzohairy, 2016). The most active compound present in neem is azadirachtin, but it also contains other compounds such as nimbolinin, nimbin, nimbidin, nimbidol, sodium nimbinate, salannin, gedunin, and quercetin. Several biological and pharmacological qualities have been attributed to neem trees, such as antibacterial (Hashmat et al., 2012), anti-fungal, anti-pyretic, anti-histamine, anti-ulcer, analgesic, anti-malarial, anti-hormonal, anti-inflammatory (S. Kumar et al., 2015) and antioxidant properties (Campos et al., 2016; Chaudhary et al., 2017; Gonzalez-Coloma et al., 2013; Tiwari et al., 2014). Neem compounds can also inhibit cancer growth and development through multiple molecular and cellular mechanisms by suppressing the proliferation and growth of cancer cells, triggering cell cycle arrest and apoptosis, interfering with growth factor signaling, inhibiting angiogenesis and decreasing tumor cell inversion (Hao et al., 2014). Extracts of neem leaves and seed kernels have effective anti-fungal compounds that can be used against fungi infection. Due to its antibacterial and anti-fungal properties, neem is extensively used in shampoos to cure dandruff as well as in soaps and lotions to treat skin

problems, for instance, acne, psoriasis, and athlete's foot (Gupta et al., 2017). In complement to its medical usefulness, neem has attracted interest in many other areas. For example, it is also used in cosmetics and hygiene sectors, such as in the composition of face masks, lotions, sunscreens, soap, toothpaste, serum, and facial wash (Hashmat et al., 2012; Mathur & Kachhwaha, 2015).

Azadirachta indica is also used as a source of natural pesticides, and it may help in sustainable development and solving the agricultural pest control issues (Lokanadhan et al., 2012). This is due to the natural properties of neem as a potent insect growth regulator. It affects other organisms, such as nematodes and fungi, and acts as plant fertilizer (Brahmachari, 2004). According to CABI (2019), in a farm trial in Sind, Pakistan, the extracts prepared from *Azadirachta indica* seeds were used to control insect pests in the stored grain. It gives more than 80 % of the controlling effect on the major insect pests, which results in reducing grain damage for up to 6 months, and the remaining effect will last to 13 months (CABI, 2019). This biopesticide produced from *Azadirachta indica* is highly effective, environmentally safe, and cheap (CABI, 2019). Besides, azadirachtin has been approved by the USA and many European countries as an insecticide that can be used for organic agricultural production. Scientific research has been done on neem that shows it is safe for workers, can be handled without risks, and may be utilised for crop production to control pests (Boeke et al., 2004) and nutrition source to plants (Campos et al., 2016; Sujarwo et al., 2016). Therefore, this has garnered the interest of many scientists to mass-produce this plant through micropropagation to increase its production (Gehlot et al., 2017; Houllou et al., 2015a).

2.3 Plant secondary metabolites

A large number of secondary metabolites have been produced by the plant, which does not directly contribute to plant growth and development, yet, they play a critical role in plant-environment interactions (Fazili et al., 2022). The plant needs these metabolites to protect itself from different environmental factors, such as defense against herbivores and microorganisms, unfavorable growing conditions, allelochemical attraction, and pollination (Twaij & Hasan, 2022; War et al., 2012; Yue et al., 2022). These chemicals are generated in low traces because they do not contribute to plant growth and are often used in plant defense systems. Three main groups of plant secondary metabolites exist based on the metabolic pathway (Figure 2.3). First, the phenolic component consists of simple sugars and benzene rings, followed by an organic molecule containing nitrogen known as an alkaloid. Lastly, the largest group, terpenes, comprise primarily carbon and hydrogen (Twaij & Hasan, 2022). A schematic overview of the route involved in the formation of secondary metabolites is provided in Figure 2.4.

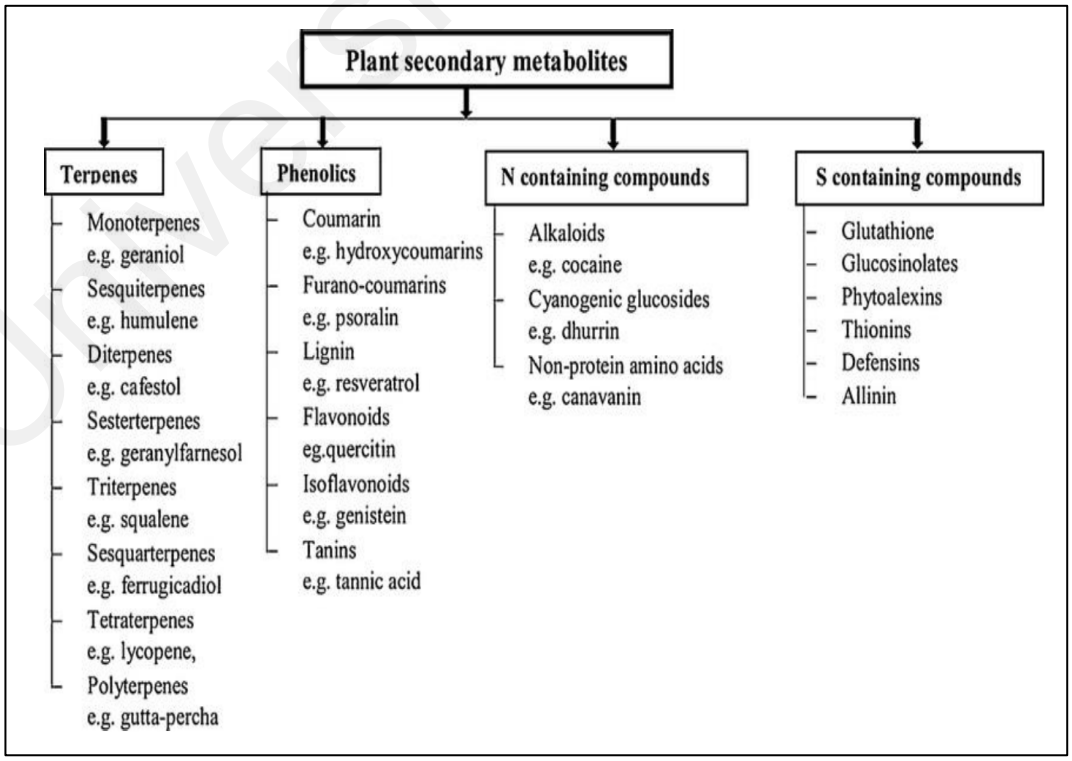

Figure 2.3: Types of secondary metabolites. Adapted from Twaij and Hasan (2022)

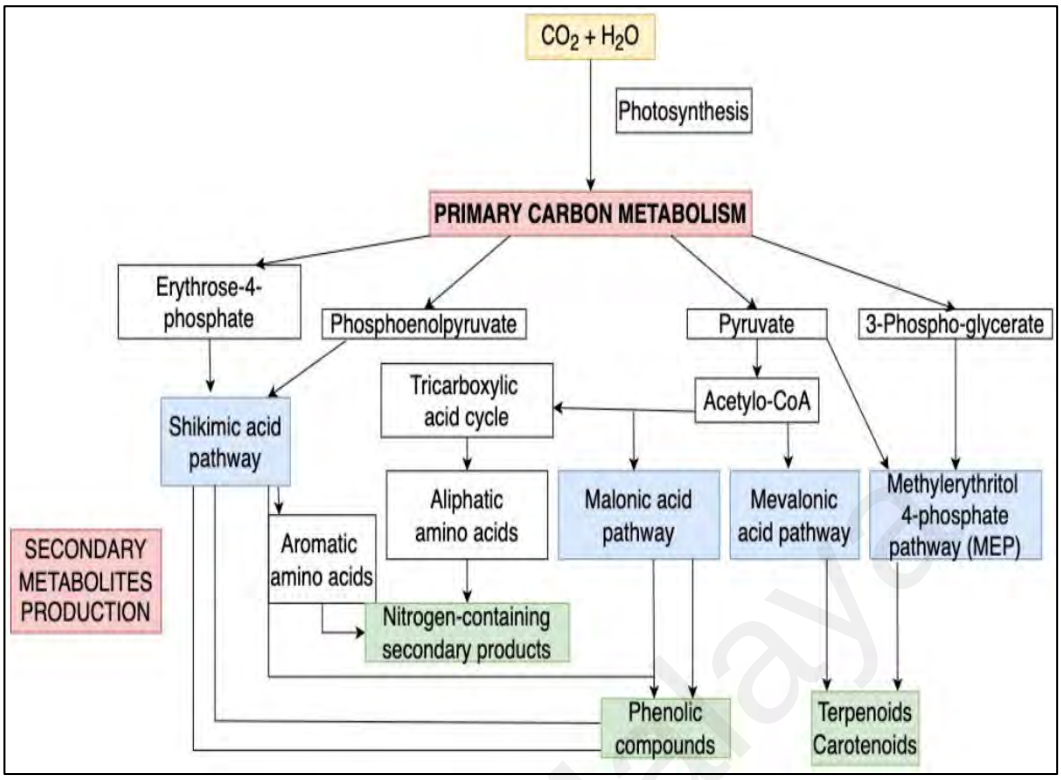

Figure 2.4: Illustrated diagram of the pathway involved in the production of secondary bioactive compounds. Adapted from Nawrot-Chorabik et al. (2022)

Many as 300 compounds have been extracted from every part of neem plant (Benelli et al., 2017), and 150 bioactive triterpenoids have been identified (Morgan, 2009). These chemical compounds are chemically varied and have highly complex structures (Gupta et al., 2017). The extracts are separated into terpenoid and non-terpenoid compounds. Protolimonoids, limonoids, pentatriterpenoids, hexatriterpenoids, and C-secomeliacins are the most prevalent terpenoids (Adhikari et al., 2020; Gupta et al., 2017). In contrast, hydrocarbon, proteins, fatty acids, steroids, phenols, and flavonoids are categorised as non-terpenoid substances (Adhikari et al., 2020).

2.3.1 Azadirachtin

Azadirachtin is one of the most abundant bioactive compounds found in neem, as it can protect the plant from insect attacks as it possesses antifeedant activity (Allan et al., 1999; Almeida et al., 2010). Azadirachtin is tetraterpenoid belonging to the class of

limonoids with the chemical formula $C_{35}H_{44}O_{16}$ and molecular weight of 720.71 $g\ mol^{-1}$ (Figure 2.5) (Fernandes et al., 2019; Morgan, 2009). This compound may be detected in all parts of neem trees; however, it is most often extracted from the leaves and seeds.

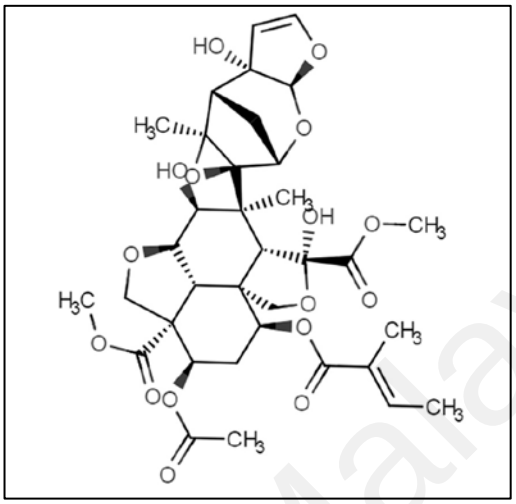

Figure 2.5.: Chemical molecule of azadirachtin Adapted from Fernandes et al. (2019)

The first researchers to isolate azadirachtin were Butterworth and Morgan (1968), and it took some time before the structure of the compound could be discovered due to the large number of oxygen atoms in the molecule (Allan et al., 1999). Azadirachtin is a highly oxidized compound with exceptionally complicated properties with rigid conformation and has a multitude of oxygenated functionalities (Veitch et al., 2007) owing to the existence of internal hydrogen carbon bonds and an abundance of reactive functional groups in close proximity (Fernandes et al., 2019; Jauch, 2008; Morgan, 2009); hence, the synthesis of azadirachtin was achieved in 71 steps and with a yield of 0.00015% after 22 years of work by Ley and his team at Imperial College, University of London (Jauch, 2008). There are 16 contiguous stereogenic centres, seven tetrasubstituted carbon atoms, and nine disubstituted carbon atoms in the chemical structure of azadirachtin (Fernandes et al., 2019; Veitch et al., 2007). The biosynthesis of azadirachtin is a complicated biosynthetic pathway with a steroid (tirucallol) serving as the primary

precursor (Figure 2.6) (Fernandes et al., 2019). The breakdown of tirucallol led to the biosynthesis of azadirachtin, which was followed by a sequence of oxidation reactions that ended in the loss of four carbon atoms (Allan et al., 1999).

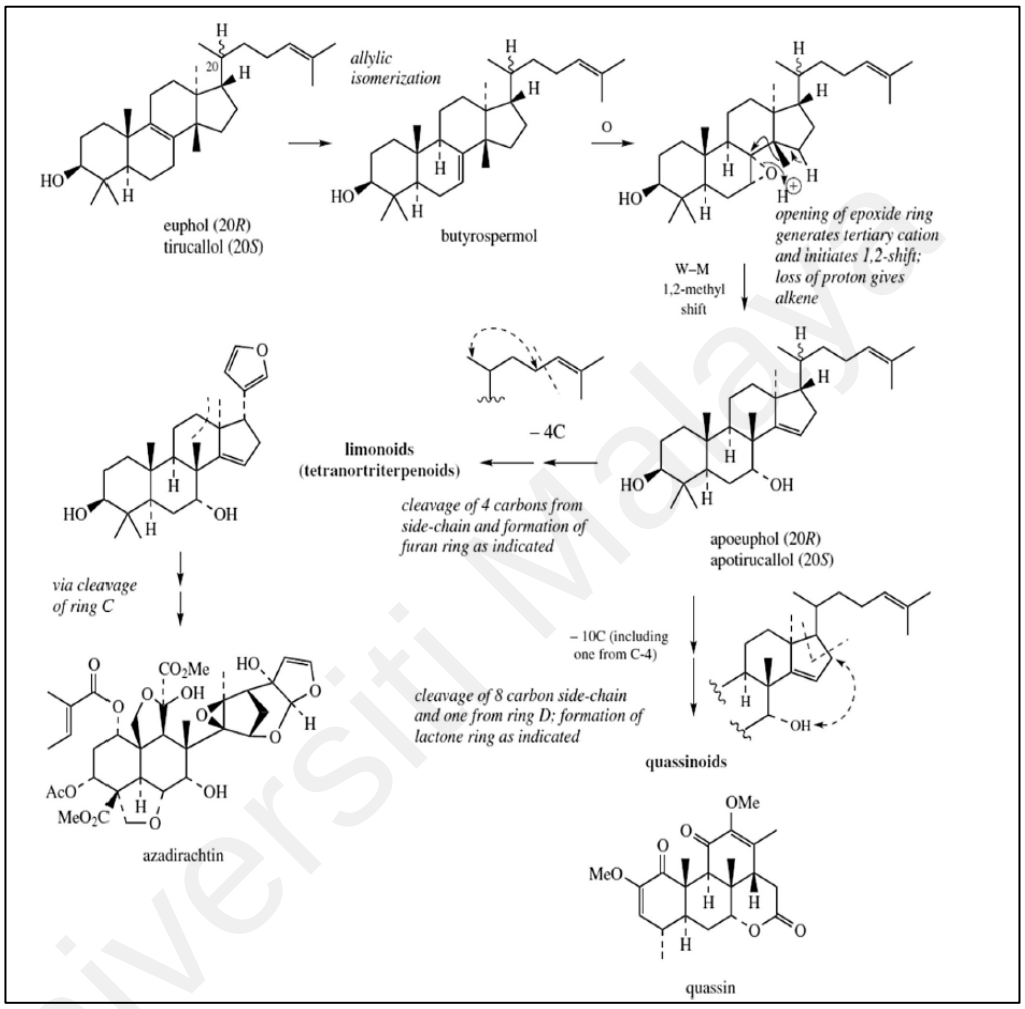

Figure 2.6: Biosynthesis pathway that leads to azadirachtin formation. Adapted from Fernandes et al. (2019)

Neem trees have been used as biopesticides and insecticides due to the compound's unique properties present in this plant. According to the study done by Salako (2002), there are a few advantages of neem that make people interested in studying it. Neem is commonly accessible and reasonably priced. Besides, the bioactive components of neem, such as azadirachtin, have managed to bring about a remarkable alteration in various parts of the life cycle and physiology of insects, making it unfeasible for pests to build a tolerance to it. In addition, due to neem's systemic activity, plant protection is its primary

function (Morgan, 2009). It has been protecting various crops from destructive pests for up to 10 weeks, such as rice, sugarcane, cotton tomatoes, and more. Among these traits, the large spectrum of action on the insects and low toxicity toward mammalian species, humans, and the environment stand out more (Fernandes et al., 2019). It has no negative impacts on organisms that seem useful in agriculture, such as earthworms (Adhikari et al., 2020).

Therefore, we may infer that azadirachtin has varied effects on insects since it has proved effective against over 540 species of hemipterans, lepidopterans, dipterans, coleopterans, and homopterans (Kilani-Morakchi et al., 2021; Mordue et al., 2010), in addition to having insecticidal effects on fungi, viruses, nematodes, and protozoa (Adhikari et al., 2020). First, azadirachtin affects the insect's physiology by altering or suppressing two hormones (20-hydroxyecdysone (20E) and juvenile hormone (JH)) which are responsible for the control of growth and development, resulting in a decrease in growth, a molting malfunction, and a mortality rise (Mordue et al., 2010). Besides, the compound is well known for its antifeedant mode of action, which inhibits feeding capacity (Morgan, 2009) when an insect ingests a plant part containing the compound and prevents the insect from swallowing food (Adhikari et al., 2020), eventually leading the insect flying away. In addition, azadirachtin was reported to disrupt the reproductive system by hindering egg production (vitellogenesis) and corpus allatum activity (Mordue et al., 2010), restricting the formation of ecdysteroids (Adhikari et al., 2020) and preventing male insect cell division in developing spermatocytes, contributing in oviposition deterrents and insect sterility (Kilani-Morakchi et al., 2021). Therefore, these findings indicate the possible application of azadirachtin in commercial biopesticides such as Azamax, Neemix 4.5, BioNeem, Margosan-O, and Neemazad (Adhikari et al., 2020) as an eco-friendly alternative measure of pest management.

2.4 Plant tissue culture technique

Neem has been commercially recognized because of its useful biological and pharmacological capabilities. However, due to inadequate natural resources to accommodate the needs of the present, the commercialization of neem products continues to develop. Neem has usually been grown from seeds; however, it is recalcitrant in nature, depending on climate, and tends to lose seed viability (Houllou et al., 2015a; Satdive et al., 2006). The heterozygous character of the seeds is a challenge that might create a drawback in producing the bioactive compounds due to cross-pollination, diverse geographical, climate and habitat regions. Therefore, some researchers have proposed a micropropagation technique to replace the conventional techniques, thus ensuring homogenous production of the compounds in clonal plants (Satdive et al., 2006).

In plant tissue culture (PTC), a small piece of plant tissue, organ, or cell has been cultured in a synthetic formulation media containing macro and micronutrients, hormones and vitamins to support the growth of cells or tissue under a sterile and controlled environment (Bhoite & Palshikar, 2014). In 1902, a scientist, Gottlieb Haberlandt, successfully isolated the plant cell and observed a formation of cell form on the leaves in response to wounding for the first time (Haberlandt, 2003; Hussain et al., 2012). Further investigation revealed that this cell has the potential to regenerate into a whole plant, which may be achievable since it has been put in an optimal culture medium with regulated nutrients and under ideal environmental circumstances (Eibl et al., 2018; Haberlandt, 2003; Hussain et al., 2012). This hypothesis is also referred to as totipotency, which is a core concept in the PTC method. In this approach, a single cell has the ability to divide and produce all of the plant's differentiated cells (Fehér, 2019). In addition, plasticity is the capability of a plant to modify its metabolism, growth, and development in response to environmental changes by initiating cell division (Barbulova et al., 2014;

Gehlot et al., 2017). Thus, it is necessary to have knowledge of both plasticity and totipotency in order to comprehend plant tissue culture.

Several factors affect PTC in selected plants, including genotype, explant, culture media, plant growth hormone (PGR), media pH, and incubation condition (Bhatia, 2015). Genotypes are considered vital sources of diversity in *in vitro* culture because a plant's genetic information is required to produce a complete part from a piece of cell (Espinosa-Leal et al., 2018). The choice of explant depends on the purpose of culture, types of culture to initiate, and species of plant; thus, the correct choice of explant material might affect the success rate of generation (George et al., 2008). Besides, the composition of culture media should consist of organic and inorganic nutrients that are needed to provide nutrients for plant growth (Figure 2.7) (Saad & Elshahed, 2012). The pH of media regulates developmental processes in the plant, such as shoot regeneration, adventitious root formation, and more, by influencing the solubility of media, which affects the absorption of nutrients and PGR (Chen et al., 2014). PGRs such as auxin and cytokinin are utilized to regulate and enhance plant development in *in vitro* culture; therefore, the ratio of these two substances may affect morphogenesis (Sehgal & Joshi, 2022). Lastly, incubation parameters such as temperature, photoperiod, and relative humidity are critical for providing a regulated environment for plant development and secondary metabolite production (Efferth, 2019).

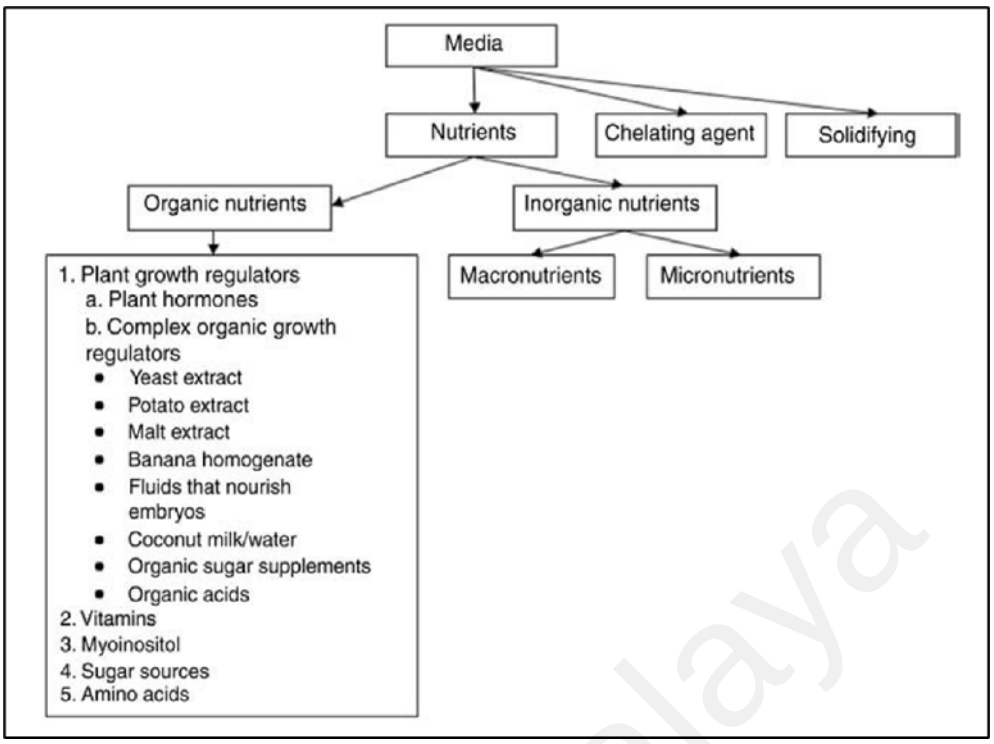

Figure 2.7: The media composition for plant tissue culture. Adapted from Bhatia (2015)

In vitro propagation helps in generating large-scale multiplication of plant materials in a short period under sterile conditions while, in contrast to conventional propagation techniques, which take a longer time, are less efficient, and sometimes even fail to produce the desired results. Besides, tissue culture methods can also be used in genetic improvement, the production of high-quality bioactive compounds, and the production of disease-free plants (Ashrafuzzaman et al., 2008; Máthé et al., 2015; Rath & Puhan, 2009; Rout et al., 2000; Tasheva & Kosturkova, 2012). Moreover, during PTC, only a piece part of the plant is used instead of the whole plant, which can solve the problem of over-harvesting (Efferth, 2019), especially the rare and endangered species of plants (Hussain et al., 2012), and the production of the cell can be continued irrespective of seasonal change and location (Eibl et al., 2018).

Several studies have been done to develop tissue culture protocols for neem. Based on a study by Phukan et al. (2017), somatic embryogenesis has been utilized to regenerate neem trees to boost their multiplication rate. It was reported that the production of calli

from leaf and stem explants is different based on the plant growth hormones that were provided. Callus generation from inoculation of explants was maximized in MS medium supplemented with BA (0.5 mg/L and 1.0 mg/L) and 2, 4-D (0.1 mg/L).

In this study, callus cultures were initiated from neem leaves at the start of the experiment by an excised small piece of leaf and then transferred into the media containing nutrients and PGR. Callus cultures are clumps of cells created by injuring or cultivating tissues in the presence of PGR (Bhatia, 2015; Ikeuchi et al., 2013). Callus is an unorganised tissue made up of both differentiated and undifferentiated cells. It forms on the cut edges of leaves when cells multiply in a disorganised way (George et al., 2008). It is also believed to be a defensive reaction developed by the plant to protect the injured tissue (Ikeuchi et al., 2017). Callus is diverse in form and texture, ranging from hard to friable cell mass, as well as shape and colour, depending on the plant species (Figure 2.8) (Bhatia, 2015).

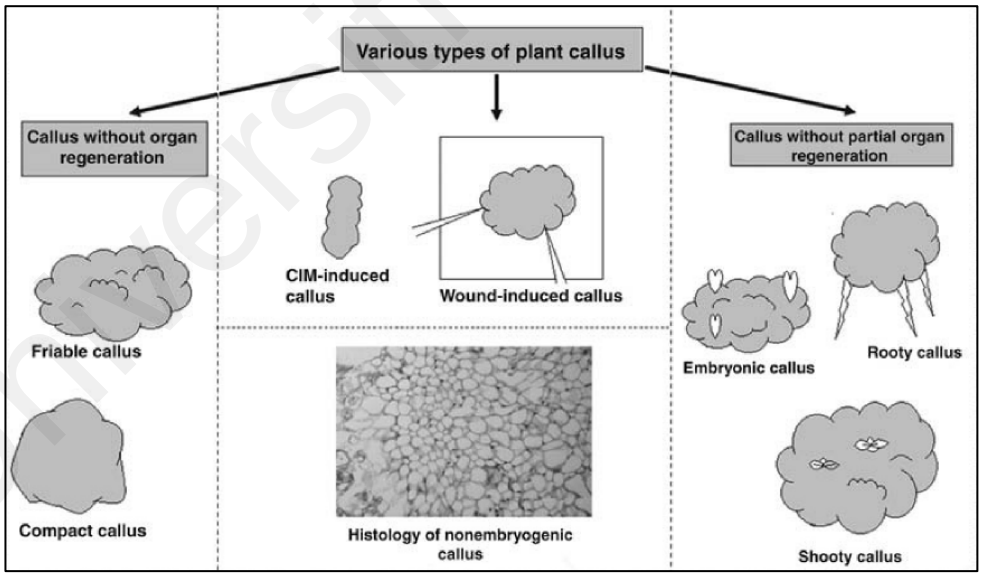

Figure 2.8: Types, histology, and different form of callus from *in vitro* tissue culture. Adapted from (Bhatia, 2015)

2.5 Abiotic stress

Elicitors are characterized as biotic and abiotic stressors that promote more excellent de novo production of secondary metabolites in PTC grown *in vitro* (Naik & Al-Khayri, 2016). Plant secondary metabolites are crucial for adaptation and defense systems against environmental change (Suzuki et al., 2014). Secondary metabolites may build up as a consequence of environmental challenges such as pathogen infection, dehydration, salt stress, bright sunlight, wounding, nutritional shortages, and UV radiation (Akula & Ravishankar, 2011). Thus, the concentration of diverse secondary metabolites is affected by the growing condition and has an influence on the metabolic pathways involved in the creation of associated natural products (Akula & Ravishankar, 2011). Table 2.2 summarised the latest findings on the effects of abiotic stressors (JAS and NaCl) on the synthesis of bioactive chemicals in diverse plant species.

Salinity is one of the stressors that restrict plant growth and production of the plant in arid and semi-arid climates, with high levels of salt being linked to lower photosynthesis and transpiration rates (Debnath et al., 2011; Sami et al., 2016). There are two types of salinity: natural and man-made. Natural salinity is caused by the long-term buildup of salt in soil or surface water by weathering, rock with soluble salt, or water carried by wind and rain. Man-made salinity is caused by activities like irrigation without a proper drainage system, using too much fertilizer, flooding with salt-rich water, and industrial waste (Hasanuzzaman et al., 2013; Rasool et al., 2013). In research on salt tolerance, NaCl is often employed. In this experiment, Na^+ has been investigated as the toxicity agent and elicitor of plant responses. Sodium will inhibit cell wall elongation and can cause competition, and inhibits the uptake of Ca^+ and K^+ (Rain & Epsstein., 1966). Cellular dehydration brought on by salt stress may result in osmotic stress and water loss from the cytoplasm (Bartels & Sunkar, 2005).

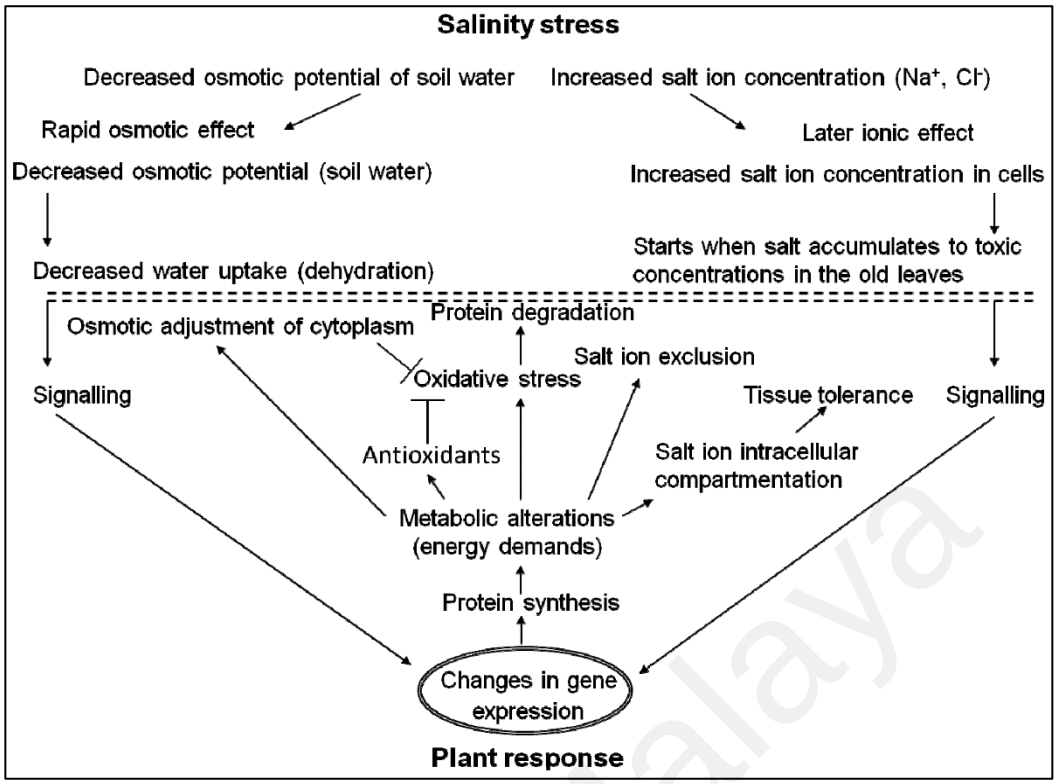

Figure 2.9: The impact of salt stress on the plant and the plant's overall reaction to salinity results in an alteration in protein expression and plant metabolism in order for the plant to adapt to salt stress. Adapted from Kosová et al. (2013)

Other than that, this study will also focus on the effect of jasmonic acid (JAS). JAS is a signalling molecule involved in abiotic and biotic stress responses in plants. (Akula & Ravishankar, 2011). JAS is a naturally occurring growth regulator that is usually found in higher plants (Creelman & Mullet, 1995; Creelman et al., 1992). The concentration of JAS in plant tissues fluctuates according to tissue function, development, and the presence of exogenous stimuli (Du et al., 2013). Gene expression is regulated by JAS, which also affects certain elements of plant growth and development such as leaf abscission, and inhibits germination in response to abiotic and biotic stresses (Awan et al., 2017; Pedranzani & Vigliocco, 2017). Jasmonate will interact with the receptors in plant cells that initiate a signalling cascade, which results in modifications in transcription, translation and others (Debnath et al., 2011). At a certain concentration of JAS, it can promote senescence in PTC and cause leaf excision. Chlorophyll depletion, chloroplast protein breakdown, such as that of ribulose biphosphate carboxylase, and an

accumulation of new proteins are all components of the senescence response (Awan et al., 2017; Pedranzani & Vigliocco, 2017). Besides, jasmonate has other vital roles, including defense against insects and pathogens and in reproductive development. JAS occurs in various modified forms such as glycosyl ester, methyl ester (MeJA) and amide-linked conjugates with several amino acids (Staswick & Tiryaki, 2004).

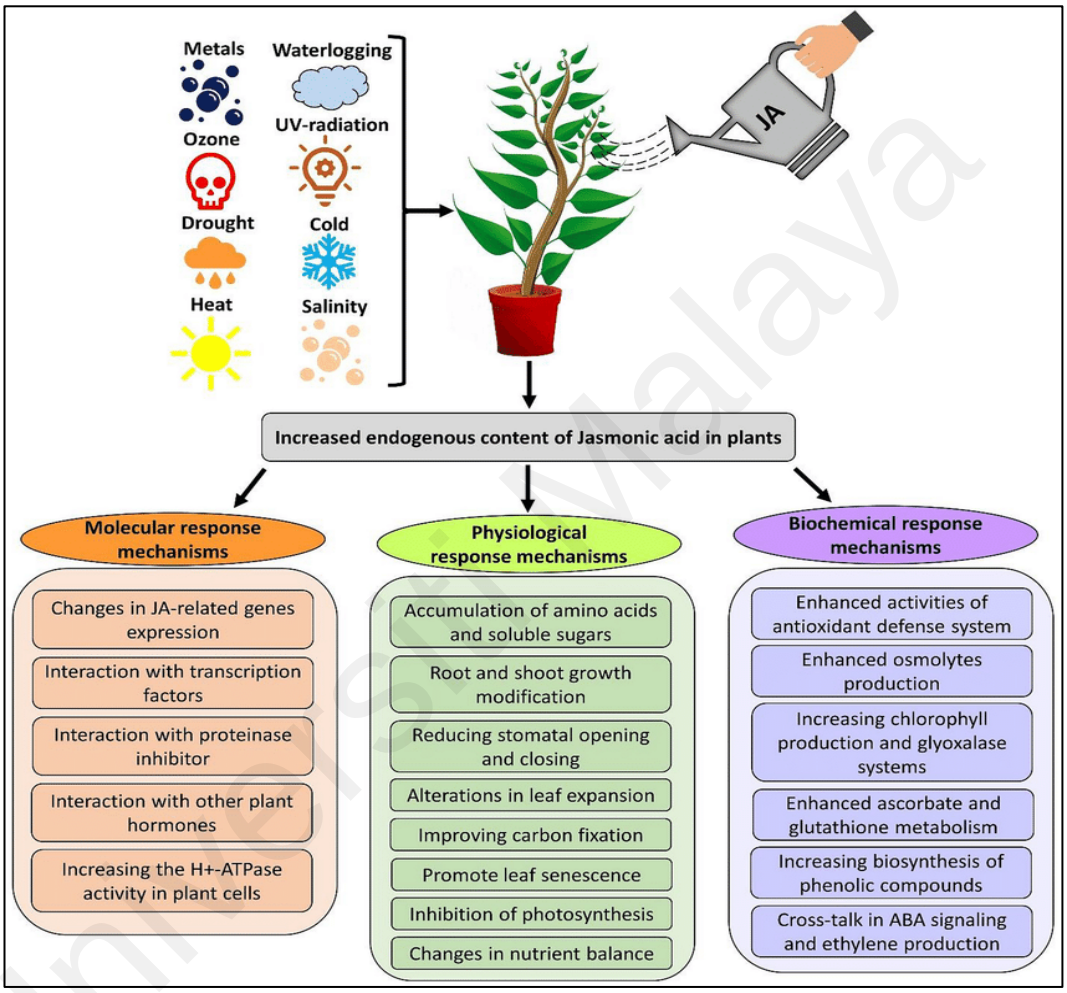

Figure 2.10: The main physiological, molecular and biochemical as plant response of JAS to adapt from abiotic stressors. Adapted from Raza et al. (2021)

Table 2.2: Elicitation of JAS and NaCl on the synthesis of certain bioactive compounds in various plant species.

Plant species	Culture type	Growth condition	Compound elicited	References
Gossypium hirsutum L.	Seedlings	50, 100, 150 and 200 $mmol.L^{-1}$NaCl	Gossypol and tannin	(Ma et al., 2021)
Carum copticum L.	Calli, and seedlings	0, 25, 50, 100 and 150 mM NaCl	Phenolic, Anthocyanin, Essential oil (Thymol, γ-terpinene, α-pinene, ρ-cymene)	(Razavizadeh et al., 2022)
Cucumis sativus and *Solanum lycopersicum*	Seedlings	25, 50, 100 and 200 mM NaCl	Flavonoid, Phenolic, Saponin, Proline	(Abdel-Farid et al., 2020)
Nigella sativa L.	Callus	0, 84, and 250 mM NaCl	Phenolic, flavonoid, flavonols, anthocyanin, thymol	(Golkar et al., 2020)
Catharanthus roseus	Callus	0, 25, 50, 75, and 100 mM NaCl	Vinblastine and vincristine	(Fatima et al., 2015)
Salacia chinensis L.	Callus	25-125 µM JAS, 50-250 µM MeJAS, 100-500 mg/l yeast extract	Mangiferin, flavonoid and phenolics	(Chavan et al., 2021)
Eclipta prostrata L.	Hairy root	100 and 140 µM JAS, 100 and 140 µM MeJAS	Flavonoids, coumestans, wedelolactone, demethylwedelolactone, and caffeoylquinic acid derivativates	(Maciel et al., 2022)
Brassica oleracea	Seedlings	100 µM JAS	Glucisinolates and sulforaphane	(Guo et al., 2017)
Opuntia streptacantha, Opuntia megacantha, and Opuntia ficus-indica	Callus	25 and 50 µM JAS, UV-C irradiation, 1.25-5% PEG (Water stress)	Phenolic, flavonoids	(Camarena-Rangel et al., 2017)
Hamelia patens	Seedlings	0.05, 0.5- and 1-mM JAS	Monoterpernoid indole alkaloids (MIAs) and monoterpenoid oxindole alkaloids (MOAs)	(Flores-Sanchez et al., 2016)
Hibicus sabdariffa Linn	Callus	0, 50 and 100 µM JAS	Anthocyanin, phenolic,flavonoid	(Jirakiattikul et al., 2021)
Vitis vinifera L.	Cell suspension	200-500 µM MeJAS, 200 µM 2HJA, 200 µM cJA	Stilbenes (trans-piceid, dimeric Ɛ- and ó-viniferin).	(Sák et al., 2021)

In the proposed study, *in vitro* culture technique is used to observe the ability of the plants to withstand abiotic stresses (salt stress and jasmonic acid) in a relatively short time under controlled conditions. These stress factors may cause changes in physico-biochemical mechanisms which may impair its development and establishment. The plants respond to stress by inducing several plant defence mechanisms, which will enhance the synthesis of intracellular solutes and oxidative enzymes.

2.6 Proteomic studies

Proteomics studies serve as another approach to studying the structural abundance and the interaction of proteins, for example, post-translational modification, protein-protein interaction, tissue and subcellular localisation, and phenotype-dependent silencing (Kosová et al., 2013; Parihar et al., 2015). The term "proteome" originates from PROTEins that are expressed by the genOME. Therefore, proteomics is the comprehensive analysis of all proteins in the cell, tissue, or whole organism at a particular time and under certain conditions (Eldakak et al., 2013). Even though genomic studies are beneficial, it stays unaltered to a considerable degree. Still, the proteins in any cell vary rapidly when genes are switched on or off in changing environmental conditions. This may occur because protein is directly implicated in treated and untreated circumstances; hence, a comprehensive knowledge of plant response may be achieved by examining the protein in treated plant cells (Cho, 2007).

When plants experience abiotic stress, it will cause them to decline in crop production. As a result, in order for plants to restore from the detrimental effects of abiotic stress, several adaptation methods at the cellular and metabolic levels have evolved in them (Aghaei & Komatsu, 2013; Liu et al., 2019). It is crucial to investigate the plant's reaction to stress conditions at the protein level because proteins are also responsible for the execution of cellular functions and maintaining cellular homeostasis (Li et al., 2020; Liu

et al., 2019). Additionally, proteins are also directly engaged in the production of new plant phenotypes by modulating physiological features for plants to adapt to changes in their environment, which makes the involvement of proteins in the biotic stress response of plants very significant (Liu et al., 2019). Proteomics studies may thus provide tools to elucidate the vital physiological processes behind plant stress tolerance. Therefore, to understand the molecular processes regulating protein levels in plants due to stress, it is critical to recognize which proteins are engaged in biological activities and are differently abundant under various situations (Liu et al., 2019).

Nowadays, numerous salt-responsive proteins, including heat shock, pathogen-related protein, protein kinase, osmotin, and some transcription factors, have been identified in certain plants, giving them the capacity to tolerate salt stress (Aghaei & Komatsu, 2013). According to Kim et al. (2015), stress-related proteins such as pathogen-related protein PR-1 (PR-1 and PR-10) and abscisic ripening protein 2-like protein (ASR2, spot7) PR were affected by drought stress. The study reported that the affected stress-related proteins were mainly up-regulated in response to drought stress, implying that the stress-related proteins may play essential roles in plant tolerance toward drought stress. Therefore, researchers may use the proteomic approach to discover differentially abundant proteins in stress-treated plants. Salinity stress and exogenous JAS treatment were used in this investigation. Table 2.3 provides an overview of past research done in various plants to examine the impact of salt stress and JAS elicitation on plant development and changes in differential protein abundance in response to stress.

Table 2.3: Representative research on plant protein differential abundance in response to exogenous JAS and NaCl stress

Species/ Treatment	Proteomic approach	Protein regulation	Protein identified	Protein functions	References
Arabidopsis thaliana 250µM MeJAS for 8 hours	iTRAQ-Quantification based	Up	DHAR1, ATCOR47, PDE334, ATHM1, Nuclear pore anchor mutants nua-1 and nua-4, Profilin-3, Photosystem I reaction center subunit N, Photosystem I protein P, Photosystem II subunit Q-1 and Q-2	Stress and defense, pollen development, photosynthesis.	(Qi et al., 2020)
		Down	40S ribosomal proteins, 60S ribosomal proteins, 30S ribosomal protein, 50S ribosomal proteins	Proteins synthesis	
Solanum tuberosum L. 0.5, 5 and 50µM JAS for 40 days	iTRAQ-Quantification based	Up	Vesical transport v-SNARE, Rubisco, transaldolase, transketolase, heterogeneous nuclear ribonucleoproteins, DEAD-box ATP-dependent RNA helicases, MAR-binding protein, glyceraldehyde 3-phosphate dehydrogenase	Carbon metabolism, cytoskeleton formation, cell wall synthesis, protein metabolism	(Yuan et al., 2022)
		Down	UDP-glucose 4-epimerase, alpha-1,4-glucan protein synthase, α-tubulin, β-tubulin, actin depolymerizing factor, pyruvate kinase, ATP citrate synthase, ribosomal proteins, secretion-associated RAS superfamily, proteasome, pyruvate dehydrogenase, phosphoglucomutase	Cell wall synthesis, cytoskeleton formation, energy and carbohydrate metabolism, protein metabolism,	
Brassica sprouts 100µM JAS for 5 days	iTRAQ-Quantification based	Up	Cytochrome P450, Glutathione S-transferase, sulfotransferase, 3-isopropylmalate dehydratase, myrosinase 1 and 2, protochlorophyllide reductase, isocitrate lyase, malate dehydrogenase, pectate lyase, pectin acetylesterase 7, alpha-galactosidase I and II, phosphoserine aminotransferase I, peroxidase, superoxide dismutase, catalase, proteasome, ubiquitin.	Secondary metabolites, carbohydrate metabolism, pectin degradation process, cell wall, amino acid metabolism	(Guo et al., 2017)

Table 2.3, continued.

Species/ Treatment	Proteomic approach	Protein regulation	Protein identified	Protein functions	References
		Down	Photosystem I and II, ferredoxin-NADP reductase, beta carbonic anhydrase, serine-glyoxylate aminotransferase, glycerate dehydrogenase HPR, chlorophyll a-b binding protein, ATP synthase, ATPase, V-type proton, 40S ribosomal protein, 60S r-protein, 30S r-protein, 50S r-protein	Energy metabolism, photosynthesis, protein synthesis	
Trigonella foenum-graecum L. 100µL^{-1} of MeJAS for 1, 3 and 6 days	2-D gel electrophoresis	Up Down	Chlorophyll a-b binding protein, photosystem II, glyceraldehyde 3-phosphate dehydrogenase, fructose-bisphosphate aldolase, NO-associated protein 1, alcohol dehydrogenase, glutamine synthase, vegetative lectin, ATP synthase, Kunitz-type trypsin inhibitor, thioredoxin Rubisco, sedoheptulose-1,7-bisphosphatase, oxygen-evolving complex, Triose-phosphate isomerase	Photosynthesis, protein metabolism, stress and defense, energy and carbohydrate metabolism Energy and carbohydrate metabolism, respiration	(Ciura et al., 2017)
Helianthus tuberosus L. 50, 100, 150 and 200 mM NaCl for 1,3,5, 7 days.	Tandem mass tag (TMT) labelling and LC-ESI-MS/MS analysis	Up	Peroxidase, superoxidase dismutase, glutathione S-transferase, phospholipase D alpha isoform X1, glycerol-3-phosphate acyl transferase, choline kinase 1, endoglucanase, carbonic anhydrase, phosphoenolpyruvate carboxylase, pyruvate phosphate dikinase, glyceraldehyde-3-phosphate dehydrogenase, pyrophosphate-fructose 6-phosphate 1-phosphotransferase	Defense and stress, lipid synthesis, carbohydrate metabolism,	(Song et al., 2021)

Table 2.3, continued.

Species/ Treatment	Proteomic approach	Protein regulation	Protein identified	Protein functions	References
		Down	Ribosomal RNA-processing protein, ribosomal proteins, tubulin-folding cofactor B, cullin family protein, mitogen-activated protein kinase kinase (MEKK1 and MKK2)	Protein synthesis and cell division, retinoblastoma-related protein, signal transduction pathway.	
Musa paradisiaca 60 mmol/L NaCl for 0,12 and 24 hours	iTRAQ-Quantification based	Up	Disulfide isomerase, cysteine protease, ribosomal proteins, Oxygen-evolving enhancer protein 2, Rubisco, ribose-5-phosphate isomerase, allene oxide cyclase, lectin, germin like protein, glyceraldehyde-3-phosphate dehydrogenase, V-type proton ATPase, beta-galactosidase, fructokinase, glucan endo-1, 3-beta-glucosidase, peroxidase, catalase, polyphenol oxidase, chaperone, tubulin, phospholipase, Acyl-CoA binding protein	Photosynthesis, protein synthesis and degradation, defense and stress, lipid metabolism, energy and carbohydrate metabolism	(Ji et al., 2019)
		Down	Thioredoxin, glutathione S-transferase, 60S ribosomal protein L3, 40S ribosomal protein S30, ATP synthase, galactinol synthase, 4-alpha-glucanotransferase, ADH-dehydrogenase, ACC synthase, actin	Defense and stress, protein synthesis, energy and carbohydrate metabolism	
Brassica juncea L Czern and Coss 50, 100, 150, 200 mM NaCl for 30 days	2-D gel electrophoresis	Up	Light-harvesting complex, zinc finger protein, ubiquitin, thioredoxin h-like protein, Fd-NADP reductase, PR10-proteins, signal recognition particle 50 kDa pro, bZIP transcription factor, polyubiquitin, PII like, Remorin family protein, sucrose, synthase 2, heme oxygenase 3	Defense and stress, protein degradation and synthesis, signal transduction, photosynthesis, nitrogen metabolism.	(Yousuf et al., 2017)

Table 2.3, continued.

Species/ Treatment	Proteomic approach	Protein regulation	Protein identified	Protein functions	References
		Down	Granule bound starch synthase, malate dehydrogenase, protein phosphatase 2c, fructokinase, Rubisco, glycerate kinase, transketolase, Ribosomal protein S19homolog, ATP synthase F0 subunit beta, photosystem II protein I, isocitrate lyase, Ribosomal protein S4, Ribulose-1,5-biphosphate carboxylase/oxygenase, glyceraldehyde-3 phosphate, dehydrogenase, NADP isocitrate dehydrogenase.	Energy and carbohydrate metabolism, protein synthesis, signal transduction, photosynthesis	
Abelmoschus esculentus L. 300 mmol L^{-1} NaCl for 48 hours.	TMT labelling & LCMS/MS based	Up Down	Peroxidases, catalases, pathogenesis-related protein (PR10-5), mitogen-activated protein kinase, heat-shock protein, chaperone, seed maturation protein Major latex like protein, peroxidase,	Defense and stress, signal transduction Stress and defense	(Zhan et al., 2019)

2.6.1 LCMS/MS

Methodologies and technology for proteomics have progressed from the point of view based on two-dimensional gel electrophoresis to gel-free analysis throughout the last several decades. According to Quirino et al. (2010), label/gel-free proteomics technique has flourished with the assistance of mass spectrometry and their capacity to link with genomic data. With significant advancement of mass spectrometry-based proteomic technique, qualitative proteome analysis may now be able to do more than identify proteins but also provide accurate and reliable quantitative analysis (Liu et al., 2019).

Bottom-up proteomics, often known as "shotgun proteomics," involves the identification and characterization of proteins by their amino acid sequence and post-translational modification by proteolytic digestion and subsequent mass spectrometric analysis (Abdallah et al., 2012). Gel-free proteomics requires some primary stages, including preparing the sample, separating it with liquid chromatography, and subsequent analysis by tandem mass spectrometry and data interpretation (Karpievitch et al., 2010). The process of sample preparation began with the extraction of proteins from the sample, followed by fractionation to eliminate impurities and trypsin digestion of the proteins to peptides (Bowman, 2014; Karpievitch et al., 2010). The separation of peptide mixtures using nano-LC, carried out in either 1-D mode or 2-D mode, is the next step (Bowman, 2014). This enables the resolution of peptides across a broad hydrophobicity and pI range. Lastly, by comparing the discovered properties to entries in databases of hypothetical or previously recognized peptides, proteins are identified. According to Liu et al. (2019) tandem mass spectrometry with rapid sequencing rates, high mass accuracy, high sensitivity, and the capacity to sort out multiple peptide-derived ions simultaneously is needed for proteomics and quantification.

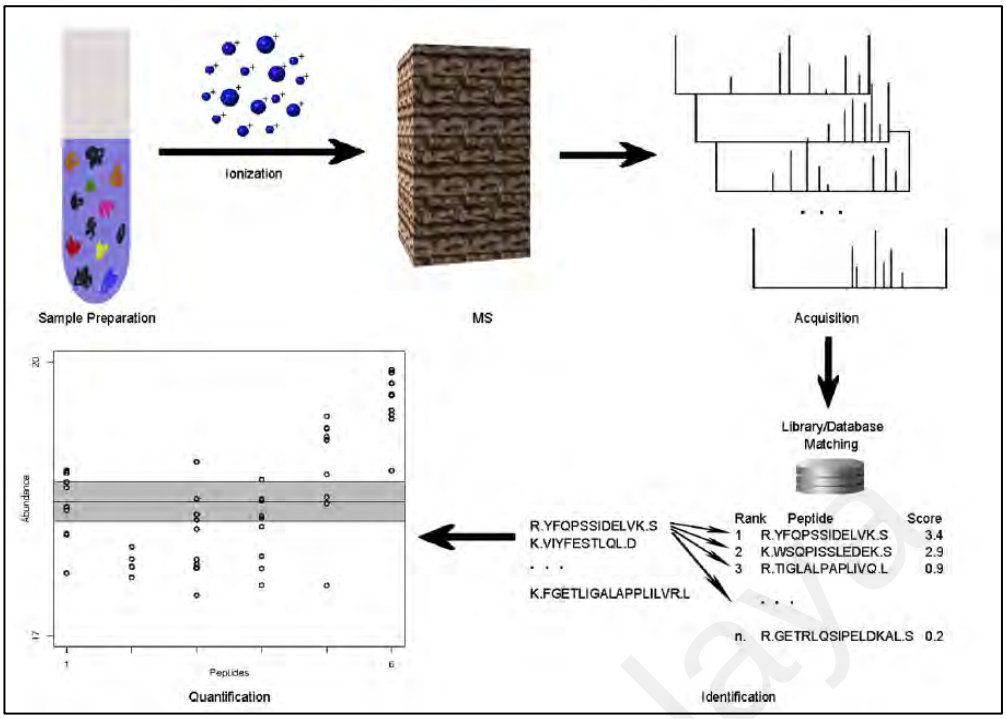

Figure 2.11: Illustration of LCMS/MS-based proteomics technique. The process began with sample preparation and analysis by mass spectrometry. Database searching is often used to discover peptides based on observable spectral information. Adapted from Karpievitch et al. (2010)

CHAPTER 3: METHODOLOGY

3.1 Methodology Flowchart

Figure 3.1 shows the methodology outline for this research project starting from the sample collection of *A. indica* until the final statistical data analysis.

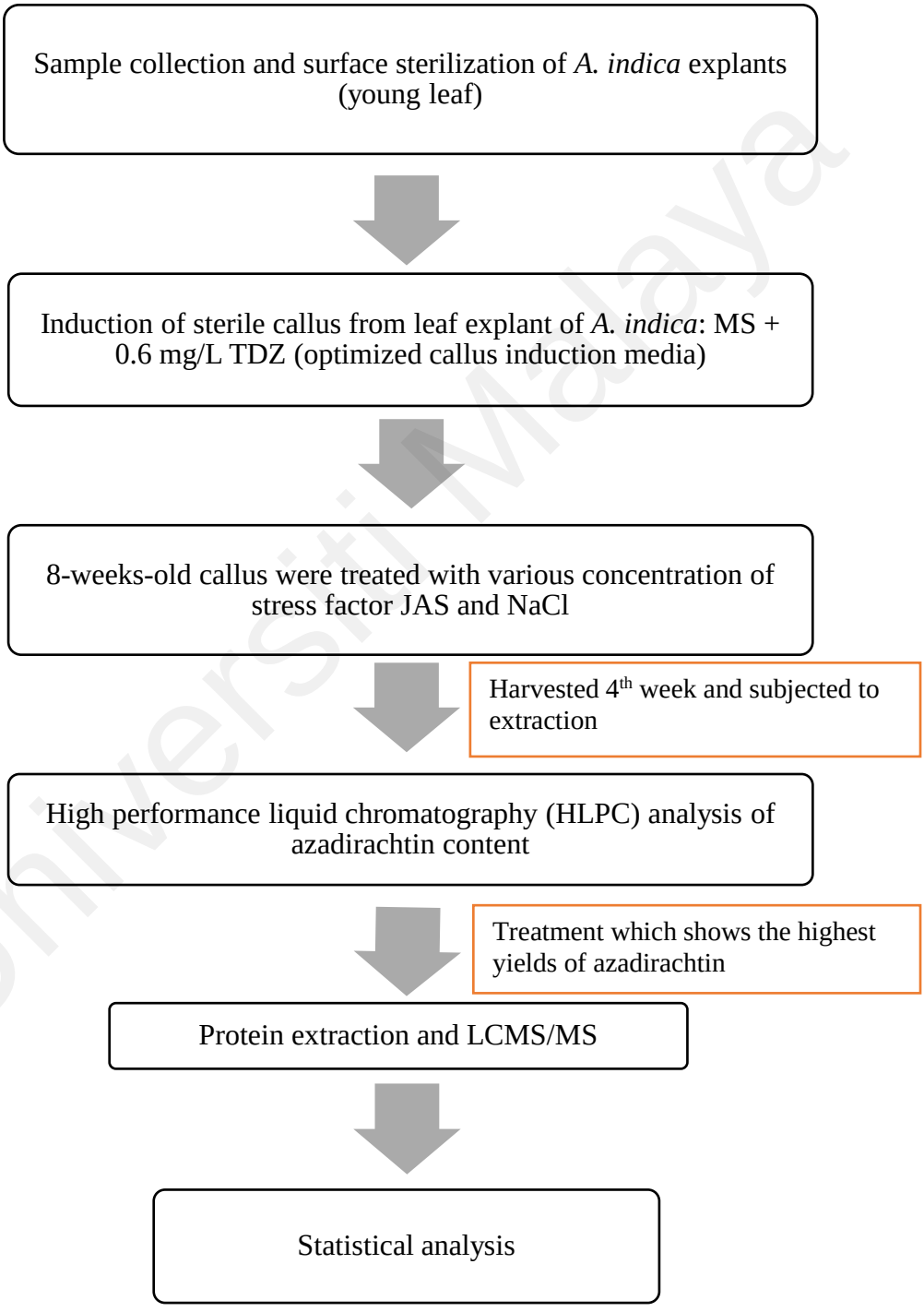

Figure 3.1: Methodology applied in this research

3.2 Establishment and maintenance of neem callus cultures *in vitro*

Newly collected young leaves were thoroughly rinsed under tap water for two hours prior to surface sterilization. The surface sterilization technique was performed as described by Houllou et al. (2015b) with minor modification by immersing the explants into 70% (v/v) of commercially available bleach, Clorox (5.25% (w/v) sodium hypochlorite) (Ashokhan et al., 2020) solution for 10 minutes with an addition of 2 drops of Tween 20. Washing the explants three times with sterile distilled water was necessary to remove all traces of detergent, and inoculating the explants was done aseptically in a laminar flow hood to ensure sterility (Daud et al., 2012).

Leaf explants were blotted dry after surface sterilization steps. The leaves were cut into small squares consisting of the midrib and veins of the leaves (Ashokhan et al., 2020; Daud et al., 2012). To induce the formation of sterile callus from *Azadirachta indica* leaf explants, optimized callus induction media (OCM) was utilized, which consisted of 4.4 g/L Murashige & Skoog media (Murashige & Skoog, 1962) with 30 g/L sucrose, 2 g/L gelrite (Duchefa Biochemie, Netherlands) and 0.6 mg/L thidiazuron (TDZ) (Ashokhan et al., 2019; K AL-Mallah, 2006). The OCM was adjusted to a 5.7-5.9 pH range before autoclaving (at 121 °C and 1kPa for 20 min) by adding 0.1 M NaOH and 0.1 M HCl (Ashokhan et al., 2020; Ashokhan et al., 2019). Sterile tubes containing leaf explants were incubated in the dark condition in the culture room at 25 ± 1 °C (Ashokhan et al., 2020).

3.3 Induction of abiotic stresses on the callus cultures

Two different types of abiotic stress factors such as salinity and JAS (a plant molecule involved in abiotic stress signalling) were used to study the effect of abiotic stress on *Azadirachta indica* callus cultures. Callus cultures with the same inoculum age 8-weeks-old) were sub-cultured onto OCM medium supplemented with several concentrations

(0.05, 0.1, 0.2, 0.5, 1.0, and 1.5% w/v) of NaCl and (1, 2, 3, 4, 5 and 6 mg/L) of JAS (Sigma, USA).

The treated cultures and non-treated cultures were incubated under controlled conditions for additional 4 weeks at 25 ± 1°C in the culture room in a dark condition before being harvested and subjected to further protein and metabolite screening and identification (Ashokhan et al., 2020; Binte Mostafiz & Wagiran, 2018; Khan et al., 2006). The culture performance was observed in terms of callus morphology; for instance, colour changes, texture, survival rate, and fresh weight of the callus were recorded. To evaluate the growth productivity of *A.indica* callus, the growth index (GI) of the callus was analysed (Bhatia, 2015). The growth index was measured based on the callus's final and initial fresh weight. The growth index (GI) was calculated using the following formula (Bhatia, 2015; Sahraroo et al., 2014):

$$\boldsymbol{GI} = \frac{(\boldsymbol{W_I} - \boldsymbol{W_O})}{\boldsymbol{W_O}} \tag{3.1}$$

Where,

W_O = initial fresh weight of callus before treatment

W_I = final fresh weight of callus after the treatment period

3.4 High-Performance Liquid Chromatography (HPLC) analysis

3.4.1 Extraction of azadirachtin

Extraction of azadirachtin and standard curve preparation was done as reported by Singh and Chaturvedi (2013) with few modifications. Samples were subjected to freeze-drying using Labconco freeze dryer (Labconco Corporation, MO 64132 United States) at -50 °C. The mass of callus was weighed by using AS 220/X electronic balance (RADWAG Wagi Elektroniczne, Poland). To improve the extraction process, dried

samples were pulverized in a cold mortar and pestle with the addition of liquid nitrogen. The whole procedure was carried out under as minimum light exposure as possible since the compounds are sensitive to light and heat (Ashokhan et al., 2020). 1g of powdered dry samples were immersed overnight in methanol at a temperature of 4 °C. The following day, the mixtures were vortexed and centrifuged for 10 minutes at 13500g using a refrigerated centrifuge (Universal 32 R centrifuge Hettich Zentrifugen D-78532 Germany).

The supernatant was collected and transferred into foil-covered 50mL graduated polypropylene centrifuge tubes. Next, water was added at the ratio of 40:60 (40% water and 60% methanol). Extraction of azadirachtin was done by adding an equal amount of dichloromethane (DCM) to the sample mixture in separating funnels. After that, the mixture was adequately mixed and was allowed to separate two immiscible solvents (methanol+ water and DCM). After the separation process, the top layer of methanol and water was discarded, while the bottom layer was recovered and transferred into new tubes. The bottom layer, which contained DCM, was evaporated to dryness using a vacuum concentrator (Thermo Scientific, Waltham, MA, USA). The dry materials were weighed and redissolved in HPLC grade methanol at desired concentration before being filtered through a 0.22 µm nylon membrane filtered and kept in amber vials. The vials were then covered and sealed with parafilm prior to HPLC analysis.

3.4.2 Quantification of azadirachtin content

Quantification of azadirachtin content was performed using Agilent 1200 series HPLC system (Agilent Technologies, USA) as described by Ashokhan et al. (2020). This system consists of a binary pump with an autosampler injector, micro vacuum degassers, a thermostat column compartment, and a diode array detector (Ashokhan et al., 2020). A reverse-phase column ZORBAX SB-C18 end-capped (5 µm, 4.6 x 250mm) (Waters,

Milford, MA, USA) was used as a stationary phase for separation (Yaacob et al., 2018). The mobile phase consisted of 9:1 v/v methanol: water pumped at the flow rate of 0.5 ml^{-1} (Singh & Chaturvedi, 2013). The column was allowed to re-equilibrate for 10 minutes before the next sample injection while the temperature was maintained at 20 °C and in with 10 µL injection volume. The analytical wavelength for azadirachtin was monitored and identified at 210 nm.

The chromatographic peaks of the analytes were determined by comparing the retention time of the samples and the azadirachtin standard (≈ 95%). The presence of azadirachtin compounds in the samples was verified by analysing the spectral characteristics shown by the standard following co-chromatography and detected by a photo-diode array detector. The calibration curve of azadirachtin was constructed with a standard compound at various concentrations. The accumulation of azadirachtin in the samples was calculated and estimated based on the standard curve of the compound and compared to their peak areas. The amount of azadirachtin was expressed as milligram per gram dry weight (mg/g DW) of callus. The NaCl and JAS treatments which resulted in the highest content of azadirachtin in the callus samples were identified, and used in subsequent experiments.

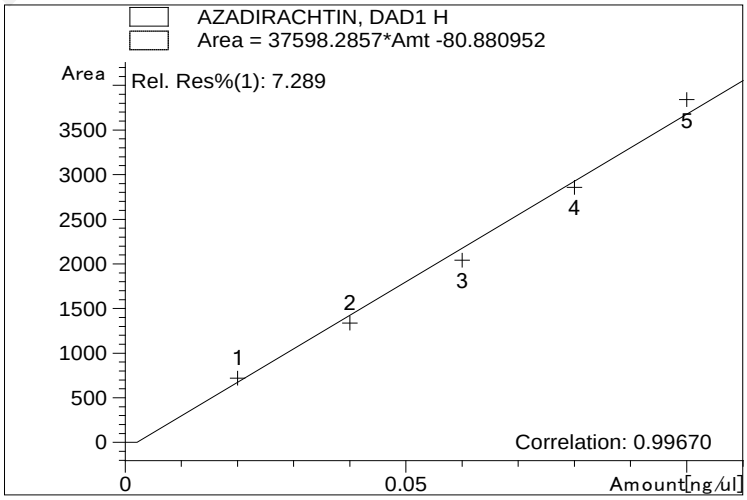

Figure 3.2: Azadirachtin standard curve as reference for HPLC analysis. Each value is the mean ± standard error (SE) consisting of three replicates.

3.5 LCMS/MS

3.5.1 Protein extraction and precipitation

4-weeks-old callus was harvested from OCM, 1.5% NaCl and 6 mg/L JAS media and subjected to a freeze-drying process using a Labconco freeze dryer (Labconco Corporation, MO 64132 United States). Dried callus was ground with chilled mortar and pestle until it became fine powder in the presence of liquid nitrogen to extract the soluble protein. Then, 0.2 - 0.5 g of callus powders were allocated into a sterile 2 ml microcentrifuge tube (Ngara et al., 2012). These tissue disruption steps are crucial to reduce the proteolysis and protein modification which may occur. The fine powder was stored in a -80 °C freezer for further extraction (Wu et al., 2014).

In this study, TCA (trichloroacetic acid) – acetone precipitate (with PVPP) (TCAP) method was chosen based on the previous studies. TCA-acetone precipitation is based on protein denaturation under acidic and hydrophobic conditions, which may aid in concentrating proteins and removal of impurities.

TCAP precipitation was performed according to a method described by Tan et al. (2013) with some modifications. 200 mg fine callus powder from neem was dissolved in 1.5 ml of freshly prepared cold precipitation solution 10% (v/v) TCA, 0.2 mM DTT, 1% (w/v) polyvinylpyrrolidone (PVPP) in acetone) and incubated at -20 °C for overnight. Then, the homogenate was vortexed (Stuart Scientific, UK) and centrifuged using Select Spin ™ 17R Refrigerated Microcentrifuge (Select BioProducts, Korea) at 15000g for 15 minutes at 4 °C. The supernatant was discarded, and the pellet was washed by adding 1 ml cold wash solution (acetone and 0.2 mM DTT) and stored at -20 °C for 3-4 hours. A few washing steps were repeated with different incubation times (2 hours and 30 minutes), and during each washing step, the sample was centrifuged until the pellet and supernatant got discoloured. During the last rinse, the supernatant was discarded. Pellet was air-dried for a few minutes at room temperature. Air-dried protein pellets were re-

suspended in lysis buffer with the addition of 0.2 mM DTT, nuclease, and protein inhibitor. The mixture was vortex thoroughly followed by incubation at room temperature for 30 minutes before centrifuged at 15000g, at 23 °C for 20 minutes. The supernatant was transferred into a new microcentrifuge tube and kept at -80 °C for further use while the pellet was discarded.

3.5.2 Protein quantification

The concentration of soluble protein in callus neem samples was evaluated based on the Bradford protein assay as previously described by Bradford (1976). The reactions were carried out in a 96-well plate under dim light. 5 µl of various known concentrations of bovine serum albumin (BSA) standard set (Bio-Rad Laboratories, US) (0.125, 0.25, 0.5, 0.75, 1, 1.5, and 2.0 mg/ml) and 250 µl Bradford dye reagent (Bio-Rad Laboratories, US) was pipetted into each well in triplicate. The plate was mixed carefully using a plate shaker and incubated for 5 minutes at 500 rpm. The absorbance was taken at 595nm by using a microplate reader. A standard curve of absorbance value against the concentration of BSA was established. This step was repeated by replacing concentration BSA with protein samples. The protein concentration of the samples was then measured in triplicate.

3.5.3 In-solution peptide digestion

Protein digestion was executed as reported by Lau and Othman (2019). A total of 25 µg of precipitated proteins were re-suspended in 100 µL of 50 mM ammonium bicarbonate and 1 M urea. The reduction and alkylation were done using 100 mM tris (2-carboxyethyl) phosphine and 200 Mm iodoacetamide, respectively. Before digestion, sodium deoxycholate (1% w/v) was added to the protein solution containing 50 mM ammonium bicarbonate in order to improve peptide solubility at 37 °C for 10 minutes. Trypsin/Lys-C mix mass spectrometry grade (Promega, Madison, WI, USA) was used as

tryptic digestion and was performed at 37 °C for 16 hours. Acidification on peptide mixtures was done using 0.5% formic acid to remove sodium deoxycholate from the solution through centrifugation at 14000g (Eppendorf Thermo Scientific) at room temperature for 15 minutes. A centrifugal evaporator (CentriVap Concentrator, Labconco, MO, USA) was used to dry the remaining solvents and acids. The dried peptide pellets were re-suspended in 100 µL of 0.1% formic acid and mixed thoroughly before peptide purification. An Empore solid phase extraction disk (3M Purification, Inc, MN, USA), conditioned with acetonitrile, methanol, and 0.1% formic acid, was added into peptide solution and incubated at ambient temperature with slightly agitation for 4 hours to bind the peptide. Elution of peptides from C18 membrane disks was done consecutively using 50% ACN in 0.1% formic acids (FA) for 2.5 hours. This solvent was then removed (Abu Bakar et al., 2022).

3.5.4 Liquid chromatography-mass spectrometry analysis (LCMS)

Peptides were reconstituted in 30 µL of 0.1% FA, and 5% ACN. 2 µL of digests were loaded onto an Acclaim PepMap 100 C18 column (2 µm, 0.075 ×150 mm) (Thermo Scientific, MA, USA). The reverse-phase column was equilibrated with 0.1% FA (mobile phase A) and 80% of ACN containing 0.1% FA (mobile phase B). Gradient of 5-35% mobile phase B in 75 min, at a flow rate of 300 nL/min, was applied for elution of peptide. Separation of the peptides was executed using EASY-nano liquid chromatography (EASY-nLC) 1200 System (Thermo Scientific, MA, USA). An online Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer system (Thermo Scientific, MA, USA) was used to generate the peptide ions with a spray voltage of 1900 V in positive mode. Precursor ion scan was performed with a resolution of 70,000 and a mass range of m/z 310-1800. Precursors containing charge states from 2 + to 8 + were fragmented further. The fragmentation was done via collision-induced and high-energy collision-induced

(CID and HCD) at normalized energy of 28%, correspondingly. The resolution, isolation window, and ion injection time were set at 17,500, 0.7 Da, and 60 ms, respectively. Scanned precursor mass range was set at m/z 110-1800 (Lau et al., 2020).

3.5.5 Protein identification and bioinformatic analysis

Mass spectra of the peptides were retrieved using Tune (Ver. 2.11 QF1 Build 3006) (Thermo Scientific, MA, USA) and deconvoluted with Proteome Discoverer (Ver. 2.4) (Thermo Scientific, MA, USA) to generate the peptide mass list. SEQUEST HT search engine, incorporated in the Proteome Discoverer, was used to match the generated mass list against *Viridiplantae* FASTA sequences downloaded from NCBI. Mass tolerance for the peptides and fragments was fixed at 10 ppm and 0.02 Da, respectively. Trypsin was indicated as the digestion enzyme used, with up to two missed cleavages allowed during the search. Carbamidomethylation modification on cysteine residues was set as a fixed modification, while variable amino acid modifications included deamidation (asparagine and glutamine residues) and oxidation (methionine residues). The mass list was also searched against a decoy database generated from the randomized protein sequences. The identified proteins were filtered with at least a Rank 1 peptide and a false discovery rate of 1% in order to be accepted. Spectra that matched the sequences were further confirmed with Percolator algorithm (Ver. 2.04) with q -value at 1% false discovery rate (Lau et al., 2020).

Data were subjected to Principal Component Analysis (PCA) and was done with the statistical analysis component in Proteome Discoverer to analyse the clustering behaviour of the samples. The clustering method used was a simple agglomerative hierarchical clustering method (UPGMA). The distance measure applied was Euclidean in logarithmic scale for rows. Distance between clusters was computed with Ward's method.

Venn diagrams were constructed to compare the identified proteins within all treatments. Protein abundance values were used to calculate the $\log_2$ ratios of JAS: OCM and NaCl: OCM. Abundance expressed proteins were identified based on the data of abundance ratio ($\log_2$). The $\log_{10}$ P-value was plotted against the $\log_2$ fold change with fold change threshold 1.5 and p-value <0.05. Gene ontology (GO) annotations were retrieved from UniProtKB database (http://www.uniprot.org/blast/).

3.6 Statistical analysis

The callus cultures were observed weekly to detect any changes or contamination. All analyses were carried out in triplicate, and the data were expressed as means ± standard error (SE). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) by SPSS 23 software was used to determine the significant difference between means. Correlations were obtained by Pearson's correlation coefficient (r) in bivariate linear correlation. *P* values <0.05 were considered as statistically significant (IBM SPSS Statistics version 23, Windows). Total protein content from TCAP extraction method was determined from a standard curve plotted using bovine serum albumin, and the value represents the mean of three biological replicates.

CHAPTER 4: RESULT

4.1 Fresh weight and growth index (GI)

4.1.1 Effects of NaCl on callus fresh weight and growth index (GI)

The effect of varying concentrations of NaCl added in OCM media was studied in order to have a better knowledge of their impact on callus performance and morphology (Figure 4.1). The fresh callus weight (FW) and growth index (GI), as illustrated in Tables 4.1, were measured, and used to evaluate callus development and growth.

The addition of NaCl to the media also influenced the colour of the callus. A noticeable browning reaction of callus was seen in all salt stress concentrations compared to OCM (Figure 4.1). Callus in OCM and those added with low levels of NaCl were dark green and slightly yellowish, while the callus became yellowish green with mixed of brown in colour as the NaCl concentration increased. 0.05, 0.1, 0.2 and 1.5 NaCl were observed in a compact texture while 0.5 and 1.0 were in friable texture. The size of callus on higher NaCl supplemented media found to be smaller compared to other treatments. According to the results of these experiments, a higher concentration of NaCl has a detrimental effect on the development of callus.

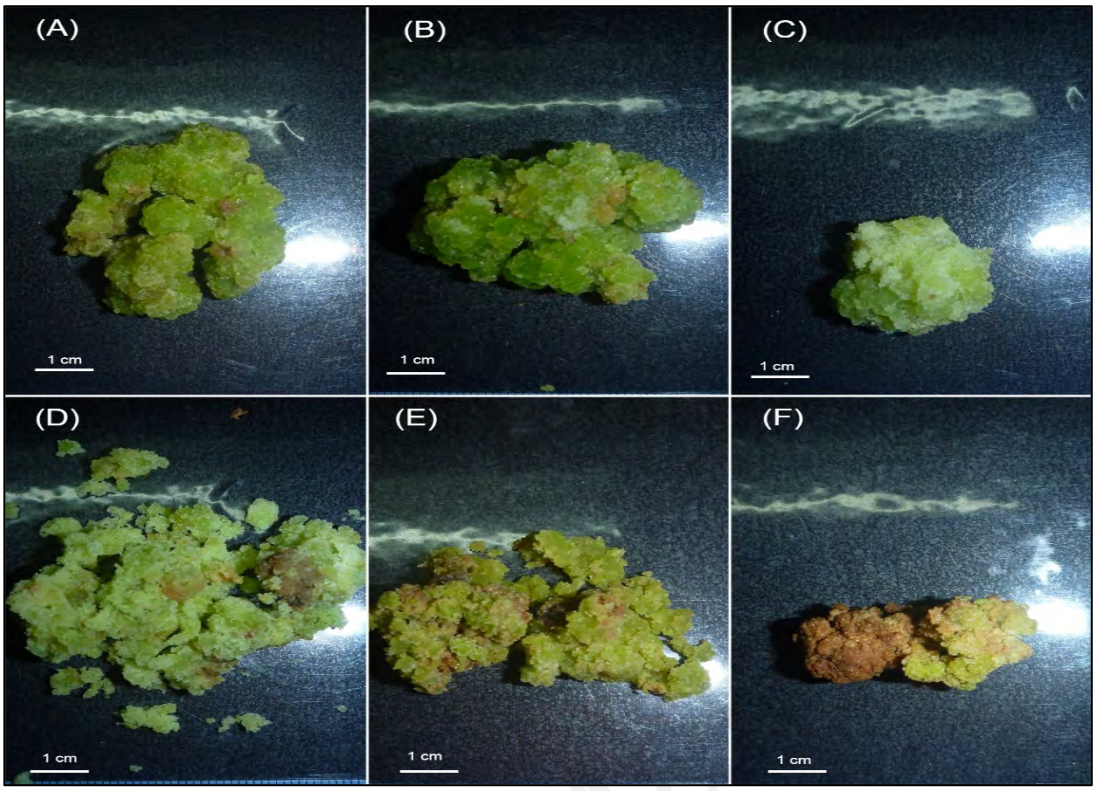

Figure 4.1: Morphology of callus produced on optimized callus induction media supplemented with different salinity stress concentrations. (A) 0.05 NaCl (B) 0.1 NaCl (C) 0.2 NaCl (D) 0.5 NaCl (E) 1.0 NaCl (F) 1.5 NaCl

To evaluate the effect of salinity on fresh weight and growth index, neem callus was exposed to 0.05, 0.1, 0.2, 0.5, 1.0, and 1.5 of NaCl for 4 weeks, and their growth parameters were evaluated (Table 4.1). The increase in salinity levels was observed to affect the callus weight. NaCl has a substantial impact on the biomass accumulation of callus culture, particularly when the concentration of NaCl reaches 150 mM. Effect of salinity indicated that the fresh weight did not significantly increase at 0.05 NaCl and 0.1 NaCl compared to OCM media. However, the fresh weight of callus decreased significantly as salinity level increased from 0.5 NaCl to 1.0 NaCl and 1.5 NaCl. The results of this study revealed that 0.05 NaCl was the most effective in enhancing callus regeneration capacity after exposure to salt stress with callus fresh weight and growth index of 2.156 ± 0.229 g and 2.197 ± 0.225 g, respectively, followed by 0.1 NaCl, 0.2 NaCl, and 0.5 NaCl as compared to OCM media. Afterwards, a sharp decline in fresh weight and growth index was noticed at 1.0 NaCl and 1.5 NaCl, where the fresh weight

and growth index went from 0.592 ± 0.076 g, 0.479 ± 0.051 g at 1.0 NaCl to 0.548 ± 0.0734 g, 0.281 ± 0.031 g at 1.5 NaCl. The fresh weight of the NaCl-treated neem callus ranged from 0.548 ± 0.0734 g to 2.156 ± 0.229 g, and arranged in a decreasing order is 0.05 NaCl > 0.1 NaCl > OCM > 0.2 NaCl > 0.5 NaCl > 1.0 NaCl > 1.5 NaCl. To put it briefly, we can conclude that a low level of salinity in culture media would enhance the growth index of callus, and simultaneously, a high salinity level would cause the callus fresh weight and growth index to decrease.

Table 4.1: Effect of TDZ and NaCl concentrations on callus fresh weight and growth index

Salinity stress			
Sample ID	**Treatment**	**Fresh weight (g)**	**Growth index (GI)**
OCM	0.6 mg/L TDZ	1.805 ± 0.116^{bcd}	1.355 ± 0.081^{b}
0.05 NaCl	0.6 mg/L TDZ + 0.05% NaCl	2.156 ± 0.229^{d}	2.197 ± 0.225^{c}
0.1 NaCl	0.6 mg/L TDZ + 0.1% NaCl	1.937 ± 0.106^{cd}	1.987 ± 0.140^{c}
0.2 NaCl	0.6 mg/L TDZ + 0.2% NaCl	1.723 ± 0.127^{bc}	1.918 ± 0.108^{c}
0.5 NaCl	0.6 mg/L TDZ + 0.5% NaCl	1.516 ± 0.134^{b}	1.186 ± 0.073^{b}
1.0 NaCl	0.6 mg/L TDZ + 1.0% NaCl	0.592 ± 0.076^{a}	0.479 ± 0.051^{a}
1.5 NaCl	0.6 mg/L TDZ + 1.5% NaCl	0.548 ± 0.0734^{a}	0.281 ± 0.031^{a}

Data expressed as mean values ± standard error (SE) of 16 replicates callus cultures. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).
*O*CM*: optimized callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *NaCl*: sodium chloride; *GI*: growth index;

4.1.2 Effects of JAS on callus fresh weight and growth index (GI)

Table 4.2 shows the result of fresh weight and growth index of callus produced from media supplemented with various concentrations of JAS. Data analysis revealed that the addition of JAS at different concentrations significantly influence the callus fresh weight and growth index, where the addition of 1 mg/L of JAS yielded the highest callus fresh

weight (2.767 ± 0.193 g) and highest growth index (1.790 ±0.074 g) (Table 4.2). Afterwards, decline in growth index was noticed as JAS concentration increased compared to OCM. The fresh weight of callus after elicitation with JAS ranged from 1.320 ± 0.209 g to 2.767 ± 0.193 g. Organized in decreasing order of fresh weight from highest to lowest is: 1 JAS > 5JAS > OCM > 2JAS > 3JAS > 6JAS > 4JAS. Meanwhile, the growth index of neem callus cultures ranged from 1.016 ± 0.132 g to 1.790 ± 0.074 g, and arranged in a decreasing order is 1JAS > 2JAS > OCM > 3JAS > 5JAS > 6JAS > 4JAS. Therefore, a low concentration of JAS added in media able to improve growth and production of biomass in neem callus, yet, an increased concentration of JAS could reduce the callus fresh weight and eventually reduce growth index reading.

Table 4.2: Effect of TDZ and JAS concentrations on callus fresh weight and growth index

Jasmonic acid elicitation			
Sample ID	**Treatment**	**Fresh weight (g)**	**Growth index**
OCM	0.6 mg/L TDZ	1.805 ± 0.116^{ab}	1.355 ± 0.081^{b}
1JAS	0.6 mg/L TDZ + 1 mg/L jasmonic acid	2.767 ± 0.193^{c}	1.790 ± 0.074^{c}
2JAS	0.6 mg/L TDZ + 2 mg/L jasmonic acid	1.637 ± 0.177^{ab}	1.407 ± 0.101^{b}
3JAS	0.6 mg/L TDZ + 3 mg/L jasmonic acid	1.681 ± 0.193^{ab}	1.322 ± 0.084^{b}
4JAS	0.6 mg/L TDZ + 4 mg/L jasmonic acid	1.320 ± 0.209^{a}	1.016 ± 0.132^{a}
5JAS	0.6 mg/L TDZ + 5 mg/L jasmonic acid	2.034 ± 0.233^{b}	1.233 ± 0.095^{ab}
6JAS	0.6 mg/L TDZ + 6 mg/L jasmonic acid	1.532 ± 0.189^{ab}	1.143 ± 0.099^{ab}

Data expressed as mean values ± standard error (SE) of 16 replicates callus cultures. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).
*O*CM*: optimized callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JAS*: jasmonic acid; *GI*: growth index

Meanwhile, the addition of JAS into the media also influenced the colour and callus morphology. A noticeable browning colour was seen on almost all callus in different JAS concentration (Figure4.2). The callus produced with on OCM added with JAS at varying concentrations showed a reduced green colour. The callus was observed to be slightly brown and yellowish light green compared to OCM. Callus exhibited compact texture in all treatments. 6JAS showed less browning reaction and darker green compared to other treatment with different JAS concentration.

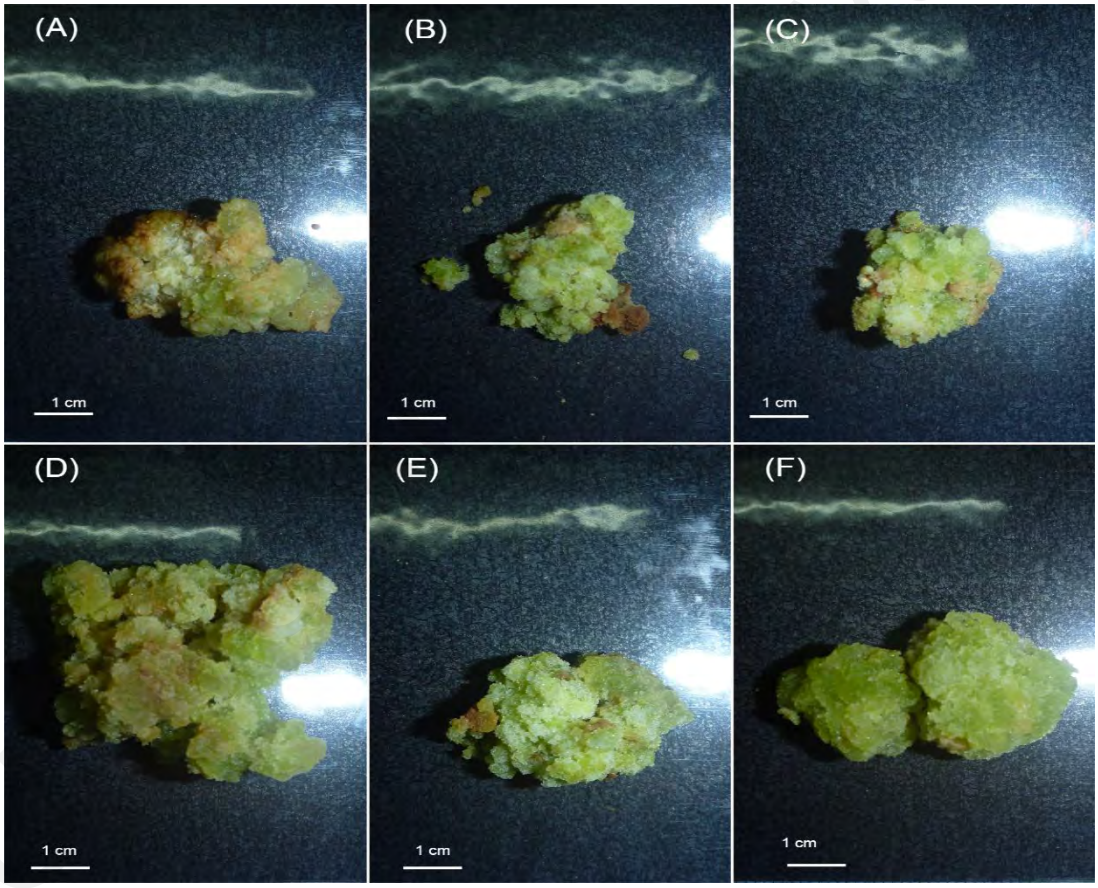

Figure 4.2: Morphology of callus produced on optimized callus induction media supplemented with different JAS concentrations. (A) 1 mg/L JAS (B) 2 mg/L JAS (C) 3 mg/L JAS (D) 4 mg/L JAS (E) 5 mg/L JAS (F) 6 mg/L JAS

4.2 Quantification of azadirachtin content of *Azadirachta indica* callus cultures produced

HPLC was used to determine the presence and to quantify the amount of azadirachtin in the callus samples. The content of azadirachtin in neem callus extract was evaluated after it was treated with various concentrations of NaCl and JAS. The azadirachtin content for each treatment was calculated using the standard curve obtained.

4.2.1 Effects of NaCl on production of azadirachtin content

According to the results, the production of azadirachtin in callus culture responded well to an increase in salt concentrations (for all concentrations). Therefore, the maximum accumulation was observed in 1.5 NaCl extract. Callus extract of 1.5 NaCl sample exhibited the highest amount of azadirachtin (10.847 ± 0.037 mg/g DW) (Figure 4.3). It was significantly 2.1-fold higher compared to callus cultured on OCM. Among the treatment, OCM media showed the lowest azadirachtin content (5.143 ± 0.022 mg/g DW). Each azadirachtin content in the callus extracts showed a gradual increase from the lowest to the highest salt concentration, however a decreasing amount from 0.5 NaCl to 1.0 NaCl was observed, but the amount was still higher than OCM. The azadirachtin contents in the salt-treated neem callus ranged from 5.143 ± 0.022 mg/g DW to 10.847 ± 0.037 mg/g DW. The following is arranged in decreasing order of azadirachtin content from highest to lowest 1.5 NaCl > 0.5 NaCl > 0.2 NaCl > 0.1 NaCl > 0.05 NaCl > 1.0 NaCl > OCM. Since the maximum accumulation of azadirachtin was observed in 1.5 NaCl, 1.5 NaCl treatment was selected for subsequent analysis. Figure 4.3 displays the representative HPLC chromatograms presenting the peak area of the detected azadirachtin compounds.

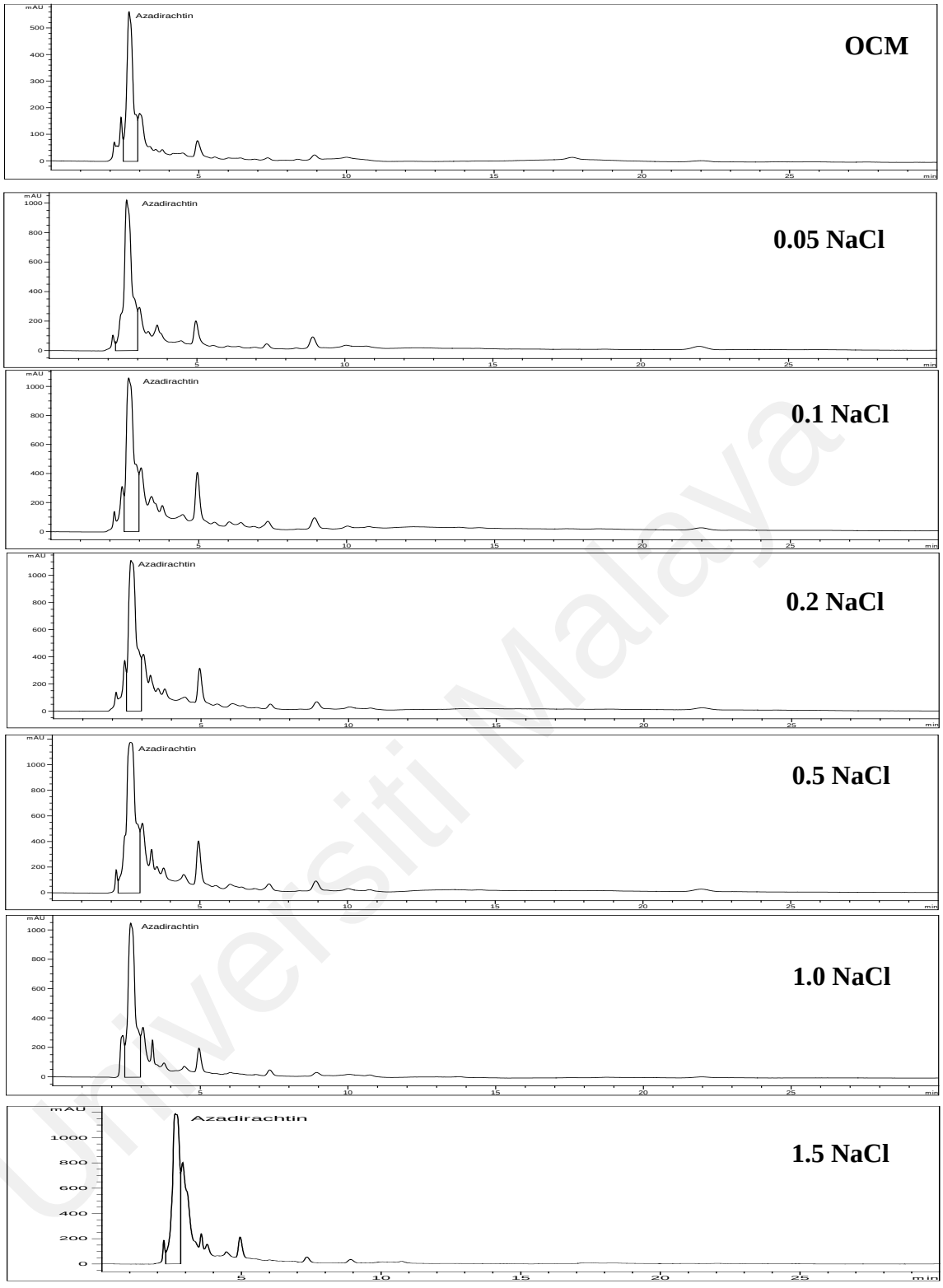

Figure 4.3: Azadirachtin content of *A. indica* callus cultures produced from media treated with various concentrations of NaCl.

*O*CM*: optimized callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *NaCl:* sodium chloride*; 0.05 NaCl:* OCM media supplemented with 0.05 % of NaCl*; 0.1 NaCl:* OCM media supplemented with 0.1 % NaCl*; 0.2 NaCl*: OCM media supplemented with 0.2 % NaCl*; 0.5 NaCl:* OCM media supplemented with 0.5 % NaCl*; 1.0 NaCl: OCM media supplemented with 1.0 % NaCl; 1.5 NaCl:* OCM media supplemented with 1.5 % NaCl.

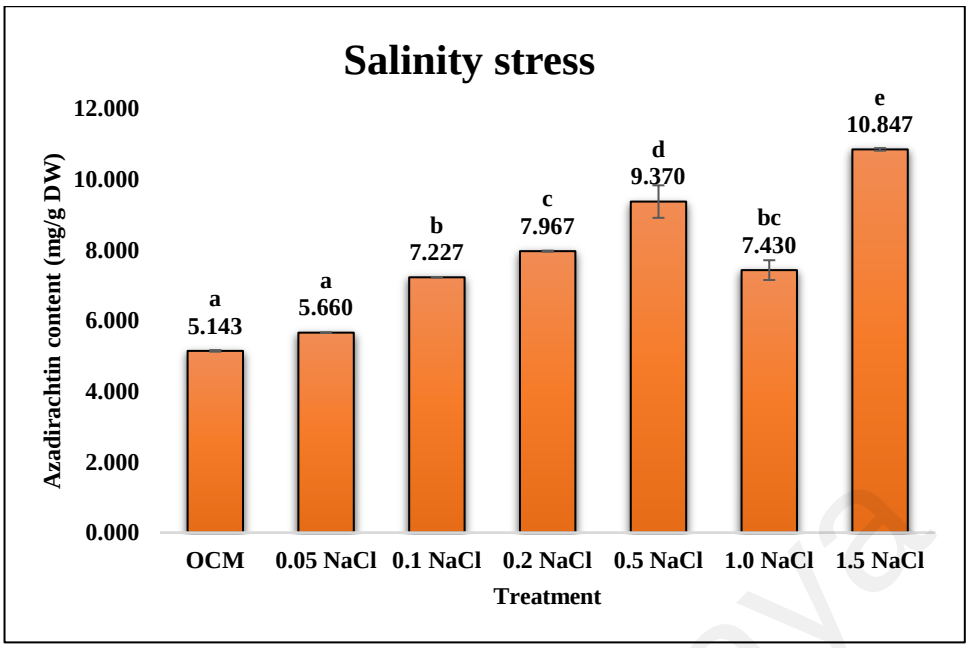

Figure 4.4: Azadirachtin content of *A. indica* callus cultures produced from media treated with various concentrations of NaCl.

Data expressed as mean values ± standard error (SE) of 3 replicates callus cultures. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

*O*CM*: optimized callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *NaCl:* sodium chloride*; 0.05 NaCl:* OCM media supplemented with 0.05 % of NaCl*; 0.1 NaCl:* OCM media supplemented with 0.1 % NaCl*; 0.2 NaCl*: OCM media supplemented with 0.2 % NaCl*; 0.5 NaCl:* OCM media supplemented with 0.5 % NaCl*; 1.0 NaCl: OCM media supplemented with 1. 0% NaCl; 1.5 NaCl:* OCM media supplemented with 1.5 % NaCl.

4.2.2 Effects of JAS on production of azadirachtin

The azadirachtin content in JAS was determined and was expressed as mg per g dry weight. An increase in azadirachtin content was noticed after elicitation with JAS. Similarly, the maximum accumulation of azadirachtin was observed at significantly higher JAS concentration (6JAS: OCM media supplemented with 6 mg/L jasmonic acids) which were 8.193 ± 0.387 mg/g DW, followed by 5JAS, 4JAS, 3JAS, 2 JAS, OCM and 1JAS. Azadirachtin content obtained from 6JAS treated callus was 1.6-fold higher compared to OCM. Figures 4.3 and 4.6 represent HPLC chromatograms showing the peak area of detected compounds. Interestingly, despite the increase in the level of JAS in the growth medium, the amounts of azadirachtin reported in callus supplemented with JAS were relatively close to one another. In general, the azadirachtin content of *A. indica*

callus culture increased with increased JAS concentrations (Figure 4.5) but was not significantly different from each treatment. However, the amount of azadirachtin calculated at 1JAS was the lowest (5.057 ± 0.007 mg/g DW) despite the callus was elicited with JAS.

Previous data analysis advocated that media supplemented with 1.5% of NaCl and 6 mg/L of JAS are the most optimum media for accumulation of azadirachtin as both produced the highest amounts of azadirachtin compared to other treatments. Thus, the samples produced from both treatments were used in subsequent experiments, where they were subjected to LC-MS/MS analysis to determine and identify the differentially abundant proteins (DAPs) compared to control.

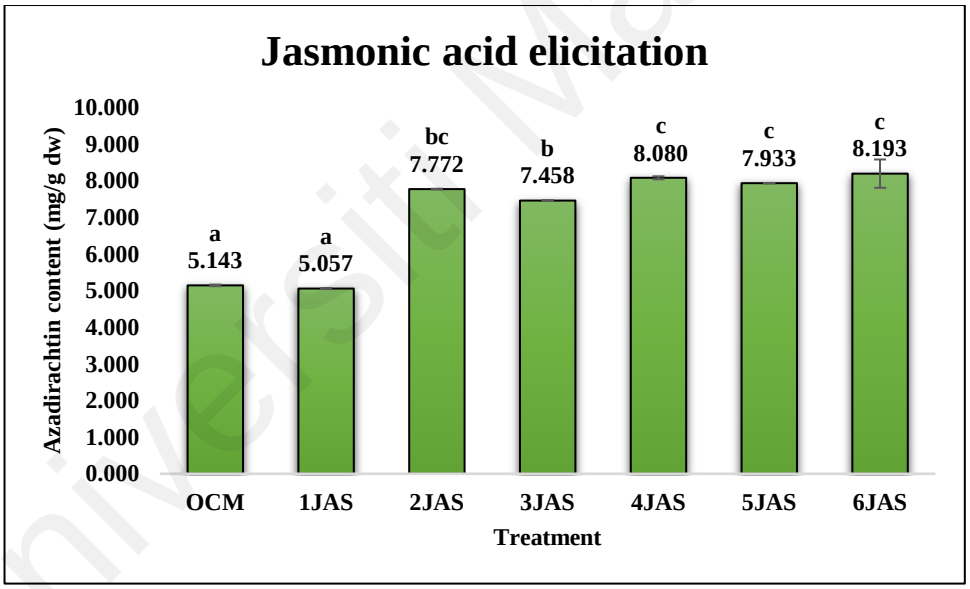

Figure 4.5: Azadirachtin content of *A. indica* callus cultures produced from media treated with various concentrations of JAS

Data expressed as mean values ± standard error (SE) of 3 replicates callus cultures. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

*O*CM*: optimized callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JAS:* jasmonic acid*; 1JAS:* OCM media supplemented with 1 mg/L of JAS*; 2JAS:* OCM media supplemented with 2 mg/L of JAS*; 3JAS* OCM media supplemented with 3 mg/L of JAS*; 4JAS:* OCM media supplemented with 4 mg/L of JAS*; 5JAS:* OCM media supplemented with 5 mg/L of JAS; *6JAS:* OCM media supplemented with 6 mg/L of JAS

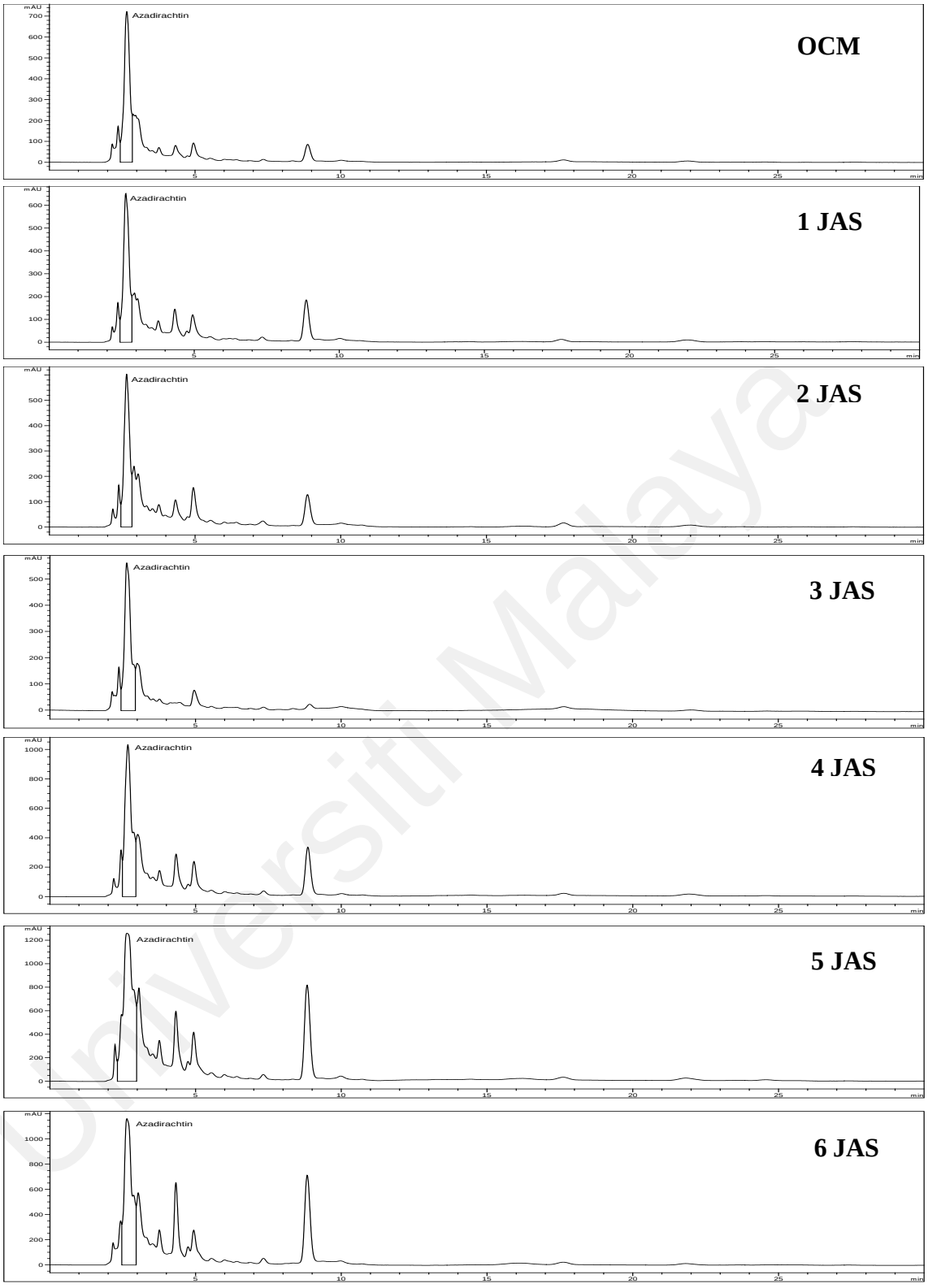

Figure 4.6: Azadirachtin content of *A. indica* callus cultures produced from media treated with various concentrations of JAS.

*O*CM*: optimized callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JAS:* jasmonic acid*; 1JAS:* OCM media supplemented with 1 mg/L of JAS*; 2JAS:* OCM media supplemented with 2 mg/L of JAS*; 3JAS* OCM media supplemented with 3 mg/L of JAS*; 4JAS:* OCM media supplemented with 4 mg/L of JAS*; 5JAS:* OCM media supplemented with 5 mg/L of JAS; *6JAS:* OCM media supplemented with 6 mg/L of JAS

4.3 Correlation analysis

4.3.1 Pearson's correlation analysis in NaCl-treated callus

With reference to Table 4.3, Pearson's correlation coefficient analysis was carried out to analyse the correlations or relationships among the salt concentrations, fresh weight, growth index and azadirachtin content of *A. indica* callus culture.

As observed Table 4.3, correlation studies based on Pearson's correlation coefficients between the variables showed that fresh weight was significantly strongly correlated with growth index in callus culture media supplemented with salt ($r = 0.822$, $p<0.01$), where an increase in fresh weight in callus culture will significantly increase the growth index of treatment. In contrast, the correlation between growth index and fresh weight with salt concentration was moderate, negative and significant; the correlation coefficient was $r = -0.588$ and $r = -0.524$, respectively ($p< 0.01$). These results showed an inverse relationship between fresh weight and growth index with salt concentration. When the salt concentration increased, callus cultures' fresh weight and growth index would significantly decrease.

A positive and significantly strong correlation was seen between azadirachtin content and salt concentration ($r = 0.771$, $p<0.01$), which indicates that azadirachtin content would increase with increasing salt concentrations. At the same time, growth index and fresh weight showed a negative and significantly strong correlation with azadirachtin content, and the correlation coefficients were $r = -0.591$ and $r = -0.752$, respectively ($p<0.001$).

Table 4.3 Pearson's correlation coefficients between variables in salt stress treatment

	Growth index	Fresh weight	Salt concentration	Azadirachtin content
Growth index	1			
Fresh weight	0.822^{**}	1		
Salt concentration	-0.588^{**}	-0.524^{**}	1	
Azadirachtin Concentration	-0.591^{**}	-0.752^{**}	0.771^{**}	1

**. Correlation is significant at the 0.01 level (2-tailed).

4.3.2 Pearson's correlation analysis in JAS-treated callus

Pearson's correlation analysis was also conducted to determine the relationship between the JAS concentration with the callus fresh weight, growth index and azadirachtin content, and the results are exhibited in Table 4.4.

Data analysis revealed that there was a significant correlation between the parameters analysed. The strongest correlation was found between growth index and fresh weight ($r=0.720$, $p<0.001$), suggesting that growth index was contributed by the fresh weight of callus. This finding is parallel with the observation made in the experiment, where when the fresh weight increased, the growth index would also be significantly increased. However, a significantly weak and negative correlation was observed between JAS concentration with the growth index ($r= -0.105$, $p<0.01$). This indicates that the growth index values would decrease with increasing JAS concentration in the media. In contrast, a positive and weak correlation was found between fresh weight and JAS concentration, but this correlation was insignificant.

Hence, in Pearson's correlation coefficients, we observed an insignificant, weak correlation between azadirachtin content and JAS concentration (Table 4.4). Where, the increase in JAS concentration did not really affect the production on azadirachtin, however it still produced higher amounts of azadirachtin compared to OCM. In contrast,

the growth index (r= -0.516) and fresh weight (r= -0.679) showed a significant, negative moderate correlation with azadirachtin content ($p<0.01$).

Table 4.4: Pearson's correlation coefficients between variables in jasmonic acid elicitation.

	Growth index	**Fresh weight**	**Jasmonic acid concentration**	**Azadirachtin concentration**
Growth index	1			
Fresh weight	0.720^{**}	1		
Jasmonic acid concentration	-0.105^{**}	0.025	1	
Azadirachtin Concentration	-0.516^{**}	-0.679^{**}	0.131	1

**. Correlation is significant at the 0.01 level (2-tailed).

4.4 Protein content of *Azadirachta indica* extracts.

Eight-weeks-old *A. indica* callus grown on OCM media supplemented with 1.5% of NaCl and 6 mg/L of JAS for four weeks was chosen based on previous HPLC analysis, which showed that these stress media produced the highest accumulation of azadirachtin. All samples were extracted by using TCA-Acetone precipitate with PVPP (TCAP). Table 4.5 shows the total yield from all the treatments. OCM recorded the highest protein yield (1.442 ± 0.091 mg/g) compared to 1.5 NaCl (1.374 ± 0.138 mg/g) and 6JAS (1.101 ± 0.165 mg/g).

Table 4.5: Total protein yield in callus extracts obtained from different treatments.

Sample ID	Treatment	Protein yield (mg/G of DW)
OCM	0.6 mg/L TDZ	1.442 ± 0.091^{a}
1.5 NaCl	0.6 mg/L TDZ + 1.5% NaCl (w/v)	1.374 ± 0.138^{a}
6JAS	0.6 mg/L TDZ + 6 mg/L jasmonic acid	1.101 ± 0.165^{a}

Data expressed as mean values ± standard error (SE) of 3 replicates callus cultures. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

*O*CM*: optimized callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JAS* : jasmonic acid; *NaCl:* sodium chloride; *6JAS:* OCM media supplemented with 6 mg/L of JAS; *1.5 NaCl:* OCM media supplemented with 1.5 % NaCl (w/v).

4.5 Protein profiling of *Azadirachta indica*

Proteomic profiling was carried out using LCMS/MS analysis. Eight hundred forty-nine proteins were identified with high confidence from OCM, JAS and NaCl treatments. A list of proteins was provided in Supplementary 1 as an attachment.

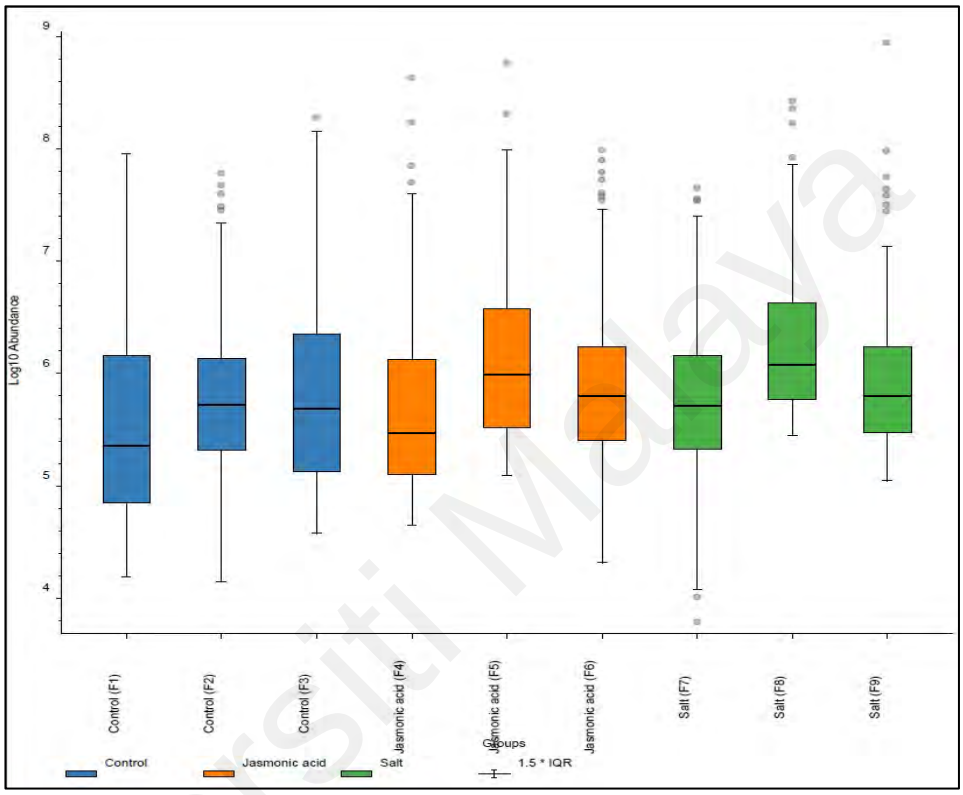

Figure 4.7: Variability of replicates in protein samples content in different treatment

Principle Component Analysis (Figure 4.8) was conducted on the nine samples. The percentage of the total variance accounted for the first principal component was 55.8%, while for the second principal component was 15.8%. Based on the data obtained, most treatments were found in a similar cluster and one of biological replicates of every treatment was found in a different cluster.

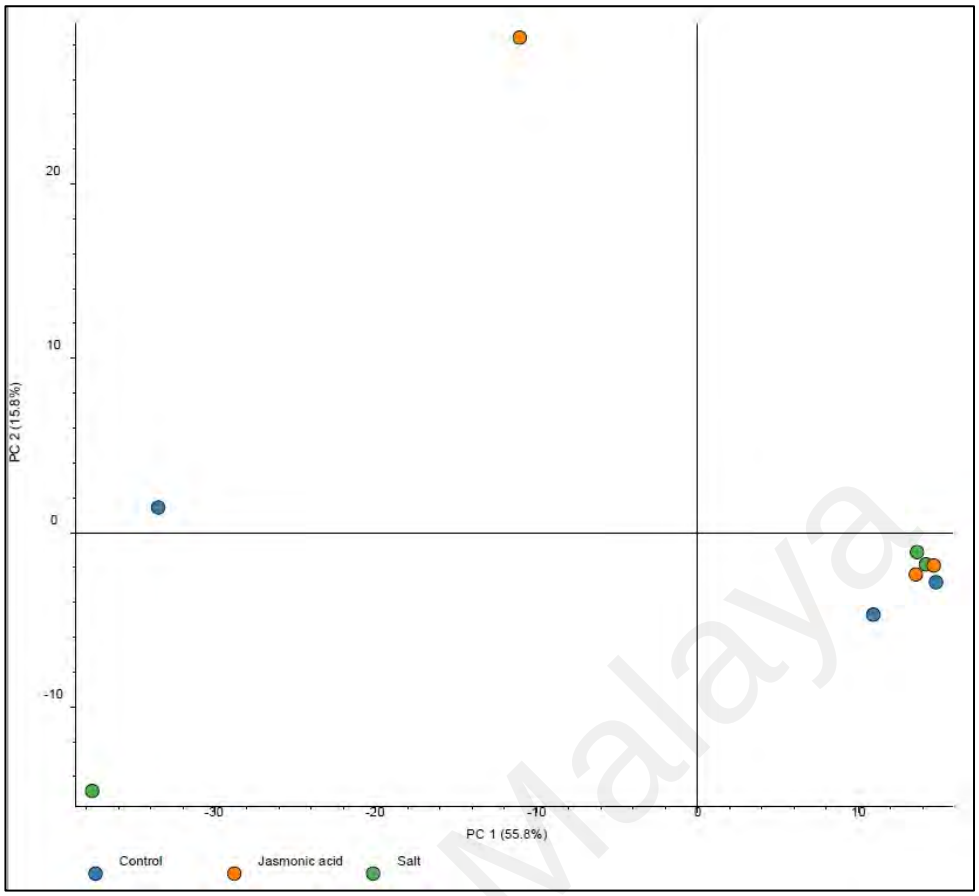

Figure 4.8: Principal component analysis plot representing proteomic data from comparative analysis of *Azadirachta indica* callus.

The enzyme class was determined by inputting each assigned protein identification (NCBI accession number) into UniprotKB database (http://www.uniprot.org/blast/). Overall, 237 out of 883 proteins were successfully mapped to UniProt IDs. Seven enzyme classes were identified from mapped protein IDs (Figure 4.9). Most of these were involved in oxidoreductase (32.34%) and transferase (32%). The small proportions involved hydrolases (14%), lyases (11%) and isomerase (11%). Then, the remaining class formed carbon-nitrogen bonds (6%) and catalysed the hydrons' translocation (2%).

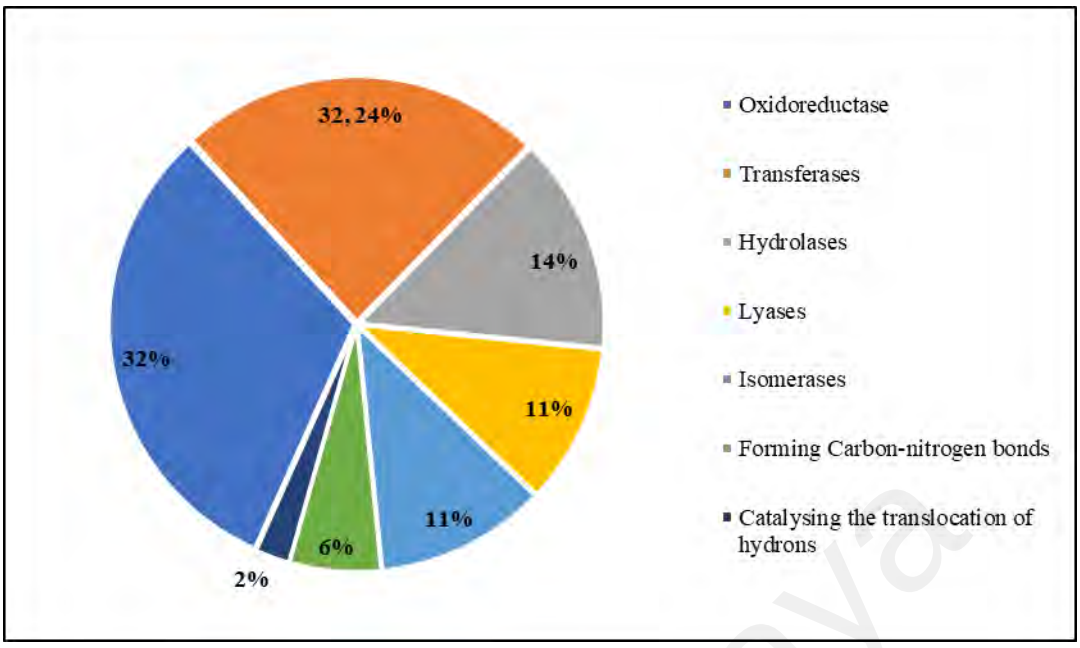

Figure 4.9: Pie chart of enzyme classes from total proteome.

The proteomic profiling from each treatment was further analysed using a Venn diagram to determine overlapping and unique proteins identified in all treatments (Figure 4.10). Overall, 505 proteins (59.5% of the total proteome) were present in all three treatments. The remaining proteins were restricted to two treatments or unique in one treatment. The overlap between the two treatments ranged between 4-227 proteins (0.5% - 26.7%), where OCM and NaCl had the highest number of shared proteins, while JAS and NaCl had the lowest. Meanwhile, the number of unique proteins in a single treatment ranged from 18-39 proteins (2.1% - 4.6% of total proteome). OCM had the highest number of unique proteins (39 proteins), while NaCl and JAS had 23 and 18 unique proteins, respectively. The list of unique proteins from each treatment is listed in Table 4.6.

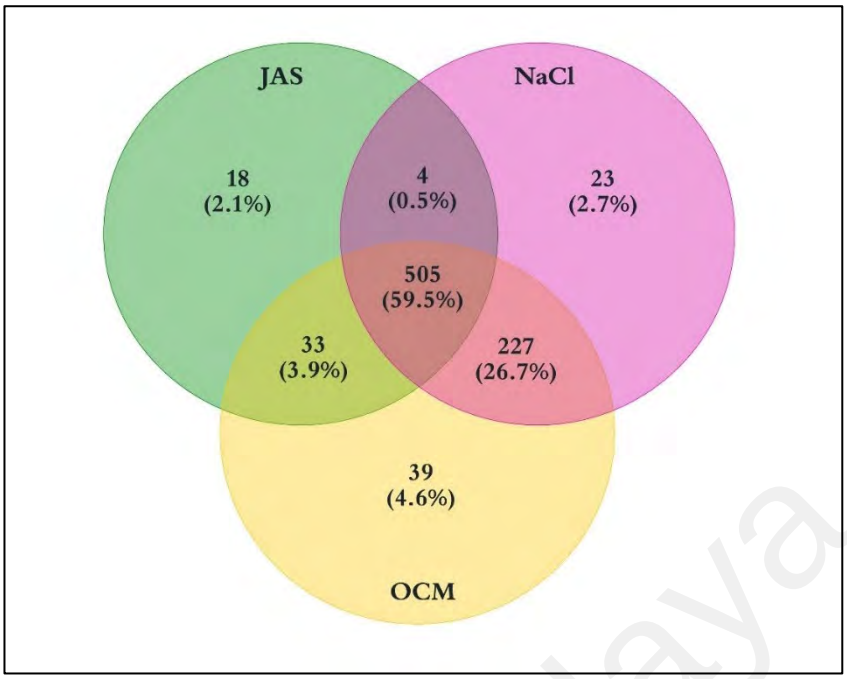

Figure 4.10: Venn diagram showing overall numbers of overlapping and unique proteins (% of protein identified) according to their treatment a) NaCl; salinity stress b) JAS; jasmonic acid elicitation c) OCM; control.

***O*CM*: optimized callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JAS* : jasmonic acid; *NaCl:* sodium chloride; *6JAS:* OCM media supplemented with 6 mg/L of JAS; *1.5 NaCl:* OCM media supplemented with 1.5% NaCl.**

Table 4.6: List of unique proteins from OCM and stress treatments.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
Found in JAS only								
75297911	S-adenosylmethionine synthase;		13	1	393	42.9	6.05	6.64
1841651170	14-3-3-like protein 16R	*Prunus dulcis*	15	1	262	29.5	4.86	6.64
1835393204	hypothetical protein F8388_015154	*Cannabis sativa*	3	1	939	104.1	6.54	6.64
75311776	Probable fructokinase-4		7	1	326	35	5.36	6.64
193805975	Cytochrome f		7	1	320	35.4	8.29	6.64
1847838864	uncharacterized protein At2g34160-like	*Vitis riparia*	10	1	130	14.5	5.35	6.64
1845108458	ubiquitin receptor RAD23b-like	*Setaria viridis*	2	1	368	39.6	4.73	6.64
1833572340	unnamed protein product	*Digitaria exilis*	3	1	373	41.2	6.58	6.64
1833620931	unnamed protein product	*Digitaria exilis*	6	1	185	19	10.18	6.64
26397553	Peroxidase 40		3	1	348	37.4	4.61	6.64
670358752	uncharacterized protein LOC100272631 isoform X1	*Zea mays*	7	1	163	18	5.19	6.64
17366771	Glucose-6-phosphate isomerase		2	1	560	61.7	6.77	6.64
1833622462	unnamed protein product	*Digitaria exilis*	2	1	429	47.3	5.5	6.64
1833619193	unnamed protein product	*Digitaria exilis*	3	1	262	29.9	6.06	6.64
1835394895	hypothetical protein F8388_019761	*Cannabis sativa*	5	1	187	21.3	6.39	6.64
1841646796	ubiquitin domain-containing protein DSK2b-like isoform X1	*Prunus dulcis*	2	1	543	58	4.75	6.64
1847893909	beta-galactosidase 9 isoform X1	*Vitis riparia*	1	1	882	99	6.98	6.64
1835400280	hypothetical protein F8388_011329, partial	*Cannabis sativa*	1	1	861	95.4	5.22	6.64
Found in NaCl only								
1841606548	heat shock cognate 70 kDa protein 2	*Prunus dulcis*	24	1	649	71.1	5.25	6.64
1847848939	peroxidase 4-like	*Vitis riparia*	11	1	321	34.5	9.2	6.64
1835395314	hypothetical protein G4B88_014226, partial	*Cannabis sativa*	5	1	469	50.9	6.98	6.64
1847870967	HMG1/2-like protein	*Vitis riparia*	8	1	153	17.2	8.79	6.64
75169728	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit alpha 2		5	1	617	67.5	7.23	6.64

Table 4.6, continued.

1835390612	hypothetical protein F8388_014588	*Cannabis sativa*	2	1	575	63.3	7.21	6.64
1847860491	probable pectinesterase/ pectinesterase inhibitor 17	*Vitis riparia*	3	1	520	57.6	9.29	6.64
1847912063	cytochrome P450 98A2	*Vitis riparia*	2	1	508	57.4	7.96	6.64
1731736220	laccase-7	*Cannabis sativa*	2	1	568	63	6.65	6.64
1847843786	peroxisomal and mitochondrial division factor 2	*Vitis riparia*	3	1	327	37.8	4.78	6.64
310947092	High mobility group B protein 4; Nucleosome/chromatin assembly factor group D 4		9	1	138	15.4	8.22	6.64
21263645	Glutathione reductase, cytosolic;		4	1	502	54	6.3	6.64
1841619370	60S ribosomal protein L17-2-like	*Prunus dulcis*	5	1	183	20.7	10.26	6.64
1833617778	unnamed protein product	*Digitaria exilis*	3	1	323	35.5	9.8	6.64
1162442738	ATP synthase subunit gamma, mitochondrial	*Zea mays*	3	1	375	40.5	9.13	6.64
116347	Endochitinase; Flags: Precursor		4	1	328	35.4	8.06	6.64
1841634826	peroxidase 73-like	*Prunus dulcis*	4	1	329	35.5	7.96	6.64
1848383116	hypothetical protein Mp_3g09050	*Marchantia polymorpha subsp. ruderalis*	7	1	144	15.9	10.04	6.64
1847891711	protein DJ-1 homolog D	*Vitis riparia*	3	1	386	41.4	5.74	6.64
75180810	Protein PELPK1; Proline-rich protein 10;		3	1	370	41.6	6.18	6.64
1835405764	hypothetical protein F8388_019316	*Cannabis sativa*	7	1	125	14.1	9.26	6.64
52783264	60S ribosomal protein L24		4	1	186	21.3	10.62	6.64
1841586809	berberine bridge enzyme-like 8	*Prunus dulcis*	2	1	542	61.1	7.08	6.64
Found in JAS and NaCl								
1841613982	heat shock 70 kDa protein-like	*Prunus dulcis*	18	1	654	71.7	5.38	6.64
1841654579	probable pectinesterase/ pectinesterase inhibitor 7	*Prunus dulcis*	3	1	560	61.6	9.07	6.64
75311568	Heat shock 70 kDa protein 7, chloroplastic; AltName:		3	1	718	77	5.3	6.64
1841619982	2-methylene-furan-3-one reductase-like	*Prunus dulcis*	4	1	322	34.2	6.96	6.64

4.6 Proteomic profiling of *A. indica* in response to salinity stress

Protein profiling in response to salinity stress were also carried out. A total of 830 proteins were identified with high confidence from *A. indica* of salt treatment in comparison to the control sample (OCM). The proteomic profiles from each treatment were further analyzed using a Venn diagram to determine protein overlaps between salinity stress and control conditions (Figure 4.11). 730 proteins (88% of total proteome detected in salt treatment) were present in both control and treatment. While the number of proteins unique was 27-73 (3.3-8.8%) found. The control treatment had the highest number of unique proteins (73 proteins), while the salt treatment had 27 unique proteins.

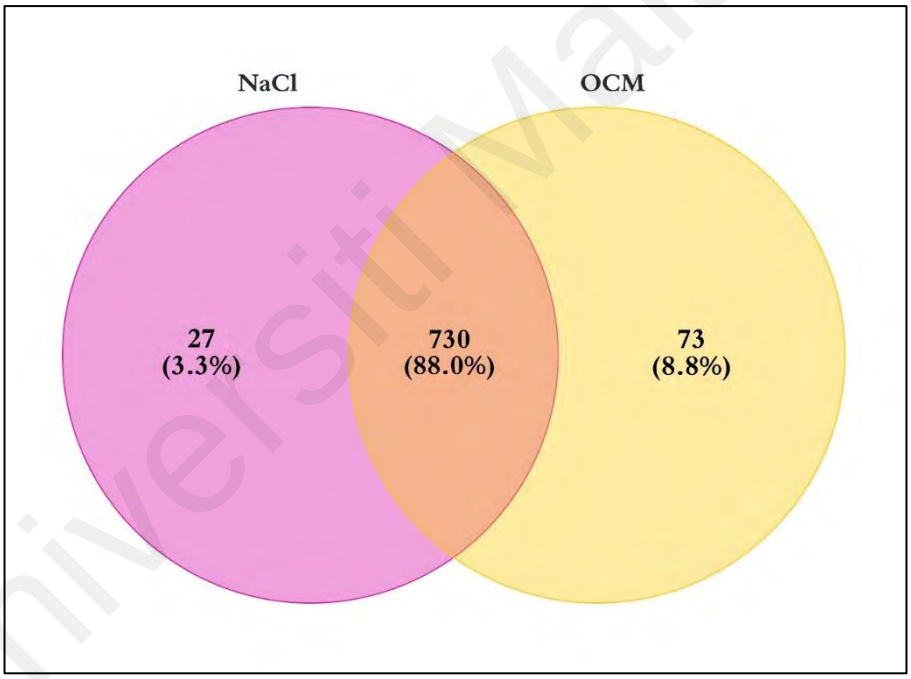

Figure 4.11: Venn diagram showing the numbers of overlapping and unique identified protein (%) between a) NaCl: Salinity stress (1.5 NaCl) treatment and b) OCM: control

***OCM*: optimized callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JAS*: jasmonic acid; *NaCl:* Sodium chloride; *6JAS:* OCM media supplemented with 6 mg/L of JAS; *1.5 NaCl:* OCM media supplemented with 1.5% NaCl.**

Determining and comparing of protein abundance under salt stress were carried out to identify differential protein abundance under salt stress. The distribution of protein

abundance identified in NaCl and OCM was shown by constructing a volcano plot (Figure 4.12). The volcano plot was constructed where fold change (FC) and p-value were log-transformed. Up-regulated proteins were proteins with fold changes (log_2 ratio Salt/Control) values of ≥ 1.5 while down-regulated with fold changes of ≤ -1.5. The red and green circles represent the proteins above the threshold. A total of 129 significantly changed abundance proteins (p-value <0.05) were identified in 1.5% NaCl. Thirty-six proteins were increased significantly, and ninety-three proteins were decreased in abundance in salt stress treatment of *A.indica* (Table 4.7 and Table 4.8) .

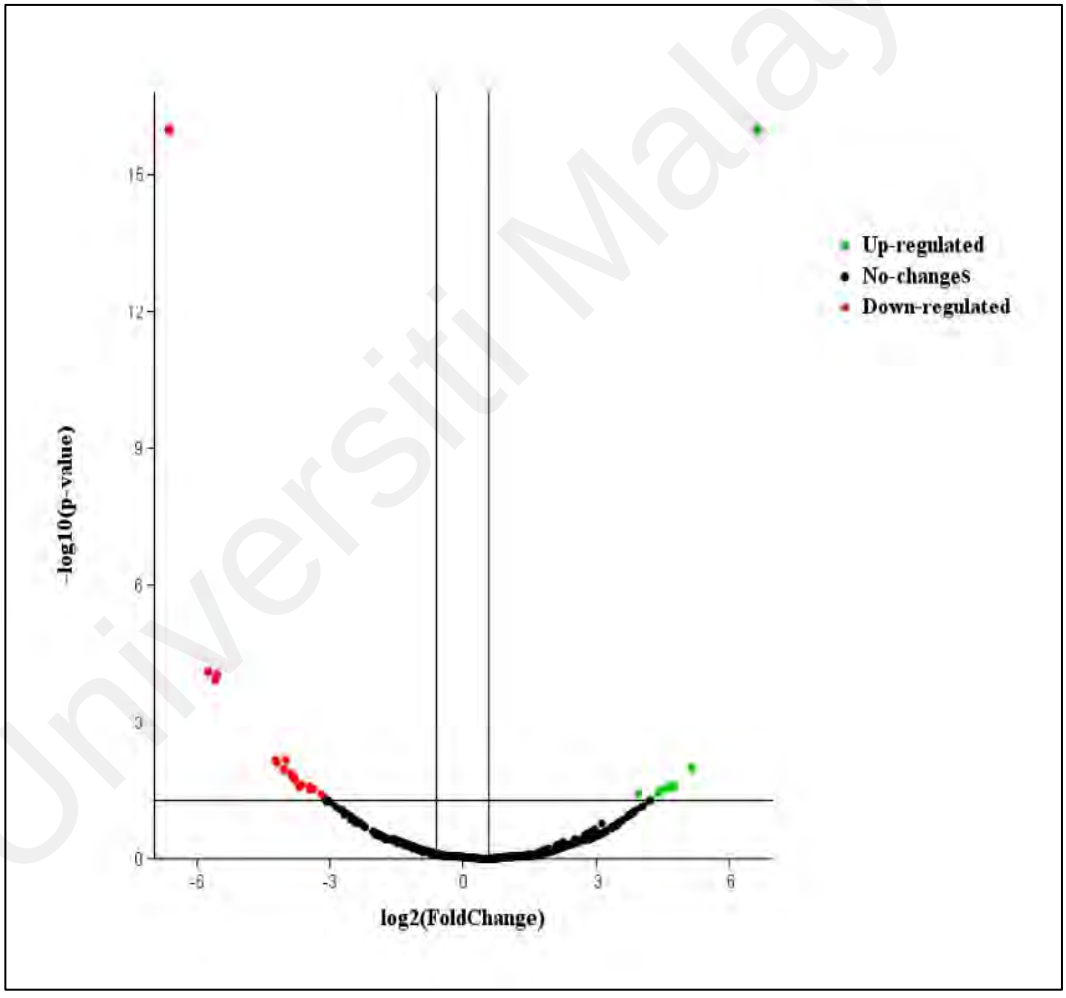

Figure 4.12: The Volcano plot of *Azadirachta indica* shows the identified protein distribution in response to salt stress. Different colours are used to represent; green: Significant and greater than the upper FC threshold; red: Significant and less than the lower FC threshold; black: non-significant

Table 4.7: List of significantly up-regulated proteins under salinity stress (fold change; $\log_2$ ratios of ≥ 1.5)

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [$\log_2$]
Upregulated Protein								
Defense and stress response related proteins								
1841606548	heat shock cognate 70 kDa protein 2	*Prunus dulcis*	24	1	649	71.1	5.25	6.64
1841613982	heat shock 70 kDa protein-like	*Prunus dulcis*	18	1	654	71.7	5.38	6.64
190358875	Thaumatin-like protein		11	2	225	24.2	7.9	4.54
1847848939	peroxidase 4-like	*Vitis riparia*	11	1	321	34.5	9.2	6.64
75311568	Heat shock 70 kDa protein 7		3	1	718	77	5.3	6.64
21263645	Glutathione reductase		4	1	502	54	6.3	6.64
116347	Endochitinase		4	1	328	35.4	8.06	6.64
1841634826	peroxidase 73-like	*Prunus dulcis*	4	1	329	35.5	7.96	6.64
1847891711	protein DJ-1 homolog D	*Vitis riparia*	3	1	386	41.4	5.74	6.64
Energy and carbohydrate metabolism related proteins								
120674	Glyceraldehyde-3-phosphate dehydrogenase		15	1	338	36.5	7.83	4.41
75169728	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit alpha 2		5	1	617	67.5	7.23	6.64
1162442738	ATP synthase subunit gamma	*Zea mays*	3	1	375	40.5	9.13	6.64
Cell wall and cell structure related proteins								
1841654579	probable pectinesterase/pectinesterase inhibitor 7	*Prunus dulcis*	3	1	560	61.6	9.07	6.64
1847860491	probable pectinesterase/pectinesterase inhibitor 17	*Vitis riparia*	3	1	520	57.6	9.29	6.64
1841645778	polygalacturonase inhibitor-like	*Prunus dulcis*	3	1	367	40.8	8.28	3.94
75180810	Protein PELPK1		3	1	370	41.6	6.18	6.64
Protein metabolism related proteins								
1847907216	60S ribosomal protein L19-2	*Vitis riparia*	13	3	208	24.4	11.43	4.63
1841619370	60S ribosomal protein L17-2-like	*Prunus dulcis*	5	1	183	20.7	10.26	6.64
52783264	60S ribosomal protein L24		4	1	186	21.3	10.62	6.64
Redox regulation related proteins								
1731736220	laccase-7	*Cannabis sativa*	2	1	568	63	6.65	6.64

Table 4.7, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
1841619982	2-methylene-furan-3-one reductase-like	*Prunus dulcis*	4	1	322	34.2	6.96	6.64
1841586809	berberine bridge enzyme-like 8	*Prunus dulcis*	2	1	542	61.1	7.08	6.64
Transcription related proteins								
1847870967	HMG1/2-like protein	*Vitis riparia*	8	1	153	17.2	8.79	6.64
310947092	High mobility group B protein 4;		9	1	138	15.4	8.22	6.64
Cell metabolism and biogenesis related proteins								
75098732	Prohibitin-1		9	1	288	31.7	9.26	4.23
Secondary metabolite related proteins								
1847912063	cytochrome P450 98A2	*Vitis riparia*	2	1	508	57.4	7.96	6.64
Photosythesis related proteins								
1841622724	plastid division protein CDP1, chloroplastic-like isoform X1	*Prunus dulcis*	3	1	392	43	5.96	4.77
Others								
1717794	Sex determination protein tasselseed-2		2	1	336	35.2	7.18	4.4
1847843786	peroxisomal and mitochondrial division factor 2	*Vitis riparia*	3	1	327	37.8	4.78	6.64
Hypothetical proteins								
1835414786	hypothetical protein G4B88_006669	*Cannabis sativa*	5	1	406	45.2	7.91	4.72
1835395314	hypothetical protein G4B88_014226, partial	*Cannabis sativa*	5	1	469	50.9	6.98	6.64
1835390612	hypothetical protein F8388_014588	*Cannabis sativa*	2	1	575	63.3	7.21	6.64
1835405764	hypothetical protein F8388_019316	*Cannabis sativa*	7	1	125	14.1	9.26	6.64
1833617778	unnamed protein product	*Digitaria exilis*	3	1	323	35.5	9.8	6.64
1835385527	hypothetical protein F8388_005021	*Cannabis sativa*	1	1	1393	155.9	7.06	5.16
1848383116	hypothetical protein Mp_3g09050	*Marchantia polymorpha subsp. Ruderalis*	7	1	144	15.9	10.04	6.64

Table 4.8: List of significantly down-regulated proteins under salinity stress (fold change; $\log_2$ ratios of ≤ -1.5).

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [$\log_2$]
Downregulated Protein								
Energy and carbohydrate metabolism related proteins								
75141370	Malate dehydrogenase		17	1	332	35.5	6.09	-5.75
3023813	Glyceraldehyde-3-phosphate dehydrogenase		18	1	340	36.7	6.89	-6.64
1841638167	V-type proton ATPase subunit G-like	*Prunus dulcis*	21	1	110	12	9.31	-3.64
113377	Alcohol dehydrogenase 2		9	1	379	41	6.06	-3.37
1845171029	pyruvate kinase 1, cytosolic-like	*Setaria viridis*	5	1	527	57.4	6.11	-6.64
12585472	UTP--glucose-1-phosphate uridylyltransferase		6	1	471	51.5	6.29	-6.64
12585489	UTP--glucose-1-phosphate uridylyltransferase		7	1	467	51.3	5.68	-6.64
2493045	ATP synthase subunit delta'		8	1	203	21.5	6.7	-6.64
1731680144	ferritin-3, chloroplastic	*Cannabis sativa*	5	1	276	30.5	5.92	-6.64
12585427	V-type proton ATPase subunit G1		21	1	110	12.4	6	-6.64
75169075	Gamma carbonic anhydrase 2		5	1	278	30	7.24	-6.64
Protein metabolism related proteins								
657341073	Prolyl 4-hydroxylase 2		7	1	299	33	6.35	-6.64
1847880588	T-complex protein 1 subunit epsilon	*Vitis riparia*	3	1	535	59.2	5.85	-6.64
1841629920	isoaspartyl peptidase/L-asparaginase 1-like	*Prunus dulcis*	11	2	320	33.7	5.67	-4.04
1731665468	protein disulfide-isomerase	*Cannabis sativa*	3	1	506	56.6	4.93	-4.2
670371654	proteasome subunit beta type isoform X1	*Zea mays*	6	1	270	31	9.32	-6.64
1841613443	26S proteasome non-ATPase regulatory subunit 2 homolog A isoform X2	*Prunus dulcis*	2	1	892	98.2	5.22	-3.45
1841616922	monothiol glutaredoxin-S15, mitochondrial-like isoform X1	*Prunus dulcis*	8	1	171	18.8	7.33	-3.81
20140765	Ubiquitin-fold modifier 1		38	1	93	9.9	9.25	-6.64
1841608100	aspartyl protease family protein 2-like	*Prunus dulcis*	4	1	479	51.2	9.32	-6.64

Table 4.8, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
75097043	Aspartyl protease AED3		2	1	449	47.6	7.52	-6.64
11386828	Glutamine synthetase, chloroplastic/mitochondrial		6	1	430	47.4	6.87	-6.64
1731680683	40S ribosomal protein SA isoform X1	*Cannabis sativa*	28	1	305	33.5	5.19	-6.64
1847911206	40S ribosomal protein S2-3-like	*Vitis riparia*	9	3	273	30	10.21	-3.85
1847854041	translationally-controlled tumor protein homolog	*Vitis riparia*	20	1	168	19.1	4.73	-6.64
Transcription related proteins								
1847905447	multiple organellar RNA editing factor 2, chloroplastic-like	*Vitis riparia*	5	1	240	27.3	9.19	-6.64
1847896355	heterogeneous nuclear ribonucleoprotein 1	*Vitis riparia*	3	1	464	46.7	8.07	-6.64
1847881581	nuclear transport factor 2B	*Vitis riparia*	11	1	123	13.6	6.11	-6.64
1841620007	L-ascorbate oxidase homolog	*Prunus dulcis*	3	1	542	60.1	8.19	-6.64
1847857952	RGG repeats nuclear RNA binding protein A-like isoform X1	*Vitis riparia*	4	1	361	38.7	7.05	-6.64
1731671536	glutamate--tRNA ligase, chloroplastic/mitochondrial	*Cannabis sativa*	2	1	578	64.4	6.49	-6.64
122230604	Deoxyuridine 5'-triphosphate nucleotidohydrolase;		6	1	171	17.3	5.31	-6.64
122194125	Phosphomannomutase;		7	1	252	28.6	6.05	-6.64
Amino acid related proteins								
8134569	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase		10	1	764	84.5	6.51	-6.64
30580330	Caffeoyl-CoA O-methyltransferase 1;		18	2	259	29.1	5.29	-5.59
1847870297	phospho-2-dehydro-3-deoxyheptonate aldolase 1, chloroplastic	*Vitis riparia*	5	1	548	60.1	8.18	-3.2
1711381	Phosphoserine aminotransferase, chloroplastic		12	1	430	47.1	8.06	-6.64
223635326	S-adenosylmethionine synthase 1;		14	1	396	43.2	5.92	-6.64

Table 4.8, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
1847889967	3-dehydroquinate synthase, chloroplastic	*Vitis riparia*	3	1	456	49.4	8	-6.64
Defense and stress response related proteins								
416755	Catalase		11	1	492	56.8	7.3	-6.64
26397792	Peroxidase 72;		5	2	336	37.4	8.43	-6.64
75116022	Probable glutathione S-transferase DHAR2		4	1	272	29.7	7.96	-6.64
1847856074	peroxidase 5-like	*Vitis riparia*	2	1	332	36.1	8.22	-6.64
1847873352	probable glucan 1,3-beta-glucosidase A	*Vitis riparia*	2	1	527	58.8	7.06	-6.64
Photosynthesis related proteins								
132098	Ribulose bisphosphate carboxylase small chain SSU11A, chloroplastic;		8	1	180	20.3	8.12	-3.11
671726284	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)	*Odontoschisma fluitans*	12	1	458	50.8	6.44	-6.64
11134054	Oxygen-evolving enhancer protein 1, chloroplastic;		10	1	332	35.2	5.96	-6.64
92090800	Thylakoid lumenal 19 kDa protein, chloroplastic		4	1	229	25	7.47	-3.69
677286725	Delta-aminolevulinic acid dehydratase, chloroplastic		3	1	432	46.2	6.55	-6.64
Transport related proteins								
1847853797	mitochondrial import receptor subunit TOM20	*Vitis riparia*	8	1	201	22.5	6.43	-6.64
1475928797	aquaporin PIP1-3/PIP1-4	*Zea mays*	3	1	292	31	8.63	-6.64
75219328	Protein TIC110, chloroplastic;		1	1	996	109.9	6.4	-6.64
1847843841	potassium transporter 7 isoform X1	*Vitis riparia*	1	1	840	93.6	6.02	-6.64
Cell wall and cell structure related proteins								
464850	Tubulin beta-1 chain		26	2	443	49.7	4.97	-6.64
576017879	Profilin-5		19	1	131	14.1	5.71	-6.64

Table 4.8, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
14423872	Profilin		19	1	131	14	4.84	-6.64
1847872457	probable beta-D-xylosidase 7	*Vitis riparia*	1	1	774	84	6.58	-6.64
259585708	Probable beta-D-xylosidase 7		1	1	767	83.8	8.03	-6.64
Lipid metabolism related proteins								
17380471	Biotin carboxyl carrier protein of acetyl-CoA carboxylase 1, chloroplastic;		3	1	280	29.6	9.06	-6.64
1847901001	protein YLS3-like	*Vitis riparia*	4	1	189	19.8	7.88	-6.64
Redox regulation related proteins								
75316039	Quinone-oxidoreductase QR2		12	1	205	22.1	6.95	-6.64
1841646005	glyoxylate/succinic semialdehyde reductase 1	*Prunus dulcis*	4	1	294	31.1	7.39	-6.64
Secondary metabolites related proteins								
166233972	Chalcone--flavonone isomerase 3;		6	1	226	24.7	6.38	-6.64
Others								
1028325835	Dormancy-associated protein 1;		10	1	132	14.4	9.29	-3.8
Hypothetical related proteins								
1835425193	hypothetical protein G4B88_005308	*Cannabis sativa*	35	1	447	49.2	9.07	-6.64
1848379843	hypothetical protein Mp_2g09390	*Marchantia polymorpha subsp. Ruderalis*	29	1	443	49.6	4.87	-6.64
1833593700	unnamed protein product, partial	*Digitaria exilis*	21	1	378	39.8	9.48	-6.64
1835383686	hypothetical protein F8388_024498	*Cannabis sativa*	6	1	467	52.4	8.21	-6.64
1835407507	hypothetical protein F8388_015860	*Cannabis sativa*	10	3	445	47.5	8.13	-3.45
1847908656	uncharacterized protein LOC117919548	*Vitis riparia*	38	3	55	6.6	10.4	-3.88
1835423624	hypothetical protein G4B88_015692	*Cannabis sativa*	10	1	290	32.6	6.71	-6.64
1847909645	uncharacterized protein LOC117919923	*Vitis riparia*	5	2	385	41.5	8.91	-3.99
1835388133	hypothetical protein F8388_025922, partial	*Cannabis sativa*	4	2	339	37.4	4.88	-5.55
1835389342	hypothetical protein G4B88_004500	*Cannabis sativa*	3	1	483	53. 5	6.13	-3.47

Table 4.8, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
1835431525	hypothetical protein G4B88_013723	*Cannabis sativa*	1	1	1455	162.7	8.69	-6.64
1845115716	uncharacterized protein LOC117843217	*Setaria viridis*	1	1	1506	162.2	6.15	-6.64
1841623145	uncharacterized protein LOC117624836	*Prunus dulcis*	6	2	425	46.1	4.67	-6.64
1835388914	hypothetical protein G4B88_009656, partial	*Cannabis sativa*	5	1	510	56.1	9.52	-6.64
1833592023	unnamed protein product	*Digitaria exilis*	2	1	747	82.4	6.01	-6.64
1833581303	unnamed protein product	*Digitaria exilis*	8	1	238	26.9	8.59	-6.64
1833599650	unnamed protein product	*Digitaria exilis*	9	1	291	31.7	8.5	-6.64
1835402033	hypothetical protein G4B88_029565	*Cannabis sativa*	2	1	600	66.2	6.13	-6.64
1833567825	unnamed protein product	*Digitaria exilis*	6	1	370	38.6	8.78	-3.79
1833622269	unnamed protein product	*Digitaria exilis*	2	1	1068	117.4	8.12	-6.64
1841591686	uncharacterized protein LOC117624249	*Prunus dulcis*	3	1	441	48.7	5.57	-6.64
1835388824	hypothetical protein F8388_006336	*Cannabis sativa*	4	1	424	46.9	9.25	-6.64
1835427927	hypothetical protein G4B88_017930	*Cannabis sativa*	2	1	538	60.1	8.54	-6.64
1835420514	hypothetical protein G4B88_004044	*Cannabis sativa*	3	1	431	48.5	6.02	-6.64
1835421359	hypothetical protein F8388_012490	*Cannabis sativa*	1	1	757	81.2	6.52	-6.64
1835428433	hypothetical protein G4B88_011028	*Cannabis sativa*	2	1	481	53	6.7	-6.64
1835378467	hypothetical protein G4B88_030252	*Cannabis sativa*	9	1	224	24.7	9.17	-6.64
1731673617	uncharacterized protein C594.04c	*Cannabis sativa*	3	1	332	39.3	9.07	-4.22
1835393252	hypothetical protein F8388_020029	*Cannabis sativa*	5	1	240	27	7.46	-6.64

Protein function was determined by inputting each assigned protein identification which is NCBI accession number into UniProtKb databases and detecting Gene Ontology (GO) terms and annotations. The GO for up-regulated and down-regulated proteins are shown in Figure 4.13, where the proteins were classified into three categories according to biological processes, molecular function, and cellular components. The results revealed that up-regulated proteins were involved mainly in cellular process, metabolic process, response to stimulus, biological regulation, and localization. In contrast, proteins with reduced abundance were involved in eleven biological processes, including cellular process, metabolic process, response to stimulus, biological regulation, localization, developmental process and more. Moreover, most differentially abundant proteins are related with catalytic activity and binding abilities for molecular function. Last but not least, differently abundant proteins annotated with cellular components are cellular anatomical entity and protein containing complex.

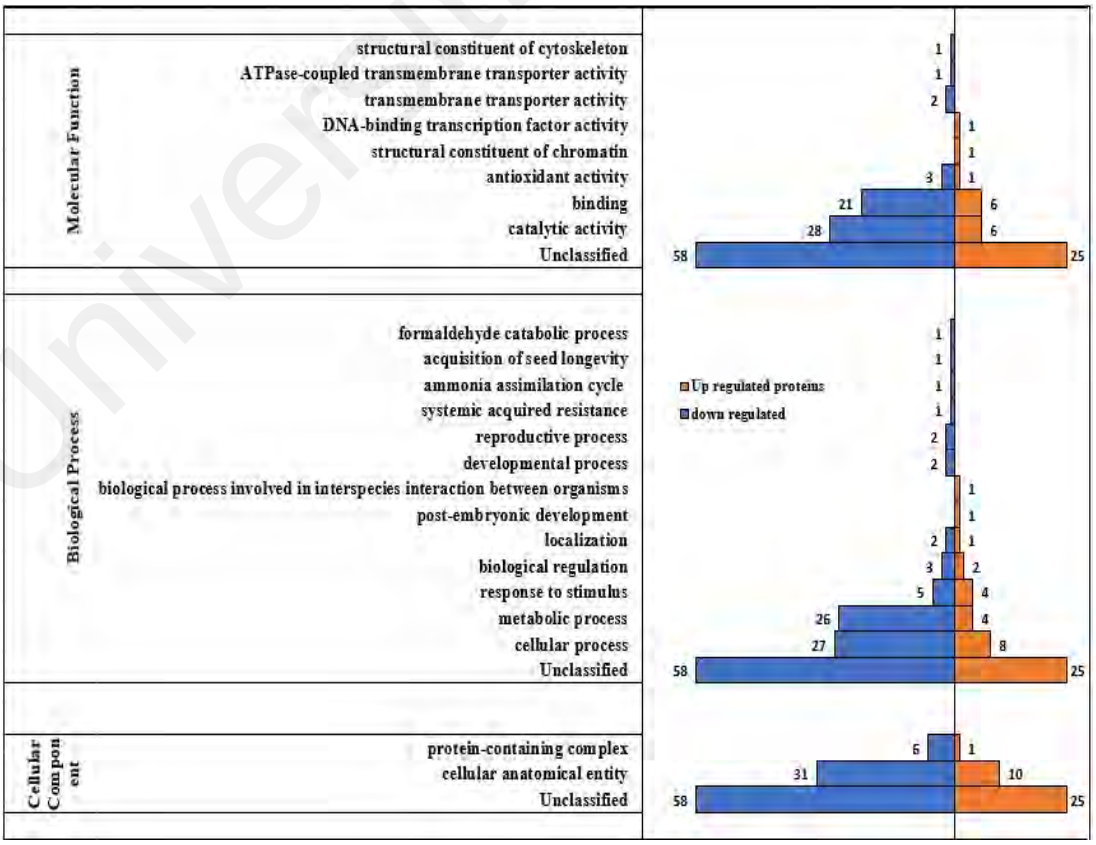

Figure 4.13: Gene ontology (GO) of differentially abundant proteins in salt stress treatment of *Azadirachta indica* based on the UniProt information available.

4.7 Proteomic profiling of *A. indica* in response to jasmonic acid stress

Protein profiling in response to JAS stress-induced callus were carried out. A total of 819 proteins were identified with high confidence ($p<0.01$) from *A. indica* of JAS treatment in comparison to the control sample (OCM). The proteomic profiles from each treatment were further analyzed using a Venn diagram to determine protein overlaps between JAS stress and control conditions (Figure 4.14). 531 proteins (64.8% of total proteome detected in JAS treatment) were present in both control and treatment. While the number of proteins unique was 22-266 (2.7 - 32.5%) found. The control treatment had the highest number of unique proteins (266 proteins), while the JAS treatment had 22 unique proteins.

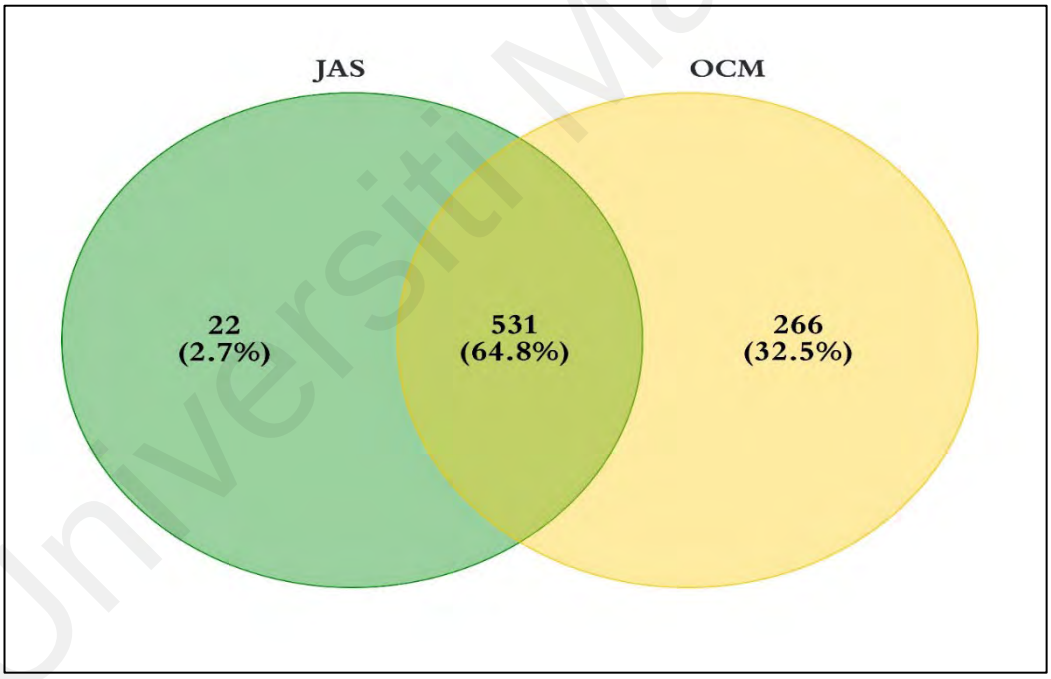

Figure 4.14: Venn diagram showing the numbers of overlapping and unique identified protein (%) between a) JAS: Jasmonic acid stress (6JAS) and b) OCM: control

***OCM*: optimized callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JAS*: jasmonic acid; *6JAS:* OCM media supplemented with 6 mg/L of JAS.**

Protein abundance was measured and compared to find differences in protein abundance caused by the JAS treatment. By making a volcano plot, the distribution of

protein abundance found in JAS/OCM was shown (Figure 4.15). The volcano plot was constructed where fold change (FC) and p-value were log-transformed. The red and green circles represent the proteins above the threshold (FC 1.5 coupled with $p < 0.05$). Among quantified proteins, 320 proteins were identified as differentially abundant proteins between JAS-treated and untreated callus culture based on the criteria: the ratio ≥ 1.5 fold changes (up-regulated) and ≤-1.5 fold changes (down-regulated). Of the differentially abundant proteins, only thirty-four were significantly up-regulated, and two hundred and eighty-six were significantly down-regulated compared to control (p-value <0.05) (Table 4.9 and Table 4.10).

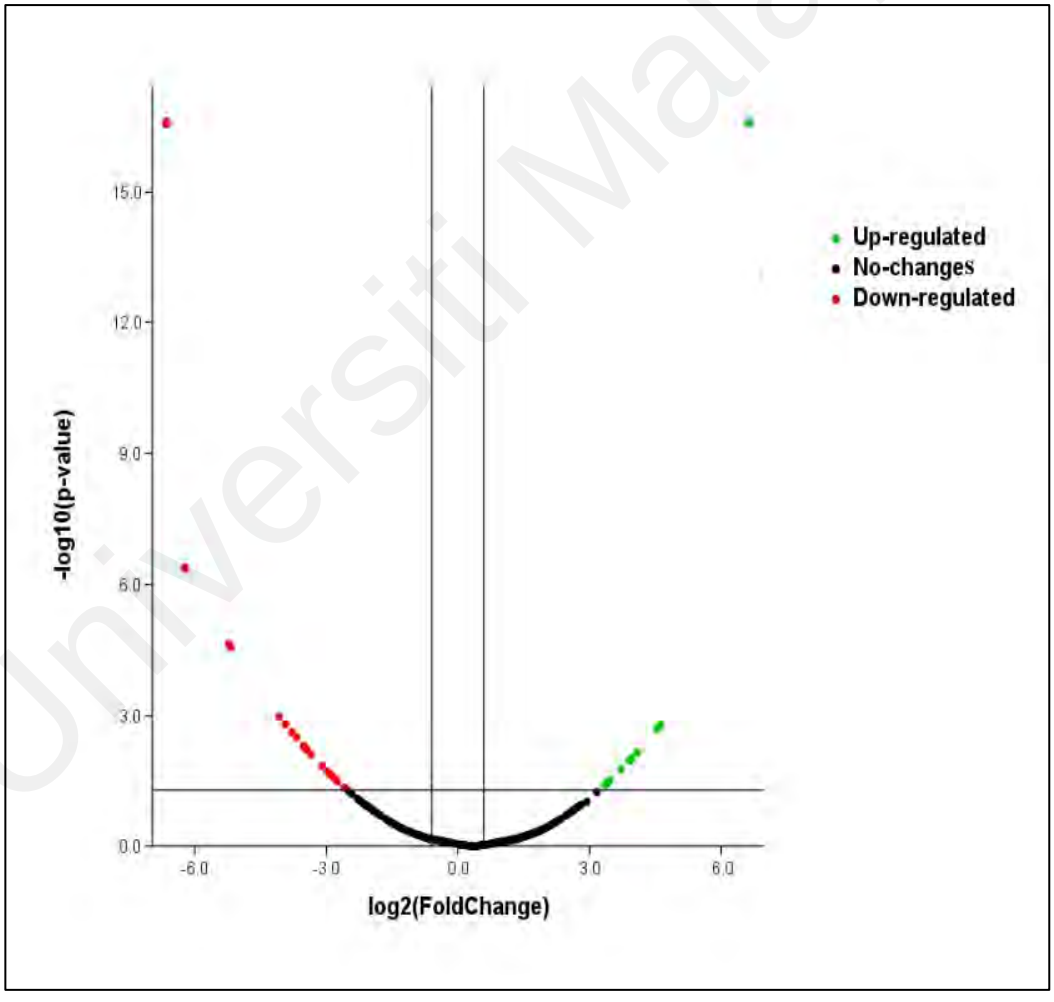

Figure 4.15: The Volcano plot of *Azadirachta indica* shows the identified protein distribution in response to jasmonic acid elicitation. Different colours are used to represent; green: Significant and greater than the upper FC threshold; red: Significant and less than the lower FC threshold; black: non-significant.

Table 4.9: List of significantly up-regulated proteins under jasmonic acid stress (fold change; $\log_2$ ratios of ≥ 1.5).

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [$\log_2$]
Upregulated Protein								
Defense and stress response related proteins								
1841613982	heat shock 70 kDa protein-like	*Prunus dulcis*	18	1	654	71.7	5.38	6.64
75311568	Heat shock 70 kDa protein 7, chloroplastic		3	1	718	77	5.3	6.64
416569	1-aminocyclopropane-1-carboxylate oxidase 1		10	2	318	36.1	5.4	3.43
26397553	Peroxidase 40		3	1	348	37.4	4.61	6.64
Energy and carbohydrate metabolism related proteins								
136063	Triosephosphate isomerase, cytosolic		10	1	253	27	5.68	3.35
75311776	Probable fructokinase-4		7	1	326	35	5.36	6.64
1731695664	ATP synthase subunit epsilon, mitochondrial	*Cannabis sativa*	16	1	70	7.9	8.51	3.41
193805975	Cytochrome f;		7	1	320	35.4	8.29	6.64
17366771	Glucose-6-phosphate isomerase, cytosolic		2	1	560	61.7	6.77	6.64
Protein destination and storage (folding, modification and degradation) related proteins								
1841656724	subtilisin-like protease SBT3.3	*Prunus dulcis*	8	1	441	50.1	5.16	3.72
1845108458	ubiquitin receptor RAD23b-like	*Setaria viridis*	3	1	560	61.6	9.07	6.64
20140765	Ubiquitin-fold modifier 1; Flags: Precursor		1	1	882	99	6.98	6.64
1841646796	ubiquitin domain-containing protein DSK2b-like isoform X1	*Prunus dulcis*	2	1	543	58	4.75	6.64
Cell wall and cell structure related protein								
1841602335	probable UDP-arabinopyranose mutase 1 isoform X2	*Prunus dulcis*	8	1	441	50.1	5.16	3.72
1841654579	probable pectinesterase/pectinesterase inhibitor 7	*Prunus dulcis*	3	1	560	61.6	9.07	6.64
1847893909	beta-galactosidase 9 isoform X1	*Vitis riparia*	1	1	767	83.8	8.03	2.81
Signal transduction related proteins								
1841651170	14-3-3-like protein 16R	*Prunus dulcis*	15	1	262	29.5	4.86	6.64

Table 4.9, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
1841631617	probable calcium-binding protein CML13	*Prunus dulcis*	22	2	147	16.5	5.05	3.46
Redox regulation related proteins								
1841619982	2-methylene-furan-3-one reductase-like	*Prunus dulcis*	4	1	322	34.2	6.96	6.64
20455364	CBS domain-containing protein CBSX3,		4	1	206	22.7	9.01	3.96
Amino acid metabolism related proteins								
75297911	S-adenosylmethionine synthase;		13	1	393	42.9	6.05	6.64
Cell metabolism related proteins								
75098732	Prohibitin-1, mitochondrial;		9	1	288	31.7	9.26	4.54
Hypothetical and other related proteins								
1835392927	hypothetical protein G4B88_015834	*Cannabis sativa*	21	1	169	20.1	6.13	3.41
1835393204	hypothetical protein F8388_015154	*Cannabis sativa*	3	1	939	104.1	6.54	6.64
1835424357	hypothetical protein F8388_008931	*Cannabis sativa*	5	2	430	47.4	10.37	4.62
1847838864	uncharacterized protein At2g34160-like	*Vitis riparia*	10	1	130	14.5	5.35	6.64
1841645293	uncharacterized protein LOC117634123	*Prunus dulcis*	3	1	328	36.2	4.91	3.4
1835400280	hypothetical protein F8388_011329, partial	*Cannabis sativa*	1	1	861	95.4	5.22	6.64
1833572340	unnamed protein product	*Digitaria exilis*	3	1	373	41.2	6.58	6.64
1833620931	unnamed protein product	*Digitaria exilis*	6	1	185	19	10.18	6.64
670358752	uncharacterized protein LOC100272631 isoform X1	*Zea mays*	7	1	163	18	5.19	6.64
1833622462	unnamed protein product	*Digitaria exilis*	2	1	429	47.3	5.5	6.64
1833619193	unnamed protein product	*Digitaria exilis*	3	1	262	29.9	6.06	6.64
1835394895	hypothetical protein F8388_019761	*Cannabis sativa*	5	1	187	21.3	6.39	6.64

Table 4.10: List of significantly down-regulated proteins under jasmonic acid stress (fold change; $\log_2$ ratios of ≤ -1.5).

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [$\log_2$]
Downregulated Protein								
Energy and carbohydrate metabolism								
1841650990	V-type proton ATPase subunit B2	*Prunus dulcis*	18	2	490	54.5	5.11	-6.64
1841591980	transaldolase	*Prunus dulcis*	17	1	441	47.9	6.81	-6.64
75141370	Malate dehydrogenase, cytoplasmic		17	1	332	35.5	6.09	-6.64
120674	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic		15	1	338	36.5	7.83	-6.64
1847876217	phosphoglycerate kinase, chloroplastic	*Vitis riparia*	10	1	478	50.1	8.68	-6.64
1031986003	ADP, ATP carrier protein 1, mitochondrial	*Gossypium hirsutum*	11	1	386	42.1	9.86	-6.64
1841594037	ATP-citrate synthase beta chain protein 2	*Prunus dulcis]*	9	1	608	65.9	7.87	-6.64
1847911284	enolase	*Vitis riparia]*	11	1	444	47.9	5.8	-6.64
1841638167	V-type proton ATPase subunit G-like	*Prunus dulcis]*	21	1	110	12	9.31	-6.64
1847910580	V-type proton ATPase subunit G-like	*Vitis riparia]*	13	2	110	12.2	9.01	-6.64
30316342	2,3-bisphosphoglycerate-independent phosphoglycerate mutase 1		6	1	557	60.5	5.53	-6.64
1841640011	V-type proton ATPase subunit E2	*Prunus dulcis]*	17	1	231	26.1	8.44	-6.64
122064255	Isocitrate dehydrogenase NAD] catalytic subunit 6, mitochondrial		9	1	374	40.6	7.17	-6.64
1847889187	UTP--glucose-1-phosphate uridylyltransferase	*Vitis riparia]*	5	1	465	51.2	7.11	-6.64
1841636951	phosphoenolpyruvate carboxykinase (ATP)-like isoform X1	*Prunus dulcis]*	6	1	686	75.8	7.23	-6.64
1773401178	ferritin-3, chloroplastic	*Vigna unguiculata]*	10	2	256	28.5	5.8	-6.22
1841626886	isocitrate dehydrogenase NADP]-like isoform X3		9	1	386	43.5	7.74	-6.64
1845171029	pyruvate kinase 1, cytosolic-like	*Setaria viridis*	5	1	527	57.4	6.11	-6.64
75225265	Pyruvate dehydrogenase E1 component subunit beta-1, mitochondrial		7	1	374	39.9	5.36	-6.64

Table 4.10, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
11135282	Thioredoxin H-type		19	1	118	13	5.85	-6.64
663434108	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit alpha		2	1	616	67.3	7.43	-6.64
1847860315	cytochrome b5	*Vitis riparia*	12	1	134	15.1	5.31	-6.64
12585472	UTP--glucose-1-phosphate uridylyltransferase		6	1	471	51.5	6.29	-6.64
1847858276	probable fructokinase-7	*Vitis riparia*	7	1	371	40.2	7.96	-6.64
12585316	Phosphoglucomutase, cytoplasmic		5	1	583	63.4	6.44	-6.64
1847877753	NAD(P)H dehydrogenase (quinone) FQR1	*Vitis riparia*	13	2	203	21.7	6.19	-6.64
1841614378	UTP--glucose-1-phosphate uridylyltransferase isoform X1	*Prunus dulcis*	6	1	471	51.6	6.23	-6.64
1847841872	dihydrolipoyllysine-residue acetyltransferase component 2 of pyruvate dehydrogenase complex, mitochondrial-like isoform X1	*Vitis riparia*	4	1	550	59.7	8.32	-6.64
1346735	2,3-bisphosphoglycerate-independent phosphoglycerate mutase		3	1	556	60.8	5.82	-6.64
1847855109	formate dehydrogenase, mitochondrial	*Vitis riparia*	8	1	383	42	7.17	-6.64
12585489	UTP--glucose-1-phosphate uridylyltransferase		7	1	467	51.3	5.68	-6.64
1706326	Pyruvate decarboxylase 1		4	2	593	63.9	5.72	-6.64
1847856494	fumarate hydratase 1, mitochondrial-like isoform X2	*Vitis riparia*	7	1	495	53.8	8.19	-6.64
1731723640	NADH dehydrogenase ubiquinone] 1 beta subcomplex subunit 7		18	1	100	11.7	7.78	-6.64
1847837513	pyruvate kinase, cytosolic isozyme	*Vitis riparia*	4	2	510	55.4	7.77	-6.64
1731680144	ferritin-3, chloroplastic	*Cannabis sativa*	5	1	276	30.5	5.92	-6.64
12585427	V-type proton ATPase subunit G1		21	1	110	12.4	6	-2.98
1845132169	probable aldehyde dehydrogenase	*Setaria viridis*	2	1	610	67.2	8.24	-6.64

Table 4.10, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
1845132169	probable aldehyde dehydrogenase	*Setaria viridis*	2	1	610	67.2	8.24	-6.64
75225383	Pyruvate dehydrogenase E1 component subunit alpha-1, mitochondrial		2	1	390	42.7	7.77	-6.64
1845115144	ATPase ASNA1 homolog	*Setaria viridis*	2	1	363	40.6	4.94	-6.64
75316008	Cytochrome B5 isoform D;		11	1	140	15.1	5.14	-6.64
12585428	V-type proton ATPase subunit G 1;		8	1	110	12.3	6.32	-6.64
1841647478	NADP-dependent malic enzyme isoform X1	*Prunus dulcis*	3	1	668	73.9	7.58	-6.64
136707	Cytochrome b6-f complex iron-sulfur subunit, chloroplastic		5	1	230	24.2	8.29	-6.64
1847864818	enolase 1, chloroplastic	*Vitis riparia*	2	1	472	50.5	6.64	-6.64
75169075	Gamma carbonic anhydrase 2		5	1	278	30	7.24	-6.64
Protein metabolism related proteins								
1841620385	elongation factor 2	*Prunus dulcis*	13	2	843	94.1	6.11	-6.64
1841599311	40S ribosomal protein S15-4	*Prunus dulcis*	32	3	152	17.2	10.43	-5.18
1173256	40S ribosomal protein S4		12	3	262	29.6	10.21	-6.64
1162439859	60S ribosomal protein L10-3 isoform X1	*Zea mays*	10	2	245	27.9	10.05	-6.64
27735239	60S ribosomal protein L32-1		17	1	133	15.5	10.89	-6.64
1845184613	nascent polypeptide-associated complex subunit alpha-like protein 1	*Setaria viridis*	6	1	220	23.5	4.42	-6.64
730456	40S ribosomal protein S19		5	1	146	16.4	10.04	-6.64
1841608100	aspartyl protease family protein 2-like	*Prunus dulcis*	4	1	479	51.2	9.32	-6.64
1847845980	40S ribosomal protein S17-like	*Vitis riparia*	8	1	144	16.2	9.98	-6.64
38503417	60S ribosomal protein L7-2		6	1	242	28.2	9.94	-6.64
1731731220	eukaryotic translation initiation factor	*Cannabis sativa*	1	1	802	87.7	7.84	-6.64
1847903388	50S ribosomal protein L7/L12-like	*Vitis riparia*	5	1	165	17.9	9.19	-6.64
1841644237	60S ribosomal protein L28-1	*Prunus dulcis*	6	1	150	16.7	11.22	-6.64
1841588827	60S ribosomal protein L17-2-like	*Prunus dulcis*	5	1	200	22.5	10.08	-2.74
1847913084	60S ribosomal protein L21-1-like	*Vitis riparia*	5	1	165	18.8	10.36	-6.64

Table 4.10, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
17369182	60S ribosomal protein L30		14	1	112	12.3	9.38	-6.64
12229936	Proteasome subunit alpha type-7		19	3	249	27.1	7.37	-6.64
266743	Protein disulfide-isomerase		7	2	512	57.1	5.1	-6.64
657341073	Prolyl 4-hydroxylase 2		7	1	299	33	6.35	-6.64
1847900952	proteasome subunit beta type-4	*Vitis riparia*	6	1	245	27.6	7.18	-6.64
1841595688	T-complex protein 1 subunit beta	*Prunus dulcis*	4	2	527	57.1	6.05	-6.64
1841656968	proline iminopeptidase isoform X1	*Prunus dulcis*	4	2	425	47.6	6.21	-6.64
1841613443	26S proteasome non-ATPase regulatory subunit 2 homolog A isoform X2	*Prunus dulcis*	2	1	892	98.2	5.22	-6.64
1847905405	subtilisin-like protease SBT1.7	*Vitis riparia*	2	1	767	81.1	6.18	-6.64
1841616922	monothiol glutaredoxin-S15, mitochondrial-like isoform X1	*Prunus dulcis*	8	1	171	18.8	7.33	-3.68
1847887754	cysteine protease RD19A	*Vitis riparia*	3	1	380	41.7	5.77	-6.64
1841634386	ubiquitin-conjugating enzyme E2 7	*Prunus dulcis*	7	1	167	18.7	5.19	-6.64
1841621974	grpE protein homolog 2, mitochondrial-like isoform X1	*Prunus dulcis*	3	1	339	37.1	6.76	-6.64
1841591636	26S proteasome non-ATPase regulatory subunit 11 homolog	*Prunus dulcis*	3	1	422	47.1	6.48	-6.64
73622182	Thiol protease aleurain-like		4	1	358	39.5	6.68	-6.64
90185101	Philibertain g I		39	1	23	2.5	8.25	-6.64
1841601143	ubiquitin carboxyl-terminal hydrolase 2	*Prunus dulcis*	3	1	334	38.4	6.43	-6.64
1847839145	glutaredoxin-C4	*Vitis riparia*	7	1	140	15.1	5.87	-6.64
1841625257	subtilisin-like protease SBT5.4	*Prunus dulcis*	1	1	775	82.6	7.69	-6.64
1845107161	probable cytosolic oligopeptidase A	*Setaria viridis*	1	1	1081	120.4	8.4	-6.64
Defense and stress response related proteins								
75326437	Heat shock 70 kDa protein BIP4		8	1	687	74.3	5.22	-6.64
26397792	Peroxidase 72		5	2	336	37.4	8.43	-6.64
1847851975	germin-like protein 5-1	*Vitis riparia*	13	2	217	22.7	8.41	-6.64
1239396279	Peroxiredoxin-2		21	3	162	17.4	6.19	-6.64

Table 4.10, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
75104682	Pathogenesis-related thaumatin-like protein 3.6		7	1	245	26.5	5.34	-6.64
50401194	Monodehydroascorbate reductase 3		3	1	441	48.3	5.27	-6.64
1731736268	tetrahydrocannabinolic acid synthase-like	*Cannabis sativa*	2	1	546	61.8	9.09	-6.64
1836290959	monodehydroascorbate reductase	*Camellia sinensis*	3	1	434	47.2	6.35	-6.64
1841587587	thaumatin-like protein 1	*Prunus dulcis*	4	1	248	26	6.1	-3.48
1847838509	temperature-induced lipocalin-1	*Vitis riparia*	9	2	185	21.5	7.18	-3.43
351721352	superoxide dismutase [Fe]		4	1	248	27.8	5.87	-6.64
1847836599	obg-like ATPase 1	*Vitis riparia*	4	1	394	44.4	6.64	-6.64
1170368	Heat shock 22 kDa protein		6	1	202	22.9	8.31	-6.64
1731737306	universal stress protein PHOS32	*Cannabis sativa*	5	1	196	21.7	6.06	-6.64
1031987033	glutathione S-transferase U8-like	*Gossypium hirsutum*	4	1	225	25.9	6	-6.64
25090885	Peroxiredoxin-2F, mitochondrial		4	1	201	21.4	8.9	-6.64
1847859556	glutathione transferase GST 23-like	*Vitis riparia*	4	1	230	25.8	6.01	-6.64
1731685433	monodehydroascorbate reductase	*Cannabis sativa*	3	1	434	47.1	5.92	-6.64
Amino acid metabolism related proteins								
1841591324	serine hydroxymethyltransferase 4	*Prunus dulcis*	12	1	471	51.8	7.3	-6.64
1711381	Phosphoserine aminotransferase,		12	1	430	47.1	8.06	-6.64
223635326	S-adenosylmethionine synthase 1		14	1	396	43.2	5.92	-6.64
1847910666	S-adenosylmethionine synthase 3	*Vitis riparia*	12	1	440	48.5	6.71	-6.64
1847841122	aspartate aminotransferase, cytoplasmic	*Vitis riparia*	9	1	411	45	7.09	-6.64
1841644107	methylenetetrahydrofolate reductase 2-like	*Prunus dulcis*	5	2	595	67	6.55	-2.59
1847889967	3-dehydroquinate synthase, chloroplastic	*Vitis riparia*	3	1	456	49.4	8	-6.64
11131901	Cysteine synthase		4	2	325	34.3	5.41	-6.64
1847847935	adenylosuccinate lyase-like	*Vitis riparia*	3	1	553	62	7.02	-6.64

Table 4.10, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
1841593774	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase 2 isoform X1	*Prunus dulcis*	7	1	202	23.9	5.21	-6.64
1731681793	IAA-amino acid hydrolase ILR1-like 4	*Cannabis sativa*	3	1	445	48.4	6.68	-6.64
1847864268	dihydropyrimidine dehydrogenase (NADP(+)), chloroplastic	*Vitis riparia*	2	1	489	53.8	6.62	-6.64
2493895	Cysteine synthase		2	1	325	34.3	6.61	-6.64
Transport related proteins								
1731706407	acyl-CoA-binding protein isoform X1	*Cannabis sativa*	21	2	91	10.3	5.24	-2.91
1172556	Mitochondrial outer membrane protein porin of 36 kDa		14	2	276	29.4	8.06	-6.64
75264036	Ran-binding protein 1 homolog a;		9	1	228	25.6	4.94	-6.64
1172555	Mitochondrial outer membrane protein porin of 34 kDa		9	1	276	29.6	8.6	-6.64
1847901198	mitochondrial dicarboxylate/ tricarboxylate transporter DTC	*Vitis riparia*	8	2	299	32	9.35	-6.64
75220197	Copper transport protein CCH		12	2	121	13	4.93	-6.64
1841616994	clathrin light chain 1	*Prunus dulcis*	3	1	413	45.4	5.15	-6.64
12230205	Mitochondrial import inner membrane translocase subunit TIM10		29	2	83	9.3	7.74	-3.78
75270222	Signal peptidase complex subunit 3B;		5	1	167	19.2	9	-6.64
1847900915	clathrin heavy chain 2	*Vitis riparia*	1	1	1705	192.8	5.49	-6.64
1845123400	mitochondrial import inner membrane translocase subunit TIM8-like	*Setaria viridis*	12	1	78	8.9	8.51	-3.35
75219328	Protein TIC110, chloroplastic		1	1	996	109.9	6.4	-6.64
75334062	Nuclear transport factor 2		2	1	460	49.5	5.96	-6.64
20138095	Exportin-2		1	1	972	108.6	5.59	-6.64
1841607819	nuclear pore complex protein NUP62-like	*Prunus dulcis*	1	1	696	70.2	4.84	-6.64
1847843841	potassium transporter 7 isoform X1	*Vitis riparia*	1	1	840	93.6	6.02	-6.64
146325057	Aquaporin PIP2-1		3	1	290	30.2	7.84	-6.64

Table 4.10, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
Transcription related proteins								
1847844385	aconitate hydratase, cytoplasmic	*Vitis riparia*	5	2	987	107.4	7.55	-6.64
7387727	Histone H2B		31	1	145	16	10.11	-6.64
1847885994	L-ascorbate oxidase homolog	*Vitis riparia*	6	2	542	60.2	9.36	-6.64
1847847700	adenosine kinase 2-like isoform X1	*Vitis riparia*	11	2	341	37.8	5.45	-6.64
1841618342	nucleosome assembly protein 1;4 isoform X1	*Prunus dulcis*	6	1	386	43.8	4.44	-6.64
1847905447	multiple organellar RNA editing factor 2, chloroplastic-like	*Vitis riparia*	5	1	240	27.3	9.19	-6.64
1847902866	apoptotic chromatin condensation inducer in the nucleus isoform X1	*Vitis riparia*	4	2	691	76.3	5.27	-6.64
1847896355	heterogeneous nuclear ribonucleoprotein 1	*Vitis riparia*	3	1	464	46.7	8.07	-6.64
75216820	Nucleosome assembly protein 1		7	1	379	43.5	4.44	-6.64
122248036	Protein DNA-damage inducible 1		3	1	414	45.3	4.93	-6.64
1841642213	serine/arginine-rich splicing factor SR30 isoform X1	*Prunus dulcis*	4	1	297	34.1	10.42	-6.64
1731671536	glutamate--tRNA ligase, chloroplastic/mitochondrial	*Cannabis sativa*	2	1	578	64.4	6.49	-6.64
308191500	Adenylosuccinate synthetase 2,		2	1	501	54.4	8.16	-6.64
1841633650	GMP synthase glutamine-hydrolyzing		3	1	534	59.3	6.8	-6.64
Photosynthesis related proteins								
132098	Ribulose bisphosphate carboxylase small chain SSU11A, chloroplastic;		8	1	180	20.3	8.12	-6.64
671726284	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)	*Odontoschisma fluitans*	12	1	458	50.8	6.44	-5.22
11134054	Oxygen-evolving enhancer protein 1, chloroplastic;		10	1	332	35.2	5.96	-3.47
1172664	Photosystem I reaction center subunit III, chloroplastic;		10	2	232	25.2	9.29	-6.64

Table 4.10, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
1168738	Carbonic anhydrase 1		3	1	330	35.6	6.04	-6.64
45477188	Photosystem II reaction center protein H;		23	1	74	7.9	8.57	-6.64
1847837566	photosystem I reaction center subunit III, chloroplastic	*Vitis riparia*	5	1	221	24.2	9.66	-6.64
677286725	Delta-aminolevulinic acid dehydratase,		3	1	432	46.2	6.55	-6.64
1847875596	protochlorophyllide reductase, chloroplastic	*Vitis riparia*	2	1	396	42.7	9.33	-6.64
11386828	Glutamine synthetase, chloroplastic/mitochondrial;		6	1	430	47.4	6.87	-6.64
Cell wall and cell structure related proteins								
1847863899	UDP-arabinopyranose mutase 1	*Vitis riparia*	13	2	358	40.8	6.68	-6.64
1845137651	sucrose synthase 1	*Setaria viridis*	3	1	802	91.7	6.28	-6.64
75262663	Beta-D-xylosidase 4		2	1	784	84.3	7.66	-6.64
1841610548	actin-depolymerizing factor 2	*Prunus dulcis*	9	1	139	16	5.71	-6.64
30580342	Caffeoyl-CoA O-methyltransferase;		5	1	245	27.6	6.3	-6.64
1847841349	beta-galactosidase 15-like	*Vitis riparia*	1	1	835	92.8	5.64	-6.64
1847838241	pectinesterase	*Vitis riparia*	2	1	528	58.2	8.43	-6.64
38257776	Fasciclin-like arabinogalactan protein 10; Flags: Precursor		3	1	422	43.6	5.82	-6.64
Redox related proteins								
12229785	Glutamate dehydrogenase A;		8	1	411	44.8	7.08	-6.64
1847892486	aspartate aminotransferase, cytoplasmic	*Vitis riparia*	8	1	450	49	9.11	-6.64
1731707291	glutamate dehydrogenase 2	*Cannabis sativa*	10	2	411	44.9	6.8	-6.64
75316039	Quinone-oxidoreductase QR2;		12	1	205	22.1	6.95	-6.64
1841610160	NADP-dependent D-sorbitol-6-phosphate dehydrogenase-like	*Prunus dulcis*	3	1	309	34.7	6.54	-6.64
75163759	Probable acylpyruvase FAHD1, mitochondrial		4	1	223	24.3	6.95	-2.83
1168493	Arginase 1, mitochondrial		4	1	342	37.3	6.55	-6.64

Table 4.10, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
Lipid metabolism related proteins								
1847868732	non-specific lipid-transfer protein-like 1 isoform X1	*Vitis riparia*	10	2	124	13.6	9.58	-6.64
1845121532	glycolipid transfer protein 1-like	*Setaria viridis*	7	1	202	22.3	8.41	-6.64
17380471	Biotin carboxyl carrier protein of acetyl-CoA carboxylase 1		3	1	280	29.6	9.06	-6.64
1731704507	peroxisomal acyl-coenzyme A oxidase 1	*Cannabis sativa*	2	1	664	74.5	7.71	-6.64
1841599379	erlin-2-B	*Prunus dulcis*	3	1	371	42.1	5.76	-6.64
1847901001	protein YLS3-like	*Vitis riparia*	4	1	189	19.8	7.88	-6.64
Cell metabolism related proteins								
75273705	Prohibitin-4, mitochondrial;		13	1	279	30.6	7.56	-6.64
1841594256	patellin-3-like	*Prunus dulcis*	5	3	619	69	4.81	-6.64
1847910359	cell division cycle protein 48 homolog	*Vitis riparia*	14	1	806	89.5	5.26	-6.64
Signal transduction related proteins								
670428157	phospholipase D family protein isoform X1	*Zea mays*	4	2	812	92.2	5.69	-6.64
115492	Calmodulin-related protein		26	3	184	21.1	4.78	-6.64
11131469	Calnexin homolog		2	1	551	62.5	4.94	-6.64
1847852284	protein RALF-like 33	*Vitis riparia*	7	1	124	13	7.93	-6.64
Secondary metabolites related proteins								
1705846	Chalcone synthase;		5	2	393	42.7	6.25	-6.64
1840527225	p-coumarate-3-hydroxylase	*Rehmannia glutinosa*	2	1	509	57.9	8.73	-6.64
1841637356	isopentenyl-diphosphate Delta-isomerase I	*Prunus dulcis*	3	1	299	34.1	6.05	-2.29
166233972	Chalcone--flavonone isomerase 3;		6	1	226	24.7	6.38	-6.64
Others								
1847894323	protein FAM136A-like	Vitis riparia	9	1	149	17.1	6.52	-6.64
1168493	Arginase 1, mitochondrial;		4	1	342	37.3	6.55	-6.64
1717794	Sex determination protein tasselseed-2		2	1	336	35.2	7.18	-6.64
Hypothetical related proteins								

Table 4.10, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
1848379843	hypothetical protein Mp_2g09390	*Marchantia polymorpha subsp. Ruderalis*	29	1	443	49.6	4.87	-6.64
1833588461	unnamed protein product, partial	*Digitaria exilis*	13	1	766	84.6	6.14	-6.64
1833593700	unnamed protein product, partial	*Digitaria exilis*	21	1	378	39.8	9.48	-6.64
1835396341	hypothetical protein G4B88_004191, partial	*Cannabis sativa*	17	1	524	58.2	5.05	-6.64
1833568948	unnamed protein product	*Digitaria exilis*	7	1	666	73.4	5.16	-6.64
1833613113	unnamed protein product	*Digitaria exilis*	6	1	672	74	5.27	-6.64
1835406355	hypothetical protein G4B88_030061	*Cannabis sativa*	5	1	525	58.2	8.81	-6.64
1835425992	hypothetical protein G4B88_001874	*Cannabis sativa*	3	1	472	53.4	6.44	-6.64
1835428499	hypothetical protein F8388_008728	*Cannabis sativa*	9	1	618	68.3	9.28	-6.64
1833621682	unnamed protein product	*Digitaria exilis*	5	3	621	67.9	10.13	-6.64
1848390176	hypothetical protein Mp_5g16740	*Marchantia polymorpha subsp. Ruderalis*	11	1	388	42	9.74	-6.64
1835413200	hypothetical protein F8388_024767	*Cannabis sativa*	18	2	214	24.3	9	-6.64
1848384344	hypothetical protein Mp_3g19060	*Marchantia polymorpha subsp. ruderalis*	6	1	674	73.7	6.77	-6.64
1835422592	hypothetical protein F8388_009911	*Cannabis sativa*	20	3	210	24	8.05	-6.64
1835412285	hypothetical protein F8388_023585	*Cannabis sativa*	7	2	529	57.5	6.32	-6.64
1833609277	unnamed protein product, partial	*Digitaria exilis*	13	3	200	24	10.96	-4.07
1835429759	hypothetical protein F8388_004847	*Cannabis sativa*	12	1	259	29	9.25	-6.64
1835423624	hypothetical protein G4B88_015692	*Cannabis sativa*	10	1	290	32.6	6.71	-6.64
1835434945	hypothetical protein G4B88_017549	*Cannabis sativa*	25	3	146	16.8	10.9	-6.64
1835380294	hypothetical protein F8388_000245	*Cannabis sativa*	3	1	1144	126.7	6.35	-6.64
1835426894	hypothetical protein G4B88_026007	*Cannabis sativa*	4	1	554	62.1	8.22	-6.64
1835396626	hypothetical protein G4B88_029145	*Cannabis sativa*	5	1	431	48.5	8.37	-6.64
1847854349	uncharacterized protein LOC117930626	*Vitis riparia*	4	2	455	50.5	10.17	-6.64
1835407066	hypothetical protein G4B88_026306, partial	*Cannabis sativa*	2	1	646	73.8	9.66	-6.64
1833587809	unnamed protein product, partial	*Digitaria exilis*	11	2	520	56.3	6.67	-6.64

Table 4.10, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
1835381047	hypothetical protein F8388_021799	*Cannabis sativa*	5	1	262	30	9.82	-6.64
1835417715	hypothetical protein F8388_010536	*Cannabis sativa*	8	2	275	31.5	6.06	-6.64
1835392650	hypothetical protein F8388_020589	*Cannabis sativa*	3	1	636	69.2	8.84	-6.64
1835435421	hypothetical protein G4B88_012296	*Cannabis sativa*	3	1	371	41.4	9.85	-3.52
1835389342	hypothetical protein G4B88_004500	*Cannabis sativa*	3	1	483	53.5	6.13	-6.64
1835429126	hypothetical protein F8388_019796	*Cannabis sativa*	4	1	302	34.3	5.34	-6.64
1835381800	hypothetical protein G4B88_002334 (mitochondrion)	*Cannabis sativa*	2	1	619	69.7	9.23	-6.64
1835417606	hypothetical protein G4B88_000226	*Cannabis sativa*	1	1	1093	120.9	8.46	-6.64
1841623145	uncharacterized protein LOC117624836	*Prunus dulcis*	6	2	425	46.1	4.67	-6.64
1835433373	hypothetical protein G4B88_023266	*Cannabis sativa*	4	1	317	36	7.09	-2.56
1835388914	hypothetical protein G4B88_009656, partial	*Cannabis sativa*	5	1	510	56.1	9.52	-6.64
1835384103	hypothetical protein F8388_021830	*Cannabis sativa*	7	1	262	27.5	5.49	-6.64
1835418059	hypothetical protein G4B88_026561	*Cannabis sativa*	2	1	748	81.5	8.29	-6.64
1833592023	unnamed protein product	*Digitaria exilis*	2	1	747	82.4	6.01	-6.64
1833581303	unnamed protein product	*Digitaria exilis*	8	1	238	26.9	8.59	-6.64
1835389753	hypothetical protein F8388_002381	*Cannabis sativa*	5	1	253	26.4	9.17	-3.1
1835379128	hypothetical protein G4B88_002946	*Cannabis sativa*	2	1	535	59.3	6.25	-6.64
1833599650	unnamed protein product	*Digitaria exilis*	9	1	291	31.7	8.5	-6.64
1835403568	hypothetical protein F8388_002864	*Cannabis sativa*	8	1	153	17.2	8.05	-6.64
1848381574	hypothetical protein Mp_2g23250	*Marchantia polymorpha subsp. ruderalis*	5	2	263	29.7	10.18	-6.64
1833620929	unnamed protein product	*Digitaria exilis*	3	1	420	44.3	4.79	-6.64
1847895816	uncharacterized protein LOC117914560	*Vitis riparia*	4	1	317	34.9	4.82	-6.64
1848375376	hypothetical protein Mp_1g03870	*Marchantia polymorpha subsp. ruderalis*	2	1	488	52.1	6.48	-6.64
1835429072	hypothetical protein G4B88_020834	*Cannabis sativa*	1	1	1428	157.4	6.74	-6.64
1835402033	hypothetical protein G4B88_029565	*Cannabis sativa*	2	1	600	66.2	6.13	-6.64

Table 4.10, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
1833604786	unnamed protein product	*Digitaria exilis*	4	1	239	26.5	10.59	-6.64
1835422922	hypothetical protein G4B88_005022	*Cannabis sativa*	18	2	82	9.2	7.28	-6.64
1835400933	hypothetical protein F8388_019108, partial	*Cannabis sativa*	8	1	131	14.3	9.51	-6.64
1835378403	hypothetical protein G4B88_006371	*Cannabis sativa*	4	1	322	34.4	7.24	-6.64
1833581296	unnamed protein product	*Digitaria exilis*	11	1	112	12.3	9.55	-6.64
1833584959	unnamed protein product, partial	*Digitaria exilis*	3	1	498	52.7	6.92	-6.64
1848391493	hypothetical protein Mp_6g02580	*Marchantia polymorpha subsp. ruderalis*	2	1	548	60.3	5.47	-6.64
1848387040	hypothetical protein Mp_4g15150	*Marchantia polymorpha subsp. ruderalis*	10	1	167	18.2	6.33	-6.64
1833622269	unnamed protein product	*Digitaria exilis*	2	1	1068	117.4	8.12	-6.64
1835417042	hypothetical protein G4B88_016838	*Cannabis sativa*	7	1	138	16.1	8.34	-3.93
1833585569	unnamed protein product	*Digitaria exilis*	2	1	628	69	9.63	-6.64
1833593019	unnamed protein product	*Digitaria exilis*	4	1	364	38.4	7.61	-6.64
1835420224	hypothetical protein F8388_011576	*Cannabis sativa*	2	1	551	63.1	9.03	-6.64
1835417686	hypothetical protein F8388_010507	*Cannabis sativa*	3	1	256	28.3	8.59	-6.64
1833593552	unnamed protein product	*Digitaria exilis*	2	1	402	44.5	5.25	-6.64
1835422266	hypothetical protein G4B88_006512	*Cannabis sativa*	1	1	736	80.9	6.18	-6.64
1835397500	hypothetical protein F8388_001776	*Cannabis sativa*	5	1	266	30.2	10.05	-6.64
1841591686	uncharacterized protein LOC117624249	*Prunus dulcis*	3	1	441	48.7	5.57	-6.64
1835394812	hypothetical protein F8388_012190	*Cannabis sativa*	2	1	577	63.2	5.86	-6.64
1833568304	unnamed protein product	*Digitaria exilis*	2	1	454	50.8	6.04	-6.64
1835427927	hypothetical protein G4B88_017930	*Cannabis sativa*	2	1	538	60.1	8.54	-6.64
1835437063	hypothetical protein G4B88_014604	*Cannabis sativa*	7	1	237	27.6	11.22	-6.64
1833570306	unnamed protein product	*Digitaria exilis*	8	1	111	11.9	10.04	-6.64
1835420514	hypothetical protein G4B88_004044	*Cannabis sativa*	3	1	431	48.5	6.02	-6.64
1835421359	hypothetical protein F8388_012490	*Cannabis sativa*	1	1	757	81.2	6.52	-6.64
1731734463	uncharacterized protein LOC115695925	*Cannabis sativa*	36	1	53	5.5	10.17	-6.64

Table 4.10, continued.

Accesion Number	Protein name	Species	Coverage [%]	#Unique Peptides	#AAs	MW [kDa]	Calc. pI	Fold change [log_2]
1835385527	hypothetical protein F8388_005021	*Cannabis sativa*	1	1	1393	155.9	7.06	-6.64
1835428433	hypothetical protein G4B88_011028	*Cannabis sativa*	2	1	481	53	6.7	-6.64
1835394337	hypothetical protein G4B88_024509	*Cannabis sativa*	3	1	294	34	9.55	-6.64
1835418656	hypothetical protein F8388_010429	*Cannabis sativa*	1	1	821	91.6	9.31	-6.64
1835425543	hypothetical protein G4B88_029434	*Cannabis sativa*	3	1	279	30.1	6.52	-6.64
1835431478	hypothetical protein G4B88_013676	*Cannabis sativa*	6	1	138	16	9.55	-6.64
1848375764	hypothetical protein Mp_1g06890	*Marchantia polymorpha subsp. ruderalis*	1	1	2178	246.4	5.66	-6.64
1833571644	unnamed protein product, partial	*Digitaria exilis*	8	1	145	15.9	10.9	-6.64
1848387614	hypothetical protein Mp_4g19810	*Marchantia polymorpha subsp. ruderalis*	2	1	379	42.8	5.87	-6.64
1833608193	unnamed protein product	*Digitaria exilis*	3	1	357	38.3	5.34	-6.64
1833593785	unnamed protein product, partial	*Digitaria exilis*	4	1	248	27.4	6.43	-6.64
1835419781	hypothetical protein G4B88_024562, partial	*Cannabis sativa*	2	1	471	52.5	6.4	-6.64
1835381087	hypothetical protein F8388_024234, partial	*Cannabis sativa*	8	1	143	16.1	9.39	-6.64
1731673617	uncharacterized protein C594.04c	*Cannabis sativa*	3	1	332	39.3	9.07	-6.64
1835393252	hypothetical protein F8388_020029	*Cannabis sativa*	5	1	240	27	7.46	-6.64
1835382409	hypothetical protein G4B88_027811	*Cannabis sativa*	2	1	370	39.7	8.94	-6.64
1835431525	hypothetical protein G4B88_013723	*Cannabis sativa*	1	1	1455	162.7	8.69	-6.64
1835397923	hypothetical protein F8388_012808	*Cannabis sativa*	4	1	180	20.5	9.52	-2.9
1835407807	hypothetical protein F8388_017298	*Cannabis sativa*	5	1	159	17.6	9.42	-6.64
1845117902	uncharacterized protein LOC117844133	*Setaria viridis*	1	1	840	94.2	9.67	-6.64
1848395379	hypothetical protein Mp_7g12690	*Marchantia polymorpha subsp. ruderalis*	1	1	581	62.7	6.62	-6.64
1833590055	unnamed protein product, partial	*Digitaria exilis*	2	1	332	37.5	8.15	-6.64

Figure 4.16 depicts the gene ontology of protein with significantly differential abundant protein in response to JAS treatment. The significantly up-regulated protein can be divided into three categories: biological process, molecular function, and cellular component. Within those main categories, the significant protein enriched GO terms were catalytic activity, binding, cellular process, metabolic process, and cellular anatomical. Similar protein enriched GO terms can be found for significantly down-regulated proteins.

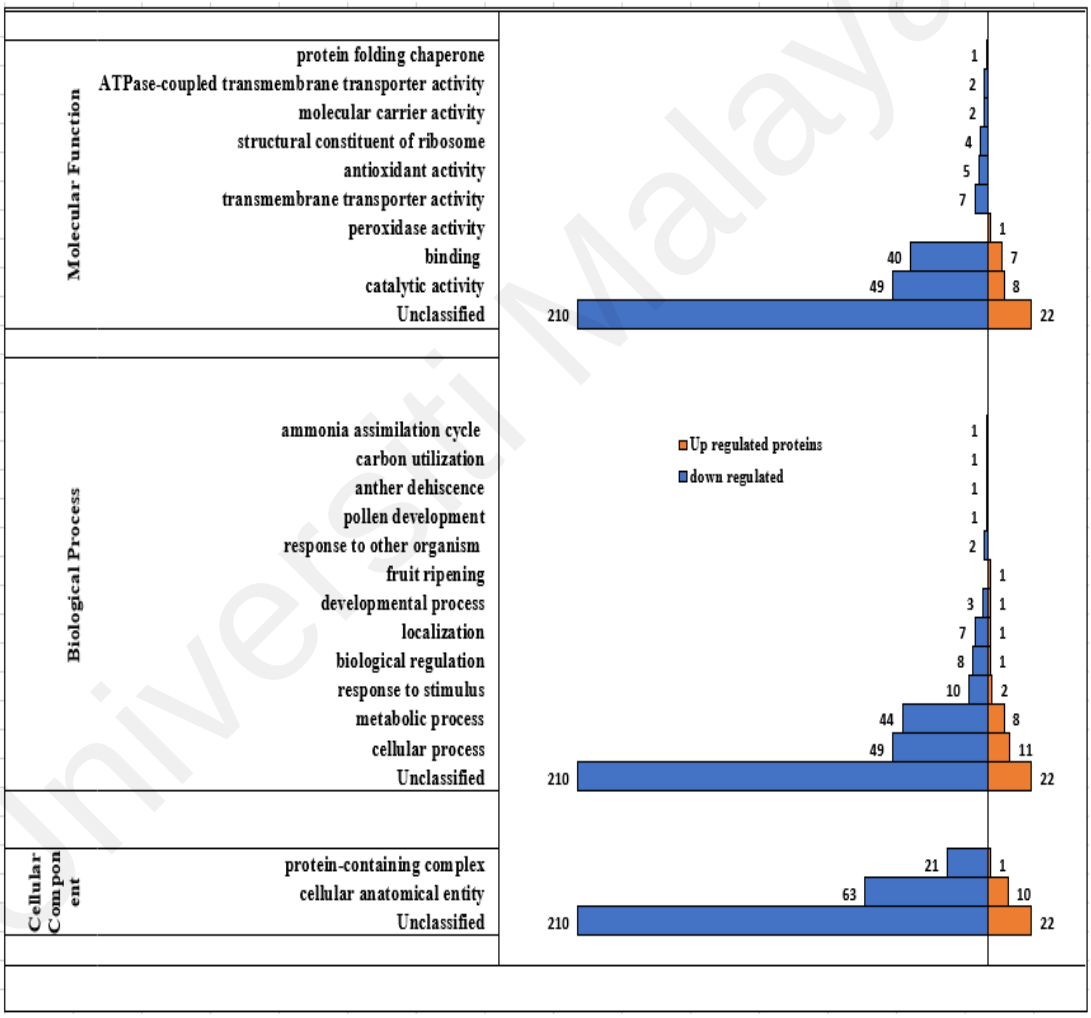

Figure 4.16: Gene ontology of DEPs in JAS vs OCM based on UniProt information.

CHAPTER 5: DISCUSSION

5.1 Effect of TDZ as a growth regulator on the growth of *Azadirachta indica* callus

Based on the findings of previous studies (Akula et al., 2003; Ashokhan et al., 2020; Ashokhan et al., 2019), TDZ was used as a plant growth regulator (PGR) in order to induce callus formation in *A. indica*.

In this report, no callus was seen to form from leaf explants grown on MS media because there were no growth regulators. On the other hand, the formation of coloured calli can be seen in cultures of leaf explants grown on MS media treated with a variety of PGR (TDZ, 2,4-D). Callus grown on MS media containing TDZ was observed to produce the highest amount of azadirachtin compared to other treatments (Ashokhan et al., 2020). Besides, a similar result has been recorded on callus formation from wild and hybrid blueberries after being cultured in basal media containing TDZ (Ghosh et al., 2017). Callus started to develop after four weeks of induction and fully formed at 12 weeks on media supplemented with TDZ, while basal media without PGR did not show any changes. These observation has been evaluated in several plants such as strawberry (Chung & Ouyang, 2020), primrose flower (Hayta et al., 2016), Chinese sage (Tsai et al., 2016), *Pelargonium sidoides* (V. Kumar et al., 2015), *Cymbidium* (Huan & Tanaka, 2004) and more. Therefore, based on the result from Ashokhan et al. (2020), TDZ at a concentration of 0.6 mg/L was found to be the best treatment used for the proliferation and development of callus from *A. indica* leaves explants.

5.1.1 Effect of NaCl on the growth of *Azadirachta indica* callus

Salinity stress can cause physiological and biochemical changes that directly affect plant growth and establishment (Abid et al., 2020; Gupta & Huang, 2014). Salinity stress can also cause a reduction in the availability of water (osmotic stress), and salt absorbed

by plants can accumulate to a lethal level in a particular tissue (ionic stress) (Hussain et al., 2019; Safdar et al., 2019).

As previously stated, the presence of NaCl in the medium alters the colour variations of neem callus, with a notable browning response seen as elicitor concentration increased. With increasing NaCl content, the neem callus displayed three distinct colour patterns: green, yellowish green, and yellowish green mixed with brown. Yongxue Zhang et al. (2019) reported a similar colour change in callus of *Puccinellia tenuifora* after 28 days of salt treatment, where 50 mM Nacl calli colour became darker and 150 mM NaCl calli colour appeared brown. Previous research revealed comparable findings for *Zea mays* (Suhartanto et al., 2022*), Carum copticum* (Razavizadeh et al., 2022), *Salacia chinensis (*Chavan et al., 2021), *Paeonia lactiflora* (Cai et al., 2020), *Limnophila and Bacopa monnieri* (Dogan, 2020). These findings might be attributed to the oxidative browning phenomenon, which often happens in plant tissue culture and is induced by the production, build-up, and oxidation of phenolic compounds as a result of plants' response to salt stress (Cai et al., 2020; Gemechu & Amante, 2021; Jones & Saxena, 2013; Suhartanto et al., 2022). Therefore, the rate of browning might be a good indicator that can show the sensitivity of callus to stress (Mahmood et al., 2012). However, over-accumulation of phenol compounds in callus tissue may harm plant cells, leading to cell death and a darkening of the callus due to its toxicity to plant cells (Cai et al., 2020; Jones & Saxena, 2013; Saputro et al., 2017). In addition, Andaryani et al. (2019) note that the brown callus signifies the formation of phenol compounds, whereas the green callus shows the existence of chlorophyll in the tissue. It has been suggested that the chlorophyll content of neem callus is influenced by salinity stress (Saputro et al., 2017) as the colour of the callus shifts from green to brown. This may be because the chlorophyll content decreases with increasing NaCl concentration (Al-Shorafa et al., 2014; Andaryani et al., 2019).

In the current study, successfully produced callus on OCM media were transferred to NaCl-containing media after 8 weeks of culture development. After 4 weeks of salt treatment, observation of the callus showed a decrease in the fresh weight of the stressed callus with an increase in salt levels, and a sharp decline was noticed at higher levels of salt stresses (1.0NaCl and 1.5NaCl), where almost 3-fold less compared to control. This finding agrees with the observation made by Ahmad et al. (2009), where 1-month-old rice calli (Basmati 385 and Basmati-Karnal) were exposed to 0, 50, and 100 mol m^{-3} in liquid LS basal media supplemented with 2,3 D and kinetin for 30 days. The results have shown that the relative growth rate of fresh calli was decreased with an increase in NaCl concentration and decreasing in callus dry weight. Similar observations were reported in the previous investigation for barley (*Hordeumvulgare* L.) (Haque et al., 2017), safflower (*Canthamus tinctorius* L.) (Soheilikhah et al., 2013), sugarcane (*Saccharum* sp.) (Errabii et al., 2007), tomato (*Solanum lycopersicon* L.) (Aazami et al., 2021) and potato (*Solanum tuberosum* L.) (Forooghian & Esfarayeni, 2013).

The observation could have resulted from the changes in the water potential gradient between media and callus cells (Lokhande et al., 2010; Shahbazi et al., 2021). High salinity concentration in a plant will cause a reduction in osmotic potential. This condition is called hyperosmotic stress (Gupta & Huang, 2014; Lokhande et al., 2010), where plants lose water and reduce the water availability to cells in the media (Golkar et al., 2020). The water availability reduction will result in turgor pressure loss (Golkar et al., 2017). Plant cells require and consume energy (Ma et al., 2017) to maintain turgor and ionic equilibrium, which eventually leads to callus growth decline (Golkar et al., 2019). Reduction of callus growth is essential to help plants save energy for defense purposes and limit the risk of heritable damage (Lokhande et al., 2010). The result was similar to those reported in *Nigella sativa* L. (Golkar et al., 2020), *Plantago ovata* (Golkar et al.,

2017), Safflower (Golkar et al., 2019), and *Sesuvium portulacastrum* (Lokhande et al., 2010).

According to Munns and Tester (2008), there are two stages to the decrease of plant growth when exposed to salt stress which is a quick response to the rise in external osmotic pressure and a slow response to the accumulation of Na+ in plants. Therefore, a decrease in callus growth in response to salt stress might be because salt ions like Na+ and Cl- interfere with crucial nutrients for uptake and translocation processes, causing a nutritional imbalance (Errabii et al., 2007). Both Na+ and Cl- enter the cells, causing a severe ion imbalance, and excessive uptake might lead to serious physiological problems (Golkar et al., 2019). Plant cells need high potassium ions, lower sodium ions, and a high cytosolic K+/Na+ ratio during normal physiological circumstances to control osmotic balance (Lokhande et al., 2010). However, when plant cells are exposed to high NaCl levels, ion ratios will be altered due to unrestrained uptake of Na+ through K+ pathways. A high concentration of Na+ will inhibit the uptake of K+ ions into plant cells and contribute to ion toxicity (Soheilikhah et al., 2013). Thus, when NaCl concentration increase, it may enhance sodium intake and reduce callus growth because K+ ions are necessary for plant growth and development (Golkar et al., 2019; Lokhande et al., 2010).

Basically, many results have shown significantly increased sodium content of callus tissues with an increased salt level in media (Ahmad et al., 2009). A previous study observed a maximum build-up of sodium ions in Basmati 385 and Basmati Karnal at 100 mol m-3 with decreased potassium ions content. Similar observations were reported in several other plants, such as Safflower (Golkar et al., 2019), *Carthamus tinctorius* (Soheilikhah et al., 2013), and *Saccharum* sp. (Errabii et al., 2007). In a nutshell, we may link the decrease in callus growth caused by induced salt stress to the osmotic and ionic adverse affects of salt.

5.1.2 Effect of JAS on the growth of *Azadirachta Indica* callus

It revealed that the combination of TDZ and JAS had no significant influence on the fresh weight of callus produced and growth index. However, the use of JAS as a stress factor did not result in detrimental impacts on callus development, indicating that it might be employed as an elicitor to stimulate the production of secondary metabolites in callus (Rawat et al., 2020). By carefully examining the data, it was found that neem callus inoculated on low concentrations (1 mg/L) of JAS showed the highest growth index. In contrast, the fresh weight of the callus was observed to decline as concentrations of JAS increased.

A previous study reported on the effects of JAS on callus production from 5-week-old globe artichoke and milk thistle during callus induction on MS media supplemented with NAA+Kin+GA3 and elicited with different concentrations of JAS (0, 25, 50, 75, 100µM) (Bekheet et al., 2014). Results on milk thistle revealed that 50µM was the most suitable for callus growth because it produced a higher fresh weight in both explants. Al-Qatrani et al. (2021) reported that a combination of JAS and salt stress improve in yield of fresh weight of date palm callus (*Phoenix dactylifera L.).* However, adding JAS alone did not significantly affect the date palm callus's growth parameters. A concentration of 25µM JAS in date palm callus resulted in an increase in growth parameters but recorded less compared to the growth rate in media absence of stress factors. In contrast, a high concentration of 75 µM JAS inhibited callus development and reduced the relative water content of palm date but did not result in callus mortality (Al-Qatrani et al., 2021).

Callus treated with JAS could induce oxidative stress due to excessive production of reactive oxygen species (ROS) (Chong et al., 2005; Kamińska, 2021), leading to lipid peroxidation in plant membranes (Danaee et al., 2015; Jirakiattikul et al., 2021). Peroxidation of membrane lipids causes the destruction on the cell membrane and homeostasis imbalance (Kamińska, 2021); therefore, to overcome this problem, plants

might start to stimulate their defense mechanism by increasing the production of secondary metabolites. These stress conditions possibly lead to reduced plant biomass, which may be due to plants slowing down their primary metabolic processes while simultaneously boosting the secondary metabolic processes (Alsoufi et al., 2019; Chong et al., 2005; Jirakiattikul et al., 2021). These perhaps explain the current outcomes of growth rate decline in neem callus as JAS concentrations increased.

A recent study revealed the effect of 50 µm and 100 µm JAS elicitation with different exposure periods in two roselles (*Hibiscus sabdariffa Linn.*) accession: HS003 and HS005 (Jirakiattikul et al., 2021). Parallel with these findings, Jirakiattikul et al. (2021) agreed that the growth index of elicited roselle callus reduced after exposure to JAS while the maximum accumulation of phenolic and flavonoid were exhibited by 21-days HS003 and 28-days HS005 roselles treated with JAS, respectively. Meanwhile, a study by Miclea et al. (2020) had reported the negative effect of high JAS concentration (1 mg/L) on growth of *in vitro* shoot and root of *Lavandula angustifolia Mill*, where there are no effect on shoot production was observed and this could be indicated that the use of JAS could probably induced growth inhibition but did not showed signs of necrosis. Similar growth suppression due to elicitation with high JAS concentration could be observed in other plants such as *Mentha x piperita* L. (Krzyzanowska et al., 2012), groundnut (*Arachis hypogaea* L.) (Jyothsna Kumari & Sudhakar, 2003), *Morinda elliptica* (Chong et al., 2005), *Phylanthus pulcher* (Danaee et al., 2015) and *Calendula officinalis* (Alsoufi et al., 2019). It has been reported that JAS plays a role in leaf senescence by accelerating the breakdown of chlorophyll (Kamińska, 2021). Exposure to JAS causes damage to chloroplast and decreases photosynthesis activity due to accelerated Rubisco degradation and increased destruction of cell membrane structure in the lipid peroxidation process (Kamińska, 2021). These reviews agreed with the observed morphological responses in this study, where JAS were regarded as inhibitors of several plants' cytokinin-induced

plant growth by decreasing biomass (Attaran et al., 2014; Hibara et al., 2016; Kamińska, 2021; Sivanandhan et al., 2013).

5.2 Quantification of azadirachtin in abiotic stress-induced callus

Plant secondary metabolites are molecules that play a vital part in a plant's interaction with environmental stresses for adaptation and defense (Akula & Ravishankar, 2011). They may perform the functions of a signal molecule or a regulator, an optimal solute, an antioxidant, or a defensive mechanism against infections. Plant metabolites give plant cells the ability to withstand adverse conditions (Sachdev et al., 2021).

Plant tissue culture techniques have been used as an alternative way to commercially produce bioactive compounds from plants (Chandran et al., 2020). However, this technique is still insufficient to scale up bioactive compound production due to low secondary metabolites yield (Espinosa-Leal et al., 2018). Therefore, the study of abiotic and biotic stress is needed to improve the productivity of the target compound (Chandran et al., 2020).

Presence of JAS and NaCl have been reported to improve the production of azadirachtin in induced callus culture than in untreated culture. In the present study, *A. indica* callus was subjected to NaCl stress, and JAS elicitation showed accumulation in azadirachtin content. It was observed that increased NaCl and JAS resulted in an increase of azadirachtin in callus culture compared to OCM (5.143 ± 0.02186 mg/g DW).

5.2.1 Effect of NaCl on the production of azadirachtin content of *Azadirachta indica* callus culture

Adding NaCl (1.5 % w/v) in the medium resulted in maximum enhancement of azadirachtin content, where 2.1-fold higher (10.847 ± 0.0037 mg/g DW) than the OCM (5.143 ± 0.022 mg/g DW). Azadirachtin content in untreated callus cultures was

significantly lower than in callus subjected to stress. Therefore, it was possible to draw the conclusion that the optimal concentration of NaCl to induce the synthesis of secondary metabolites in the current investigation was 1.5 NaCl. Previous studies have documented a positive relationship between salt stress treatment on the production of azadirachtin.

Similar observations have been noted by Garoosi et al. (2016), where supplementation of 100 mM of NaCl in the cell suspension of neem yielded a maximum amount of azadirachtin content (0.0332 mg/g DW) with 2.59 fold higher than control after 12 days of incubation time. From this observation, it could be inferred that the production of azadirachtin will increase significantly with increased NaCl concentration levels and more prolonged incubation treatment. Parallel with the current findings, Wang et al. (2015) agreed that salinity stress affected the accumulation of secondary metabolites in Bt (*Bacillus thuringiiensis*) transgenic upland cotton varieties (SRC 28 and K638). A recent study revealed the effect of salt stress, which was analyzed in SRC28 and K836 of cotton varieties (Ma et al., 2021). The highest accumulation of gossypol and tannin in SCR28 was exhibited by 200 mM of NaCl with 34.63 and 40.63 %, respectively. While in K839, the gossypol and tannin content increased by 30.91 % and 34.87 % at a similar salt concentration as SCR28 compared to the control (Ma et al., 2021). Another study demonstrated that salt stress of cotton varieties with increasing salinity levels was proven to significantly enhance the production of secondary metabolites (gossypol, tannin, and flavonoid) after seven days of incubation compared to the control (Wang et al., 2015). Meanwhile, in a different study, the addition of NaCl at 129.3 mM showed nearly 1.5 and 1.3 fold enhancement in the accumulation of hyoscyamine and scopolamine in root cultures of *Datura metel* L, respectively (Ajungla et al., 2009). A positive effect of salt stress on the accumulation of secondary metabolites was also reported in *Catharanthus roseus* (Thakore et al., 2012), *Solanum lycopersicum* (Ahanger et al., 2019), *Sesamum indicum* L. (Khademian et al., 2019), and bell pepper plants (Ellenberger et al., 2020).

Being exposed to salt stress may trigger the production of secondary metabolites in plants (Isah, 2019), which afterwards may have a protective function on cells to counteract the oxidative damage brought on by the build-up of reactive oxygen species (ROS) (Ogbe et al., 2020). Therefore, in order for the plants to minimize the damaging effect of ROS and maintain the osmotic equilibrium in cells, they need to synthesize antioxidant compounds and various secondary metabolites to act as ROS scavengers (Garoosi et al., 2016).

5.2.2 Effect of JAS on the production of azadirachtin content of *Azadirachta indica* callus culture

According to the results, azadirachtin content obtained from 6JAS treated callus was 1.6-fold higher (8.193 ± 0.387 mg/g DW) compared to OCM (5.143 ± 0.022 mg/g DW). By carefully observing the data, it was found that the application of jasmonic acid in callus media caused an increase in the biosynthesis of azadirachtin content of neem extract. Thus, it could be concluded that 6JAS was the optimal concentration of JAS to boost the accumulation of secondary metabolites in the current study.

JAS is a plant signalling molecule that is involved in various physiological and molecular responses (Wang et al., 2020), such as plant growth, development and defensive response (Ho et al., 2020). It is also involved in signal transduction systems that trigger specific enzymes of secondary metabolic pathways to create defence chemicals, for instance, polyphenols, terpenoids, flavonoids and alkaloids (Ho et al., 2020). Application of exogenous JAS may induce the accumulation of reactive oxygen species (ROS) as a plant defence mechanism response against wounds and stress (Jaiswal et al., 2021; Kamińska, 2021; Nabi et al., 2021). When the quantity of reactive oxygen species (ROS) in a plant's cell membrane surpasses the normal level, the plant may experience toxicity and oxidative stress (Ho et al., 2020). Consequently, plants control

the defence mechanism by initiating and stimulating signal transduction (Nabi et al., 2021), which leads to the regulation of gene expression (Ali, 2021) and the enhancement of antioxidant enzyme activity (Wang et al., 2020), resulting in an increase in secondary metabolite synthesis (Jaiswal et al., 2021; Jirakiattikul et al., 2021). Antioxidant enzymes and non-enzymatic antioxidants inside the cell are required for ROS scavenging and detoxification in order to prevent further damage (Ho et al., 2020; Jirakiattikul et al., 2021). Increased production of secondary metabolite in treated callus with JAS might be needed to eliminate ROS generated under stresses condition.

A similar enhancement of azadirachtin accumulation in hairy root culture of Azadirachta indica after the addition of 100 mM JAS was observed by Satdive et al. (2007), showed a 6-fold (0.095% DW) enrichment in accumulation of azadirachtin over control cultures (0.015% DW). Balaji et al. (2003) and Rodrigues et al. (2014) revealed that methyl jasmonate promoted the biosynthesis of azadirachtin in neem culture and yielded 6.92+ 0.11 mg/L and 0.2470 µg g^{-1} of azadirachtin, respectively. This finding agrees with a previous study (Alsoufi et al., 2019), where the biosynthesis of oleanolic acid was increased 11-fold and 20-fold, respectively, in samples of CC16 and CH2 lines of *Calendula officinalis* hairy root culture treated with 100µM JAS. In reaction to JAS, plants will increase the biosynthesis of triterpenoid compounds while simultaneously decreasing the biosynthesis of primary metabolites such as sterols (Alsoufi et al., 2019). These may potentially be due to the plants prioritizing the production of defensive chemicals more than metabolites involved in primary metabolism, as a plant response mechanism under stressful situations (Alsoufi et al., 2019; Jirakiattikul et al., 2021). Therefore, the effect of JAS as a potent elicitor of triterpenoid production is relevant due to the positive relationship between JAS concentration and the accumulation of secondary metabolites.

Recent evidence showed that accumulation of secondary metabolites (polyphenolic, chlorophyll, and carotenoid) was observed in 21-days old of *Lavandula angustifolia Mill* induced with jasmonic acid and salicylic acid (Miclea et al., 2020). Explants grown on media supplemented with elicitors produced a higher amount of polyphenol and chlorophyll compared to non-treated; thus, 0.5 mg/L JAS and 1.5 mg/L of SA+ CA were chosen as suitable media for accumulation of polyphenol in *Lavandula angustifolia Mill*. Similar observations were recorded by Mu et al. (2009), where *Lycoris chinensis* treated with biotic and abiotic elicitor successfully accumulated the production of galanthamine (GAL), an essential drug for Alzheimer's disease. The production of GAL in media presence with MeJA, yeast elicitor (YE), and sodium nitroprusside as NO donor resulted in increased accumulation of GAL in 3-month-old seedlings. The highest amount of lycorine (GAL-related alkaloid) was obtained in seedlings treated with 100 µM of MeJA and p.p1 g/l YE on the 10^{th} day of cultivation (Mu et al., 2009). Moreover, the elicitation of *M. piperita* with 100µM methyl jasmonate and 200µM in cell suspension was proven to increase the amount of rosmarinic acid significantly; 117.95 mg g^{-1} DW and 110.12 mg g^{-1} D, respectively compared to the control (Krzyzanowska et al., 2012). Above all, it has been suggested that JAS is a suitable stress-induced elicitor for enhancing the production of bioactive compounds in *Azadirachta indica* callus.

5.3 TCA- acetone precipitation with PVPP extraction method of stress-induced callus of *Azadirachta indica*

Neem callus protein samples were subject to extraction using TCA-acetone precipitation with PVPP. The plant produces several secondary metabolites such as terpenoids, phenolics, tannins, flavonoids, and aromatic compounds in response to stress as adaptive strategies to protect themselves in harsh environments (Charoenkit et al., 2018). During protein extraction, secondary metabolites form a strong hydrogen bond

with protein and build irreversible complexes, which can severely affect protein extraction and analysis (Wang et al., 2008). Therefore, removing interfering compounds is essential to producing high-quality protein samples (Faoro & Stanta, 2011). According to Wang et al. (2008), adding PVPP has improved extracts' recovery and protein yield. This study used PVPP to remove phenolic and tannins compounds in the extracts (Charoenkit et al., 2018). PVPP can interact with phenolic compounds, form hydrogen bonds, and prevent enzyme deactivation (Loomis et al., 1979) due to water-insoluble properties (Caronni et al., 2021). Hence, it has been suggested as an efficient way to remove phenolics because phenolic binds to PVPP and forms insoluble complexes, which can be removed from the solution by centrifugation (Charoenkit et al., 2018; Isaacson et al., 2006).

TCA/acetone precipitation is a standard protein extraction method that relies on complete pulverization to a fine powder with the presence of liquid nitrogen and repeated rinsing of extract powder until it becomes colorless (Niu et al., 2018; Wang et al., 2006). These steps were needed to minimize protein degradation (Niu et al., 2018) and allow the inactivation of protease activity (Sagu et al., 2021) as well as to remove the interfering substances or contaminants such as phenolic, salt, and more (Charoenkit et al., 2018). The result of the study by Sagu et al. (2021) revealed that high protein concentration was recorded in almonds, cashew, hazelnut, and pistachio, while moderate protein concentration was obtained in peanut and walnut with TCA/acetone precipitation extraction method. However, prolonged exposure to TCA/acetone will make the protein difficult to redissolve (Alias et al., 2017; Niu et al., 2018) and could probably cause protein degradation (Wang et al., 2006) as well as may lead to modification of protein (Simpson & Beynon, 2010). Therefore, it could affect the outcome of MS/MS analysis.

5.4 Identification of differentially expressed proteins in stress-induced callus of *A. indica*

According to previous studies, there are several functional groups of affected proteins by salt stress and jasmonic acid elicitation, including proteins related to photosynthesis, stress-related protein, energy metabolism, protein metabolism, and others.

5.4.1 Proteins related to photosynthesis

One of the physiological processes most vulnerable to environmental changes, particularly salt stress, is photosynthesis. In salinity stress, ROS production increase will cause oxidative damage to plant cells, restricting water and mineral uptake (Sachdev et al., 2021). This eventually causes sodium ion toxicity, where excess sodium and chloride ions are present in the media , hence decreasing the potassium ion intake into the plant (Jalil & Ansari, 2020; Sachdev et al., 2021). Salt stress in plants will provide a drought-like condition to the cell due to the reduction of water absorption in plants (Jalil & Ansari, 2020). Hence, the plants will decline in stomatal conductance and disrupt photosystem and photosynthesis-related enzymes that contribute to ROS production (Hasanuzzaman et al., 2018; Sachdev et al., 2021). A decline in photosynthesis efficiency due to the accumulation of ROS in chloroplast will affect the growth and productivity of the plant. Similar observations were observed by Yu et al. (2013), where, leaf photosynthesis of mangrove plants decline with salt induced treatment coupled with exogenous hydrogen sulfide treatments.

This experiment identified photosynthesis-related proteins: Ribulose bisphosphate carboxylase (Rubisco), Photosystem I subunit III, Photosystem II 10 kDa phosphoprotein, Oxygen-evolving enhancer protein 1 (OEE1), Carbonic anhydrase, and Thylakoid luminal 19 kDa protein. Five photosynthesis-related identified proteins, including two Rubisco, an oxygen-evolving enhancer protein 1, a thylakoid luminal protein, and delta-

aminolevulic acid dehydrase were found to decrease in abundance as a response to salt stress. While, ten proteins including two Rubisco, an OEE1, a carbonic anhydrase, two photosystem I (PSI), a photosystem II, a delta-aminolevulic acid dehydrase, a protochlorophyllide reductase, and glutamine synthetase also show significant decrease in response to jasmonic acid treatment in *A. indica*.

RuBisCO is the main enzyme involved in the carbon fixation of atmospheric carbon dioxide, resulting in glucose production necessary for growth and development in most photosynthetic organisms (Cummins et al., 2018; Vitlin Gruber & Feiz, 2018). The results were compared with a previous report where Cho et al. (2007) found that two Rubisco large subunit and four RuBisCO small subunit spots decreased in the shoot of jasmonic treated rice (cv. Nipponbare), which also led to a reduction in plant growth. It may result in the inactivation of RuBisCO. In brief, salt stress enhances oxidation and lowers carboxylation activities of RuBisCO because of the sensitivity toward chloride ions, ultimately reducing carbon dioxide fixation efficiency (Komatsu et al., 2014). Kang et al. (2016) noted that 20-day-old Alfalfa (*Medicago sativa* L.*)* treated with 200 mol L-1 NaCl for 48 hours showed downregulation of Rubisco, which leads to a decrease in photosynthesis efficiency. Therefore, from the observation, we can say that decreasing of Rubisco enzyme can decrease Rubisco activity and eventually decrease the efficiency of photosynthesis.

Recent studies have found that the oxygen-evolving enhancer (OEE) protein or 33 kDa subunit of oxygen-evolving system of photosystem II plays a significant role in photosystem II (PS II) stability (Ji et al., 2019) and is found to be involved in the light reaction of photosynthesis. During the light reaction process, light-harvesting complex is responsible for capturing light and then allowing oxygen-evolving enhancer protein to split the water (Zhang et al., 2012). These proteins enhance the manganese cluster during the water oxidation process and protect the reaction centre proteins from oxidative

damage caused by oxygen radicals generated under light reactions (Heide et al., 2004). Downregulation of OEE proteins were observed in cold-tolerant winter turnip rape (*Brassica rapa* L.) compared with a cold-sensitive variant in response to cold stress. This might be one of the mechanisms to reduce the photodamage triggered by increased free radicals levels in the thylakoid membrane of winter turnip rape during cold stress (Xu et al., 2018). Interestingly in this study, OEE proteins were found to be present at lower abundance in response to salt stress and jasmonic acid elicitation, suggesting that the loss of OEE proteins induced changes in the activity of photosystem II (PS II) and might contribute to a reduction of the water oxidation process in coping to stress condition.

5.4.2 Proteins related to energy and metabolism

Energy is usually gained from photosynthesis and the plant's carbon fixation process in the form of ATP, which will be used for plant growth, development, and maintenance (Bandehagh & Taylor, 2020; Jacoby et al., 2011). However, when plants are exposed to salinity stress, energy production processes decrease due to the decline in photosynthesis rate. The plant has used most of the energy obtained during the stress exposure period to activate its defence and tolerance mechanisms as a response toward stress (Bandehagh & Taylor, 2020). Suppression of photosynthesis activity under stress conditions often leads to change in carbohydrate metabolism (Shi et al., 2013) because plants start to save energy to prevent additional damage to the cell due to the imbalance production of reactive oxygen species (Jiang et al., 2007; Møller, 2001). Thus, it is possible to see the downregulation of energy and carbohydrate metabolism, which may be an energy conservation plan and a sign that indicates plants are shifting from growth development to a defense mechanism in response to stress (Bandehagh & Taylor, 2020; Liu & Howell, 2010). Following this analysis, 11 and 46 proteins related to energy and carbohydrate metabolism were noticed to be downregulated in response to NaCl and JAS stress,

respectively. Meanwhile, it was discovered that three and five proteins were upregulated in response to NaCl and JAS stress.

It was shown that many of the differentially abundant proteins in neem callus participated in fundamental processes such as glycolysis, Calvin cycle and carbon fixation. The enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plays a crucial role in both glycolysis and gluconeogenesis pathway. During glycolysis, it mainly breaks down glucose to supply energy for the plant's metabolism. During reduction phase of the Calvin cycle, GAPDH catalyses the conversion of glyceraldehyde-3-phosphate by NADPH to 3-phosphoglycerate (GAP) (Song et al., 2021). To better deal with salt stress, RB855536 tolerant sugarcane variant showed higher levels of the enzyme GAPDH, which possibly has been shown to play a direct role in photosystem repair and hence boost photosynthetic activity in sugarcane under salt stress (Passamani et al., 2017). Another study reported that GAPDH proteins increased abundance in the (G441) salt-tolerant sesame genotype during salt treatment (Yujuan Zhang et al., 2019). In contrast, GAPDH-deficient mutants display a reduced respiration rate and ATP generation (Passamani et al., 2017; Rius et al., 2008). This might happen since H+-ATPases need ATP as a substrate; the reduction in ATP generation affects cellular energy metabolism (Passamani et al., 2017). In this study, GAPDH enzyme was upregulated in response to both stresses. This finding agrees with previous studies of *Helianthus tuberosus* L (Song et al., 2021) and *Musa paradisiaca* (Ji et al., 2019) under stress conditions. Therefore, the build-up of this protein might be a sign of the stimulation of catabolic processes aiming at energy gain from stored carbohydrates, as a response to increasing energy demands in response to stress.

5.4.3 Proteins related to defense and stress response

Fourteen and twenty-two differentially abundant proteins associated with salt stress and jasmonic acid elicitation fall in the defense and stress-related protein category. Plants responding to abiotic and biotic stressors produce several proteins, most of which are considered essential components of the plant's self-defense system (Cho et al., 2007). Under salt stress and jasmonic acid elicitation, ROS production increases, resulting in an abundance of ROS scavenging enzymes produced in plant cells, such as superoxide dismutase (SOD), peroxidase (POD), peroxiredoxin, catalase (CAT), glutathione peroxidase (GPX) and more. SOD functioning in the inactivation of superoxide radicals into oxygen and water, while CAT will converts H_2O_2 into water and oxygen (Komatsu et al., 2014). Due to their function, these two proteins' activities have been monitored to predict the production of ROS in plant cells as they respond by detoxifying ROS (Yang & Poovaiah, 2002). In a previous study, Yu et al. (2013) found that the antioxidant activity of SOD, CAT, GPX, APX, DHAR, and GR were increased with the application of exogenous hydrogen sulfide in salt stress cucumber hypocotyl and radicle. Another study on comparative proteomics of *Abelmoschus esculentus* L. seedlings grown in the presence of salt stress recorded an increase in POD, POD precursor, and CAT protein abundances which may be an antioxidant defence system of orka seedlings in response to salt stress (Zhan et al., 2019). Interestingly in this study, monodehydroascorbate reductase (MDAR), germin-like protein 5-1, peroxiredoxin, and peroxidase were found to be decreased in abundance in JAS stress. However, heat shock proteins, peroxidase 40 and 1-aminocyclopropane-1-carboxylaste oxidase were noticed to be increased in response to JAS stress. A similar result can be observed in *Arabidopsis* mutant opr3-1 with exogenous MeJA treatment, showing a POD reduction in mutant opr3-1. Yet, the production of POD in mutant opr3-1 was higher than in *Arabidopsis* wild type (Qi et al., 2020).

Besides, heat shock proteins acting as chaperones for protective functions in plant cells increased abundantly in salt stresses. Most of the heat shock proteins in this study are Hsp70 and Hsp90. Heat shock proteins (Hsps) are involved in many normal cellular activities, including protein folding, assembly, translocation, and degradation (Wang et al., 2004). They can protect plants from stress by refolding proteins to restore standard protein structure and cellular homeostasis (Wang et al., 2004; Zhu et al., 2012). Hsp70 works with co-chaperones to assist with protein folding, preventing aggregation, and assisting in refolding of non-native protein under normal and stressful conditions. They are also involved in the import and translocation of the precursor protein from the cytoplasm into the mitochondrial matrix, facilitate proteolytic degradation, and assist in folding de novo synthesized polypeptide (Wang et al., 2004). This finding agrees with the previous study, where heat shock protein 70 was abundantly increased in Alfalfa plant treated with 200 mol L-1 and PEG4000 (Kang et al., 2016). Long et al. (2020) have shown that the abundance of heat shock protein 70 kDa increases in one-month-old *Medicago sativa* cv. Zhongmu-1 root treated with 300 mM NaCl. A study conducted in K. candel seedlings showed that upon exposure to high salinity levels, the accumulation of HSP, HSC70, H SP60, non-cell autonomous heat shock cognate protein 70 and high molecular weight HSP were noticeable. Similar results can be observed in other plants such as *Bruguiera gymnorrhiza (*L.) Lam (Zhu et al., 2012), *Sesamum indicum* L. (Yujuan Zhang et al., 2019) and *Arabidopsis* (Jiang et al., 2007). Therefore, it may be suggested that this protein has an essential role in supporting the folding and assembly of protein in response to salinity stress.

5.4.4 Proteins related to protein metabolism

Under salinity stress, the level of three proteins related to protein biosyntheses, such as 60s ribosomal proteins (L19-2, L17-2, and L24) found to be elevated in the callus of

A.indica. In contrast, three proteins consisting of 40s ribosomal proteins (SA isoform and S2-3-like) and glutamine synthase were down-regulated in salt-treated *A.indica* callus compared to the control. Based on the result, we can see that plants increase proteins related to protein translation and synthesize new proteins to strive against salinity stress. Ribosomes are composed of a small subunit of the 40S and 60s ribosomal proteins, which are the large subunits necessary for protein synthesis and translation (Ghulam et al., 2019). 40s ribosomal protein plays a role in decoding the genetic message, while 60s ribosomal proteins catalyze peptide bond formation (Gregory et al., 2019). In contrast, under jasmonic elicitation, sixteen proteins related to protein biosyntheses, including 40s ribosomal proteins (S15-4, S4, S19, and S17-like), 60s ribosomal proteins (L10-3, L32-1, L7-2, L28-1, L17-2, L21-1 and L30), eukaryotic translation initiation factor protein and elongation factor 2 observed to be decreased in callus induced in jasmonic acid. These results can be seen in *Arabidopsis* mutant opr3-1, where the 40s, 60s, 30s, and 50s ribosomal proteins showed a decline in abundance (Qi et al., 2020). This suggests that elicitation of MeJa in mutant opr3-1 will inhibit protein synthesis. Therefore, it is possible to propose that JAS treatment caused an inhibition in the manufacture of proteins, whereas NaCl stress caused an elevation in protein biosynthesis.

Next, protein disulfide-isomerase was down-regulated in NaCl-treated and JAS-treated neem callus culture, which involved protein folding and assembly. Disulfide isomerase protein is part of the thioredoxin (TRX) superfamily and is mainly found in the endoplasmic reticulum (Houston et al., 2005). It catalyzes the oxidation of protein thiols, restores disulfide bonds, catalyzes the transition of three types of disulfide bonds, and is involved in folding disulfide bonds during the protein folding process (Houston et al., 2005; Kang et al., 2016). Disulfide isomerase is essential in all cell types, particularly those related to protein storage and secretion. The disulfide isomerase protein's abundance was down-regulated under salt stress in alfalfa seedlings (Kang et al., 2016). It also

decreased in three-week-old soybean leaves in response to drought stress (Irsigler et al., 2007). Also, disulfide isomerase and peptidylprolyl proteins, which are essential in protein folding, are found in lower abundance in genetically modified soybean types (Benevenuto et al., 2021).

CHAPTER 6: CONCLUSION

6.1 Conclusion

The present analysis describes the elicitation with NaCl and JAS enhanced the biosynthesis of azadiractin in the callus of *Azadirachta indica*. The outcomes showed that callus could survive with increasing NaCl and JAS concentrations. However, it causes a reduction of fresh weight and growth index as stress concentration increases. Meanwhile, the level of azadirachtin in abiotic-stressed culture was recorded to be increased. The optimum JAS and NaCl concentrations that gave the highest accumulation of azadirachtin were 6 mg/L of JAS and 1.5% of NaCl, respectively. The outcome of the current investigation suggested that exogenous application of JAS and NaCl as elicitors into neem callus culture media boosts the production of azadirachtin in samples by increasing the abiotic stress concentrations. Further investigation was carried out to evaluate the two elicitors' impact on *in vitro* cultured neem callus on proteomic profiling and expression via comparative proteomics. Quantitative free-gel based was carried out to identify the differential abundance proteins present in treated neem callus in response to JAS and NaCl using LCMS coupled with mass spectrometry. 883 proteins were generated from proteomic profiling of *Azadirachta indica*. Comparative proteomic analysis revealed that 129 proteins were differentially abundant in NaCl-treated callus, with 36 proteins upregulated and 93 proteins down-regulated. Meanwhile, 320 proteins differentially abundant in JAS-treated callus extract with 34 proteins were significantly increased, and 286 proteins were decreased in response to stress treatment. Up-regulated proteins in response to salinity stress belong to the defence and stress response, energy and carbohydrate metabolism, cell wall and cell structure, and redox pathways, whereas down-regulated proteins include protein metabolism, transcription protein, amino acid metabolism, and photosynthesis-related protein. In addition, among the differentially

abundant proteins in JAS-treated callus, defence and stress response, protein synthesis, cell wall and cell structure, redox regulation, and signal transduction-related protein were found to be elevated. In contrast, energy and carbohydrate metabolism, transcription, transport, amino acid metabolism, and photosynthesis-related protein were found to be decreased. This indicated that molecular change occurred in stress-treated callus cells. However, neither of the proteins discovered in this experiment had a direct connection with the production of azadirachtin. Rather, the majority of the identified proteins were primarily associated with primary metabolism and stress-induced defence mechanisms. Protein identification in proteomic studies of medicinal plants is hindered by the lack of sequence data for both genes and proteins (Aghaei & Komatsu, 2013); consequently, cross-species approaches have been used to identify proteins in species lacking genome information. Some sample preparation procedures may result in protein degradation and impact the outcome of the MS/MS analysis. These factors could help clarify why the total number of proteins identified by LCMS/MS in the present study was low in comparison to other comparative plant stress response proteomics studies (Ciura et al., 2017; Guo et al., 2017; Yuan et al., 2022; Zhan et al., 2019).

6.2 Recommendation for Future Research

The present research discovered that JAS and NaCl had a favourable influence on azadirachtin production in neem callus. In cultured tissues of woody plants, distinct elicitor types were observed to elicit diverse responses in terms of the generation of bioactive chemicals (Akula & Ravishankar, 2011). Therefore, it is possible to increase azadirachtin production by modifying the various type of elicitors used or by combining chemical elicitors with physical factors, which leads to better secondary metabolite production. Further investigation of the scale-up of bioactive compounds on an industrial level through the utilization of bioreactors and cell suspension cultures can be conducted to optimize neem cultures. It is important to consider a range of factors in the secondary

metabolite production process, such as the selection of high-yielding cell lines, optimization of conditions for optimal culture growth, combination strategies (with elicitors or precursors), and choice of appropriate bioreactor system (e.g., stirred-tank bioreactor, bubble column bioreactor, airlift bioreactor) as well as culture parameters (flow, shear pattern, mixing efficiency, oxygen transfer, carbon dioxide exchange) (Yue et al., 2016). Meanwhile, combining genomic, transcriptomic, and proteomics research may provide a unique viewpoint on the expression of genes, proteins, and metabolites involved in producing secondary metabolites. Additional insight into the biosynthetic pathway and mechanism by which azadirachtin is synthesised in neem callus may be gained through combined investigations.

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