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INYUVESI YAKWAZULU-NATALI

Physiological and genotypic analyses of the African leafy vegetable, *Amaranthus dubius*, in response to environmental stresses and cryopreservation

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As the candidate's supervisor, I have approved this thesis for submission.

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Preface

The experimental work described in this thesis was conducted at the University of KwaZulu-Natal, School of Life Sciences, under the supervision of Dr Shakira Shaik and Professor Muhammad Nakhooda. This research was financially supported by the National Research Foundation (grant: 106409; postgraduate scholarship: MND210704619547).

This study represents the original work of the author and has not been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, they have been acknowledged.

Abstract

Africa's population is predicted to double by 2050. However, the current population's dietary demand exceeds the agricultural output capacity of arable land and irrigable water. This issue is further exacerbated by the climate crisis, causing unpredictable rainfall and weather patterns and influencing soil quality and water supply. Identifying under-utilised crops that are resistant to water-deficit, soil salinity, and high temperatures can therefore mitigate some of these challenges to secure a sustainable food supply. Furthermore, long-term preservation of identified resilient genotypes is necessary to safeguard germplasm for future research and use. It is, therefore, also necessary to investigate post-storage plant growth and development to determine viability retention and true-to-type genetic fidelity.

Amaranthus dubius, an under-utilised African leafy vegetable, thrives in southern Africa and is a nutritious food source containing many essential vitamins, minerals, and nutrients. This annual shrub can also tolerate environmental stresses and remains resilient on marginal lands. However, phenotypic variations observed in wild-type populations, including altered growth rates and biomass partitioning, result in reduced nutrient concentrations and yield, unpredictable quality, and overall agricultural inefficiency, hindering propagation and cultivation. Furthermore, the underlying genetic regulation of these stress responses has not been investigated, representing a missing step towards improving crop resilience, resource-use efficiency, and developing propagation strategies.

This study aimed to elucidate the growth, physiological and genetic responses of *A. dubius* to water-deficit, soil salinity, high-temperature, and preservation stresses, thereby identifying superior, resilient genotypes. Phenotypic responses were quantified by individually exposing *A. dubius* seedlings to each stress and measuring various growth and physiological parameters. Genetic expression was measured by quantifying mRNA transcripts of stress-responsive genes. This data was used to identify and clonally propagate superior genotypes, through cuttings and *in vitro* propagation, to conserve desired traits and increase scalability for greater agricultural capacity.

Multiple stress-tolerant specimens of *A. dubius* were identified by measuring biomass, shoot height, leaf area, water pressure potential, electrical conductivity, and contents of total chlorophyll, proline, and protein. Proteins were characterised in water-deficit and heat stress tolerant genotypes. The expression of putative Na^+/H^+ antiporter transcripts was quantified using degenerately-primed real-time qPCR, revealing a mechanism of ionic stress tolerance whereby toxic solutes were increasingly compartmentalised in roots rather than foliar tissues under salinity stress. Quantification of water-deficit, high-temperature and cryopreservation-responsive transcripts requires further optimisation. Nevertheless, this research produced water-deficit, salinity, high-temperature and preservation-tolerant clonal genotypes of *A. dubius*, aiding the struggle for food security in southern Africa. This work culminated in the expansion of indigenous knowledge and facilitates future studies regarding gene identification, sequencing, and the possible development of transgenic crops to withstand a changing climate.

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Publications

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List of abbreviations

-	minus/negative
%	percent
~	approximately
+	plus/positive
<	less than
=	equal
>	greater than
±	plus/minus
°C	degrees Celsius
°C/min	degrees Celsius per minute
°C/s	degrees Celsius per second
A	adenosine
Al	aluminium
ALV	African leafy vegetable
ANOVA	analysis of variance
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BAP	6-benzylaminopurine
bp	base pair
BSA	bovine serum albumen
C	cytosine
C_4	four-carbon
Ca	calcium
CAT	catalase
CBB	Coomassie Brilliant Blue
CBFs	cytosine-repeat binding factors
CCCH	cysteine and histidine residues
cDNA	complementary deoxyribonucleic acid
Cl	chlorine
cm	centimetre

cm^2	square centimetre
C_t	cycle threshold
Cu	copper
CV%	coefficient of variation
d	day
DM	dry mass
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DREB	dehydration-responsive element-binding protein
E	efficiency
EC	electrical conductivity
EtOH	ethanol
FAO	Food and Agriculture Organisation
Fe	iron
FM	fresh mass
g	gram
G	guanine
g/L	gram per litre
GOI	gene of interest
h	hour
H	hydrogen
HSF	heat shock factor
HSP	heat shock protein
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
K	potassium
Kbp	kilobase pair
KZN	KwaZulu-Natal
L	left
LN_2	liquid nitrogen
m	metre

M	molar
MDH	malate dehydrogenase
Mg	magnesium
mg/L	milligram per litre
min	minute
mL	millilitre
mm	millimetre
Mn	manganese
MS	Murashige and Skoog medium
MYB	myeloblastosis
m/z	mass to charge ratio
N	nitrogen
n	numeric
Na	sodium
NaOCl	sodium hypochlorite
ND	no data
ng	nanogram
NHX	sodium/hydrogen exchanger
Nm	nanometre
O	oxygen
P	phosphorous
p	probability
PCR	polymerase chain reaction
PGR	plant growth regulator
POX	peroxidase
psi	pounds per square inch
PVS2	plant vitrification solution 2
R	right
R^2	correlation coefficient
RE	relative expression
RNA	ribonucleic acid

rpm	revolutions per minute
RQ	relative quantification
RT-qPCR	real-time quantitative polymerase chain reaction
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
s	second
SD	standard deviation
SDG	sustainable development goals
SOD	superoxide dismutase
spp.	species
T	thymine
T_m	melting temperature
t-test	Student's test
UN	United Nations
USDA	United States Department of Agriculture
V	vigour
Vitamin C	ascorbic acid
v/v	volume per volume
w/v	weight per volume
Zn	zinc
µ	micro
µS	microsiemens
Ψ	water pressure potential

1. Introduction

1.1 Background and rationale

Suboptimal crop growth negatively impacts food security, resulting in food shortages and malnutrition, particularly among vulnerable groups, including children and the impoverished (Shariff and Khor 2008; Zakari et al. 2014). Inadequate nutrition predisposes children to growth disorders and developmental retardation, having lifelong repercussions for physical and cognitive maturation (Albertsson-Wikland and Karlberg 1997; Fernández et al. 2021). Furthermore, insufficient food supply drives demand, thus increasing prices, exacerbating poverty, and amplifying economic inequality (Folwarczny 2021). Worryingly, the international population is poised to surpass 8.9 billion by 2050, intensifying the demand for food (United Nations 2019). Food security is further threatened by the global climate crisis, causing increasingly frequent, severe, and unpredictable weather patterns, resulting in water scarcity, soil salinisation, and atmospheric temperature fluctuations (Lesk et al. 2016; Li and Lei 2022). These environmental stresses negatively affect crop growth and development, reducing yields and economic output (Zhu 2016). Therefore, understanding complex stress tolerance mechanisms in plants can help in the development of strategies to reduce the impact of these stresses, leading to more resilient agriculture and a sustainable food supply for the growing global population. This concern is echoed in the United Nations Sustainable Development Goals (UN SDGs) related to food security and climate change, including Zero Hunger (SDG 2) and Climate Action (SDG 13). This project specifically addresses targets 2.1 and 2.4 - end hunger and implement sustainable food production systems, respectively, and 13.1 - strengthen resilience and adaptive capacity to climate-related hazards.

Climate change exacerbates water deficit, intensifying droughts worldwide (Lindqvist et al. 2021), and escalating population growth and urbanisation amplifies competition for freshwater resources, aggravating water scarcity (He et al. 2021). Implementing efficient water management practices is a global agricultural concern, critical in optimising water utilisation and enhancing crop productivity (Mekonnen and Sintayehu 2020; Selie 2022). Additionally, high temperatures, brought on by climate change, also pose a threat to crop production as heat stress during critical growth stages results in reduced yields and crop failure (Dou et al. 2019). Soil surface temperatures, climbing above 40 °C in sun-exposed niches, elicit extreme vapour pressure gradients, amplifying evaporation (Pearson et al. 2002). Crop breeding programs have prioritised the development of heat-tolerant cultivars capable of

withstanding high temperatures while maintaining productivity (Roy et al. 2014; Li et al. 2020; Patra et al. 2022).

Another challenge impacting global crop production is soil salinity, which is the primary constraint in arid and semi-arid regions (Sangeeta et al. 2011). Soil salinisation afflicts approximately 800 million hectares of arable land, restricting plant growth and diminishing crop yields (FAO 2008). The anticipated impacts of climate change include a heightened prevalence of soil salinity, particularly in coastal regions, driven by rising sea levels and saltwater intrusion (Dasgupta et al. 2008). Mitigating the effects of soil salinity calls for strategies using salt-tolerant crop varieties and sustainable irrigation practices (Gond et al. 2015; El-Metwally et al. 2019; Baltazar-Bernal et al. 2022).

Stress-tolerant plants have evolved an intricately interconnected array of physiological, biochemical, and molecular mechanisms, ensuring resilience and continued productivity through effective stress management. Resilient crops have adapted systems to conserve water resources, including efficient water uptake, reduced transpiration rates, and heightened water-use efficiency (Takeda and Matsuoka 2008). Under salt stress, resilient crops maintain favourable ionic stases, minimising osmotic and oxidative damage (Hariadi et al. 2010). Genes undergo dynamic modulations, transitioning between states of activation and suppression, inciting shifts in transcript levels and synthesis of proteins that orchestrate adaptive responses to the prevailing environmental conditions (Son and Park 2023). Mechanisms opposing temperature stress include upregulated heat shock-responsive genes, increased antioxidant activity, and stabilised membrane integrity (Larkindale et al. 2005). Resilient species have developed efficient machinery to acquire and utilise nutrients, featuring enhanced root architecture, nutrient transport, and storage adaptations (Aerts et al. 2016). Collectively, these traits further maintain favourable yield, rendering tolerant crops appropriate for cultivation on marginal land.

Gaining insights into the physiological and molecular mechanisms, such as genetic expression, of stress tolerance can enhance the resource utilisation efficiency of crops by identifying genes, hormones, and metabolites associated with improved water and nutrient uptake (Barnabás et al. 2007). Stress-responsive gene regulation is coordinated by hormones and proteins such as transcription factors, protein kinases, and phosphatases, assuming crucial roles in complex signal transduction pathways and necessitating characterisation (Shao et al. 2015). The abscisic acid signalling pathway coordinates adaptation to osmotic stress, including water-deficit and salinity stresses (Yoshida et al. 2014). In the

context of stress tolerance, genotypic diversity causes phenotypic plasticity, resulting in varying degrees of resilience to specific environmental conditions. Some plants change leaf morphology in response to photostress or invest more energy into root growth due to water-deficit conditions. These adaptations are reversible and are triggered by environmental cues, facilitating survival and reproduction under fluctuating conditions. In contrast, constitutive, or always on, tolerance signifies the inherent ability to withstand stress without prior exposure, often emerging as the outcome of genetic adaptations accrued over time through natural selection. This phenomenon is exemplified by halophytes, wherein specific genes and transcription factors are constantly expressed or repressed, granting salt tolerance by maintaining ionic and osmotic equilibrium, thus preserving cellular integrity in saline conditions (Fan 2019).

Exploiting the genetic architecture of stress tolerance represents a critical component of effective breeding programs, focusing on acquiring and preserving novel or more productive and resilient genotypes, particularly in the context of shifting climatic patterns. Conventional methods include field gene banks and refrigeration. Protecting plant genetic traits, such as water-deficit, salinity, and high-temperature stress resistance, can be further facilitated by cryopreservation, encompassing the long-term storage of tissues at ultra-low temperatures, arresting metabolic activity while theoretically maintaining viability and genetic integrity (Niino and Arizaga 2015). Cryopreservation may be achieved through seeds, embryos, shoot tips, and pollen, enabling germplasm storage (Benson 2008). Unfortunately, cryopreservation subjects tissues to osmotic, oxidative, and mechanical stresses, necessitating mitigation to uphold germplasm viability, vigour, and genetic stability (Uchendu et al. 2013). Furthermore, freezing-induced DNA damage and the accumulation of reactive oxygen species have been shown to lead to genetic alterations, influencing gene expression, protein synthesis, and cellular functions (Bi et al. 2021). Moreover, cryopreservation can induce epigenetic modifications, altering DNA methylation and histone conformations, impacting gene expression (Patishtan et al. 2017; Singh et al. 2022; Zhang et al. 2022). Therefore, stringent protocols and quality controls must be in place to ensure the genetic fidelity of cryopreserved material in breeding programs.

Some crops, including the highly nutritious but underutilised African leafy vegetable (ALV), *Amaranthus* dubius, have demonstrated resistance to multiple environmental stresses (Ruth et al. 2021). Research on the anatomic, physiological, and genetic responses of this accessible and easy-

growing shrub to water-deficit, soil salinity, high-temperature, and cryopreservation stresses is important for its cultivation and conservation. Prior research demonstrates that the leaf water dynamics of vegetable amaranths, including *A. dubius*, undergoes fluctuations in response to soil desiccation, underpinning adaptations to water scarcity (Liu and Stützel 2002). Furthermore, adaptations to soil salinisation have been observed, particularly in coastal regions, where many *Amaranthus* species find their niche as leafy greens (Lubbe et al. 2016; Wouyou et al. 2019). High-temperature stress is another factor necessitating scrutiny since *A. dubius* is endemic to tropical and subtropical regions, representing an attractive prospect for cultivation as global climate trend projections predict increasingly frequent and intense heatwaves (Perkins et al. 2012). Lastly, cryopreservation is a favourable technique for long-term germplasm storage, capable of sustaining the vitality and fidelity of superior genotypes, but necessitates optimised protocols. Although research dedicated to the cryopreservation of *A. dubius* remains limited, investigations of its close relatives, *A. hypochondriacus* and *A. hybridus*, have demonstrated tolerance to the osmotic and oxidative stresses inherent to freezing and thawing during cryopreservation and recovery (Slabbert and Kruger 2011). This project aims to bridge this gap through the successful cryopreservation of *A. dubius,* serving as a model for further elucidation of the mechanisms granting tolerance to water-deficit, soil salinity, and high-temperature stress.

Examining the growth, physiology, and genetic dynamics of *A. dubius* will deliver many benefits for food security, agriculture, and scientific knowledge. First and foremost, the *Amaranthus* genus, including *A. dubius*, has the potential to contribute to food security, ameliorating the adverse effects of climate change on crop productivity and increasing food supply for the rapidly increasing population (Akin-Idowu et al. 2016). Furthermore, studying the genetic responses of *A. dubius* can help uncover the diversity and population structure of this species, identifying markers for valuable traits and facilitating the selection of superior genotypes with desirable traits. This study aimed to investigate stress-specific responses by screening for genotypes independently adapted to individual stresses, maximising the identification of traits and mechanisms relevant to each stress.

1.2 Research questions

The following research questions were posed for the present study:

i. How do stresses induced by water-deficit, salinity, high-temperature, and preservation affect the growth and physiology of *A. dubius*?

ii. What are the underlying genetic responses of *A. dubius* to stresses induced by water-deficit, salinity, high-temperature, and preservation?

1.3 Research aims

This project aimed to investigate the growth and physiological responses and quantify the genetic responses of *A. dubius* to water-deficit, salinity, high-temperature, and preservation stresses.

1.4 Objectives

i. To investigate the effects of variable watering and temperature schedules on the growth parameters, physiological processes, micropropagation, and molecular mechanisms in *A. dubius* followed by screening for, and micropropagation of superior genotypes.

ii. To quantify some effects of varying levels of salinity stress on the growth parameters, physiological processes, micropropagation, and molecular mechanisms in *A. dubius* followed by screening for, and micropropagation of superior genotypes.

iii. To evaluate the feasibility of multiple storage techniques for preserving *A. dubius* germplasm and assess post-preservation viability and genetic integrity.

1.5 Thesis structure

This thesis is made up of:

Chapter 1: Introduction

Contextualises the research topic, delineating its significance, outlining research objectives, and providing a scope for this study.

Chapter 2: Literature review

Reviews indigenous knowledge and modern biotechnology in addressing underutilisation of the African leafy vegetable, *Amaranthus dubius*.

Chapter 3: Water-deficit and high-temperature stress

Addresses objective (i): screening for water-deficit and high-temperature tolerant genotypes of *A. dubius* sampled in KwaZulu-Natal, South Africa.

Chapter 4: Soil salinity stress

Addresses objective (ii): quantifying the relative expression of a salinity stress-responsive Na^+/H^+ exchanger (NHX) in root and shoot tissues of *A. dubius*.

Chapter 5: Cryopreservation

Addresses objective (iv): assessing cryopreservation protocols for stress-tolerant genotypes of *A. dubius*.

Chapter 6: Conclusion

Synthesises the key aspects of this research project, emphasising its contributions to the field and offering insights into future implications and directions for the findings of this study.

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2. Literature review

2.1 Background

Unsustainable agricultural practices continue to lead to steady increases in marginal lands around the world. Climate change has been accelerating this effect, resulting in rapid land degradation and increasingly poorer-quality soils that are unsuitable for conventional agriculture. This land is defined by its soil quality, topography, climate, and water availability (Kang et al. 2013). Marginal land often exhibits poor drainage, high salinity, steep slopes, and decreased productivity compared to arable agricultural land (Rodriguez et al. 2022). Therefore, specialised management practices, including meticulous site selection, planning, and supplementations, which are often prohibitively costly, are required to support the cultivation of conventional crops on marginal land (Stoof et al. 2014).

Although marginal land is not conducive to conventional agriculture, specific food crops have adaptive abilities to each unique condition, thereby expanding agricultural output (Stoof et al. 2014). As such, farmers can introduce crops that are not vulnerable to climate-induced stresses, especially in rural regions where land is often passed down through generations or where land prices dictate farming decisions, representing a vital source of income and employment (Rini et al. 2021; Smith et al. 2019). Therefore, the identification of hardy crops and the selection of superior genotypes that are resilient to water-deficit, soil salinity, and temperature fluctuations are necessary for the optimal utilisation of marginal land to improve socio-economic conditions and mitigate rural poverty (Cossel et al. 2019).

Indigenous and rural communities have developed a profound understanding of their local environment, improving their agricultural techniques over generations by learning from best practices (Ba et al. 2018). Rooted in scientific principles, including observing the natural world, testing hypotheses, and drawing logical conclusions, traditional farming practices are inherently sustainable because they are not profit-driven (Anchirinah et al. 2001). In South Africa, small-scale farmers utilise indigenous wisdom, predict rainfall, and practice intercropping and crop rotation to enhance yield and mitigate soil degradation and nutrient depletion (Chaudhary et al. 2022; Rankoana 2022). Eco-friendly, indigenous farming inherently aids conservation and sustainable production, significantly reducing carbon emissions and improving resilience against climate fluctuations (Zondi et al. 2022).

Furthermore, meticulous seed management practices, selecting and saving seeds from top-performing plants, further enhance crop adaptation and improvement (Abay et al. 2008).

Generations of careful cultivation have preserved the rich genetic diversity of African leafy vegetables (ALVs), increasing crop yields and stress resilience (Brush 1995; Nyadanu and Lowor 2014). Leveraging these adaptations reduces reliance on external supplements, aligning with sustainable, low-input farming practices (Akinola et al. 2020). Moreover, consuming locally grown crops minimises carbon footprints from importing exotic species, strengthening local food security (Smith et al. 2005). Unfortunately, shifts in dietary preferences, market dynamics, and agricultural policies have led to a decline in the cultivation and consumption of ALVs despite their benefits (Abioye et al. 2014). Integrating these underutilised crops into diets is crucial to promoting dietary variety and reducing malnutrition and micronutrient deficiencies (M'Kaibi et al. 2016; Li et al. 2020). Furthermore, promoting the cultivation and preservation of underutilised crops reinforces the resilience of agricultural systems against climate change because they require fewer inputs like water and fertilisers than conventionally cultivated species (Chapman et al. 2022). The genetic diversity inherent in underutilised food crops represents a valuable resource for the identification and selection of stress tolerance traits, pinpointing genetic variations that confer resistance or tolerance to various abiotic (drought, salinity, and heat) and biotic (pests and diseases) stresses (Ray et al. 2009). Different genotypes within a species may exhibit varying degrees of tolerance to specific stressors, potentially leading to the identification of individuals that possess desirable traits, such as efficient water and nutrient utilisation and antioxidant capacity (Ray et al. 2009). However, exploitation of the genetic diversity of underutilised crops necessitates responsible practices, ensuring the availability of genetic resources and the preservation of biodiversity. This approach requires concerted efforts in germplasm collection, characterisation, conservation, and breeding programs.

ALVs, including many *Amaranthus, Brassica, Colocasia, Cucurbita, Solanum,* and *Vigna* species, are entwined with the heritage of indigenous African cultures and have adapted to thrive in sub-optimal conditions. These vegetables have been integral to the dietary systems throughout sub-Saharan Africa for many generations but have lost popularity due to social, political and economic reasons (Smith and Eyzaguirre 2005; Faber et al. 2010; Dweba and Mearns 2011). However, the stems, leaves, fruits, and seeds of many ALVs contain higher micronutrient concentrations than many conventional crop species

and can be utilised to alleviate micronutrient deficiency and malnutrition (Pretorius and Schönfeldt 2012; Mabhaudhi et al. 2018). Recently, ALVs have gained recognition for their potential to promote food security and nutrition, spurred by scientific enquiry (Shayanowako et al. 2021). However, extensive research remains essential for ALVs to achieve similar productivity and market viability as commercial crops.

Table 2.1 presents the mean nutritional content per 100 g leaf fresh mass (FM) of a variety of commercial and indigenous leafy vegetables available in South Africa, wherein most ALVs boast higher protein, Ca, Fe, and vitamin C than their conventionally cultivated commercial counterparts (USDA 2019). Notably, *A. cruentus* contains higher protein, Zn, Mn, Mg, and β-carotene content than all commercial crops in ***Table 2.1***. Furthermore, the Mg and Mn contents of *A. cruentus* are more than two- and four-fold greater than the next highest contents found in *B. carinata* and *Beta vulgaris* var. *cicla*. The Ca content of *A. cruentus* is the second highest, closely following *Trigonella foenum-graecum*. Moreover, the protein content of *A. cruentus* is second only to another ALV, *C. esculenta*. In the context of culinary applicability, including salads, soups, sautés, smoothies, and savoury bakes, *A. cruentus* is a suitable substitute for *Spinacia oleracea*. The amaranth contains more protein, Ca, Fe, Zn, Cu, Mn, Mg, β-carotene, and vitamin C than *S. oleracea*. The data summarised in ***Table 2.1*** underscores the significance of ALVs in diversifying diets with nutrient-rich alternatives, reinforcing food security.

Amaranthus dubius contents of Ca, Fe, and Mn are higher than the corresponding nutrient contents of all leafy vegetables in ***Table 2.1***. Furthermore, its Zn content is greater than that of commercial leafy vegetables. The Zn, Cu, and β carotene contents of *A. dubius* are marginally lower than those of *A. cruentus*, but the protein, fibre, Ca, Fe, Mn, Mg, and vitamin C measures of *A. dubius* are higher than those of its close relative. Similar to *A. cruentus*, *A. dubius* can serve as a culinary substitute for *S. oleracea* (Alegbejo 2014). However, *A. dubius* is preferred over *A. cruentus* because of its milder flavour and superior nutritional content (Alegbejo 2014). Additionally, *A. dubius* leaves have increased antioxidants, flavonoids, and phytochemicals, supporting putative health benefits (Rastogi and Shukla 2013; Dhanya et al. 2017; Bang et al. 2021). These reasons contributed to the selection of *A. dubius* for the present study.

Table 2.1: Nutritional content of common leafy vegetables available in South Africa per 100 g leaf fresh mass (FM). ND = no data, $^{+}$ denotes commercial spp., * denotes ALV.

Scientific name	**Common name**	**Protein** (g)	**Fibre** (g)	**Ca** (mg)	**Fe** (mg)	**Zn** (mg)	**Cu** (mg)	**Mn** (mg)	**Mg** (mg)	**β carotene** (mg)	**Vitamin C** (mg)	**Reference**
*Amaranthus dubius**	Sugarcane herbs	4.6	1.8	419	8.9	0.8	0.1	16.3	674	5.3	86	Alegbejo 2014
*Amaranthus cruentus**	Green herbs	4.2	1.1	334	6	2	0.2	4.2	210	5.7	62.7	(USDA 2019)
Beta vulgaris^{+}	Beet greens	2.2	3.7	117	2.6	0.4	0.2	0.4	70	3.8	30	(USDA 2019)
Beta vulgaris var. *cicla*$^{+}$	Swiss chard	1.8	1.6	51	1.8	0.4	0.2	0.4	81	3.7	30	(USDA 2019)
Brassica oleracea var. *capitata*$^{+}$	Cabbage	1.6	1.2	163	2	0.4	0.1	0.2	42	0.04	77.4	(USDA 2019)
*Brassica carinata**	Kale	3.3	1.5	135	1.7	0.4	0.3	0.	34	2.9	120	(USDA 2019)
Brassica rapa var. *chinensis**	Chinese cabbage	1.5	1	105	0.8	0.2	0.02	0.2	19	2.7	45	(USDA 2019)
*Colocasia esculenta**	*Madumbe* leaves	5.0	3.7	107	2.3	0.4	0.2	0.7	45	2.9	52	(USDA 2019)
*Cucurbita pepo**	Pumpkin leaves	3.2	1	39	2.2	0.2	0.1	0.4	38	1.9	11	(USDA 2019)
Lactuca sativa^{+}	Lettuce	1.4	1.3	36	0.9	0.2	0.03	0.3	13	4.4	9.2	(USDA 2019)
Nasturtium officinale^{+}	Watercress	2.3	0.5	120	0.2	0.1	0.08	0.2	21	1.9	43	(USDA 2019)
Spinacia oleracea^{+}	Spinach	2.9	2.2	99	2.7	0.5	0.1	0.9	79	5.6	28.1	(USDA 2019)
Trigonella foenum-graecum^{+}	*Methi* herbs	4	1.1	395	16.5	ND	0.3	ND	67	2.3	52	Rao 2004

2.2 Amaranths

Amaranthaceae is a collection of 70 genera and over 1000 species comprising the most anciently cultivated crops, with records dating back 8000 years to Mesoamerican civilisations (Alvarez-Jubete et al. 2009; Das 2011). Amaranths can be found throughout the Americas, Asia, and Africa, thriving in tropical and temperate latitudes (Mwase et al. 2014; Thapa and Blair 2018), where optimal seed germination occurs between 18 and 25 °C (Rensburg et al. 2007; Jaarsveld et al. 2014). The *Amaranthus* genus, within the *Amaranthaceae* family, is known for its genetic diversity and phenotypic adaptability, enabling resistance to environmental stressors and emerging as an excellent candidate for cultivation on marginal land (Rastogi and Shukla 2013; Jimoh et al. 2019). Some members of this genus are cultivated all over the world for use as leafy vegetables, cereal grains, or ornamental plants, while others are categorised as invasive weeds.

2.2.1 Morphology

All known members of the *Amaranthus* genus are herbaceous plants with upright growth (Rensburg et al. 2007; Thapa and Blair 2018). These short-lived annuals exhibit vigorous growth and readily adapt to environmental flux (Wang et al. 1999; Omamt et al. 2005; González-Rodríguez et al. 2019). *Amaranthus* spp. leaves are simple, lanceolate-ovate, and display an alternating arrangement on the stem. Weedy amaranths, such as *A. dubius, A. hybridus* and *A. tuberculatus*, which are products of interspecific hybridisation, produce spiky or paniculate flowers and glomerules located at the base of the inflorescence (Trucco et al. 2005; Achigan-Dako and Sogbohossou 2014). There are five sepals present in the female flowers, producing minuscule seeds (< 1 mm), which are black and circular (Achigan-Dako and Sogbohossou 2014). *Amaranthus* spp. produce densely packed catkin-like inflorescences, with a single seed head producing up to 500,000 seeds (Tucker 1986; Brickell and Kindersley 2016). This high seed production rate, in conjunction with higher macro-micronutrient levels, shorter growing periods, and multiple stress tolerance abilities, encompasses the various advantages that *Amaranthus* spp. hold over many conventionally grown crops (Odhav et al. 2007; Rastogi and Shukla 2013; Achigan-Dako and Sogbohossou 2014; Mabhaudhi et al. 2018).

2.2.2 Plasticity

Members of the *Amaranthus* genus exhibit high degrees of interspecific hybridisation and genetic variability (Steinau et al. 2003; Lymanskaya 2012; Okoye and Ani 2013; Gerrano et al. 2015; Akin-Idowu et al. 2016). This variability may affect the vertical length, branch length, inflorescence length, leaf width, petiole length, leaf number, lateral length, and the number of branches per plant (Mwase et al. 2014; Malaghan et al. 2018). Most amaranths are diploid with 32 chromosomes, with the notable exception of *A. dubius,* an allotetraploid with 64 chromosomes (Grant 1959; Grubben 2004; Stetter and Schmid 2017; Viljoen et al. 2018). These characteristics increase genotypic variation and phenotypic plasticity, bolstering resistance to environmental stresses (Pal 1972; Andini et al. 2013; Mabhaudhi et al. 2018; Lin et al. 2022).

Furthermore, numerous studies have examined the impacts of abiotic stresses on the micronutrient content of ALVs, including members of the genus *Amaranthus* (Liu and Stützel 2004; Odhav et al. 2007; Molina et al. 2011; Hoang et al. 2019). The consensus is that the micronutrient content is significantly higher in genotypes that exhibit stress tolerance than non-tolerant cultivars, which is amplified by inherent genotypic variation. This genotypic variation affects morphological and physiological characteristics such as plant colour, height, nutrient content, and stress response (Shukla et al. 2010; Achigan-Dako and Sogbohossou 2014; Gerrano et al. 2015; Areington et al. 2022; Shaik et al. 2022). The growth, physiology, and genetics of an underutilised member of this genus, *A. dubius,* are critical factors contributing to the ability of this herbaceous annual shrub to thrive on marginal land. However, many of these traits have not been comprehensively characterised in this species*,* particularly in elucidating stress-responsive genetic profiles.

2.2.3 The species of interest, *Amaranthus dubius*

Thriving in tropical and subtropical latitudes, *A. dubius* exhibits remarkable productivity and adaptability to diverse edaphoclimatic conditions, rendering it invaluable for cultivation on marginal land (Lubbe et al. 2016; Espitia-Rangel 2018), ***Figure 2.1*** encapsulates this phenomenon. Typically having a green-red stem with simple, green, and elliptic-ovate leaves that may have purple markings along the midrib, *A. dubius* is the only known allotetraploid (2n = 64) member of the *Amaranthus* genus, emerging via interspecific hybridisation between *A. spinosus* and either *A. hybridus* or *A.*

quitensis (Grant 1959; Sauer 1967; Behera 1982; Grubben 2004; Stetter and Schmid 2017; Viljoen et al. 2018; Waselkov et al. 2018). The ongoing uncertainty regarding the exact parentage of *A. dubius* arises out of the ease with which this species hybridises with other *Amaranthaceae,* obscuring efforts to identify a single set of parent species on a global scale (Andini et al. 2013; Stetter and Schmid 2017; Waselkov et al. 2018). This increase in ploidy enables greater genetic variability, contributing to myriad morphological and physiological differences that allow *A. dubius* to adapt and colonise new environments (Achigan-Dako and Sogbohossou 2014). In rural sub-Saharan Africa, *A. dubius* is commonly consumed, incorporated into carbohydrate-based dishes (Musinguzi et al. 2011; Nyonje et al. 2022).

Figure 2.1: *Amaranthus dubius*, colloquially known as the sugarcane herb, on an illegal construction and demolition debris dumpsite. This picture was taken on 18th April 2021 in Verulam, KwaZulu-Natal (29° 39' 10.4" S; 31° 03' 30.2" E).

Abundant in essential dietary constituents, including vitamins, minerals, protein, and fibre, *A. dubius* presents a viable source of nutrition, particularly in regions where access to nutrients and animal protein is limited (Andini et al. 2013; Bang et al. 2021; Ruth et al. 2021). Furthermore, elevated antioxidant and phytochemical compositions have led to this species being identified as a multipurpose plant with potential nutraceutical, medicinal, and industrial applications, in addition to its use as a traditional edible vegetable (Bang et al. 2021; Ruth et al. 2021). The utilisation of this crop carries further socio-economic implications in southern Africa, offering income-generating opportunities for small-scale farmers in rural areas, primarily because this species exhibits excellent resource-use efficiency and does not require fertilisers and pesticides (Modi et al. 2006). Furthermore, the commercialisation of underutilised ALVs, such as *A. dubius,* extends economic benefits throughout the supply chain, generating employment opportunities and fostering broader economic development.

Beyond the herb's nutritional and socio-economic promise, *A. dubius* serves other purposes, finding use as fodder for livestock (Molina et al. 2015) and ameliorating phytoremediation by accumulating heavy metals, ions, and organic solutes in its roots and shoots (Shankar et al. 2011; Mellem et al. 2012; Lubbe et al. 2016; Islam et al. 2019; Wouyou et al. 2019). Moreover, cultivating *A. dubius* on marginal land rehabilitates degraded soils, mitigates soil erosion (Atayese et al. 2010), and enables biomass production (Tripathi et al. 2016), contributing to the development of bioenergy resources and reducing dependence on fossil fuels. Additionally, aqueous leaf extracts have been used to synthesise environmentally responsible metallic nanoparticles for a myriad of biomedical and metallurgic engineering applications (Firdhouse and Pottail 2014; Muthukumar et al. 2015; Sigamoney et al. 2016; Almarhoon et al. 2022). Furthermore, methanolic extracts of *A. dubius* leaves have demonstrated antiproliferative activity against breast cancer cell lines, proffering the development of natural and novel anticancer therapies (House et al. 2020).

2.2.4 Utilisation in southern Africa

Many *Amaranthus* spp*.* have realised their potential in southern Africa, facilitating propagation and cultivation, particularly in rural communities (Slabbert and Kruger 2014). However, *A. dubius* remains underutilised in efforts to combat malnutrition and aid food security despite its many benefits (Wang and Ebert 2012; Ebert 2014). This is due to socio-economic issues, such as the ill-perceived notion that

the plant is a “poor person’s food” (Dweba and Mearns 2011). Additionally, with the popularisation of Westernised diets, many indigenous people in southern Africa disregard traditional food sources (Dweba and Mearns 2011). Increased awareness is required to promote *A. dubius* as a viable regional food source. A possible way to destigmatise the cultivation and consumption of this plant is to establish it as a model organism for ALVs in southern Africa. With the development of a viable method for *in vitro* propagation of *A. dubius* (Shaik et al. 2022), the next important step toward reaching this goal would be identifying and propagating genetically superior clones resistant to multiple environmental stresses. This process can be initiated by assessing the genetic regulation and expression of various desirable characteristics exhibited by *A. dubius.*

2.2.5 Resilience

Members of the *Amaranthus* genus exhibit significant resistance to abiotic stresses, namely drought, heat and salinity (Russell et al. 1998; Wang et al. 1999; Liu and Stützel 2004; Omamt et al. 2005; Delano-Frier et al. 2011; Nuugulu 2013; Korres et al. 2017; Kujur and Kurrey 2017; González-Rodríguez et al. 2019; Hoang et al. 2019). In conventionally bred crops, these stresses are the predominant factors leading to global crop losses, resulting in >50% reduction in yield (Bray et al. 2000; Kumar 2020). In contrast, the projected reduction in output attributed to pathogens usually falls within the range of 10–20% (Kreps et al. 2002; Mahajan and Tuteja 2005; Tuteja et al. 2011). Many members of the *Amaranthus* genus possess morphological and physiological traits that enable biomass maintenance in challenging conditions, including indurated xylem vessel morphology, Kranz anatomy, C_4 metabolism, solute accumulation, stomatal conductance, and stress-responsive gene expression, sustaining rapid growth even under water-deficit, high-temperature, and saline conditions (Modi 2007; Wouyou et al. 2019; Govender and Baijnath 2022).

The growth and development of *A. dubius* are influenced by its genetic diversity, which governs traits like nutrient uptake, stress tolerance, and biomass production. Studies leveraging nuclear and chloroplast DNA markers have identified genes and phylogenetic relationships within the *Amaranthus* genus, shedding light on its adaptation strategies and evolutionary history. Techniques like amplified fragment length polymorphism (AFLP) and genotyping-by-sequencing (GBS) have been used to explore genetic variability, aiding in identifying markers for traits essential to growth, such as photosynthetic efficiency and resilience to environmental stresses (Viljoen et al. 2018; Waselkov et al. 2018). However,

there is limited evidence documenting the response of these mechanisms, particularly gene expression, in *A. dubius* genotypes undergoing multiple environmental stresses.

Individual cells of resistant plants sense and respond to adverse environmental conditions mediated by stress signals transduced to the nuclei. This cascade leads to the expression of many genes that function to increase stress tolerance. For example, heat shock proteins (HSPs) are expressed in response to high temperatures and are conserved across numerous organisms (Kimpel and Key 1985; Vierling 1991; Bharti and Nover 2002). However, the type and amount of HSP expression varies significantly among plants (Vahala et al. 1990; Roberts and Key 1991; Kim et al. 2001; Wang et al. 2020). Another instance of a genetically mediated stress response is demonstrated by *Amaranthus* spp., which produces choline monooxygenase in water-deficit and highly saline conditions (Russell et al. 1998).

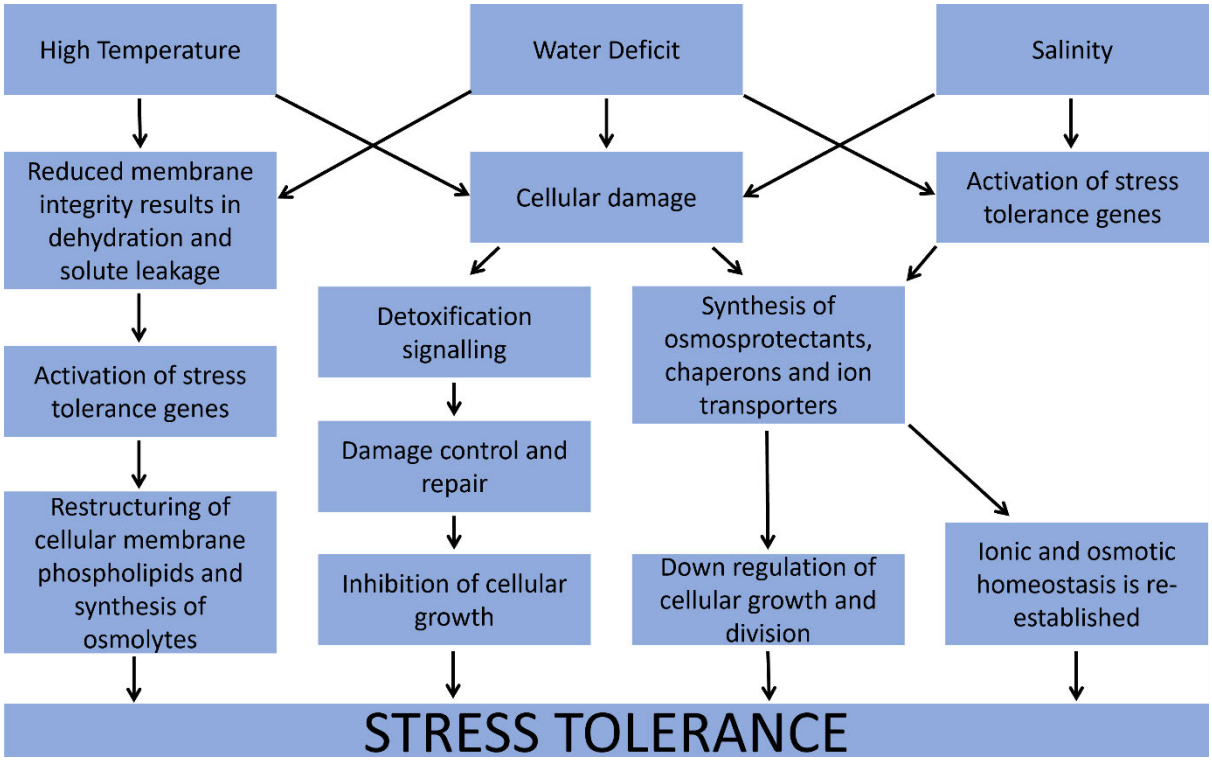
2.3 Abiotic stresses

There are over 300 highly conserved stress-responsive genes which have been identified and characterised in organisms from all Kingdoms of life, including animals (*Homo sapiens*), fungi (*Saccharomyces cerevisiae*), eubacteria (*Escherichia coli*), archaebacteria (*Halobacter* spp.), and higher plants (*A. thaliana*) (Kültz 2003). Many plants can tolerate abiotic stresses, including extreme temperatures, water shortages, and ion toxicity induced by highly saline environments (Tuteja et al. 2011). Due to the sessile nature of plants, they are unable to avoid such harsh environmental conditions. However, some have developed complex mechanisms to respond and adapt to these antagonistic growing conditions. The stress response cascade, simplified in ***Scheme 2.1***, is complex, involving numerous interconnected signal transduction pathways that regulate many cellular metabolic processes and networks (Nakashima et al. 2009). This inherent complexity enables a flexible and dynamic response to multiple stresses. Whole genome or proteome profiling has been used to assess genetic expression in the contemporary model plant *A. thaliana* (Cao et al. 2011) and conventional crop species such as *Oryza sativa* (Lachagari et al. 2019) and *Zea mays* (Tian et al. 2021). Unfortunately, this approach is not viable for all species of interest due to the high start-up costs and data processing required to extract meaningful conclusions. Microarrays significantly reduce operating costs and data processing load, although this approach is species-specific and not yet

available for most understudied and underutilised crops such as *A. dubius* due to the lack of genomic data needed to design probes for gene expression analysis.

Scheme 2.1 depicts a generalised abiotic stress response signal cascade utilised by commonly studied plants, including *A. thaliana, Z. mays,* and *O. sativa*. A response to stress begins with signal detection via receptors such as phytochromes, histidine kinases, receptor-like kinases, G-protein coupled receptors (GPCRs), and other hormone receptors. G-proteins accelerate the binding of signal molecules to trigger GPCRs, initiating subsequent cascades by transducing the signal through the cellular membrane (Russell et al. 1998; Tuteja et al. 2011). Once the signal enters the cell, a series of phosphorylation events involving protein kinases and phosphatases are engaged, which result in the production of secondary signalling molecules such as inositol phosphatase, abscisic acid (ABA), and reactive oxygen species (ROS) (Foyer et al. 1997; Mahajan and Tuteja 2005; Tuteja et al. 2011; Rasmussen et al. 2013; Pandey et al. 2017). Since the discovery of ABA accumulation in response to drought stress (Wright 1969), advancements in molecular and genomic biotechnology have been applied to further comprehend the function of ABA and other molecular signals in stress tolerance and resistance.

High-temperature, water-deficit and salinity stress trigger an osmotic stress response meted by elevated ABA concentrations (Swamy and Smith 1999). ABA-responsive transcription factors (TFs) then regulate the expression of proteins by binding to *cis*-acting DNA sequences, which promote the downstream expression of other TFs or functional stress-responsive genes (Choi et al. 2000). Secondary signalling molecules regulate intracellular Ca^{2+} concentrations, which initiate a multitude of protein phosphorylation cascades synchronised by Ca^{2+} dependent-protein kinases (CDPKs) (Martí et al. 2013). Mitogen-activated protein kinases (MAPKs) have been characterised in response to water-deficit, high-temperature, and high salinity stress (Cardinale et al. 2002; Peng et al. 2006; Sun et al. 2017). These cascades ultimately induce transcriptional control mechanisms, which activate functional stress-responsive genes (Foyer et al. 1997; Mahajan and Tuteja 2005; Pandey et al. 2017).



Scheme 2.1: Generic cascade of salinity, water-deficit, and high-temperature stress response in plants adapted from Daie and Campbell 1981; Ünlükara et al. 2015; Cheng et al. 2021; Bilal et al. 2023.

2.3.1 Water-deficit

Plant adaptations that enable resistance to water deficiency stress can further direct breeding programs, aiming to foster the creation of crop varieties endowed with heightened tolerance to such stress. Moreover, insights into plant water use efficiency promote the advancement and optimisation of irrigation and soil monitoring systems (Chaves 2003). Water absorption and movement are crucial for plant growth, supporting osmotic balance and nutrient uptake (Hodge 2004). Changes in root architecture under water-deficit stress, including elongated roots and altered branching patterns, influence water absorption (Lynch 2013). Exploration of dicot species, including the broad bean (*Vicia faba*) and sunflowers (*Helianthus annuus*), has underscored the role of root adaptations in enhancing resilience to water-deficit and drought (Hsiao 1973; Passioura 1983; Hartmann et al. 2002).

Root hairs are among the endogenous structures of the plant that convey a degree of tolerance to water-deficit. These elongated projections emerging from root epidermal cells effectively amplify the cell's surface area, facilitating the absorption of water and nutrients, resulting in swifter water transport through specialised cell wall channels (Zhu 2015). Specific aquaporin channel proteins

facilitate the flux of water and nutrients into the root system by enhancing the permeability of cell membranes (Chrispeels et al. 1999). In response to water-deficit stress, the expression and function of aquaporins are upregulated, permitting the adjustment of water transport to preserve cellular water equilibrium and the overall turgidity of the plant (Maurel et al. 2008). In the root's epidermis and xylem parenchyma cells, aquaporins enable the unhindered movement of water (Chaumont and Tyerman 2014). In contrast, aquaporins within the root cortex and endodermis regulate water absorption and distribution throughout the root system (Maurel et al. 2008).

In the leaves, aquaporins influence the management of stomatal closure by facilitating the rapid influx of water into guard cells, effectively governing water loss through the process of transpiration (Liu 2015; Hachez et al. 2017). In response to signal transduction pathways, levels of ABA surge in reaction to water-deficit stress and bind to ABA-responsive elements within the promoter regions of aquaporin genes in stomatal guard cells, triggering gene expression (Maurel et al. 2008). Moreover, sucrose non-fermenting 1-related protein kinase 2 (SnRK2) phosphorylates aquaporins in response to water stress, instigating changes in aquaporin activity and cellular traffic (Hachez et al. 2017). TFs with dehydration-responsive element binding/C-repeat binding factor interact with dehydration-responsive elements within gene promoters. This interaction triggers the activation of genes integral to cultivating tolerance to water-deficit stress (Yamaguchi-Shinozaki and Shinozaki 2006). Additionally, the presence of water-deficit stress gives rise to the production of ROS, functioning as messengers of stress that govern gene expression, cellular redox equilibrium, and the antioxidant defence mechanisms upheld by superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) (Mittler 2004). Hormonal signalling pathways, including ABA, ethylene, jasmonic acid, salicylic acid, and gibberellins, further contribute to the orchestration of adaptive responses and the regulation of plant physiology in response to water-deficit conditions (Ranty 2016).

2.3.2 Salinity

The repulsion of ions, such as Ca^{2+}, Na^{+}, and Cl^{-}, to lipids presents a barrier to their passive movement through a cell's lipid layers, potentially giving rise to substantial gradients in ion concentrations on either side of these membranes. Salinity stress disrupts the equilibrium of ionic and osmotic states in plant cells, resulting in a stressed state (Mahajan and Tuteja 2005; Tuteja et al. 2011). Consequently, the overabundance of ions and imbalances in osmotic pressure, notably turgor pressure, are the main

instigators of signalling pathways involving ions and osmosis. The intricacy of salt tolerance in dicot plants involves numerous mechanisms, encompassing selective ion exclusion, osmotic adaptation, and protective antioxidative measures (Munns and Tester 2008). Selective ion exclusion enables plants to selectively assimilate essential ions like K^+ while keeping out harmful ions like Na^+, governed by transporter activities controlling ion flow across the plasma membrane and tonoplasts (Zhu 2002). Osmotic adaptation entails the accumulation of beneficial solutes, such as proline, glycine betaine, and trehalose, which facilitate the maintenance of cell pressure and counteraction against desiccation (Flowers and Colmer 2008). Antioxidant defence mechanisms centre on the removal of ROS produced by salinity stress, facilitated by enzymes like SOD, CAT, and POX (Munns and Tester 2008).

The resilience of plants to salinity stress is multifaceted and influenced by genetic and physiological factors. Strategies that facilitate salinity resilience incorporate ion transporters like salt overly sensitive proteins and Na^+/H^+ exchangers (NHX) and enzymes for scavenging ROS like SOD and CAT (Xu et al. 2018). Furthermore, physiological factors like root structure, water utilisation efficiency, and carbon distribution contribute to salinity tolerance in dicots. Plants with deeper roots can access water and nutrients from lower soil layers, potentially mitigating salinity stress. Similarly, plants exhibiting higher water use efficiency can sustain turgor pressure amidst salinity stress, and those with increased carbon allocation to roots can amplify salt exclusion via ion transporters.

The objective is to maintain equilibrium, culminating in stress endurance. Salinity stress is evident in observable harm such as chlorosis and necrosis and can also hinder cell division, leading to growth retardation (Mahajan and Tuteja 2005; Tuteja et al. 2011). Diminished shoot growth, predominantly leaves, confers advantages to plants by curtailing exposed surface area, thus restricting transpiration and conserving water. Plants may discard older leaves as an adaptive response to stress. The ramifications of stress-induced damage include denaturation of cellular enzymes, ROS generation, Na^+ toxicity, and perturbation of membrane stability. The response to stress-triggered injuries causes a detoxification process, encompassing modulation of chaperone expression, proteinases, ROS-scavenging enzymes, and other detoxifying proteins to mend stress-induced harm (Russell et al. 1998; Tuteja et al. 2011; Rasmussen et al. 2013; Hoang et al. 2019).

Numerous plant species have been extensively investigated to examine the characteristics of passive and active ion transport proteins, represented in ***Figure 2.2***. These proteins coordinate the regulation

of ionic flow across cellular membranes, ensuring the equilibrium of solute concentrations within aqueous cellular compartments and adjacent tissues. Active transport processes instigate the establishment of electrochemical gradients across these membranes, which is imperative for diverse metabolic functions. Furthermore, turgor pressure is regulated through the active accumulation of ions within cellular vacuoles, offering structural integrity and support to the entire plant structure. Ultimately, ionic transport is a foundational mechanism that upholds cytoplasmic equilibrium and generates vitality within chloroplasts and mitochondria.

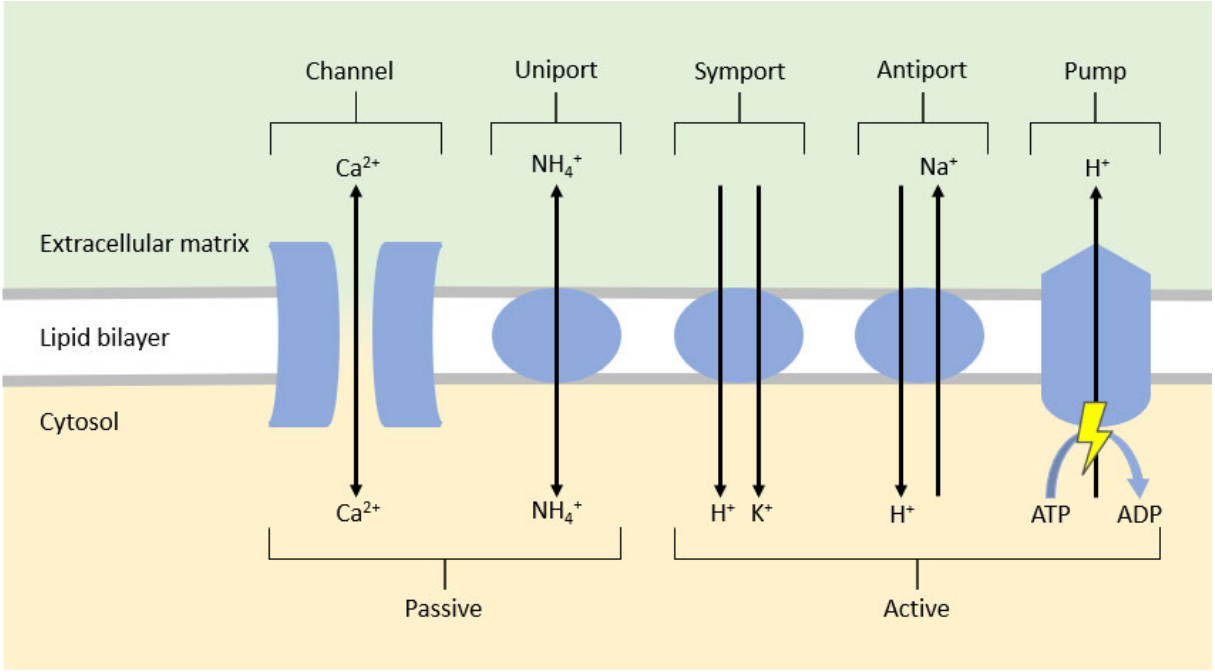


Figure 2.2: Types of intramembranous ionic transport proteins. From L to R: passive (divalent cation channel, $NH4^+$ uniport) and active (K^+/H^+ symport, Na^+/H^+ antiport and H^+ ATPase) adapted from Foster and Miklavcic (2017).

The mediation of active ionic transport involves many membrane-anchored proteins that either enzymatically hydrolyse energy-rich substrates (denoted as "pump" in ***Figure 2.2***), such as ATP, to propel specific ions in opposition to the inherent electrochemical gradient or operate through the initiation of a primary ion (typically H^+) which expedites the energetic transfer of secondary ions (referred to as "symport" and "antiport" in ***Figure 2.2***). However, these mechanisms are intertwined, as the provision of ATP or pyrophosphatase is necessary to energise protein pumps responsible for moving the primary ions in opposition to their electrochemical gradients.

2.3.3 Heat

Environmental challenges like temperature stress disturb the integrity of cellular membranes, resulting in desiccation and imbalances in osmotic pressure (Djanaguiraman et al. 2018). The process of heat acclimation, caused by an array of genes, induces a transformation in cellular membranes by modifying their lipid composition and producing protective osmolytes (Gao et al. 2012; Niu and Xiang 2018). These adaptive reactions slow cellular desiccation and boost resilience to high-temperature stress (Tuteja et al. 2011; Rasmussen et al. 2013). Plants undergo additional physiological alterations when confronted with distressing ambient temperatures. These transformations encompass shifts in the transcriptome, proteome, and metabolome, culminating in physiological, biochemical, and molecular adjustments (Rykaczewska 2015; Nievola et al. 2017; Liu et al. 2019; Urban et al. 2021; Ahmad et al. 2021; Pradhan et al. 2022). Physiological indications like leaf curvature and changes in canopy temperature are often observed at the onset of high-temperature stress (Ahmad et al. 2021; Pradhan et al. 2022). Detection of temperature fluctuations in plants involves multiple and interconnected pathways and processes. A spectrum of functional proteins, regulatory proteins, and non-coding RNAs intricately impact plant responses to elevated temperatures (Huang et al. 2022). For instance, C-repeat binding factors (CBFs) initiate reactions to temperature by activating distinct genes (Li et al. 2022). Additionally, epigenetic processes, including changes in histone structure and the presence of unique histone variants, enable plant adaptations to increased ambient temperatures (Hou et al. 2022).

Heat Shock Proteins (HSPs), known for aiding proper protein folding and preventing untimely protein denaturation, become particularly influential in stressful conditions (Sung and MacRae 2011; Sadura et al. 2020). This family of proteins contribute to various physiological processes, encompassing growth, ageing, environmental acclimatisation, and the immune response (Sung and MacRae 2011). The expression of HSPs is pivotal for enhancing the heat tolerance of crops (Kumar et al. 2020), with their upregulation supporting resistance to heat stress. Furthermore, HSPs serve as molecular chaperones for other proteins, ensuring proper folding and hindering protein aggregation (Sadura et al. 2020). An examination of the foxtail millet (*Setaria italica*) genome pinpointed potential genes for enhancing crop endurance to environmental pressures (Singh et al. 2016). Transcriptome analysis in the turfgrass, *Agrostis stolonifera*, uncovered genes responsive to heat and drought stresses (Xu and

Huang 2018). Genetic engineering methods focusing on HSPs promise to boost plant productivity during high-temperature stress (Askari-Khorasgani and Pessarakli 2019). Moreover, the synthesis and accumulation of HSPs offer insight into the proteome of heat-induced stress resilience (Ergin et al. 2016).

Transcription factors (TFs) attach themselves to the promoter region of target genes, influencing expression and shaping reactions to stress (Yanagisawa 1998; Shaik and Ramakrishna 2013; Guo et al. 2016; Baillo et al. 2019; Bi et al. 2022). NAC, an acronym denoting NAM, ATAF1/2, and CUC2, TFs exert influence across diverse biological processes by governing gene expression through hetero- or homodimeric formations (Lee and Park 2012; Ailizati et al. 2022). WRKY TFs are named and distinguished by the highly conserved tryptophan (W), arginine (R), lysine (K), and tyrosine (Y) amino acid sequence at their N-terminus and a zinc-finger motif (Kwon and Hwang 2014), which act by binding to *cis*-acting elements in target gene promoter regions (Song et al. 2018). Myeloblastosis (MYB) TFs are an expansive cluster of TFs with one or more MYB DNA-binding domains characterised by approximately 50 amino acid imperfect repeats (Wang et al. 2022). In the model plant, *A. thaliana,* heat shock factor (HSF) genes are downregulated by HSFB1 and HSFB2b TFs, heightening resistance to high temperatures (Ikeda et al. 2011). Likewise, strategies in genetic engineering that centre around genes integral to heat stress signalling pathways have been examined for enhancing the tolerance of crops to heat stress and amplifying agricultural output (Wang et al. 2022).

Amplified cytosolic Ca^{2+} levels serve as one of the initial reactions of plant cells when faced with temperature stress, attributed to the increased activity of Ca^{2+}-binding channel proteins (Hou et al. 2014). Stress-triggered Ca^{2+} channels transmit stress cues to Ca^{2+}-dependent downstream sequences (Hou et al. 2014). Furthermore, jasmonic acid, which increases in both biotic and abiotic stress reactions, including heat stress, is complicit in stress signalling (Yang et al. 2019). Moreover, crosstalk involving salicylic acid, melatonin, ethylene, and other hormones contributes to high-temperature stress resilience (Albacete 2020; Poór et al. 2021). Manipulating auxin and cytokinin signalling pathways also contributes to tolerance against heat stress (Bielach et al. 2017). The impact of high-temperature stress extends to an array of post-transcriptional and post-translational regulatory mechanisms. Alternative splicing, a post-transcriptional regulator, generates multiple mRNA isoforms from a single gene in response to temperature cues (Xue et al. 2023). Additionally, CCCH zinc-finger

proteins operate in post-transcriptional regulation, fostering plant adaptation to high-temperature stress.

2.3.4 Preservation

Conventional plant germplasm storage protocols encompass a variety of methods aimed at preserving the genetic diversity of plant species for future use in breeding, research, and conservation efforts. These protocols typically involve the collection, preparation, and long-term storage of seeds, tissues, or whole plants under controlled conditions to maintain their viability and genetic integrity. Seeds are one of the most commonly used germplasm storage materials due to their relative ease of collection, handling, and storage (Liu et al. 2005). Most seeds can be stored under low-temperature and low-moisture conditions to minimise metabolic activity and prolong viability. Common seed storage methods include drying seeds to low moisture content levels (e.g., 5-10%), storing them in airtight containers at temperatures below freezing (e.g., -18°C or lower), and cryopreservation in liquid nitrogen (LN_2) (Yan 2016).

Cryopreservation can also cause stresses, with dehydration and ice crystallisation as predominant antagonists, both detrimental to plant tissues (Volk and Waters 2006). The initial stages of cryopreservation subject plant tissues to desiccation, thereby eliciting dehydration stress. Subsequently, the formation of ice crystal structures inflicts mechanical harm through expansive crystalline growth, causing disruption to cellular frameworks, organelles, and membranes, resulting in irreparable damage (González-Arnao and Engelmann 2006). Compromised membranes lead to cell death, attributed to lipid peroxidation, fluctuations in membrane fluidity, and cytoplasmic component leakage. Rapid ice crystallisation and the size of ice crystals affect the survivability of plant tissues during cryopreservation. Therefore, successfully mitigating these stressors is necessary for the effective preservation of plants, maintaining genetic fidelity, and enhancing regrowth capabilities (Panis and Lombardi 2015; Reed and Hummer 2018).

Oxidative stress emerges as an outcome of cryopreservation, demonstrated by the generation of ROS and the shielding mechanisms of antioxidants, mitigating cellular impairment and facilitating post-cryopreservation regrowth (Popova et al. 2023). Notably, the cryopreservation process disrupts cellular metabolic pathways, culminating in diminished energy reserves and negatively impacting

biochemical pathways (Popova et al. 2023). The result of this cascade is reduced viability and vigour (Poobathy et al. 2013; Popova et al. 2023). To offset these implications, cryopreservation protocols often integrate preliminary treatments, such as osmoprotection, cold acclimatisation, and hormonal manipulation (Sershen et al. 2019). A diverse range of cryoprotectants, including glycerol, dimethyl sulfoxide, and polyethylene glycol, have been harnessed in the cryopreservation of plant tissues, with the aim of mitigating ice crystal formation while also alleviating dehydration stress by replacing water within cells (Nadarajan and Pritchard 2014; Oliveira et al. 2022). Long-term preservation of germplasm necessitates techniques that prevent somaclonal variations originating from the ongoing propagation within controlled environments (Duncan 1996). Cryogenic conservation is particularly useful due to its resilience against genetic infidelity (Towill et al. 2004). Although other amaranths, such as *A. hybridus*, have effectively undergone conservation through cryopreservation (Adu-Gyamfi et al. 2014), it has been understudied in *A. dubius*. An *A. dubius* seed storage study conducted by Reddy (2022) assessed multiple cryoprotectants, cooling rates, and germination conditions, finding that LN_2 immersion resulted in the highest germination % and subsequent vigour. However, this study did not investigate conventional seed storage methods or long-term (> 24 h) storage independent of cryoprotectant treatments (Reddy 2022). The undocumented genetic response of *A. dubius* to the cryopreservation process adds an additional layer of complexity.

Within the spectrum of cryopreservation techniques, gradual cooling methods encompass controlled-rate and vitrification approaches. The former involves a stepwise reduction in tissue temperature, facilitating the gradual evacuation of water from cells (Sakai and Yoshida 1967). In contrast, vitrification strategies leverage elevated concentrations of cryoprotectants to catalyse the glass-like solidification of water molecules, bypassing the formation of ice crystals (Kirichek et al. 2015; Zamecnik et al. 2021). The controlled-rate cooling mechanism is instrumental in curtailing the formation of intracellular ice crystals, thereby minimising mechanical injury to cells during cryopreservation. Controlled-rate cooling, coupled with hormone priming, has been observed to modulate antioxidant and glyoxalase systems, facilitate osmolyte accumulation, and influence the expression of stress-linked proteins, including HSPs (Hossain et al. 2017; Omidbakhshfard et al. 2020). Of pivotal importance for the successful rejuvenation of cryopreserved plant tissues are thawing and rehydration protocols, which are needed to remove cryoprotectants and avert osmotic shock (Popova

et al. 2023). *In vitro* propagation methodologies, spanning shoot induction, root generation, and somatic embryogenesis, present an effective way to revitalise cryopreserved tissues (Uchendu et al. 2019; Popova et al. 2020).

2.4 Micropropagation

In vitro tissue culture (micropropagation) is efficient for preserving and propagating genotypes with superior germplasm (Thorpe 2007). This approach can be used to maintain living germplasm and produce large quantities of plants (with the desired germplasm) within a controlled environment using different plant parts (explants) that are manipulated using plant growth regulators (PGRs) viz., auxins or cytokinins, to induce tissues and organs via direct or indirect (intervening callus phase) organogenesis or somatic embryogenesis (Reed et al. 2011; Lebedev et al. 2018; Fehér 2019). This technique has been widely applied for the mass propagation of plants under *in vitro* conditions, providing a reliable method to produce genetically uniform and healthy plants provided that callus formation and somaclonal variation are reduced or prevented (Abiri et al. 2020). *In vitro* cultures have also become an alternative method for the production of secondary metabolites of pharmaceutical importance, highlighting the potential of *in vitro* culture systems to produce compounds under controlled conditions (Baskaran et al. 2012).

The stages of micropropagation involve shoot multiplication, elongation, and rooting, and the successful completion of these stages is essential for the rapid production of disease-free clones in a controlled environment (Muniz et al. 2013). The process begins with the selection and maintenance of field-grown stock plants, followed by the initiation of *in vitro* propagation using explants from meristematic tissue (Kaur and Sandhu 2014). Shoot multiplication involves several passages on specific culture media containing PGRs to promote the proliferation of shoots (Kaur and Sandhu 2014). Sometimes, an elongation step is required in which culture media are supplemented with reduced or different auxins or cytokinins to promote internode development. The rooting of the shoots *in vitro* is the final step in the micropropagation process and is necessary for the successful acclimatisation of the plantlets and their establishment in soil (Finti et al. 2013; Yegorova et al. 2021).

The utilisation of PGRs can increase productivity, control genetic alterations, and minimise biological contamination during the micropropagation process (Bidabadi and Jain 2020; Teixeira et al. 2021).

PGR manipulation can facilitate clonal propagation of stress-tolerant plant genotypes but necessitates optimisation for the species of interest (Mamun et al. 2015; Bhaskarla et al. 2020). The optimisation of growth medium composition and environmental culture conditions can enhance the activity of antioxidant enzymes, recover hyperhydric shoots, and improve rooting *in vitro*, thus contributing to the development of an efficient micropropagation protocol for upscaling production (Mohamed et al. 2023). The culture media composition, PGRs, and growth conditions for different stages of micropropagation have been extensively studied, emphasising the importance of these factors to the successful micropropagation of various plant species (Clemente-Moreno et al. 2011; Hiti-Bandaralage et al. 2017). Moreover, specific culture media and growth regulators have been shown to cause genome-wide changes in DNA methylation, highlighting the relevance of these factors in the selection of micropropagation systems (Kitimu et al. 2015). Additionally, the impact of *in vitro* culture conditions on the physiology and anatomy of micropropagated plants underscores the significance of optimising culture conditions for successful micropropagation (Martins et al. 2020).

In the context of screening for stress tolerance, micropropagation can efficiently produce many clonal genotypes for use in stress application assays, facilitating the rapid identification of genotypes tolerant to stresses (Rao et al. 2008). This highlights the significance of *in vitro* culture in providing a platform for evaluating stress tolerance across different developmental stages of plants. The integration of stress-tolerant genotypes with micropropagation offers a promising approach to enhance the large-scale production of healthy and resilient plant material. Stress-tolerant genotypes possess inherent mechanisms to withstand adverse environmental conditions, making them valuable candidates for micropropagation. For instance, the identification of genotypes tolerant to salinity and alkalinity stresses in rice has been achieved through rigorous analysis, enabling the selection of suitable genotypes for micropropagation (Rao et al. 2008). Similarly, the evaluation of drought-tolerant genotypes in wheat and chickpeas has provided insights into the physiological and biochemical traits associated with stress tolerance, offering valuable information for the selection of genotypes for micropropagation (Vessal et al. 2020; Sunil et al. 2021).

The benefits of using micropropagation instead of conventional propagation methods include pathogen-free, rapid clonal multiplication with year-round production capabilities, reduced acclimatisation period *ex vitro*, and improved survival of micropropagated plantlets after transfer to

ex vitro conditions, ultimately reducing the cost of individual plants (Bhatia et el. 2015). The latter is one of the main advantages of micropropagation (Soumaré et al. 2021). Additionally, micropropagation has been shown to yield more crops compared to traditional methods of propagation, making it a more efficient technique, streamlining the overall propagation process and reducing the time required for plantlet production (Ovono et al. 2007; Valliath and Mondal 2023). Micropropagation maximises space efficiency and requires fewer resources like land and water, aiding studies in genetics, physiology, and biotechnology (Umate 2010). Most important for the present study are direct pathways of morphogenesis, which theoretically yield true-to-type clones.

Unfortunately, clonal propagation of stress-tolerant genotypes is not without limitations. Somaclonal variation poses a risk to the genetic fidelity of micropropagated plants, which may impact the uniformity of the propagated genotypes (Rani and Raina 2000). Additionally, adventitious shoot regeneration and somatic embryogenesis through a callus (indirect morphogenesis) phase can result in abnormal growth patterns, impacting the overall efficiency of propagation when true-to-type clones are desired (Debnath and Arigundam 2020). Variation can arise from the choice of explants, culture conditions, and PGRs, requiring rigorous monitoring and evaluation to ensure the reproductive and performance of clones throughout the micropropagation process (Kumar et al. 2012).

2.5 Measuring stress

External stresses can detrimentally influence the development, growth, and overall vitality of a plant. These stresses encompass an assortment of abiotic factors, such as extreme temperatures, insufficient water availability, elevated salinity, and toxic heavy metals (Naing and Kim 2021). Therefore, the measurement of plant responses to stress offers insights into the resilience of plants and their capacity to adapt to adverse conditions. Elucidating the intricate machinery behind these responses not only unveils the mechanisms fostering resilience but also assists in identifying genotypes that can withstand stress, cultivating stress-resistant crops, and forming sustainable agricultural models against climate change (Li et al. 2014; Suzuki et al. 2014; Fortes and Gallusci 2017; Zandalinas et al. 2021). Consequently, methods that accurately and dependably quantify these stress reactions are indispensable for making informed choices and effective strategies that underpin global food security (Raza et al. 2019).

2.5.1 Biophysical water potential

The concept of water potential enables the quantification of stress responses and plant health. Two widely employed strategies for quantifying water potential are osmotic potential evaluation and the pressure chamber technique. Osmotic potential assessment hinges upon the fundamental principle of osmosis, whereby water molecules traverse a selectively permeable barrier to equate solute concentrations on opposing sides. The application of osmotic potential assessment is widespread within plant physiology research, serving to gauge the water potential of plant tissues and appraise the aptitude of plants to manage water uptake and uphold turgor pressure (Cochrane 1994). This assessment is helpful in investigating how plants respond to water-deficit, heat, and other ecological factors (Falcone et al. 2015).

The pressure chamber technique, also known as the pressure bomb, emerged in the 1960s to directly measure plant water potential (Scholander et al. 1964). Pressure is applied to detached plant tissue or leaf samples within a sealed chamber, and the ensuing water loss or gain is monitored to measure sap pressure within the xylem (Scholander et al. 1964). This method is grounded in the fact that the pressure required to move water through the xylem equates to the water potential of that plant tissue. This method is useful for evaluating plant water status and responses to environmental constraints, particularly in scrutinising drought resilience and water-use efficiency (Shackel 2011). The pressure chamber technique has a notable benefit - the capacity to yield direct and precise plant water potential measurements to assess overall plant water status, thus informing decisions regarding irrigation and water management tactics (Mira-García et al. 2020).

This technique can be implemented in natural habitats, allowing field measurements and represents a relatively uncomplicated and cost-effective choice versus other methodologies like the thermocouple psychrometer or hygrometer-based techniques (Murphy and Smith 1994). However, the pressure chamber technique is not without limitations and constraints, as accurate implementation requires specialised equipment and training. This approach is intrusive, damaging plant tissue and limiting repeat usage on the same specimen. Detaching plant samples for the assessment introduces potential distortions and could modify the plant's physiological state. The method only offers a snapshot of plant water potential at a specific instant and lacks the capability to continuously monitor water status. This technique also demands substantial labour and time

investments, with each measurement necessitating meticulous sample preparation and pressurisation. The pressure chamber method is also restricted to xylem water potential measurement and might not provide a complete assessment of plant water status across all tissues.

2.5.2 Electrical conductivity

A standard method to measure electrical conductivity (EC) involves using EC meters (portable devices that measure the EC of a solution), enabling ion concentration quantification. This method has been used to measure agricultural soil solutes (Corwin and Lesch 2005) and the impact of salinity stress on watermelon (*Citrullus lanatus*) (Silva et al. 2019), wheat (*T. aestivum*) (Abebe et al. 2003; Zhu et al. 2015; Bharti et al. 2016), palm lily (*Cordyline fruticose*) (Plaza et al. 2019), tobacco (*Nicotiana tabacum*) (Tarczynski et al. 1993), and barley (*Hordeum vulgare*) (Angessa et al. 2017). Measuring EC can evaluate the impact of salinity stress on plant physiology and determine the effectiveness of different breeding strategies, such as genetic modifications, substrate supplements, or plant growth-promoting microbes, in enhancing salinity tolerance.

Kwok et al. (1992) reviewed the limitations associated with conductivity measurements, highlighting challenges such as electrode polarisation, sensitivity to temperature, and the need for calibration. Variables affecting electrical conductivity measurements, such as temperature and electrode types, can have a significant impact on the accuracy and reliability of the measurements. Temperature influences the conduction properties of materials, while the choice of electrode material and its interaction with the measured material can impact the accuracy and reliability of the measurements. Therefore, considering and controlling these variables are crucial to obtain accurate and meaningful conductivity data. The effects of plant species and tissue types on electrical conductivity can vary due to their inherent physiological and structural characteristics.

Munns (2002) highlighted the differences between salt-tolerant and salt-sensitive plants concerning their ability to transport Na^+ and Cl^- ions to tissues and their capacity to compartmentalise these ions in vacuoles, which subsequently influence the EC of tissues and contribute to EC variations among different species. Deinlein et al. (2014) discussed the accumulation of organic osmolytes, such as proline, glycine betaine, sugar alcohols, polyamines, and proteins, in plant cells as a mechanism to maintain low intracellular osmotic potential and mitigate the harmful effects of stressors. Measuring

the EC of stressed plants can provide insights into the compartmentalisation of ions and the accumulation of osmolytes, which can guide genotype screening and selection efforts.

2.5.3 Chlorophyll spectrophotometry

The spectrophotometric method for measuring the chlorophyll content of leaves is a widely used technique in plant and environmental sciences, involving extracting chlorophyll using ethanol (EtOH) and then quantifying the chlorophyll content through spectrophotometric analysis (Wintermans and Mots 1965). The use of EtOH as an extraction solvent has been shown to yield higher chlorophyll contents compared to other solvents and has also been applied to assess the phytochemical content and antioxidant activities of plant extracts (Loots et al. 2007; Nejatzadeh-Barandozi 2013; Nobossé et al. 2018; Olatunde et al. 2018; Tagrida and Benjakul 2020). The spectrophotometric method allows for the accurate measurement of chlorophyll content in plant samples, providing information for understanding physiological responses to environmental constraints, particularly in scrutinising water-use efficiency (Ertani et al. 2016; Jeyadevi et al. 2012; Novac et al. 2022). Furthermore, the spectrophotometric determination of chlorophyll content in EtOH leaf extracts has been compared with other methods, such as portable fluorescence meters, verifying the reliability and accuracy of the spectrophotometric method in quantifying chlorophyll content (Zhang et al. 2022).

2.5.4 Proline quantification

Proline is a thoroughly characterised stress-responsive amino acid that accumulates in plants under abiotic stress conditions, serving multiple functions, including osmotic adjustment, membrane stabilisation, ROS scavenging, and signal transduction (Siddique et al. 2018). Therefore, measuring proline content provides a reliable biochemical marker for assessing plant stress responses and tolerance mechanisms. Water-deficit stress induces proline accumulation as part of the adaptive response to maintain cellular homeostasis and sustain metabolic functions under limited water availability (Szabados and Savouré 2010). Similarly, high-temperature stress triggers proline synthesis, aiding in the maintenance of protein stability and preventing heat-induced denaturation (Kavi Kishor et al. 2005). Quantitative estimation is commonly performed using spectrophotometric methods based on the reaction of proline with ninhydrin, providing sensitivity and reproducibility (Bates et al. 1973).

2.5.5 Protein quantification and characterisation

The Bradford protein quantification method has been widely used to measure plant stress responses (Bantscheff et al. 2007; Howell 2013; Ma et al. 2016). This assay is based on the principle of protein-dye binding, precisely, Coomassie Brilliant Blue (CBB) (Bradford 1976). A sample is mixed with CBB, and the colour intensity of the resultant solution is proportional to the protein concentration, which is quantified spectrophotometrically at a wavelength of 595 nm (Bradford 1976). Total protein quantification in plant stress response studies provides insights into the overall protein abundance, turnover, and potential stress-responsive proteins, assessing the impact of stress on protein concentration (Ndimba et al. 2005). The Bradford method of protein quantification offers several advantages over the Lowry and bicinchoninic acid assays, making it a widely used technique in various fields of research. Firstly, the Bradford assay is relatively simple and quick, allowing for rapid quantification of protein samples (Cui et al. 2019) and facilitating high-throughput experiments. Additionally, this method is compatible with a wide range of protein concentrations, making it suitable for both high and low-abundance proteins (Redmile-Gordon et al. 2013), detecting protein concentrations as low as 1 µg/mL. Furthermore, the Bradford assay is compatible with various sample types, including soil extracts and plant tissues (Redmile-Gordon et al. 2013; Moragues-Saitua et al. 2018).

While stems and roots have crucial roles in plant physiology, focusing on the protein content of leaves in stress studies is favoured due to the central role of photosynthesis and metabolic activity in these organs (Liu et al. 2011; Dien et al. 2019). Protein content in stems and roots may also change under stress, but the dynamics can be influenced by factors unrelated to photosynthesis (Dien et al. 2019). Leaves serve as the primary site for photosynthesis, thereby synthesising organic compounds, including proteins (Liu et al. 2011). The intricate interplay between chloroplasts and other cellular components in leaves ensures the synthesis of proteins critical for photosynthesis and other physiological processes (Wang et al. 2017). Monitoring leaf protein content allows researchers to gauge the impact of stress on the efficiency of protein synthesis, offering a direct reflection of photosynthetic capabilities (Wang et al. 2017). Leaves are also highly metabolically active and are the primary organs involved in nutrient assimilation and translocation (Liu et al. 2011). Changes in leaf protein content can serve as a sensitive indicator of the plant's overall cellular health and metabolic

status. Stress-induced alterations in protein content may signify adjustments in metabolic pathways or activation of stress-responsive proteins crucial for plant survival (Wang et al. 2017).

Unfortunately, the Bradford method also has some limitations that should be considered. It is susceptible to interference from certain substances, such as detergents and reducing agents (Cui et al. 2019), affecting the accuracy of protein quantification. Additionally, the Bradford assay relies on a standard curve for quantification, which necessitates the use of a known protein standard (Redmile-Gordon et al. 2013). If the protein standard does not accurately represent the proteins of interest, it can introduce errors in the quantification process. Another limitation of the Bradford method is its sensitivity to the amino acid composition of proteins. Proteins with different amino acid compositions may yield different colourimetric responses, leading to variations in quantification accuracy (Contreras-Martos et al. 2018), particularly problematic when quantifying intrinsically disordered proteins, which have distinct and variable amino acid compositions (Contreras-Martos et al. 2018). These limitations can be supplemented by more specific, accurate, and dynamic molecular-based analyses, such as mass spectrometry and real-time polymerase chain reaction.

Protein characterisation using electrospray ionisation mass spectrometry time of flight (ESI-MS-TOF) is an analytical approach to identifying and profiling proteins in complex biological mixtures. This technique couples the soft ionisation capabilities of ESI, which preserves the native state of biomolecules, with the high-resolution and accurate mass measurement of TOF analysers (Kosová et al. 2011). Such an approach enables the identification of proteins by matching observed mass spectra to theoretical data from protein databases, providing insights into the molecular responses and adaptive mechanisms of plants subjected to abiotic stress. Stress alters the protein expression profile, often leading to the synthesis of stress-responsive proteins, transcription factors, and enzymes (Zandalinas et al. 2017). Through the analysis of crude extracts, it is possible to uncover both novel and known proteins implicated in stress tolerance, contributing to a deeper understanding of stress physiology.

2.5.6 Real-time polymerase chain reaction (RT-qPCR)

RT-qPCR serves as a method to assess gene expression levels in real-time, achieved through the amplification and quantification of specific transcripts combined with fluorescent probes or DNA-

binding dyes (Bustin et al. 2009). The approach facilitates precise and sensitive gene expression quantification by tracking the accumulation of PCR products as the amplification progresses from cycle to cycle (Schmittgen 2008). The use of fluorescent probes brought RT-qPCR into prominence in the early 2000s, enabling real-time PCR amplification tracking (Cao and Shockey 2012). Over time, RT-qPCR has emerged as a standard gene expression analysis technique due to its sensitivity, specificity, and broad dynamic range (Chang et al. 2016). Accurate and reproducible measurements of specific mRNA sequences are necessary to scrutinise genetic expression. Formerly, gene expression quantification relied on Northern blot analysis, a time-intensive method demanding substantial RNA quantities (Brown and Du 2004). RT-qPCR is the most sensitive tool for identifying low-abundance mRNAs and probing tissue-specific gene expression (Bustin et al. 2009; Zogchel et al. 2021; Park et al. 2023). RT-qPCR commonly uses an internal control gene as a reference to mitigate bias. This transcriptional analysis modality proves instrumental in unravelling functional genes and enhancing gene expression quantification.

The genetic response to distinct stressors can be assessed by exposing a plant to stress, followed by quantifying resultant genetic expression through RT-qPCR. This data aids in identifying and proliferating plants showcasing desired genotypic traits, subsequently advancing the cultivation of these genetically superior cultivars. RT-qPCR effectively assesses the expression of stress-responsive genes under diverse environmental conditions, encompassing drought, salinity, temperature fluctuations, and pathogenic infestations (Wu et al. 2009; Chang et al. 2016; Gupta et al. 2016; Moebes et al. 2022; Yang et al. 2022). RT-qPCR is renowned for its sensitivity, enabling the detection of low-abundance transcripts (Chang et al. 2016). It also boasts an extensive dynamic range, allowing gene expression quantification across multiple orders of magnitude (Mortazavi et al. 2008). Furthermore, RT-qPCR proves relatively expedient and cost-effective compared to alternative gene expression analysis methods (Ahmed et al. 2022). However, RT-qPCR has certain limitations; it entails designing and optimising specific primers or probes for each target gene, an undertaking that can be time-intensive and financially demanding (Cao and Shockey 2012). Moreover, RT-qPCR is a focused approach capable of measuring the expression of a limited gene pool at any given time (Chang et al. 2016).

2.5.6.1 RNA extraction

High-quality RNA is essential for studying gene expression, identifying differentially expressed genes, and understanding the molecular mechanisms underlying plant development and responses to environmental stimuli (Sah et al. 2014; Liu et al. 2018). RNA is susceptible to degradation by ribonucleases, which are present in cells, tissues, and laboratory environments. Furthermore, high yields and purity are desired for downstream applications. However, excessive RNA purification steps may result in RNA loss. Therefore, achieving a balance between yield and purity is crucial for successful RNA extraction. Contaminants, such as genomic DNA, proteins, or chemicals from the extraction reagents, can interfere with downstream applications like reverse transcription and RT-qPCR. Several studies have developed reliable methods for RNA extraction from diverse plant species (Sah et al. 2014). These methods aimed to minimise the time and effort required for RNA extraction while maintaining the quality and integrity of the RNA.

Developed by Life Technologies (now Thermo Fisher Scientific), TRIzol™ is a monophasic solution that combines the properties of phenol and guanidine isothiocyanate for the efficient isolation of RNA, DNA, and proteins from a wide range of biological samples. The reagent is known for its efficiency in breaking down cell and tissue samples, enabling rapid lysis of cells (Rio et al. 2010). This efficiency is crucial for preserving RNA integrity since it minimises the time RNA molecules are exposed to degrading enzymes, such as ribonucleases. Furthermore, TRIzol™ enables the simultaneous extraction of RNA, DNA, and proteins from the same sample, offering a comprehensive approach to studying various aspects of gene expression regulation in parallel. RNA extracted with TRIzol™ is an appropriate starting material for a wide range of downstream applications, including reverse transcription for cDNA synthesis and RT-qPCR. The quality and quantity of the extracted RNA are typically assessed using spectrophotometry and gel electrophoresis. Key parameters evaluated include RNA concentration, A260/A280 ratio (indicative of protein contamination), and RNA integrity (Johnson et al. 2014).

2.5.6.2 Primer design

The design of high-quality primers is essential for the accurate and reliable detection and quantification of target genes (Jeon et al. 2019). Crucial considerations in primer design include the

length of the DNA fragment of interest; shorter sequences are preferred as they are less prone to degradation during processing and sample preparation, increasing reaction efficiency (Yin et al. 2022). Furthermore, optimising annealing temperatures is essential when designing primers because different primer pairs may require different annealing temperatures for optimal amplification efficiency (Yl et al. 2015). The length and GC content of primers are also important factors in primer design, ideally between 18-25 nucleotides in length (Bustin and Huggett 2017). Shorter primers may lack specificity, while longer primers can lead to non-specific amplification. Additionally, the GC content of primers should be 40-60% (Bustin and Huggett 2017), maintaining the balance between primer stability and specificity. Self-complementarity is another consideration in primer design, leading to the formation of secondary structures and primer-dimer artefacts, hindering target amplification, and resulting in false-positive signals (Bustin and Huggett 2017). Primer melting temperature (T_m), the temperature at which half of the DNA duplex is denatured, is influenced by primer length, GC content, and salt concentration. The T_m of the forward and reverse primers should be similar to ensure efficient and specific amplification (Bhosle et al. 2022).

Advancements in computational tools utilising algorithms and parallel computing have been developed to expedite the primer design process, optimising T_m, GC content, and specificity. PrimerQuest™ (Integrated DNA Technologies) and Primer3 (Whitehead Institute for Biomedical Research) can calculate T_m and optimise primer parameters (Untergasser et al. 2012). Additionally, hot-start PCR enzymes or modified primers, such as locked nucleic acids, prevent primer-dimer formation and software tools like UNAfold (Markham and Zuker 2008) or RNAstructure (Mathews Group) can be used to predict secondary structure formation. Furthermore, degraded or low-quality RNA samples can present challenges in RT-qPCR due to fragmented or partially degraded sequences, necessitating primers that target shorter amplicons or anneal closer to the 3' end, which are less likely to be affected by RNA degradation (Kaderali 2003).

2.5.6.3 Degenerate primers

Degenerate primers contain degenerate bases, allowing them to anneal to multiple related target sequences, enabling the amplification of highly conserved regions, such as homologous genes or gene families (Compton 1990). The number and placement of degenerate bases should be carefully chosen to balance specificity and coverage. Too many degenerate bases may lead to non-specific

amplification, while too few may limit the range of target sequences. The degenerate bases should also be selected based on the known sequence variation in the target region (Green et al. 2015). Experimental testing is needed to validate degenerate primers, involving performing PCR with the degenerate primers and analysing the resulting amplicons. It is also vital to optimise PCR conditions, such as annealing temperature and cycling parameters, to ensure efficient amplification (Green et al. 2015). Several software tools have been developed to aid in degenerate primer design, utilising algorithms and heuristics to generate degenerate primer sequences based on user-defined parameters and target sequence alignments (Rose and Henikoff 2003; Wang et al. 2011). Such tools include consensus degenerate hybrid oligonucleotide primer (CODEHOP) software (Boyce et al. 2009).

2.5.6.4 Primer validation

Experimental validation of primers is necessary to ensure specificity, efficiency, and reliability. Standard curve analysis is used to assess the linearity and dynamic range of the PCR assay, encompassing creating a series of known target DNA or RNA concentrations and performing RT-qPCR. The resulting cycle threshold (Ct) values are plotted against the logarithm of the initial target concentration to generate a standard curve. A well-designed primer pair will yield a standard curve with a high correlation coefficient (R^2) (Park et al. 2020). Furthermore, amplification efficiency is a measure of how effectively the primers amplify the target sequence, calculated using the equation:

$$Efficiency\ (\%) = (10^{-1/slope} - 1) \times 100$$

An amplification efficiency of 90-110% is optimal, indicating that the primers amplify the target sequence with high specificity and sensitivity (Piñol et al. 2014). Deviations from this range may suggest issues such as primer-dimer formation or non-specific amplification. Melting curve analysis is performed to assess the specificity of the PCR amplification. After the amplification step, the temperature is gradually increased, causing the DNA strands to denature. The fluorescence intensity is monitored, and the T_m is determined, representing the temperature at which half of the DNA duplex is denatured. A single, sharp peak at the expected T_m indicates specific amplification of the target sequence, while multiple peaks suggest non-specific amplification or primer-dimer formation (Ririe et al. 1997).

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3. Anatomic and physiologic screening for water-deficit and heat-tolerant genotypes of the sugarcane herb, *Amaranthus dubius,* sampled in KwaZulu-Natal, South Africa.

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Abstract

Amaranthus dubius is a highly nutritious and easy-to-grow annual shrub. Like other African leafy vegetables (ALVs), *A. dubius* can tolerate water-deficit and heat stress, enabling cultivation on marginal land. However, the widespread propagation of *A. dubius* as a stable food source has thus far not been realised due in part to the high frequency at which hybridisation occurs, resulting in high genotypic variability and unpredictable growth characteristics. To increase the agricultural output capacity of this species in water-deficit environments, particularly because South Africa is prone to periodic drought conditions and fluctuating rainfall patterns, it is important to screen, select, then clonally propagate water-deficit and heat stress tolerant genotypes. One approach to achieving this goal is to screen for robust growth and physiological responses, like high biomass, leaf water potential (Ψ_{Leaf}), and chlorophyll, proline, and protein content and their characterisation, under these stresses. The present study exposed four-week-old *A. dubius* seedlings to 4-, 8-, 12-, and 16-day variable watering schedules to determine the inherent degree of water-deficit tolerance of this species and to day cycles of 16 h at 30 (control), 35, 40, and 45 °C and night cycles of 8 h at 24 °C to determine the degree of high-temperature tolerance. Amidst the severe water-deficit interval (16 days), genotypes from Genozzano and Tongaat showed superior vigour compared to their Amanzimtoti, Verulam, and Westville counterparts. Numerous genotypes from all locations maintained Ψ_{Leaf} despite reduced watering frequencies. Notably, genotype Genozzano-1 (G1) demonstrated similar or elevated Ψ_{Leaf} when watered every 16 days across the 4-week study. Tongaat-8 (T8) and G7 genotypes retained peak leaf protein levels following the 16-day watering regimen, and Amanzimtoti-3 (A3) preserved the most chlorophyll. The genotypes which accumulated the highest proline under the most severe water-

deficit treatment were A2 (109.3 ± 8.0 µg/mL), G1 (108.0 ± 7.2 µg/mL), G5 (111.8 ± 5.7 µg/mL), and T8 (110.8 ± 5.6 µg/mL). Among the fastest-growing and highest-yielding genotypes during micropropagation and subsequent acclimatisation were A2, G7, T5, and Verulam-3 (V3). Following micropropagation through direct organogenesis and acclimatisation, selected clones maintained true-to-type traits such as similar Ψ_{Leaf}, protein, and chlorophyll as their parent plants when exposed to the 16-day watering regimen. In the high-temperature stress assay, genotype Heat-37 (H37) performed the best across the tested parameters, demonstrating consistency across the 35, 40, and 45 °C treatments in chlorophyll content (2.1 ± 0.2 µg/cm^2) and protein turnover (3.7 ± 0.8 µg/mL), and showed enhanced propagative capacity (5.4 ± 1.0 new shoots/explant). The genotypes which accumulated the highest proline under the most severe heat stress treatment were H1 (167.9 ± 18.3 µg/mL), H34 (156.9 ± 29.4 µg/mL), and H35 (164.6 ± 10.3 µg/mL). This study revealed that some *A. dubius* genotypes were highly tolerant to severe water-deficit and high-temperature stress. Mass spectrometry analysis of tolerant genotypes detected the presence of many proteins, including the RNA polymerase β subunit (accession WOK42753), ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) large subunit (accession AKG26942), ribosomal protein L16 (accession YP_009922872), ribosomal protein S16 (accession CAB45301) under water-deficit stress, and photosystem II protein D1 (accession XIF09057), putative channel protein 1 (accession AAC03555), ribosomal protein L32 (accession XED52978), and ribosomal protein S12 (accession XCH54301) under high-temperature stress. Quantifying the relative expression of transcripts was attempted using degenerately primed Real Time-qPCR but yielded no significant amplification in any of the selected genotypes, necessitating further optimisation and exploration to elucidate the underlying genetic mechanisms facilitating the observed physiological responses to water-deficit and heat stress. Further experimentation should delve into the nutritional quality of identified genotypes to determine if they retain their nutritional status following cloning.

Keywords: Amaranthaceae, Water-deficit stress, Heat stress, Physiology, Genotype screening, Micropropagation

3.1 Introduction

The South African KwaZulu-Natal province showcases diverse ecological and climatic diversity influenced by the region's biogeography (Eeley et al. 1999). The climate varies from temperate in the

elevated inland areas to subtropical or tropical along the coast, contributing to the province's ecological diversity, mirrored in the variety of ecosystems and species across many taxonomic groups (Minin and Moilanen 2014; Klopper et al. 2020). Hence, KwaZulu-Natal is a critical area for biodiversity conservation and a hub for research and conservation initiatives (Minin et al. 2013; O'Donoghue et al. 2016). Unfortunately, the province faces environmental degradation, biodiversity loss, and drought susceptibility as a result of climate change, and therefore requires strategies to mitigate these ecological crises (Nel 2021). Climate change-induced elevated temperatures, altered precipitation patterns, and increased frequency of extreme weather events limit the viability of conventional crops and agricultural land, primarily by inducing water scarcity (Khumairoh et al. 2018; Afshar et al. 2021; Huo et al. 2022). However, some plant species have demonstrated resilience to stress, presenting an opportunity for sustaining agricultural productivity and food production.

Water-deficit and elevated temperatures exhibit specific and diverse effects on ecosystems, resulting in shifts in species distribution, changes in phenology, and modifications to ecosystem dynamics, influenced by the intricate relationship between climate change and biodiversity (Singh et al. 2014). Climate trend forecasts anticipate a widespread ecological effect from shifting rainfall patterns and increasing global temperatures, altering ecosystem functions and redistributing species and zonation (Gibert et al. 2016). Moreover, the consequences of climate change prompt a decline in ecological productivity, contributing to the loss of flora and fauna (Fereja 2017; Sun et al. 2022). As resource exploitation increases, the adaptive capacity of ecosystems decreases, increasing their susceptibility to climatic shifts (Chiu et al. 2017).

Water scarcity and temperature fluctuations influence crop growth and development, often detrimentally by decreasing yield (Asseng et al. 2014; Zhao et al. 2017) and affecting pollen development and fertilisation (Zinn et al. 2010). The repercussions of these stresses on plant physiology manifest in morphological, physiological, biochemical, and molecular alterations (Hatfield and Prueger 2015; Qadir et al. 2019; Faiq and Noori 2021). The adverse effects of water-deficit and high temperatures highlight the need to cultivate 'climate-resilient crops' that can thrive and maintain yield in extreme environmental conditions (Abdullah et al. 2021). Addressing the urgency of identifying and conserving resilient crops entails recognising the impact of water-deficit and high-temperature stress on plant growth, development, and yield. This is especially necessary given the

limited availability of adaptation strategies prioritising global food security and the establishment of more resilient food production systems (Döring et al. 2015; Hatfield and Prueger 2015; Holbrook et al. 2016; Yoon et al. 2021).

The response to water-deficit and high temperatures involves regulating gene expression linked to hormone signal transduction, osmoregulation, and metabolic pathways such as fructose and mannose metabolism (Callwood et al. 2021; Alshameri et al. 2020). The heat stress response in plants engages various pathways such as the heat shock response, calcium-mediated signalling, maintenance of reactive oxygen species (ROS) equilibrium, endomembrane trafficking, and cross-membrane transport pathways (Zhao et al. 2018; Cui et al. 2020; Kudapa et al. 2023). Conversely, downregulation of genes linked to light-harvesting complexes and the photosynthetic electron transport system results in diminished photosynthetic capacity under stress, restricting energy production, ultimately affecting plant growth and productivity (Song et al. 2014; Ren et al. 2019; Yuan et al. 2022). The decrease in expression of genes related to hormone signalling, transcription factors, and metabolic pathways indicates the broad-ranging impact of these stresses on plant physiological and developmental processes (Cheng et al. 2021; Wu et al. 2022). An avenue towards developing strategies to mitigate the adverse effects of climate change begins with elucidating the adaptive responses of resilient food crops to climate-related stresses through anatomic, proteomic and genetic analyses.

Amaranthus dubius, commonly known as the sugarcane herb and *imbuya* (isiZulu) in KwaZulu-Natal, is a versatile and nutritionally rich annual allotetraploid belonging to the *Amaranthaceae* family, renowned for its edible leaves and tender stems (Kwinana-Mandindi 2014; Ruth et al. 2021). Originating in Central and South America*, A. dubius* populations have spread globally throughout tropical and subtropical latitudes (Molina et al. 2011; Espitia-Rangel 2018). Furthermore, the shrub holds cultural significance in traditional medicine and culinary practices in several regions, garnering attention for its demonstrated resilience to diverse environmental conditions and potential as a sustainable food source, but is relatively underutilised (Bang et al. 2021; Ruth et al. 2021). Therefore, studying the anatomic, physiologic, and genetic responses of *A. dubius* genotypes to water-deficit and high-temperature stress demands further exploration. While other *Amaranthus* species have been studied for water-deficit effects, specific literature on water use efficiency in *A. dubius* is sparse. Analysing how these stresses influence the growth and physiology of this species is necessary for

understanding its adaptability in arid environments. Furthermore, the genetic expression under stress in *A. dubius* remains understudied but is important for elucidating adaptive strategies and breeding resilient varieties. Investigating homologous loci evolution in related plants emphasises the necessity for similar *A. dubius* studies, especially because understanding duplicate gene evolution in natural allopolyploids can shed light on relevant genetic dynamics amidst water-deficit and heat stress.

The pressure chamber method of measuring leaf water potential (Ψ_{Leaf}) has been used to physiologically quantify the adaptive strategies of various plant species to stress, including oil palm (*Elaeis guineensis*) (Brito et al. 2023), rapeseed (*Brassica napus*) (Ali et al. 2017), sorghum (*Sorghum bicolor*) (Wahua and Miller 1978), jalapeno pepper (*Capsicum annuum*) (Deveci and Pitir 2016), soybean (*Glycine max*) (Brady et al. 1974), and apple (*Malus* sp.) (Swietlik et al. 1982). Measuring Ψ_{Leaf} quantifies the tension within the water transport system of a plant, reflecting its ability to extract water from the soil and transport it through the roots, stem, and leaves to maintain turgor pressure, showing its capacity for osmotic adjustment. This process is necessary for regulating plant growth and morphology, influencing cell enlargement and propagation, stomatal conductance, leaf development, and flower formation, while indirectly affecting vital physiological processes such as photosynthesis, metabolism, and nutrient absorption (Forouzesh et al. 2012; Chen et al. 2015; Dong et al. 2018). Correlating multiple growth parameters, such as biomass, root and shoot length, and leaf number with Ψ_{Leaf} validates the severity of stress, strengthening the reliability of conclusions drawn regarding the selection of superior genotypes and the impact of water-deficit stress on the growth and physiology of tested plants (Santos et al. 2023).

Quantifying Ψ_{Leaf} also provides insights into the ability to maintain water transport efficiency under heat stress, which often causes water loss through increased transpiration and evaporation (Ashraf et al. 2022; Tao et al. 2022). Additionally, many stresses, including high temperatures, disrupt the photosynthetic apparatus, leading to a decrease in chlorophyll content, impacting the overall capacity to generate energy and sustain growth (Rossi et al. 2017; Kaur and Thind 2017). Heat stress also causes protein denaturation, affecting overall protein composition (Vâlcu et al. 2008). Therefore, changes in total protein content reflect the response to heat stress, potentially including the synthesis of stress-responsive proteins involved in cellular protection, repair, and adaptation (Selinga et al. 2022). For example, HSPs are important molecular chaperones that help in protein folding, repair, and

maintenance of overall cellular homeostasis under stress conditions, including heat stress (Ul Haq et al. 2019).

Protein content analysis provides further insights into the adaptive responses of plants to stressful conditions, encompassing protein regulation, including enzymatic and antioxidant metabolism (Reddy et al. 2004; Ali et al. 2023). Generally, total soluble protein content decreases under water-deficit and heat stress (Crusciol et al. 2009; Silva et al. 2022). Protein degradation and turnover allow resilient plants to minimise energy expenditure and recycle amino acids for the synthesis of stress-protective proteins or other essential molecules, such as enzymes and signalling peptides (Cardoso et al. 2017; He et al. 2020; Moloi and Ngara 2023). Unfortunately, the turnover of chloroplast proteins under stress affects essential growth and developmental processes, including photosynthesis, mRNA modification, transcription, amino acid synthesis, and lipid metabolism (Wang et al. 2023). Therefore, measuring chlorophyll content also serves as an indicator of photosynthetic capacity directly affected by stress (Lin et al. 2015; Romero et al. 2017; Urban et al. 2017). Water scarcity and elevated temperatures induce chlorophyll degradation through the production of reactive oxygen species (ROS), such as O_2 and H_2O_2, leading to lipid peroxidation, which disrupts the integrity of chloroplast membranes (Mannan et al. 2017; Meher et al. 2018). The upregulation of chlorophyllase, an enzyme responsible for chlorophyll degradation, influences resource reallocation and stress-induced senescence (Gibon et al. 2000; Ayub et al. 2021).

Additionally, in response to stress, some resilient species redirect the conversion of glutamate, a chlorophyll precursor, into proline, which scavenges ROS, regulates stress-responsive gene expression, and aids in maintaining membrane integrity and osmotic balance (Bulegon et al. 2016; Beigzadeh et al. 2019). Proline content analysis quantifies this physiological marker, which can be used to assess plant responses to environmental stress, particularly in the context of water-deficit and high-temperature conditions. Quantifying proline content under controlled stress conditions facilitates screening different genotypes, identifying those with enhanced proline synthesis and accumulation capacity. Therefore, this assay can provide insights into the physiological mechanisms underlying stress tolerance, offering a rapid and reliable means to assess and select genotypes with superior resilience to abiotic stresses.

Proline, chlorophyll, and protein analyses, in corroboration with growth and Ψ_{Leaf} measurements, enable a multifaceted assessment of the water-deficit and high-temperature tolerance of different genotypes, encompassing both physiological and molecular responses. Resilient genotypes might exhibit consistent or increased protein content under stress, suggesting robust adaptive mechanisms, efficient stress-responsive protein synthesis, and the ability to maintain essential cellular functions under stress conditions. Similarly, genotypes displaying minimal fluctuations or maintaining higher chlorophyll content under stress indicate superior photosynthetic resilience and potential stress tolerance. However, in some cases, a controlled reduction in chlorophyll content indicates an adaptive strategy to allocate resources efficiently and to adjust to stress conditions. Nevertheless, a substantial and sustained decrease in chlorophyll content is typically unfavourable and is associated with decreased growth, yield, and stress tolerance. Due to this complexity, it is also necessary to evaluate the genetic mechanisms underlying these physiological responses in the species of interest.

There are gaps in knowledge regarding the response of *A. dubius* genotypes to water-deficit and high temperatures, which need to be addressed because this species can help enhance food security and dietary diversity. In particular, there is a need for more research on how *A. dubius* genotypes physiologically and genetically react to water-deficit and high-temperature stresses. Understanding the variations in responses among different genotypes is necessary for identifying and preserving resilient varieties. Additionally, the ecological and agronomic ramifications of these stresses on *A. dubius* genotypes remain relatively unexplored. While some studies have touched upon the allelopathic effects of other plant species on *A. dubius* (Musyimi et al. 2018), comprehensive examinations of the performance, growth, and resilience of various genotypes under stressful conditions are lacking. Addressing this gap would yield valuable insights into the ecological adaptation and agricultural potential of tolerant *A. dubius* genotypes.

Due to escalating global challenges associated with climate change, understanding the adaptive responses of plant species to water-deficit and heat stress is imperative for sustainable agriculture. This study aimed to quantify the effects of varying degrees of water-deficit and heat stress on the growth parameters (plant height, root length, number of leaves, leaf area, and root and shoot biomass) and physiological processes (chlorophyll, protein, proline, and Ψ_{Leaf}) in *A. dubius* followed by screening for superior tolerant genotypes. Specifically situated in the environmental context of

KwaZulu-Natal, South Africa, this research seeks to quantify these effects under variable watering and temperature schedules. Furthermore, micropropagation and protein characterisation of superior genotypes were explored.

3.2 Materials and methods

3.2.1 Growth characteristic meta-analyses

A systematic literature search was conducted for growth data of *A. dubius* to assess variability within this species. Peer-reviewed journals and theses (n = 15) were sourced from online repositories, and relevant data, including growth-related measurements, were extracted and catalogued. This approach aimed to compile a comprehensive dataset from diverse sources to enhance the understanding of the growth characteristics of *A. dubius* specimens. The coefficient of variation ($CV\%$) was calculated to quantify the variability and dispersion for each parameter using ***Equation 3.1***.

Equation 3.1: Coefficient of variation ($CV\%$):

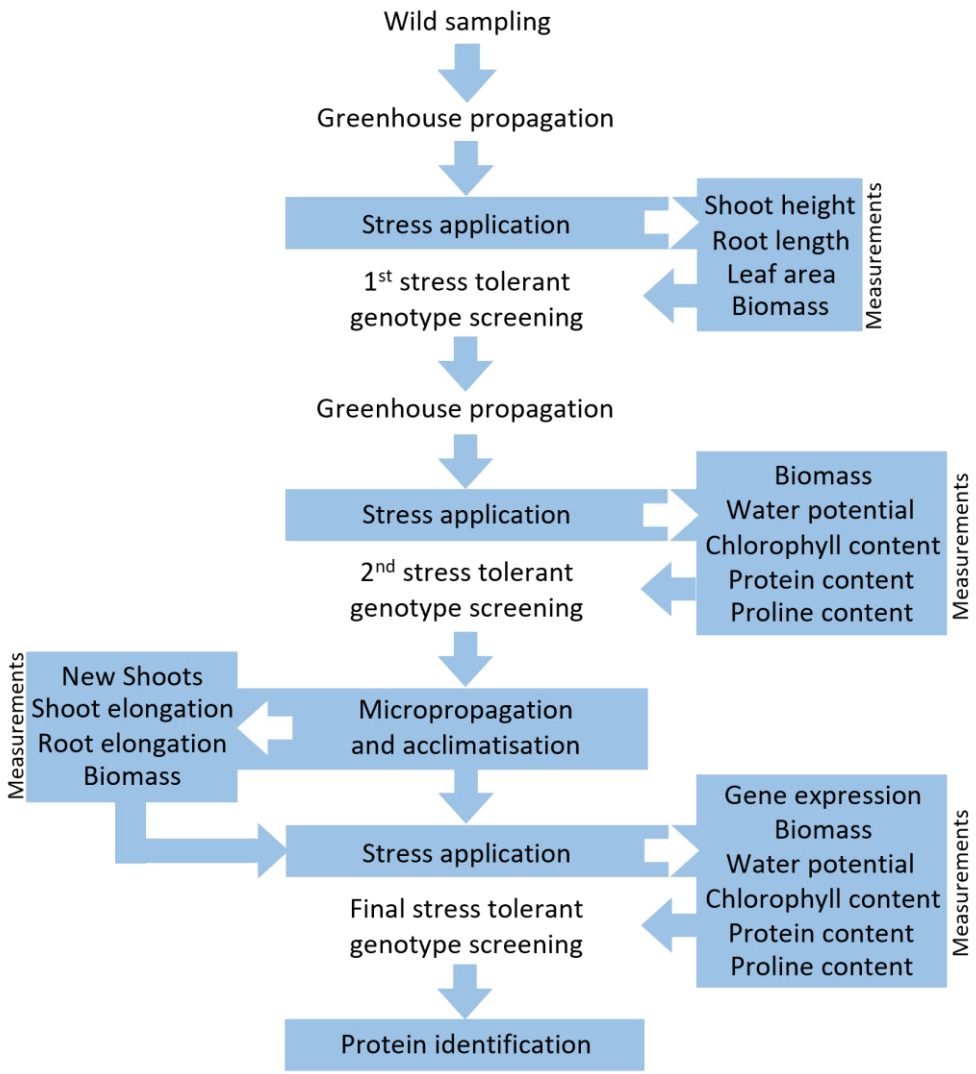
$$CV\% = \frac{SD}{Mean} x\ 100$$

Where SD = standard deviation of each parameter.

3.2.2 Plant material

Scheme 3.1 summarises the experimental design for this study, outlining the sequence of growth, physiology, and gene expression analyses and measurements used to screen and select water-deficit and high-temperature tolerant genotypes of *A. dubius* from the wild population. There were three screening steps following stress treatments wherein the best-performing genotypes were selected for further experimentation. The first two screening steps were carried out in a greenhouse. The final screening occurred following micropropagation and subsequent greenhouse acclimatisation of the identified stress-tolerant clonal genotypes.

Amaranthus dubius seeds were harvested in December 2021 from wild-type populations found in various locations around KwaZulu-Natal, South Africa (***Figure 3.1***). For verification, a specimen was deposited to the Bews Herbarium in Pietermaritzburg, KwaZulu-Natal (accession: NU0094621). Inflorescent spikes were sun-dried for two weeks under ambient conditions. Seeds were then hand-stripped and stored at 4 °C in hermetic amber containers.



Scheme 3.1: Flow diagram of the experimental design for this study to investigate the effects of water and heat stress on *A. dubius*.

For the water-deficit stress assays, 200 seeds from each location were individually sown into seedling tray inserts (37 mm width x 60 mm height) containing a 1:1 mixture of coco peat (Grovida, South Africa) and seedling mix (Grovida, South Africa). The trays were placed inside mist tents within the greenhouse at the School of Life Sciences (Westville campus, University of KwaZulu-Natal) and watered daily at 4 pm for 5 min with municipal water using an automatic system. The minimum and maximum average temperatures were 16 °C (night) and 31 °C (day), respectively, and the average relative humidity was 60%. Each insert of the seedling tray received an average of 15 mL of water per day. For the heat stress assay, seedling trays were placed inside GC-300TLH growth chambers (Lab Companion™, Korea) and subjected to 24-hour day/night cycles of 16 h at 32 °C and 8 h at 24 °C and hand-watered daily with 15 mL distilled water. After one week, following cotyledon emergence, the

seedlings were individually transferred into plastic flowerpots (90 mm diameter x 100 mm depth) with the same substrate as the seedling trays but supplemented with vermiculite (10% v/v) (Grovida, South Africa) to improve aeration, water retention, and nutrient exchange (Rajkumar et al. 2017; Xu et al. 2021. Dr Fisher's Classic Multifeed® (AECI, South Africa) solution (1.3 g/L) was applied to this substrate once every seven days at a volume of 10 mL per seedling. Before starting the stress treatments, baseline data were obtained for seedling height, measured using a 1000 mm stainless steel ruler (Tork Craft, South Africa).

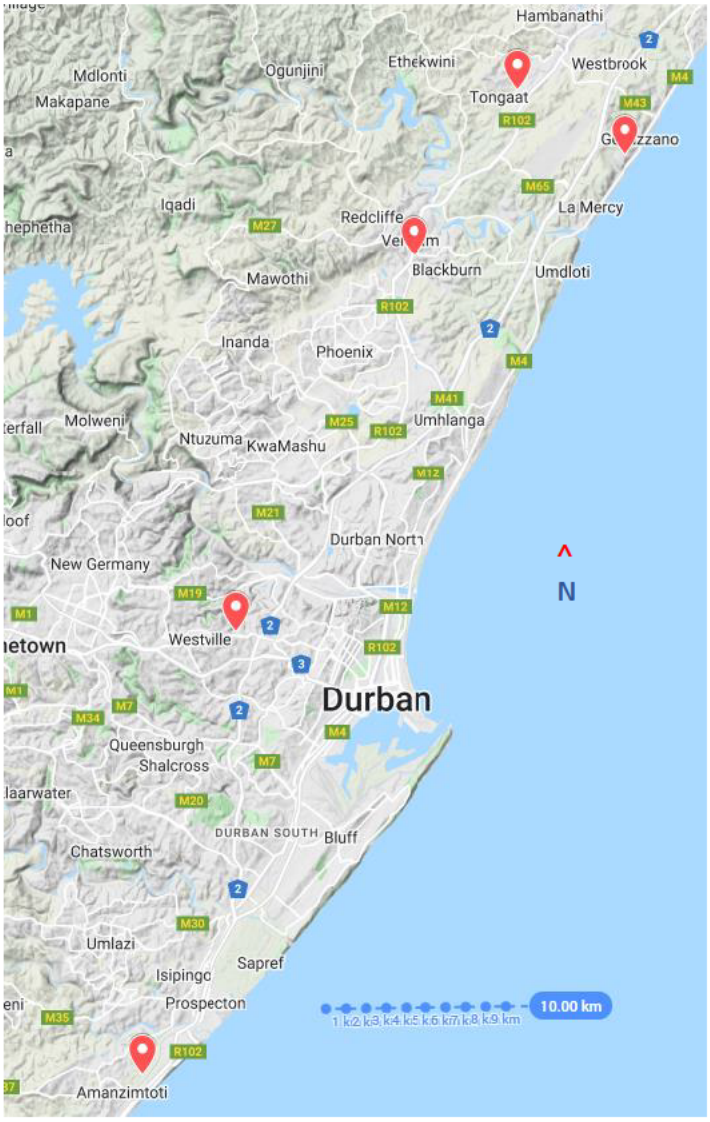


Figure 3.1: Seeds harvested from wildly growing *A. dubius* plants from pinned locations around KwaZulu-Natal, South Africa. From North to South: Tongaat (29° 58' 47.8" S, 31° 09' 38.3" E), Genozzano (29° 61' 40.4" S, 31° 14' 94.5" E), Verulam (29° 65' 93.9" S, 31°04'02.8" E), Westville (29° 82' 81.1" S, 30° 94' 87.9" E), and Amanzimtoti (30°02'68.7" S, 30°89'95.1" E). Map data from AfriGIS, 2022.

3.2.3 Stress application

Water-deficit stress was incrementally applied following seedling establishment, indicated by the emergence of the fifth or sixth true leaves, approximately three weeks post-germination. Individual seedlings representing unique genotypes (n = 50) were labelled according to their region of origin: Tongaat (T), Genozzano (G), Verulam (V), Westville (W), and Amanzimtoti (A) (***Figure 3.1***). The seedlings were then watered with 50 mL of distilled water once every 4 (control), 8, 12 or 16 days for 4 weeks. The 4-day watering interval was used as the control based on previous studies (González-Rodríguez et al. 2019; Amma and Rajalakshmi 2023) and preliminary data (not shown). Heat stress treatments were also applied after seedling establishment, approximately four weeks post-germination, on a separate set of genotypes. The seedlings were arranged randomly for each of the four treatments: day cycles of 16 h at 30 (control), 35, 40, and 45 °C and a night cycle of 8 h at 24 °C (n = 50). The pots were watered, as mentioned in **3.2.2**.

3.2.4 Growth measurements

Following stress application, seedling height, root length, and foliar and root fresh masses (FM) were measured (n = 50). Total leaf area was determined by version 4.0 of the generative pre-trained transformer (GPT-4) architecture developed by OpenAI (USA), which was prompted to identify and calculate the area of leaves in an image against an internal reference scale, adapted from Minervini et al. (2014) and Hariadi et al. (2018). Sample images were converted into hue, saturation, and value colour space components and then segmented to create a binary representation, highlighting green-coloured areas. Morphological operations such as erosion and dilation were applied to clean up the binary image and remove noise, facilitating the identification of contours to outline the boundaries of the green area. Finally, the area of all contours was summed in GPT-4 using the OpenCV function cv2.contourArea (Open Source Computer Vision, Russia) to obtain the total leaf area (n = 50).

3.2.5 Genotype tracking of stress-tolerant clones

The stress tolerance ability of treated seedlings was assessed by a vigour index rating (V) system calculated from ***Equation 3.2.***

Equation 3.2: Seedling vigour index (V):

$$V = \frac{a}{\bar{a}^c} + \frac{b}{\bar{b}^c} + \frac{c}{\bar{c}^c} + \frac{d}{\bar{d}^c} + \frac{e}{\bar{e}^c}$$

Where: a = height, b = root length, c = leaf area, d = foliar fresh mass (FM), e = root FM, and: $\bar{a}^c$, $\bar{b}^c$, $\bar{c}^c$, $\bar{d}^c$, $\bar{e}^c$ = mean values of corresponding measurements for untreated (control) seedlings.

This novel seedling growth index equation was necessary to combine multiple stress-affected growth parameters into a single rating (V) to comprehensively compare and identify the best-performing genotypes at the optimal harvestable age and condition for consumption – young and unblemished leaves and stems. Moreover, most stress tolerance and vigour rating indices incorporate a single parameter, typically foliar FM, and assume linearity between stressors and growth, inadequately quantifying and oversimplifying potentially nonlinear and complex stress responses (Ranal and Santana 2006; Castan et al. 2018). The condition in stressed genotypes where $V \geq 5$, signifying cumulatively greater than or equal performance to the control, indicated the absence of stress-induced growth constraints. However, achieving such equivalence was rare due to the overall detrimental impact of stress on growth. Therefore, a selection threshold of $V > 4$ was used to set a sufficiently stringent yet attainable standard for identifying the top-performing genotypes, thereby reducing ambiguity and maintaining sample size in the selection process.

Following data collection, shoot cuttings (5-10 cm in length, diagonally excised below the node) of the identified genotypes were maintained in distilled water containing 1% indole-3-butyric acid (IBA) (PBR Trading International, South Africa) for approximately eight days until adventitious root emergence and root growth to at least 1 cm. Whole plants (roots and shoots) were then transferred to plastic flowerpots (120 mm diameter x 150 mm depth) containing the same substrate used in flowerpots outlined earlier (**3.2.2**). These selected genotypes were then placed in greenhouse mist tents and watered daily with municipal water for 3 min in the evenings at ~80 mL per pot. Dr Fisher's Classic Multifeed® (AECI, South Africa) solution (1.3 g/L) was applied to the substrate once every seven days at 15 mL per clone until the cuttings grew to the approximate vegetative stage of the initial parent genotypes at the onset of water-deficit treatment (5-6 leaves).

3.2.6 *In vitro* propagation of selected genotypes

Nodal explants harvested from cuttings of selected superior genotypes of *A. dubius* were clonally propagated (n = 6) according to the protocol established by Shaik et al. (2022). The explants were decontaminated in 1% (v/v) sodium hypochlorite (NaOCl) (Reckitt Benckiser, South Africa) with two drops of Tween 20® (Bayer, South Africa) for 10 min (Shaik et al. 2022) and then embedded in semi-solid shoot multiplication medium (half-strength Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962), 30 g/L sucrose, 2 mg/L 6-benzylaminopurine (BAP), 0.5 mg/L indole-3-acetic acid (IAA), pH 5.7) for three weeks and then subcultured onto shoot elongation medium (half-strength MS, 30 g/L sucrose, 0.1 mg/L BAP, 0.1 mg/L IAA, pH 5.7) for two weeks and then finally transferred to rooting medium (half-strength MS, 30 g/L sucrose, 0.1 mg/L IAA, pH 5.7) for a further two weeks. All media were steam sterilised in an HL-341 autoclave (Gemmy Industrial Corporation, Taiwan) at 121 °C and 15 psi for 20 min.

The number of emergent shoots from individual explants was recorded weekly throughout the shoot multiplication period. Thereafter, the shoot elongation and root proliferation rates were measured during growth on each medium. Following acclimatisation (plantlets were potted in soil and sealed inside plastic bags for 2 weeks, then transferred to the greenhouse mist tent for 4 weeks (Shaik et al. 2022)), the yield of individual explants was recorded. Wild-type specimens which were not exposed to stress treatments were used as a control. Furthermore, upon the emergence of the fifth or sixth leaves, the clonal genotypes were subjected to the most stringent stress treatments, as outlined in section **3.2.3**.

3.2.7 Leaf water pressure potential

Leaf water potential (Ψ_{Leaf}) of cutting and *in vitro* propagated clones were measured using a modernised iteration of Scholander's pressure chamber (Scholander et al. 1964), as illustrated in ***Figure 3.2***. Leaf petioles (n = 3) were placed in clear plastic bags and excised intermittently throughout the stress treatments. The samples were excised after a 30 min stomatal equilibration phase. The cut ends were then inserted into the pressure chamber and secured with the rubber gasket, thus creating an airtight seal with the cut end of the petiole or stem exposed outside the chamber (***Figure 3.2***). Air

pressure was increased inside the chamber until fluid was visibly extruded through the exposed petiole and stem segments, at which time the pressure reading was recorded from the gauge.

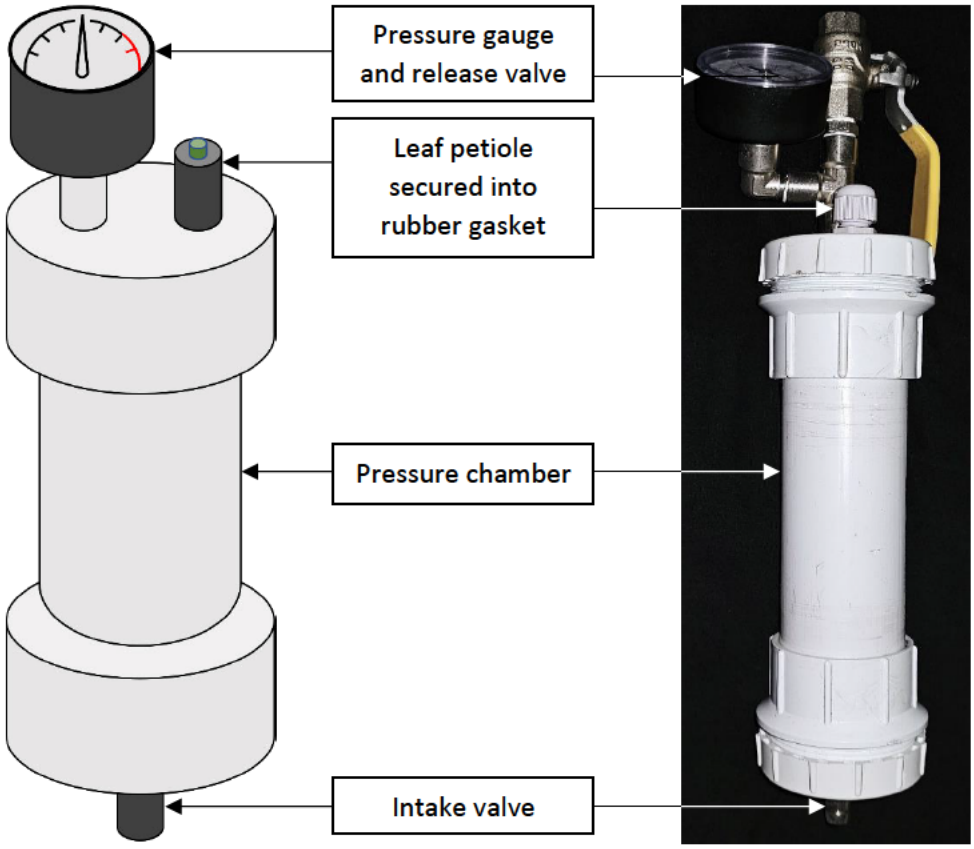


Figure 3.2: Modified Scholander pressure chamber used to measure leaf water potential (Ψ_{Leaf}).

3.2.8 Chlorophyll content

The chlorophyll content of a 1 cm^2 leaf disc excised from newly developed, unblemished leaves of selected clonal cuttings and micropropagated genotypes were spectrophotometrically quantified at the conclusion of the 2nd and 3rd screening stages (***Scheme 3.1***). The leaf discs were briefly rinsed in distilled water, individually transferred into 2 mL microcentrifuge tubes containing 1.5 mL 99.9% chilled ethanol (Protea Lab Services, South Africa) and stored on ice in the dark for 24 h. The samples were then centrifuged (Eppendorf 5710R®, Germany) for 5 min at 10,000 rpm and 4 °C. The resultant supernatant was analysed by a UV-1800® spectrophotometer (Shimadzu, Japan) at wavelengths of 660 nm (chlorophyll-a) and 640 nm (chlorophyll-b) (n = 3). Thereafter, the total chlorophyll content was calculated (Ritchie 2008).

3.2.9 Proline content

The method established by Bates et al. (1973) was used to quantify the proline content of genotypes exposed to water-deficit and heat stress treatments. Fresh leaf tissue (0.5 g) was homogenised in 5 mL of 3% sulfosalicylic acid (Merck, Germany) and centrifuged (Eppendorf 5710R®, Germany) at 10,000 rpm for 10 min to collect the supernatant (n = 3). For the assay, 2 mL of extract was mixed with 2 mL of acid ninhydrin (Merck, Germany) and 2 mL of glacial acetic acid (Protea Lab Services, South Africa). The mixture was incubated in a boiling (100 °C) water bath for 1 h, followed by immediate cooling in an ice bath. Then, 4 mL of toluene (Protea Lab Services, South Africa) was added and vortexed, and the toluene layer was separated and measured at 520 nm in a spectrophotometer (Shimadzu, Japan). A standard curve (Appendix, ***Figure 7.2***) was prepared using serially diluted L-proline (Merck, Germany) solutions (12.5, 25, 50, 75, 100 µg/mL). The proline content of each sample was calculated as µg/g of fresh weight (FW) using linear regression.

3.2.10 Protein content

The Bradford method of total protein quantification was used to assess the protein concentrations in leaves of selected clonal cuttings and micropropagated genotypes (n = 3) (Bradford 1976). Homogenisation was achieved by grinding ~100 mg of tissue in liquid nitrogen (LN_2) (Afrox, South Africa), and proteins were extracted using TRIzol™ Reagent (Life Technologies, Netherlands) according to the manufacturer's specifications. Coomassie Brilliant Blue dye (Merck, Germany) was added to each sample (n = 3), resulting in a shift in the dye's absorption spectra, measured at 595 nm using a spectrophotometer (Shimadzu, Japan). A calibration curve was established using serial dilutions (12.5, 25, 50, 75, 100, and 150 µg/mL) of the protein standard, bovine serum albumin (Merck, Germany). The unknown protein concentration of the samples was then determined using linear regression analysis of the measured absorbance values plotted against the standard curve (Appendix, ***Figure 7.1***).

3.2.11 Protein characterisation

Mass spectrometry was performed using electrospray ionisation (ESI) in positive ion mode, with the mass analyser set to detect ions within the m/z range of 100-2000. This configuration provided a platform for the characterisation of the crude proteins extracted from selected stress-tolerant genotypes. The analysis was conducted using an Acquity Premier Ultra-Performance Liquid

Chromatography (UPLC) system coupled with a Synapt XS Mass Spectrometer (Waters, USA). Chromatographic separation was achieved on a Waters Atlantis dC18 column (3 µm, 2.1 × 30 mm). The mobile phases consisted of 0.1% formic acid in deionised water (solvent A) and methanol (solvent B). The UPLC was operated at a flow rate of 1.5 mL/min, with the column temperature maintained at 30 °C. A gradient elution program was employed, starting with 5% solvent B for 0.5 minutes, increasing linearly to 80% solvent B over 10 minutes, and holding at 80% for 3 minutes before re-equilibration at 5% solvent B. A photodiode array detector monitored absorbance within the wavelength range of 210-600 nm. Following the acquisition of mass spectra, the resulting data were analysed using the National Center for Biotechnology Information protein databases to identify potential proteins present in the samples.

3.2.12 RNA extraction, quantification, and qualitative control

Foliar tissue, amounting to three unblemished young leaves, and root tissue samples were randomly excised from three of the tallest and most vigorously elongating clonal cuttings and micropropagated genotypes from each stress treatment (n = 3). The tissue samples were rinsed with distilled water for 10 s and blotted dry with autoclaved paper towel. The samples were sectioned into allotments of 100 mg, snap-frozen in LN_2 (Afrox, South Africa), and then immediately prepared for RNA extraction or stored at -80 ˚C in an ultra-freezer (NuAire, USA).

Total RNA was isolated from 100 mg (fresh mass) of foliar tissue for each stress treatment using TRIzol™ Reagent (Life Technologies, Netherlands) according to the manufacturer's specifications. The RNA concentration was quantified using the RiboGreen® protocol for the NanoDrop™ 3300 Fluorospectrometer (Thermo Fisher Scientific, USA) by linear regression of the measured fluorescence values against an RNA standard curve (Appendix, ***Figure 7.3***). RNA quality and purity were evaluated by running 5 µg of the extract on 1% Tris-Acetate-Ethylenediaminetetraacetic acid (TAE) agarose gel (Thermo Fisher Scientific, USA) supplemented with 1% NaOCl (Reckitt Benckiser, South Africa) for 30 min at 110 V (Aranda et al. 2012). All RNA samples were aliquoted and stored at -80 ˚C in an ultra-freezer (NuAire, USA) until needed for RT-qPCR analysis.

3.2.13 RT-qPCR parameters

RT-qPCR analysis was completed using the GoTaq® 2-Step RT-qPCR System (Promega, USA). The cDNA template was synthesised from 1 µg of total RNA per the manufacturer's instructions. The qPCR final reaction volume of 20 µl was composed of 1 µl cDNA template (equivalent to 1 ng starting quantity of RNA), 10 µl of GoTaq® qPCR Master Mix (2X), 1 µl of both primers (500 nM) (see **3.2.14**), and 7 µl of nuclease-free water. No template controls (NTCs) were performed with 8 µl of nuclease-free water to account for the lack of cDNA, thus ensuring a constant final reaction volume.

The RT-qPCR thermocycling reactions were performed with three biological and two technical replicates of each stress treatment using the Eco™ Real-Time PCR System (Illumina, USA). All assays were carried out under the following conditions: one cycle of 95 ˚C for two min, 40 cycles of 95 ˚C for 15 s and 68 ˚C for one min for the degenerate primer pairs (dehydration-responsive element-binding protein 1A (DREB1A), annexin 1, and HSP70) and 60 ˚C for one min for the reference gene primer pairs. A melting curve was then generated from 95 to 65 ˚C to verify amplicon specificity and identify erroneous hairpin and primer dimer formation.

3.2.14 Primer design

Degenerate DREB1A, annexin 1, and HSP70 forward and reverse primers were developed using Base-By-Base with j-CODEHOP integration (Brodie et al. 2004). Multiple known amino acid sequences of DREB1A, annexin 1, and HSP70 expressed by stress-tolerant species within the same class (Dicotyledons) as *A. dubius* were aligned using a constraint-based multiple alignment tool (COBALT) (Papadopoulos and Agarwala 2007) to identify highly conserved regions (Appendix, ***Table 7.1***, ***7.2***) and input into j-CODEHOP.

The degenerate primers used for DREB1A quantification were:

5'-CGGCGGAACTCCGGCAARTGGGT-3' (forward)

5'-CAGCCAGATCCGGGTYTTYTTRTT-3' (reverse)

The degenerate primers used for annexin 1 quantification were:

5’-CGAGATCGACTGAAGGTGATCGCGARGARTAYCA-3’ (forward)

5’-CGTCCTCGCCCAGCAGGGCCACCAGCATYTTYTCRTA-3’ (reverse)

The degenerate primers used for HSP70 quantification were:

5’- GGAGCTGAACATGGACCTGTTCCGGAARTGYATGGA-3’ (forward)

5’-CCAGCACCACGTCGTGCACGGTGGAYTTRTCCATYTT-3’ (reverse)

The reference gene used for RT-qPCR normalisation was malate dehydrogenase (MDH). Primers for this reference gene were developed by González-Rodríguez et al. (2019) with the following sequences:

5’-TGCTCCCAACTGCAAGGTTC-3’ (forward)

5’-ACCAAGTGCCCTGTTGTGAT-3’ (reverse)

3.2.15 RT-qPCR Analysis

LinRegPCR (Ruijter et al. 2009) was used to determine the fluorescence threshold and the mean RT-qPCR efficiency per amplicon. The generated quantitation cycle (Cq) values and RT-qPCR efficiencies (E) of the genes of interest (GOI), DREB1A, annexin 1, and HSP70, and the internal reference gene (MDH) were used to calculate the relative quantification (RQ) for each stress treatment as described by ***Equation 3.3*** below (Pfaffl et al. 2004). These data were then expressed as relative fold changes per stress treatment. BestKeeper was used to assess the stability of the reference gene by calculating the standard deviation (SD) and CV% (Pfaffl et al. 2004).

Equation 3.3: Pfaffl method of relative quantification:

$$RQ = \frac{(E_{unknown})^{\Delta Cq_{unknown}}}{(E_{control})^{\Delta Cq_{control}}}$$

Where: $\Delta Cq_{unknown}$ = Cq of GOI in control treatment – Cq of GOI in unknown treatment, and: $\Delta Cq_{control}$ = Cq of MDH in control treatment – Cq of MDH in unknown treatment.

The relative quantification (RQ) of the GOI was calculated by determining the RT-qPCR efficiencies (E) and differences in quantitation cycles (ΔCq) between stressed samples and the control.

3.2.16 Measurements and data analyses

Growth and physiology data of parent and micropropagated clonal genotypes were analysed using the Statistical Package for the Social Sciences (SPSS®) version 29.0 (IBM, USA). The data sets were assessed for normality (Shapiro-Wilk test) and metavariance (Levene's test) to satisfy the assumptions of the post hoc test. Significant interactions among treatments were identified by univariate ANOVAs followed by Tukey post hoc testing. In instances where the assumption of homogeneity of variances was violated, the pairwise Games-Howell post hoc test was applied, and when normality was violated, the non-parametric alternative to a one-way ANOVA, the Kruskal-Wallis test, was used. A probability of $p < 0.05$ was used as the threshold for statistical significance. A paired samples T-test was used to determine differences between pre- and post-micropropagation parameters.

3.3 Results and discussion

3.3.1 Growth data meta-analysis

Salient *A. dubius* growth parameters documented in the scientific literature are compiled in ***Table 3.1***, including the results of the present study. Notably, the CV% for all parameters exceeded the 25% threshold, demonstrating a high degree of phenotypic plasticity within the species. Leaf area was the most variable dataset, emphasising the remarkable range of leaf numbers and sizes among specimens. Interestingly, this is one of the most important constraints affecting the agronomic production potential of herbaceous vegetables such as *A. dubius*. Furthermore, significant variability was recorded within studies conducted on multiple *A. dubius* genotypes, highlighting the considerable genotypic variation exhibited by this species under controlled conditions (Sindhu 2002; Celine et al. 2007; Arti et al. 2018; Nyonje et al. 2022; Shaik et al. 2022). The maximum and minimum values are represented from studies which investigated more than 10 genotypes, further emphasising the vast extent of genotypic variability characteristic to *A. dubius* specimens. Contrastingly, root measurements exhibited the lowest variability, suggesting relatively greater consistency across studies.

Table 3.1: Meta-analysis of documented growth characteristics of multiple *A. dubius* genotypes. ND = no data, DM = dry mass.

Genotype	Age (d)	Foliar height (cm)	Root length (cm)	Leaves	Stem diameter (mm)	Leaf area (cm^2)	Foliar DM (g)	Root DM (g)	Reference
Maximum	56	35	69	22	ND	307.2	ND	4.4	
Mean	56	25.3	49.9	17.1	ND	214.5	ND	3.3	Present study
Minimum	56	17	29	12	ND	122.1	ND	2.3	
ND	20	44.4	6.7	12.2	ND	97.8	ND	ND	Amma & Rajalakshmi 2023
ND	40	30.6	14.1	19	ND	13.8	5.1	ND	Širić et al. 2023
ND	60	19.6	25.4	16	ND	ND	0.5	0.7	Shaik et al. 2022
ND	28	112.6	ND	ND	ND	ND	ND	ND	Lin et al. 2022
ND	30	39.6	ND	ND	ND	ND	ND	ND	Dwiratna et al. 2022
ND	60	27.8	14.4	12.6	1.2	12.7	ND	1.5	Oluoch 2021
AM1908	35	66.8	ND	ND	ND	1701.8	ND	ND	Nyonje et al. 2022
AM1910	35	42.4	ND	ND	ND	936.4	ND	ND	
ND	38	25.8	9.9	15.5	2.6	400	1.1	0.5	Hoang et al. 2019
IV6	60	42.7	14.1	45.4	ND	ND	ND	ND	
I7	60	22.4	10.4	28.5	ND	ND	ND	ND	
II4	60	34.3	14.6	31.6	ND	ND	ND	ND	Arti et al. 2018
III1	60	21.6	9.9	36.8	ND	ND	ND	ND	
V4	60	28.5	14.4	32.0	ND	ND	ND	ND	
ND	55	50	ND	ND	ND	506.8	4.1	1.0	Lubbe et al. 2016
ND	40	ND	14	14	ND	ND	ND	ND	Abhilash et al. 2013
Am 7	60	48.2	ND	ND	4.4	ND	ND	ND	
Am 86	60	62.9	ND	ND	4.3	ND	ND	ND	Celine et al. 2007
Am 88	60	30.7	ND	ND	5.6	ND	ND	ND	
Am 90	60	59.2	ND	ND	4.4	ND	ND	ND	
Maximum	55	58.2	ND	ND	5.9	ND	ND	ND	Sindhu 2002
Minimum	55	32.9	ND	ND	2.5	ND	ND	ND	
ND	42	4.5	ND	ND	ND	ND	ND	ND	Potluri & Persad 1998
Mean	**48**	**40**	**19**	**23**	**3.9**	**455**	**2.69**	**1.8**	
CV (%)	**28**	**53**	**75**	**45**	**39**	**114**	**72**	**74**	

For the present study: maximum, mean, and minimum values of *A. dubius* genotypes grown under the 4-day (control) watering regimen are presented (n = 250).

The lower variability in root measurements indicates that this parameter is relatively stable and consistent across different studies on *A. dubius* and, therefore, genotypes. This species exhibits a conserved or consistent response despite differing nutrient availabilities, substrates, and temperatures, implying that there are conserved genetic factors influencing the observed stability of root growth. This consistency may be attributed to shared genetic pathways or regulatory mechanisms, highlighting the potential importance of root length as a key trait in the adaptation of

this species to water-deficit stress. Plants often adjust their root architecture to optimise water uptake, and a stable root length may indicate its significance in coping with water scarcity (He at al. 2017; Bauw et al. 2018; Gao et al. 2022). Therefore, root length is positioned as a reliable biomarker for assessing the impact of water-deficit stress on *A. dubius* growth when studying the effects of water scarcity.

The stability in root length measurements also suggests that this parameter is linked to water use efficiency. Plants with a consistent root length are more efficient in utilising available water resources, contributing to their ability to withstand water-deficit conditions (Comas et al. 2013). The low variability in root length emphasises the importance of further research into the mechanisms and physiological processes associated with root development and water stress response in *A. dubius*, providing insights into the adaptive strategies of this species. Furthermore, understanding the genetic stability of specific traits can inform strategies for selecting tolerant genotypes and improving crop varieties. These findings provide a baseline for understanding the natural variation in growth among different genotypes and are valuable for future comparisons under stress conditions. The low variability further implies that certain root characteristics are vital for the optimal functioning of roots. Identifying these characteristics is necessary for understanding plant-root-stress interactions.

3.3.2 Growth characteristics

One-month-old *A. dubius* specimens are depicted in ***Figure 3.3***, each representing a set of 50 genotypes sourced from different regions across Kwazulu-Natal, South Africa. Notably, diverse phenotypic traits were observed among these specimens. Plants grown from seeds sourced in Westville exhibited advanced development, with a significant proportion (> 50%) displaying flowering (***Figure 3.3*** E), a characteristic absent in seedlings from other locales. Furthermore, an abundance of leaves was observed in many Westville genotypes, surpassing the leaf count of most specimens sourced from other regions. In contrast, the seedlings from Amanzimtoti exhibited larger leaves in comparison to other genotypes, demonstrating phenotypic differences in leaf morphology. These results suggest region- or genotype-specific traits, hinting at the influence of geographic origin and intraspecific variation on the phenotypic expression of this species.

Flowering in Westville specimens suggests potential genetic adaptative mechanisms linked to region-specific environmental parameters, including soil composition, elevation, and climate (Koltai and Kapulnik 2011; Lu et al. 2021; Barragán-Fonseca et al. 2022). From all tested seed sources, Westville was the highest elevated location, where environmental conditions such as increased weather exposure, shorter growing seasons, and decreased pollinator visitation rates possibly favoured flowering (Totland 2001; Zhao and Wang 2015; Javid et al. 2023). Additionally, self-shading is generally increased at higher elevations due to shorter flowering stalks, reducing the inflorescence display area (Fabbro and Körner 2004).

Figure 3.3: Representative morphological variability of one-month old *A. dubius* specimens sourced from 5 localities around Kwazulu-Natal, South Africa (A: Amanzimtoti, B: Genozzano, C: Tongaat, D: Verulam, E: Westville). Arrow points to inflorescent spike.

Figure 3.4 A-G summarises various growth characteristics of water-deficit-stressed *A. dubius* plants grown from seeds found throughout KwaZulu-Natal, South Africa. Seeds sourced from Genozzano and Tongaat grew into plants having the greatest height under the highest tested level of water-deficit stress (16-day watering schedule) (***Figure 3.4*** A). Seedlings from Genozzano also yielded the highest leaf and stem FM (***Figure 3.4*** B), and the Tongaat and Westville specimens had the longest roots during the 16-day watering regimen (***Figure 3.4*** D). However, Westville seedlings demonstrated the lowest root FM, while there was no significant difference for this parameter among seedlings from the other locations (***Figure 3.4*** E). Additionally, there were no statistical differences in leaf area (***Figure 3.4*** C) and root DM (***Figure 3.4*** F) among all seedlings undergoing the highest, 16-day watering schedule, indicating a common physiological response or limitation among the tested seedlings. This represents a critical threshold tolerance level beyond which water scarcity affects these parameters uniformly. Conversely, the observed standard deviations indicated high variability within the datasets of these

parameters, suggesting less consistency, posing challenges to drawing reliable conclusions, and preventing the determination of true differences in growth amongst the seed groups. Factors contributing to high standard deviations include inherent biological variability due to genotypic differences among individual specimens.

The discrepancy observed in Westville seedlings having the longest roots, but the lowest FM could indicate a distinct strategy to cope with water scarcity (Vile et al. 2011; Comas et al. 2013). Although the root length was extended, the lower FM suggests prioritisation of root elongation and surface area over biomass accumulation in response to water-deficit conditions, serving to enhance soil exploration, water absorption, and resource conservation (Zafar et al. 2019; Meyer et al. 2021). While lower root FM might indicate reduced biomass accumulation in the short term, the allocation of resources towards root elongation could confer long-term fitness advantages because elongated root systems establish deeper, more extensive networks, enhancing the plant's resilience to recurrent or prolonged water-deficit periods.

Generally, all measured growth parameters decreased as water-deficit increased. However, seedlings from Genozzano showed no statistical difference in height between the 4- (25.7 ± 3.0 cm) and 8-day (24.9 ± 2.6 cm) regimens, and the Amanzimtoti seedlings yielded no change in height between the 8- (22.3 ± 2.2 cm) and 12-day (21.5 ± 3.1 cm) regimens (***Figure 3.4*** A). Furthermore, there was no significant difference in height for seedlings from Tongaat and Westville between the 12- (20.5 ± 3.0 and 17.9 ± 2.4 cm, respectively) and 16-day (19.9 ± 2.3 and 17.1 ± 2.8 cm, respectively) treatment groups. Regarding leaf and stem FM, there were no significant differences observed among seedlings from Genozzano, Tongaat, and Verulam undergoing the 4- (53.2 ± 5.1, 46.6 ±5.1, and 42.6 ± 4.3 g, respectively) and 8-day (51.2 ± 4.7, 45.8 ± 4.0, and 43.4 ± 5.0 g, respectively) watering regimens (***Figure 3.4*** B). Moreover, the leaf area of Genozzano and Tongaat specimens did not change between the 12- (149.6 ± 35.4 and 149.0 ± 35.0 cm^2, respectively) and 16-day (137.9 ± 25.6 and 149.1 ± 37.3 cm^2, respectively) groups, and Verulam seedlings performed best during the 8-day regimen, yielding the greatest leaf area among all treatments (207.8 ± 21.8 cm^2) (***Figure 3.4*** C).

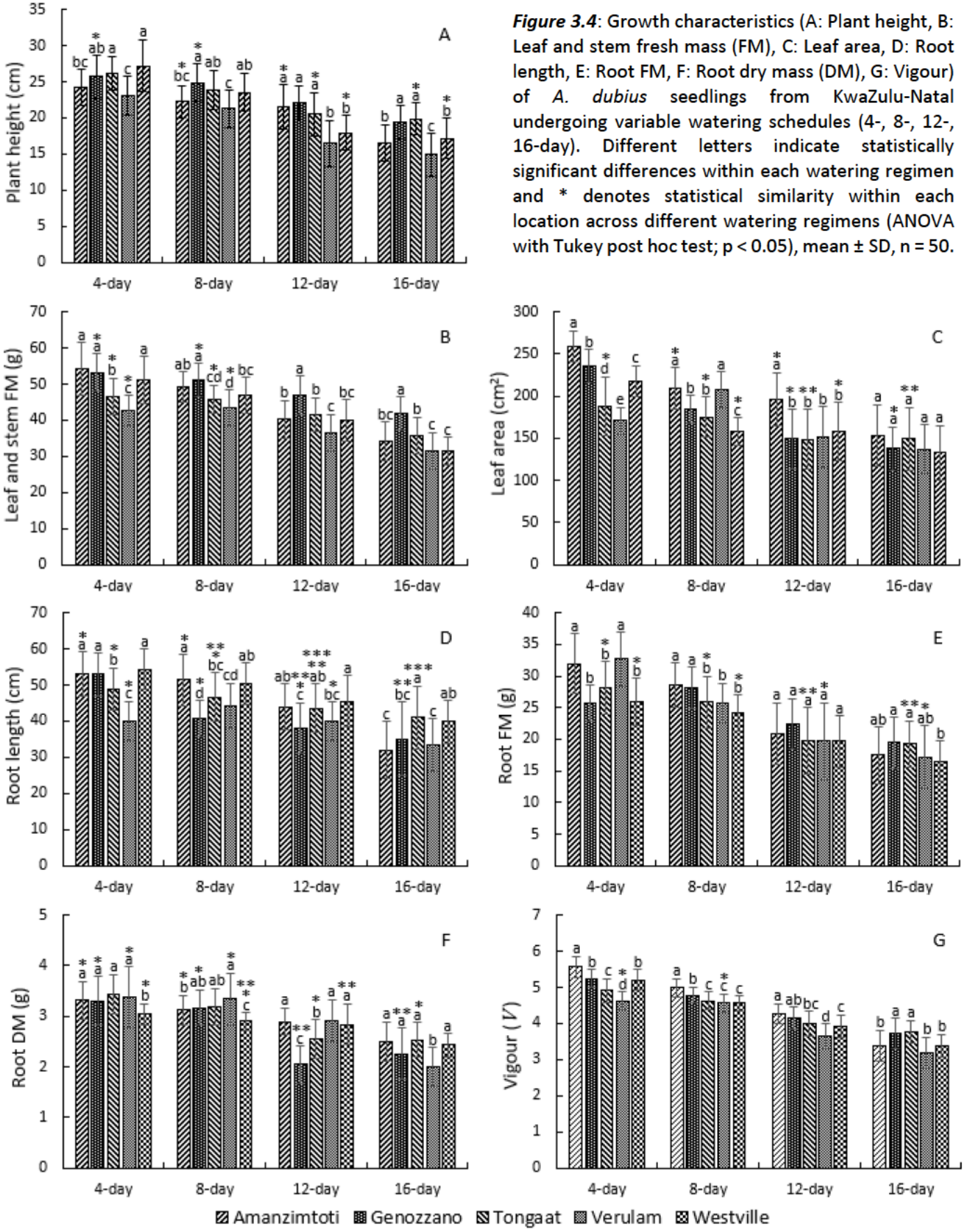


Figure 3.4: Growth characteristics (A: Plant height, B: Leaf and stem fresh mass (FM), C: Leaf area, D: Root length, E: Root FM, F: Root dry mass (DM), G: Vigour) of *A. dubius* seedlings from KwaZulu-Natal undergoing variable watering schedules (4-, 8-, 12-, 16-day). Different letters indicate statistically significant differences within each watering regimen and * denotes statistical similarity within each location across different watering regimens (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, n = 50.

A similar trend was observed in the root length of Verulam seedlings and root FM of Genozzano seedlings, wherein these cohorts, respectively, yielded the longest roots (44.4 ± 6.1 cm) (***Figure 3.4*** D) and highest FM (28.1 ± 3.3 g) (***Figure 3.4*** E) under the 8-day watering regimen. Additionally, the root lengths of Verulam seedlings were similar between the 4- (40.0 ± 5.5 cm) and 12-day (40.0 ± 5.5 cm) schedules. Moreover, the root lengths of Amanzimtoti specimens were similar between the 4- (53.0 ± 6.2 cm) and 8-day (51.4 ± 7.1 cm) regimens, Tongaat specimens showed similarity between the 4- (48.9 ± 5.9 cm) and 8- (46.6 ± 6.7 cm), 8- and 12- (43.6 ± 6.7 cm), and 12- and 16-day (41.1 ± 8.7 cm) watering schedules and Genozzano seedlings demonstrated similar root lengths between the 8- (40.8 ± 5.0 cm) and 12- (38.1 ± 6.9 cm) and 12- and 16-day (35.1 ± 10.3 cm) regimens.

The root FMs of Tongaat and Westville specimens were statistically similar between the 4- (28.0 ± 4.2 and 25.9 ± 3.7 g, respectively) and 8-day (25.9 ± 4.1 and 24.2 ± 2.8 g, respectively) groups and for seedlings from Tongaat and Verulam between the 12- (19.8 ± 5.4 and 19.7 ± 6.1 g, respectively) and 16-day (19.2 ± 3.7 and 17.2 ± 4.9 g, respectively) watering regimens (***Figure 3.4*** E). The root DMs of seedlings from Amanzimtoti, Genozzano, Verulam, and Westville were similar between the 4- (3.3 ± 0.4, 3.3 ± 0.5, 3.4 ± 0.6, and 3.0 ± 0.2 g, respectively) and 8-day (3.1 ± 0.3, 3.2 ± 0.3, 3.3 ± 0.5, and 2.9 ± 0.2 g, respectively) groups (***Figure 3.4*** F). Westville seedlings also showed similarity in root DMs between the 8- (2.9 ± 0.2 g) and 12-day (2.8 ± 0.4 g) schedules, and Genozzano and Tongaat seedlings had similar root DM between the 12- (2.0 ± 0.4 and 2.6 ± 0.4 g, respectively) and 16-day (2.3 ± 0.5 and 2.5 ± 0.4 g, respectively) watering schedules.

Overall, Genozzano and Tongaat seedlings exhibited the most adaptability to water scarcity, manifesting in sustained growth metrics under the most extreme watering regimen (16-day). These results could be linked to specific environmental factors unique to these regions, including soil compositions and microclimates favouring adaptations against water-deficit stress. Such characteristics are reflective of adaptations that enable these specimens to effectively utilise limited water resources to thrive in stressful conditions (Reich et al. 2003; Baytar et al. 2018). Furthermore, these locations were the most geographically proximal and exhibited the most abundant presence of *A. dubius* specimens compared to the other locations. This ubiquity suggests natural selection favouring traits like enhanced seed dispersal, genetic variability, and adaptability, indicating ongoing selection pressures that drive adaptations for better fitness in water-limited conditions (Reich et al.

2003; Brouillette et al. 2013; Marais et al. 2016). The collective growth response of these specimens to water-deficit stress emphasises the complex resilience strategies, warranting further exploration into the specific mechanisms employed by individual seedlings from distinct geographical origins.

The most vigorous seedlings, each representing a unique genotype, from the 12- and 16-day treatment groups from each location were determined by applying ***Equation 3.2*** and selected for further assessment. The application of ***Equation 3.2*** consolidated various stress-affected growth parameters into a single vigour rating (V), enabling direct comparisons among genotypes undergoing various levels of water-deficit in relation to the 4-day watering regimen, which served as the control. Under the highest level of water-deficit (16-day), genotypes from Genozzano (3.7 ± 0.4) and Tongaat (3.8 ± 0.3) had the highest vigour ratings (***Figure 3.4*** G). Additionally, the Verulam genotypes maintained similar vigour throughout the 4- and 8-day treatments. The condition in water-stressed genotypes where $V \geq 5$, signifying cumulatively greater than or equal performance to the control, indicated the absence of stress-induced growth constraints. However, achieving such equivalence was rare due to the overall detrimental impact of water-deficit stress on growth. Therefore, a selection threshold of $V > 4$ was used to set a sufficiently stringent yet attainable standard for identifying the top-performing water-stressed genotypes, thereby reducing ambiguity and maintaining sample size in the selection process.

The representative range of seedling growth following exposure to varying levels of heat stress for 2 weeks is depicted in ***Figure 3.5***. Some seedlings from the 35, 40, and 45 °C treatment groups exhibited stunted growth with small, discoloured, and curled leaves and wispy stems (***Figure 3.5*** B2, C2, D2). However, other specimens undergoing the same treatments showed similar growth (***Figure 3.5*** B1, C1, D1) compared to the control (***Figure 3.5*** A), indicating genotype-specific variation. Most seedlings (> 50%) from the 30 and 35 °C groups demonstrated typical *A. dubius* growth characteristics and appearance, i.e., vibrantly green, simple, alternate, and elliptic-ovate-shaped leaves (***Figure 3.5*** A).

Figure 3.5: Representative one-month old *A. dubius* seedlings after undergoing heat stress treatments for 1 week. A: 30 (control), B: 35, C: 40, and D: 45 °C.

Table 3.2 lists the average growth characteristics of *A. dubius* seedlings following each heat stress treatment. Generally, growth parameters decreased as temperatures increased, with the exception of the 35 °C treatment group, which yielded similar values for all measured parameters to the 30 °C control group. Furthermore, between the 40 and 45 °C groups, there was no significant difference in height and number of leaves. The observed decrease in growth parameters with increasing temperatures aligns with the anticipated effect of heat stress on plant development (Hwang et al. 2018), indicating the sensitivity of some *A. dubius* seedlings to elevated temperatures. The observations of the 35 °C treatment group may indicate an adaptive response or an optimal thermal range of this species for the measured parameters. The lack of significant differences in height and leaf count between the 40 °C and 45 °C groups implies a potential threshold or saturation point in the adverse effects of higher temperatures on these growth parameters, suggesting that up to 45 °C, the detrimental impact on these parameters does not significantly escalate, possibly due to an upper limit of the adaptive or tolerance mechanisms of this species. These findings are supported by investigations into the heat tolerance of other amaranths, demonstrating the wide range of thermal tolerance exhibited by this genus (Steckel et al. 2004; Ye and Wen 2017). Moreover, amaranths are C_4 plants that thrive in warm conditions, renowned for their ability to photosynthesise at high temperatures with greater water use efficiency than many C_3 plants (Maseko et al. 2017; Baturaygil et al. 2021).

Table 3.2: Growth parameters of one-month old *A. dubius* seedlings undergoing high-temperature stress treatments for 1 month. Different letters indicate statistically significant differences for each parameter (ANOVA with Tukey post hoc test; $p < 0.05$), median ± SD, n = 50. FM = fresh mass.

Treatment (°C)	**Height** (cm)	**Root length** (cm)	**Number of leaves**	**Foliar FM** (g)	**Root FM** (g)	**Leaf area** (cm^2)	**Vigour** (*V*)
30 (Control)	18.5^a ± 3.1	26.3^a ± 2.2	12.4^a ± 1.7	25.3^a ± 5.4	14.7^a ± 2.3	121.4^a ± 7.4	5.0^a ± 0.4
35	19.3^a ± 4.2	25.7^a ± 3.2	12.0^a ± 3.2	25.6^a ± 5.6	13.6^a ± 3.3	123.3^a ± 11.4	5.1^a ± 0.5
40	15.2^b ± 4.5	15.4^b ± 3.8	8.3^b ± 4.5	17.8^b ± 7.3	9.4^b ± 3.2	89.5^b ± 14.5	3.6^b ± 0.6
45	11.4^b ± 5.2	11.4^c ± 4.8	7.1^b ± 4.0	10.1^c ± 7.4	5.5^c ± 3.5	64.6^c ± 19.4	2.5^c ± 0.5

The findings of the present study demonstrate that multiple growth parameters of different *A. dubius* genotypes are significantly affected by increasing levels of heat stress and can be used to preliminarily evaluate the heat tolerance capabilities of this species to identify tolerant genotypes. However, repeated exposure to elevated temperatures and more analyses are needed to elucidate the physiological and molecular responses of the identified genotypes, supplementing these initial findings and further screening for heat tolerance. Therefore, clonal cuttings from the selected genotypes were allowed to regrow in the growth chambers for 4 weeks before day cycles of 16 h at 30 (control), 35, 40, and 45 °C and night cycles of 8 h at 24 °C were reapplied (n = 3). Subsequently, 30 clonal genotypes survived the 45 °C treatment and were therefore labelled as heat (H) tolerant. These genotypes were selected for micropropagation and further analyses.

3.3.3 Leaf water pressure potential

The graphs in ***Figure 3.6*** represent the Ψ_{Leaf} of selected *A. dubius* clonal genotypes (derived from cuttings), which survived all watering schedules (4-, 8-, 12-, 16-day) for 4 weeks. None of the genotypes showed significant differences in Ψ_{Leaf} from week 1 to week 4 when watered once every 4 days. The absence of significant differences in Ψ_{Leaf} suggests that the 4-day watering frequency provided sufficient moisture to maintain stable Ψ_{Leaf} for all tested genotypes, regardless of inherent genetic variability (Shrestha et al. 2006; Androcioli et al. 2020). During the other, more stringent, watering regimens, Ψ_{Leaf} generally decreased with time. However, during the 8-day regimen, genotypes A10, G1, T3, T10, V3, and V6, maintained similar Ψ_{Leaf} over the 4-week experiment (-8.0 ± 1.4, -10.3 ± 1.3, -6.2 ± 1.5, -7.7 ± 1.1, -8.7 ± 1.4, and -7.8 ± 1.5 psi, respectively), and T3 (-8.7 ± 1.3 psi) and V3 (-8.8 ± 1.6 psi) also maintained similar Ψ_{Leaf} throughout all 4 weeks of the 12-day watering

regimen. Furthermore, genotypes A3, A10, G1, G7, and G8 demonstrated similar or increased Ψ_{Leaf} during the 12-day watering schedule (-9.2 ± 1.3, -13.4 ± 1.0, -11.1 ± 1.6, -13.1 ± 1.6, and -10.4 ± 1.3 psi, respectively). Additionally, genotypes T8 and T10 showed an initial decrease in Ψ_{Leaf}, which then stabilised for the remainder of the experimental period of the 12-day regimen (from -6.4 ± 2.1 and -6.4 ± 1.8 to -11.9 ± 1.2 and -11.7 ± 1.8 psi, respectively).

During the 16-day watering schedule, Ψ_{Leaf} did not significantly deviate throughout the 4-week treatment period in genotypes A3 (-11.8 ± 1.4 psi), A10 (-11.5 ± 1.5 psi), G3 (-12.8 ± 2.0 psi), G5 (-10.8 ± 1.8 psi), G7 (-14.7 ± 1.7 psi), T3 (-15.2 ± 2.1 psi), T5 (-14.3 ± 2.1 psi), and W7 (-13.2 ± 1.7 psi), and similar to the 12-day regimen, genotype G1 demonstrated similar or increased Ψ_{Leaf} during 16-day treatment (-13.5 ± 2.0 psi). Genotypes A2, T10, and V3 showed an initial decrease in Ψ_{Leaf}, which then stabilised over the remaining experimental period (from -14.6 ± 0.9 to -18.9 ± 1.7, -8.4 ± 1.7 to -16.5 ± 1.7, and -13.6 ± 1.1 to -17.4 ± 1.9 psi, respectively).

When considering the performance of each genotype across watering regimens over the 4-week treatment period, a familiar trend emerged wherein Ψ_{Leaf} generally became more negative as watering frequency decreased. However, genotypes A3, T3, V6 maintained similar Ψ_{Leaf} throughout the 4-, 8-, and 12-day watering regimens (-7.7 ± 1.4, -6.9 ± 1.2, and -8.6 ± 1.5 psi, respectively) and V4 had similar Ψ_{Leaf} across the 8-, 12-, and 16-day treatments (-10.9 ± 1.7 psi). Furthermore, genotypes A2, A10, T10, and W7 demonstrated similar Ψ_{Leaf} between the 4- and 8-day regimens (-6.9 ± 1.4, -7.1 ± 1.2, -7.5 ± 1.0, and -5.4 ± 1.4 psi, respectively), A2, A9, G1, T5, V3, and W2 had similar Ψ_{Leaf} between the 8- and 12-day regimens (-9.1 ± 1.7, -10.4 ± 1.3, -10.7 ± 1.5, -10.4 ± 1.6, -8.8 ± 1.5, and -11.3 ± 1.2 psi, respectively), and A3, A6, A10, and G3 showed similar Ψ_{Leaf} between the 12- and 16-day regimens (-10.5 ± 1.4, -11.3 ± 1.4, -12.5 ± 1.2, and -13.0 ± 1.7 psi, respectively). Moreover, genotype A9 shared similar Ψ_{Leaf} between the 8- and 16-day regimens (-11.9 ± 1.5 psi). The 16-day (-10.8 ± 1.8 psi) Ψ_{Leaf} measurements for genotype G5 were statistically similar to the 8- (-9.0 ± 1.5 psi) and 12-day (-11.5 ± 1.5 psi) values, but the 8- and 12-day measurements were statistically dissimilar to each other.

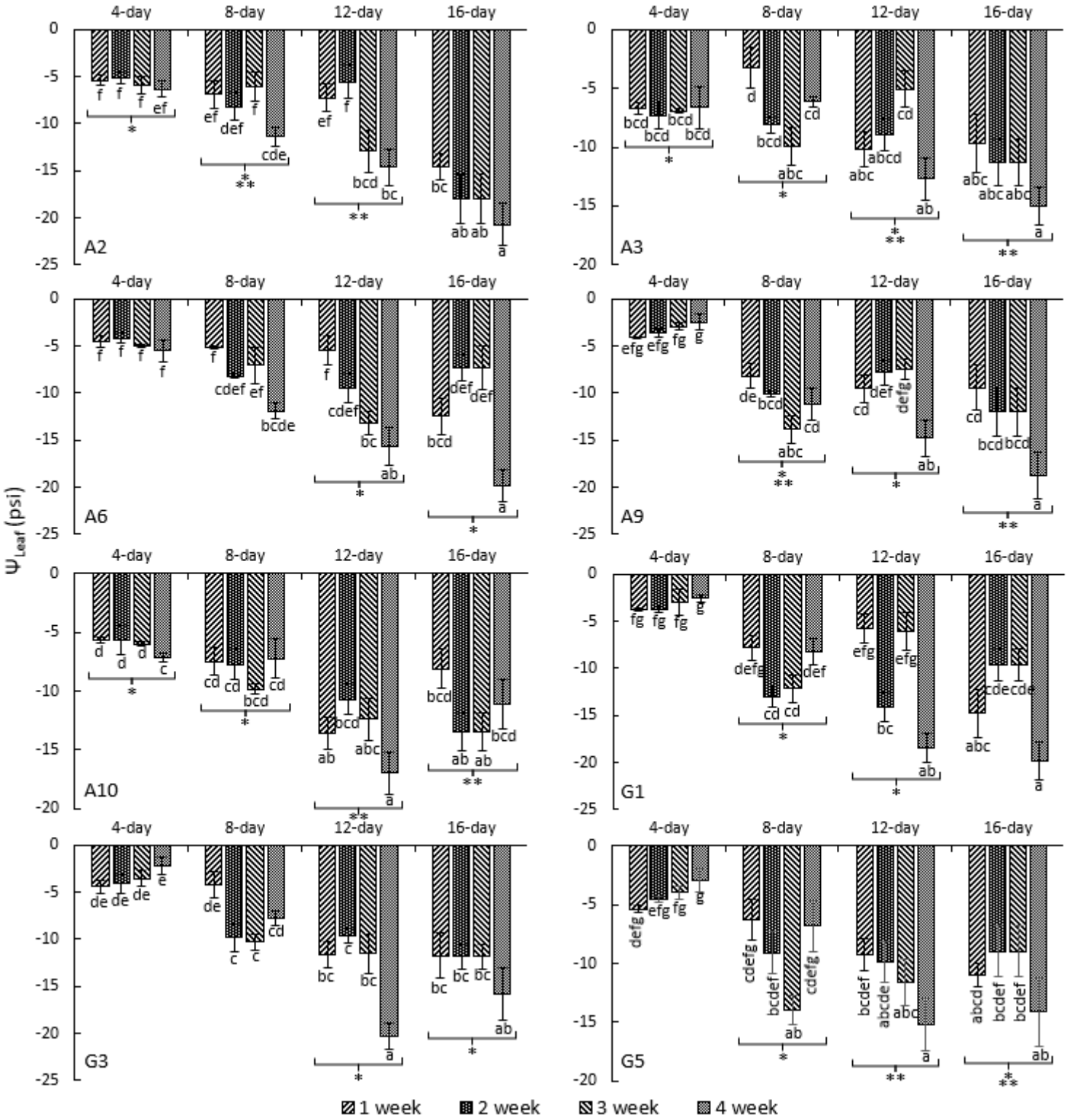


Figure 3.6: Water pressure potential in leaves (Ψ_{Leaf}) of clonal cuttings of *A. dubius* genotypes (n = 3) exposed to variable watering regimens (4-, 8-, 12-, 16-day) measured once a week for 4 weeks. Different letters indicate statistically significant differences for each parameter and * brackets denote similarity among watering regimens (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD.

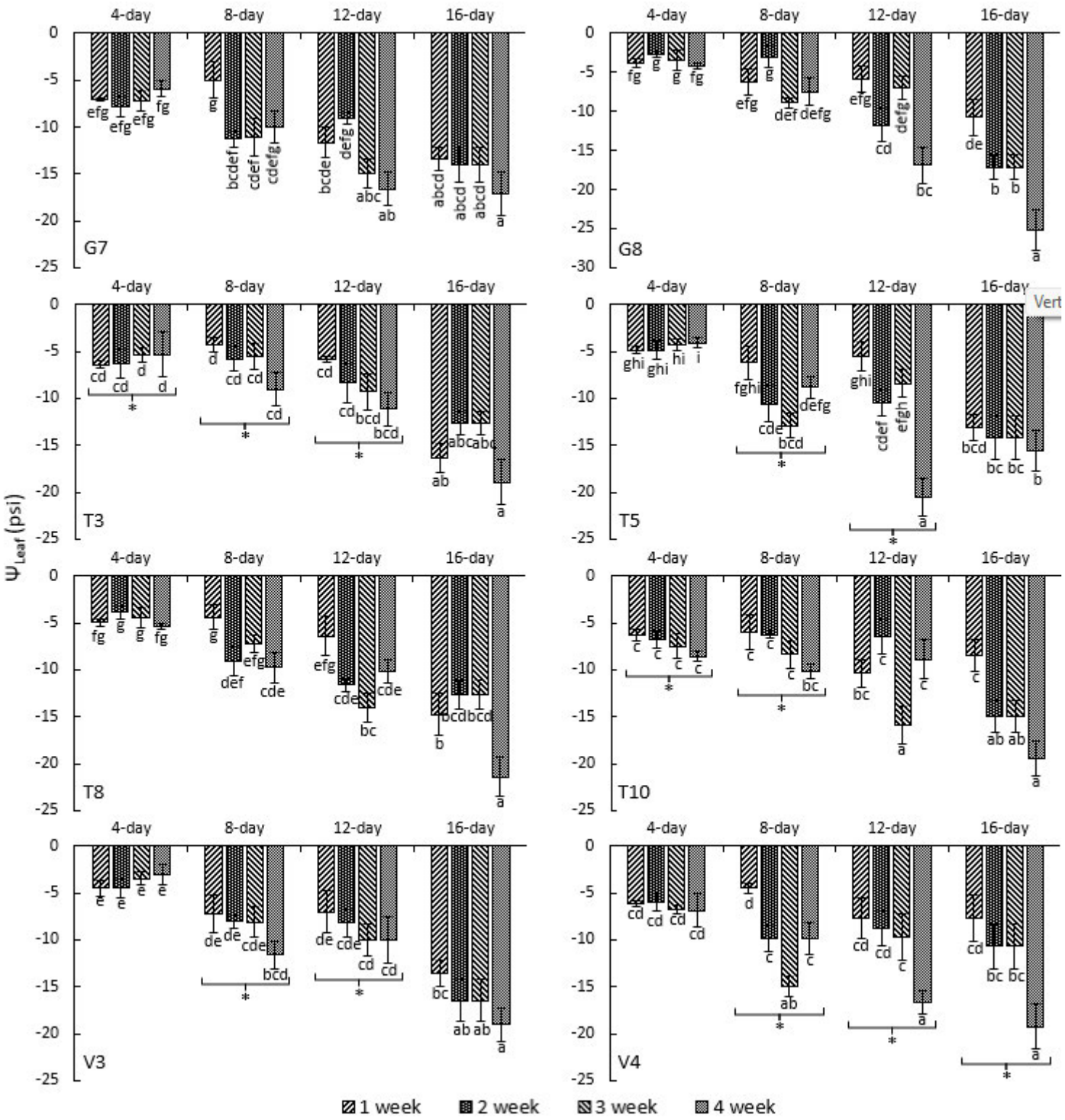


Figure 3.6 (continued): Water pressure potential in leaves (Ψ_{Leaf}) of clonal cuttings of *A. dubius* genotypes (n = 3) exposed to variable watering regimens (4-, 8-, 12-, 16-day) measured once a week for 4 weeks. Different letters indicate statistically significant differences for each parameter and * brackets denote similarity among watering regimens (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD.

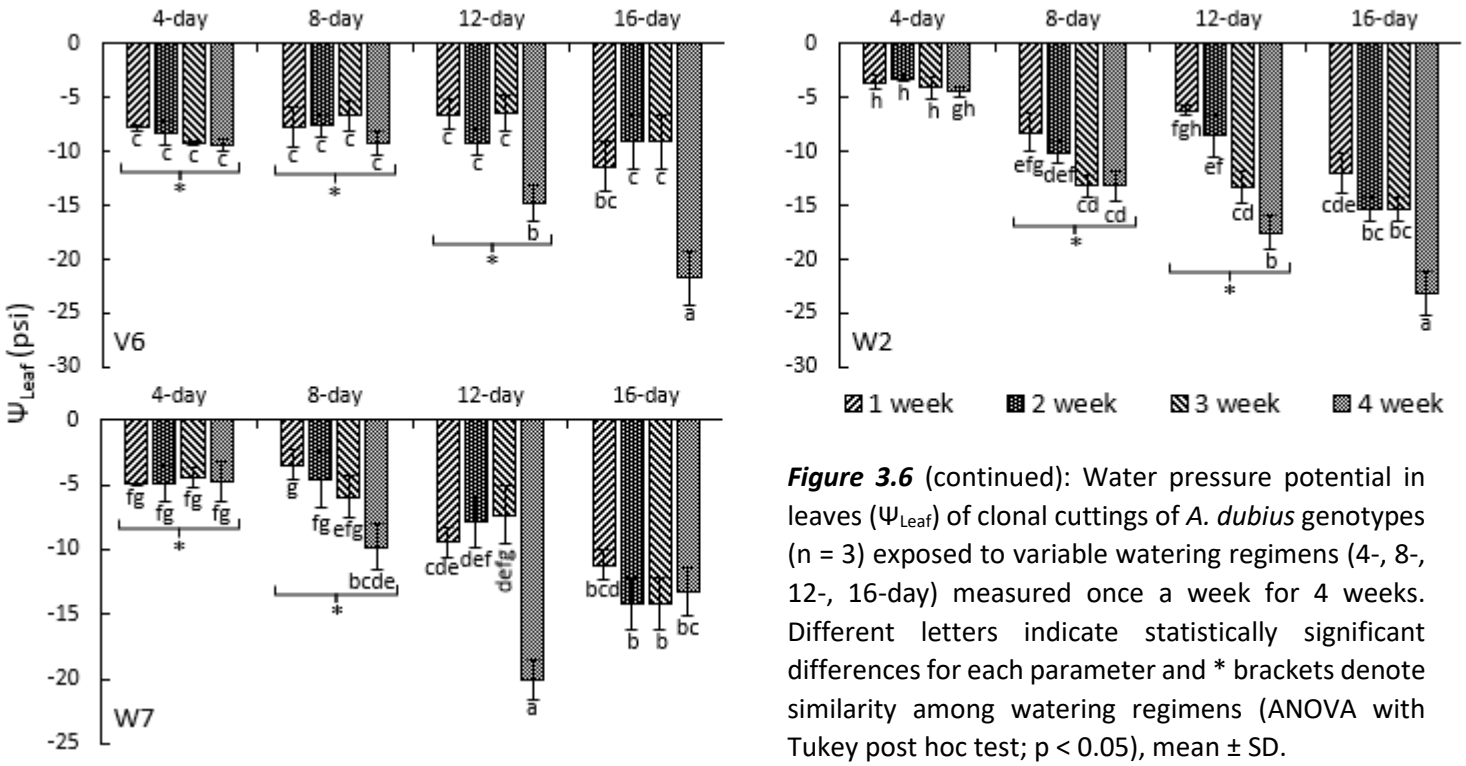


Figure 3.6 (continued): Water pressure potential in leaves (Ψ_{Leaf}) of clonal cuttings of *A. dubius* genotypes (n = 3) exposed to variable watering regimens (4-, 8-, 12-, 16-day) measured once a week for 4 weeks. Different letters indicate statistically significant differences for each parameter and * brackets denote similarity among watering regimens (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD.

The observed Ψ_{Leaf} data were a result of the varied cumulative physiological responses of each clonal genotype to water-deficit stress. The limited availability of water in the substrate reduced root water uptake, availing less water for vital physiological processes (Bavel and Cornelius 1996). Furthermore, turgor pressure decreased, resulting in decreased pressure against the cell walls, impacting overall Ψ_{Leaf}. Moreover, to mitigate water loss through transpiration, plants often close their stomata, limiting the uptake of CO_2, which is essential for photosynthesis, further affecting Ψ_{Leaf} (Monteith 1995; Hõrak et al. 2021). Additionally, tolerant plants initiate osmotic adjustments by accumulating solutes (osmolytes) within cells to maintain cellular water potential and prevent excessive water loss (Jones and Turner 1978). Varying degrees of these adjustments enable sustained cellular functions, contributing to the observed trends in Ψ_{Leaf}.

Under water-deficit conditions, cassava (*Manihot esculenta*) sheds its leaves as an adaptive response to conserve water (Liao et al. 2016). Additionally, water-deficit stress led to a reduction in the number of leaves and relative water content in the Brazilian peppertree (*Schinus terebinthifolia*) and medicinally relevant gabiroba (*Campomanesia xanthocarpa*) (Beltramin et al. 2020; Bartieres et al. 2020). Furthermore, the impact of water-deficit stress on the growth potential of peas (*Pisum sativum*) was evidenced through changes in physiological and biochemical processes, such as reduced

photosynthesis rates and altered energy assimilation (Soni et al. 2022). Water-deficit stress-induced biochemical and physiological responses in potatoes, such as changes in leaf gas exchange and photosynthetic pigment composition (Al-Selwey et al. 2023). The response of sorghum stomata to water-deficit has been shown to manipulate leaf conductance and transpiration rates, preserving water (Touré et al. 2019). Additionally, the leaf water potential of water-stressed lettuce (*Lactuca* sativa) and cowpeas (*Vigna unguiculata*) was significantly reduced during water-deficit conditions (Umebese et al. 2013; Bankole et al. 2018).

The increase or maintenance of Ψ_{Leaf} indicated that the selected *A. dubius* genotypes possess inherent adaptations (genetic traits), allowing maintenance of water potential despite prolonged water-deficit, resulting in higher tolerance through efficient water management strategies. Maintaining Ψ_{Leaf} is mediated by many physiological and anatomical traits which culminate in enhanced osmotic adjustment, including increased photosynthetic efficiency (see **3.3.5**), stomatal conductance, leaf and cuticle thickness, lower leaf area-to-volume ratios, and the production of enzymatic and non-enzymatic antioxidants (Farooq et al. 2009; Ribeiro et al. 2013; Petrov et al. 2017). Plant hormones have been shown to improve water-deficit tolerance by modulating enzymatic antioxidants and leaf gas exchange, which is mediated through increased protein synthesis (see **3.3.4**) of dehydration-responsive transcription factors (TFs), like DREB1A and other signal molecules, like annexin 1, enabling the maintenance of tissue water potential in tolerant genotypes (Pellegrineschi et al. 2004; Anjum et al. 2011; Schneider et al. 2020).

The graphs in ***Figure 3.7*** represent the Ψ_{Leaf} of selected *A. dubius* clonal genotypes measured 6 h into the night cycle at 24 °C and day cycle at 30, 35, 40, and 45 °C. Generally, Ψ_{Leaf} was similar between the 24 and 30 °C treatments, then became more negative as temperature increased. However, genotypes H2, H13, and H39 maintained Ψ_{Leaf} throughout the 30 to 40 °C treatments, and genotype H40 maintained Ψ_{Leaf} throughout the 35 to 45 °C treatments. The comparability in Ψ_{Leaf} between the 24 and 30 °C treatments signified a thermal range within which the selected genotypes maintained osmotic equilibrium. Additionally, genotypes H3, H7, H8, H20, H29, H32, H35, and H38 maintained similar Ψ_{Leaf} between the 40 and 45 °C treatments. Genotypes H13 (-3.9 ± 0.8 psi) and H33 (-3.5 ± 0.2 psi) demonstrated the least negative Ψ_{Leaf} during the 45 °C treatment, indicating the presence of an adaptive response within these genotypes, conferring thermotolerance. Among the genotypes which

demonstrated relatively stable Ψ_{Leaf} as heat increased, H8, H23, and H37 also demonstrated superior chlorophyll and protein contents when exposed to elevated temperatures.

Maintenance of Ψ_{Leaf} despite increasing temperatures underscores the potential of these genotypes as candidates with enhanced thermoregulatory capabilities, necessitating further investigation into the underlying molecular mechanisms governing this response. The ability to maintain Ψ_{Leaf} stability under high-temperature stress demonstrates the capacity to modulate cellular osmotic potential and turgor pressure, facilitated by osmoprotectants, HSPs, and antioxidant enzymes, which collectively mitigate the deleterious effects of thermal stress on cellular membranes and structures (Reiser et al. 2003; Prinsloo et al. 2009; Forouzesh et al. 2012; Yang et al. 2013). During heat stress, the likelihood of protein misfolding and denaturation increases. Fortunately, HSPs, particularly the molecular chaperones, HSP70 and HSP90, assist in folding and stabilising proteins, preventing the accumulation of misfolded or aggregated proteins that would otherwise interfere with cellular functions, including those involved in maintaining turgor pressure (Maio and Hightower 2021). Additionally, by facilitating membrane-associated protein folding, stabilisation, and solubilisation, HSPs indirectly protect cellular membranes under heat stress (Haider et al. 2021). HSPs have also been shown to interact with lipids, directly influencing the composition and fluidity of cellular membranes, maintaining the optimal physical properties of the lipid bilayer despite the disruptive effects of heat stress (Maio and Hightower 2021).

Among the genotypes which demonstrated relatively stable Ψ_{Leaf} as heat increased, H8, H23, and H37 also demonstrated superior chlorophyll and protein contents when exposed to elevated temperatures. The concurrent observation of superior chlorophyll and protein contents in these genotypes under elevated temperatures is indicative of their ability to maintain photosynthetic efficiency and protein synthesis despite heat stress. The combination of stable Ψ_{Leaf}, high chlorophyll, and protein contents underscores the resilience of these genotypes to heat stress and their potential for adaptation to changing climate conditions, suggesting that they possess genetic traits or physiological mechanisms that confer thermotolerance, possibly involving mechanisms such as efficient antioxidant defence systems, HSP regulation, or membrane stabilisation.

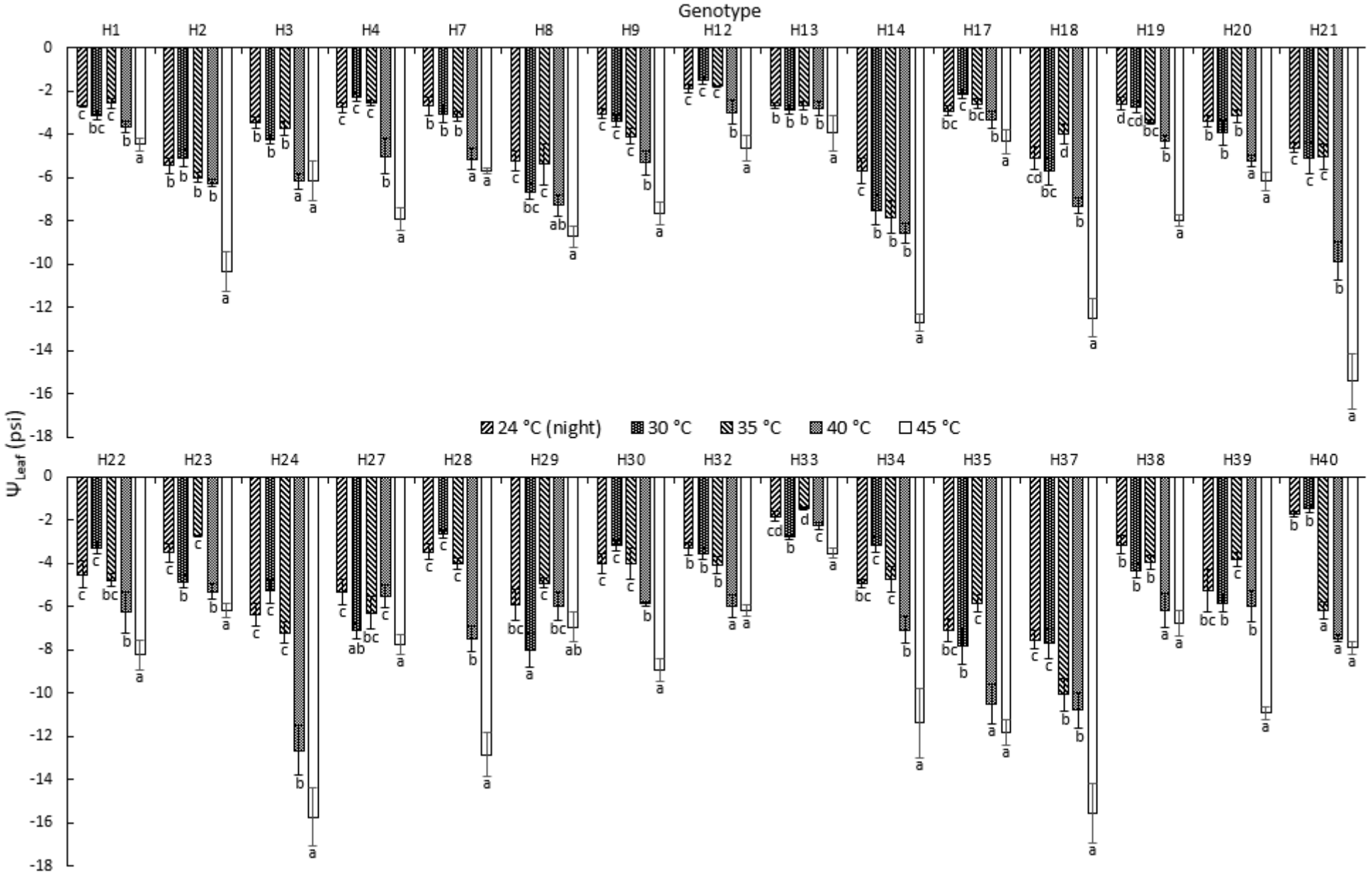


Figure 3.7: Water pressure potential in leaves (Ψ_{Leaf}) of *A. dubius* genotypes propagated through cuttings (n = 3) exposed to variable temperatures (24, 30, 35, 40, and 45 °C). Different letters indicate statistically significant differences for each parameter (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD.

3.3.4 Chlorophyll content

The chlorophyll content in *A. dubius* leaves from selected clonal genotypes propagated via cuttings and then exposed to variable watering schedules is depicted in ***Figure 3.8***. Generally, chlorophyll content decreased as water-deficit increased, aligning with the expected response and indicating the sensitivity of chlorophyll levels to water availability. Exceptions include genotype A9 in the 8-day watering regimen, which yielded significantly higher chlorophyll than the control which was watered once every 4 days. Furthermore, genotypes G1, G3, G5, G7, T3, W2, and A2 in the 8-day group yielded similar chlorophyll contents as their counterparts in the control group, and A2, A6, A10, G3, G7, T5, V4, and V6 maintained similar chlorophyll levels between the 12- and 16-day watering schedules.

Genotypes T8 and W7 maintained statistically similar chlorophyll contents throughout the 4-, 8-, and 12-day watering treatments, and A3 maintained chlorophyll content throughout the 8-, 12- and 16-day regimens and demonstrated the highest chlorophyll content (4.0 ± 0.4 µg/cm^2) among all tested genotypes which were watered once every 16 days. These results emphasise the diversity of chlorophyll content among different genotypes of *A. dubius* and highlight the capacity for resilience in genotypic responses to water-deficit stress. Genotypes which yielded resilient results in the protein and Ψ_{Leaf} assays (notably, A3, G7, and T8) also demonstrated resilience in chlorophyll content, suggesting a potential genetic basis for enhanced stress tolerance and emphasising the interconnectedness of physiological processes, which is indicative of a comprehensive adaptive response to water-deficit stress.

The maintenance of chlorophyll content in *Amaranthus* spp. under water-deficit conditions potentially involves various mechanisms, including the activation of antioxidant enzymes, such as SOD, ascorbate peroxidase, and glutathione reductase, and osmoprotectants, like proline and sugars (Slabbert and Kruger 2014). Additionally, an increase in unsaturated fatty acids in membrane lipids may protect photosystem II from photoinhibition under osmotic stress, potentially contributing to the maintenance of chlorophyll content (Jayme-Oliveira et al. 2017). Some amaranths can also suspend transpiration by reducing stomatal opening under water restriction, conserving water, and contributing to the maintenance of chlorophyll content (Soares et al. 2019). Furthermore, the ability to withstand water-deficit stress conditions suggests genotype-specific genetic adaptations

contributing to the maintenance of chlorophyll content in response to water-deficit (Sinasson and Shackleton 2023).

Dehydration-responsive element-binding (DREB) protein family TFs have been extensively studied to understand their role in regulating gene expression under various abiotic stress conditions in numerous plant species, including *Arabidopsis thaliana* (Kobayashi et al. 2012), barley (*Hordeum vulgare*) (Morran et al. 2011), cotton (*Gossypium hirsutum*) (Su et al. 2023), soybean (Zhou et al. 2020), and tobacco (Chen et al. 2022), indicating a conserved role across diverse plant taxa, and have been shown to enhance stress tolerance by orchestrating the expression of enzymes involved in chlorophyll biosynthesis and maintenance, particularly under water-deficit conditions. Unfortunately, the interaction of these elements has not been investigated in *A. dubius*, limiting insights into the myriad functions of this protein family in this species.

DREB TFs coordinate the regulation of multiple transcriptional pathways, including the expression of key antioxidant genes, such as ascorbate peroxidase, catalase, SOD, and glutathione reductase, as well as genes involved in ascorbate and glutathione biosynthesis (Christou et al. 2013). Furthermore, over-expression demonstrated enhanced chlorophyll content in transgenic plants, suggesting control of chlorophyll biosynthesis (Bouaziz et al. 2012). Additionally, DREB TFs have been associated with the regulation of carotenoid biosynthesis, further indicating their role in protecting chlorophyll content (Chen et al. 2022). However, this family of TFs have also been shown to directly activate the expression of chlorophyll degradation genes, suggesting a sensitive and selective mechanism for regulating chlorophyll levels during leaf senescence under stress conditions (Mao et al. 2017). Determining the species-specific effects of these TFs in *A. dubius* warrants further research.

While DREB factors primarily influence the regulation of gene expression and activation of stress-responsive pathways, the annexin multigene family of calcium-dependent phospholipid-binding proteins complement these efforts by contributing to cellular stability and protecting against stress-induced ROS damage (Konopka-Postupolska et al. 2009; Saad et al. 2019). Overexpression of annexin genes has been shown to enhance water-deficit tolerance in tomato (*Solanum lycopersicum*) and cotton, maintaining chlorophyll content through the modulation of ABA synthesis and scavenging of ROS (Kesanakurti et al. 2010; Ijaz et al. 2017).

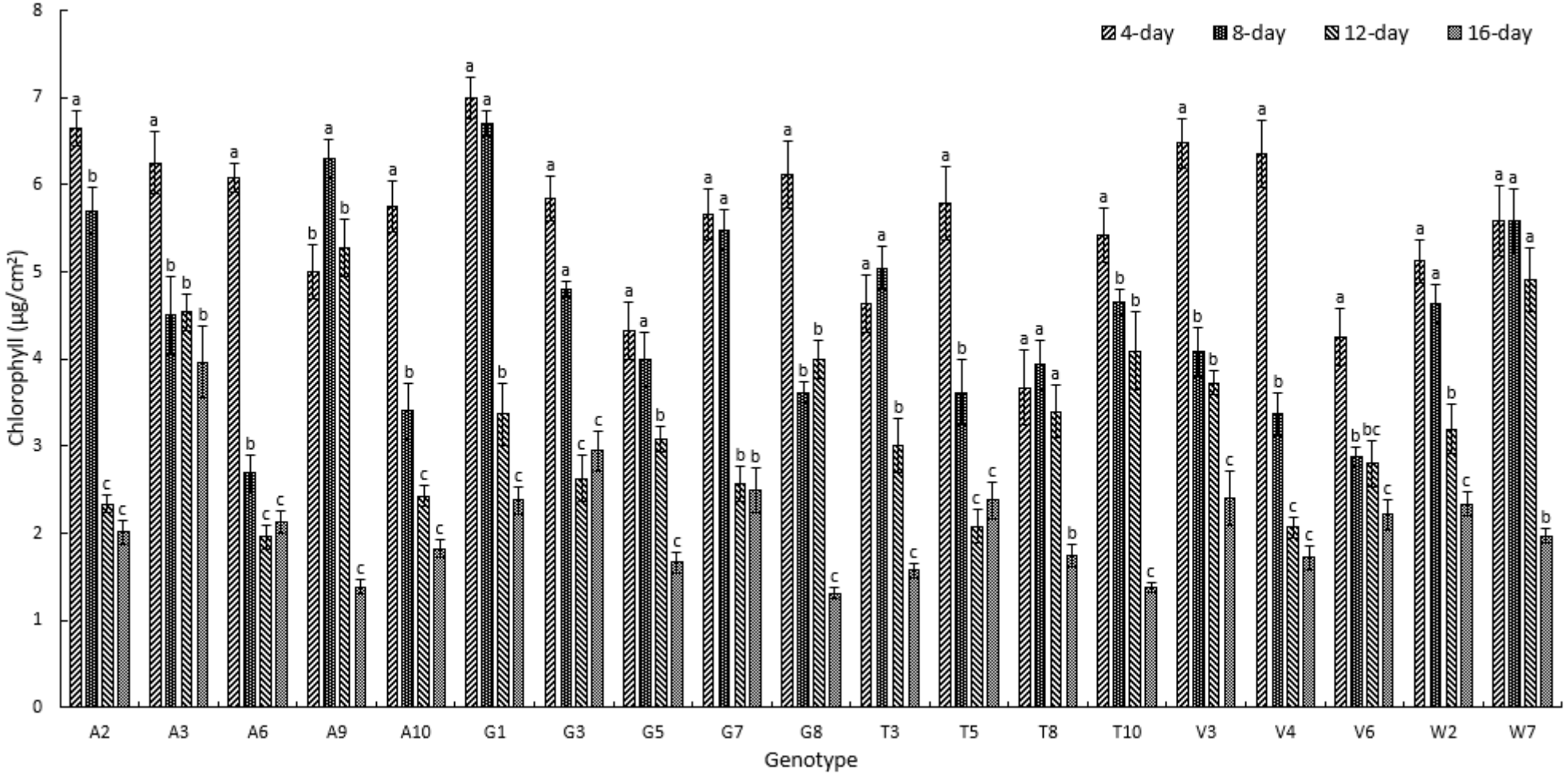


Figure 3.8: Chlorophyll content in the leaves of water-deficit tolerant clonal genotypes propagated through cuttings (n = 3) of *A. dubius* undergoing 4-, 8-, 12-, and 16-day watering regimens. Different letters indicate statistically significant differences for a genotype (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD.

Further investigation of DREB and annexin is needed to elucidate the convoluted molecular mechanisms employed by plants to tolerate water-deficit, particularly in stress-tolerant *A. dubius* genotypes, wherein this knowledge is severely lacking and enables uncovering novel signalling networks and crosstalk between different stress response pathways. Identifying the roles and functions of DREB and annexin can facilitate the development of crop varieties with enhanced stress tolerance, which is necessary for sustainable agriculture in increasingly water-limited environments. The present study attempted to quantify DREB1A and annexin 1 expression in response to water-deficit stress using degeneratively-primed RT-qPCR but was unsuccessful, necessitating further optimisation.

The total chlorophyll content in the leaves of selected *A. dubius* clonal genotypes propagated via cuttings growing under different daytime temperatures (30 (control), 35, 40, and 45 °C) is represented in ***Figure 3.9***. Generally, chlorophyll content decreased as heat increased, with the exception of genotypes H8, H21, and H23, which had more chlorophyll than the control when exposed to 35 °C daytime temperatures. Additionally, genotypes H4, H14, and H24 yielded similar chlorophyll levels throughout the 30 to 40 °C treatments, and H17, H33, and H37 maintained similar chlorophyll throughout the 35 to 45 °C treatments. Between 40 and 45 °C exposure, genotypes H2, H12, H21, H22, H27, H28, H29, H32, H34, and H35 demonstrated similar chlorophyll content. Genotype H34 maintained the greatest concentration of chlorophyll when exposed to 45 °C (3.6 ± 0.2 µg/cm^2), suggesting a robust ability in this genotype to either maintain or activate mechanisms to preserve chlorophyll integrity under heat stress. The observed diversity in chlorophyll content across genotypes underlines the genetic variability within the *A. dubius* population. While the general trend aligns with existing knowledge that higher temperatures lead to reduced chlorophyll levels, the exceptions offer intriguing insights. These genotypes may activate protective pathways or possess inherent traits that mitigate heat-induced chlorophyll loss, indicating potential candidates for further study and possibly utilisation in breeding programs for heat-tolerant cultivars. The consistent chlorophyll levels across different temperature ranges in specific genotypes, such as H4, H14, H24, H17, H33, and H37, suggest genetic stability or specific regulatory mechanisms, enabling the maintenance of chlorophyll levels despite varying heat stress.

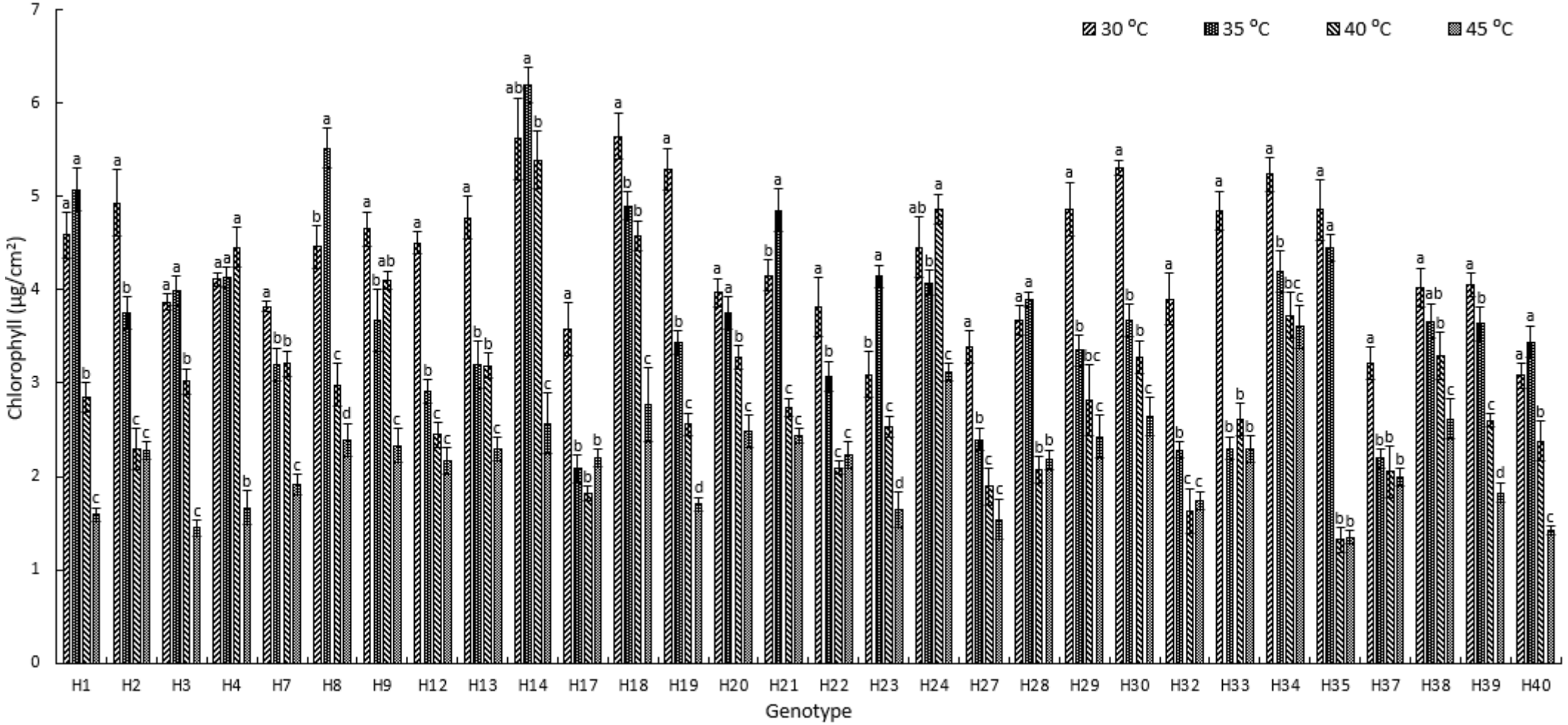


Figure 3.9: Chlorophyll content in the leaves of selected genotypes of *A. dubius* propagated through cuttings growing under varying levels of heat treatments (30 (control), 35, 40, and 45 °C). Different letters indicate statistically significant differences for a genotype (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, n = 3.

To mitigate heat-induced chlorophyll loss in heat-tolerant plants, several protective pathways and regulatory mechanisms exist, involving the activation of stress-responsive genes, hormone signalling pathways, and the modulation of photosynthetic and antioxidant systems. For instance, hormone-signalling pathways sense heat stimuli and transduce transcriptional regulation of heat stress-responsive genes (Poór et al. 2021). Additionally, the co-expression of stress-responsive regulatory genes facilitates high-temperature adaptations (Venkatesh et al. 2022). For example, melatonin has been found to improve stress tolerance in plants by directly scavenging ROS and indirectly increasing antioxidant enzyme activity and chlorophyll content (Fan et al. 2018). The regulation of non-coding RNAs has also been shown to act in regulating heat tolerance, including the maintenance of photosynthetic systems (Wang et al. 2016; Zhao et al. 2016; Ding et al. 2020). HSPs confer thermotolerance by protecting photosystem II electron transport during heat stress (Heckathorn et al. 1998; Suzuki 2023).

3.3.5 Proline content

Proline content generally increased as the watering intervals extended from 4 to 16 days, reflecting a heightened stress response across most genotypes (***Figure 3.10***). However, several genotypes displayed distinct patterns of proline accumulation that deviated from this general trend. For genotypes A2, A3, G1, G3, G5, G7, T8, V3, V4, and W2, there was no significant increase in proline content between the 4-day and 8-day watering regimens, indicating a delayed or less pronounced response to initial water-deficit conditions. In genotype V6, proline levels did not show a significant difference between the 8-day and 12-day watering intervals, suggesting that this genotype may have reached the maximum threshold for this stress response during moderate water-deficit stress. Furthermore, genotypes A9, G3, G8, and W2 displayed no significant increase in proline content between the 12-day and 16-day regimens, possibly indicating that, despite prolonged stress, these genotypes either do not require further proline accumulation for survival or have reached their maximum capacity for proline synthesis. These results highlight variability in proline accumulation among genotypes that can be used for further screening of water-deficit resilience.

The observed increase in proline content with extended watering intervals is consistent with the role of proline as an osmoprotectant, which accumulates in response to water-deficit stress (Bulegon et al. 2016; Beigzadeh et al. 2019). The function of proline in osmotic adjustment and stress tolerance has

been well-documented, as it stabilises proteins and membranes and scavenges reactive oxygen species during stress (Yamaguchi-Shinozaki and Shinozaki 2006; Merewitz et al. 2011; Saud et al. 2020; Tayyab et al. 2020). Therefore, the general trend of increasing proline levels across most genotypes aligns with the expected physiological response to water-deficit stress. The genotypes that maintained high or increasing proline levels throughout the watering treatments, especially under severe drought conditions, could be considered more tolerant to water-deficit stress compared to those genotypes that exhibited early stabilising of proline levels.

However, the lack of significant proline increases between the 4-day and 8-day watering regimens in genotypes A2, A3, G1, G3, G5, G7, T8, V3, V4, and W2 suggested that these genotypes may exhibit a delayed or decreased stress response under milder water-deficit conditions. Since these genotypes survived, this could also indicate an inherent ability to cope with moderate stress without requiring substantial proline accumulation, potentially reflecting more efficient water-use strategies or alternative mechanisms of stress tolerance. These genotypes may exhibit enhanced water-use efficiency through optimised stomatal regulation, reduced transpiration, or deeper root systems that access water from subsoil layers, reducing the need for proline accumulation (Blum 2009; Comas et al. 2013). Additionally, these genotypes could rely on other osmoprotectants, such as glycine betaine and trehalose, which also contribute to osmotic adjustment and membrane stabilisation (Ashraf and Foolad 2007). A robust antioxidant defence system involving enzymes like superoxide dismutase, catalase, and peroxidases may further mitigate oxidative stress without requiring significant proline synthesis (Mittler 2002). Alternatively, stress tolerance could also result from epigenetic modifications or transcriptional regulation, such as the activation of DREB/CBF transcription factors that modulate drought-responsive genes independently of proline biosynthesis (Agarwal et al. 2006).

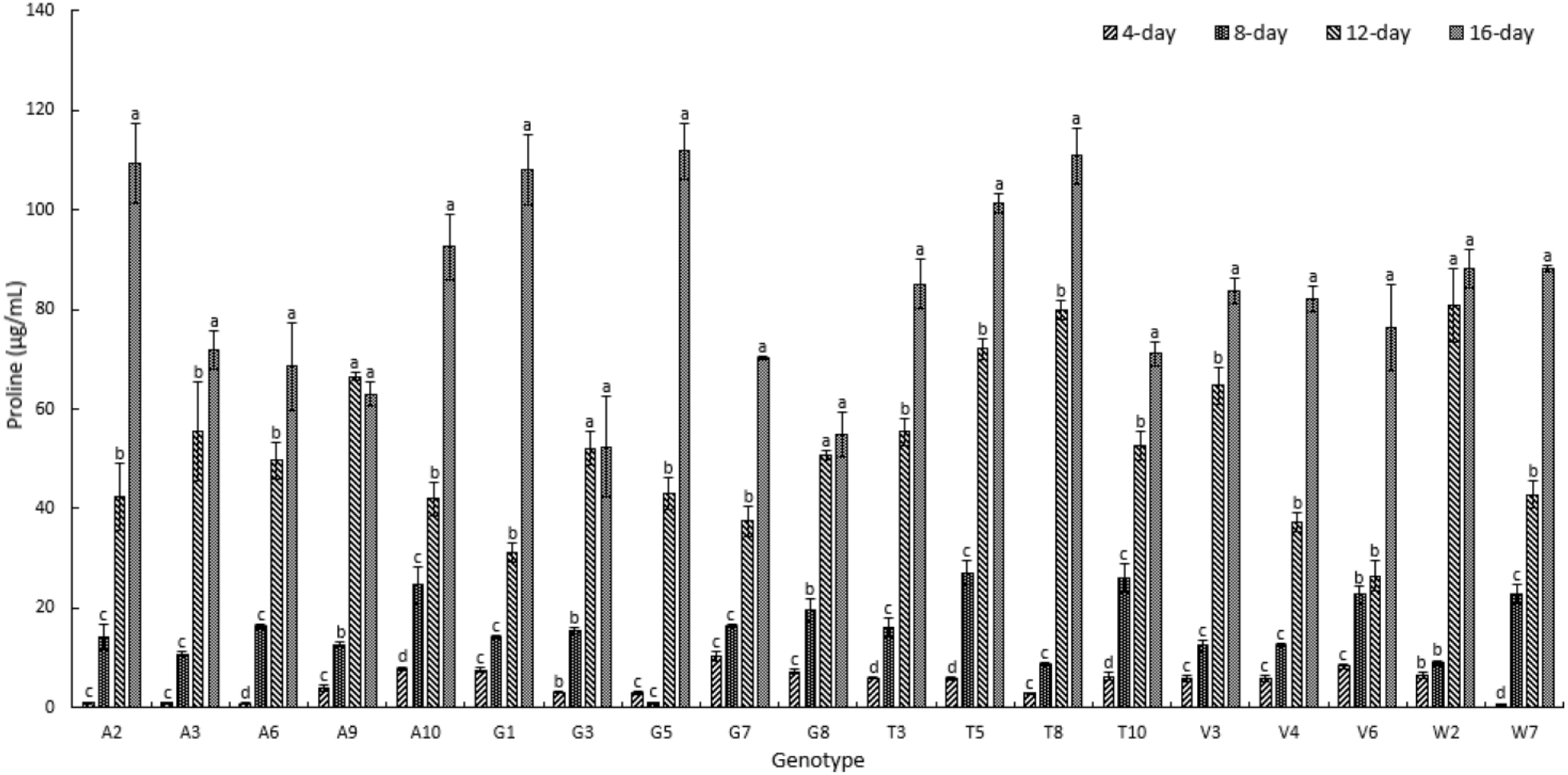


Figure 3.10: Proline content in the leaves of water-deficit tolerant clonal genotypes propagated through cuttings (n = 3) of *A. dubius* undergoing 4-, 8-, 12, and 16-day watering regimens. Different letters indicate statistically significant differences for a genotype (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD.

The proline content in leaves of heat-stressed *A. dubius* genotypes is shown in ***Figure 3.11***. Generally, proline increased as heat stress intensified from 30 to 45 °C. However, several genotypes displayed distinct proline accumulation patterns in response to specific temperature treatments. Most genotypes did not show a significant increase in proline content between the 30 (control) and 35 °C treatments, except for genotypes H9, H12, H32, H38, and H39, which exhibited an early proline response even at 35 °C. This suggests a more sensitive heat response in these genotypes compared to the others that were presently tested. These genotypes could potentially be more responsive to early-stage heat stress, making them promising candidates for further investigation into early heat acclimation mechanisms. Genotypes H9, H12, and H21 did not show a significant difference in proline accumulation between the 35 and 40 °C treatments, implying a possible plateau in proline synthesis during moderate heat stress. Similarly, genotypes H4 and H32 showed no significant increase in proline content between the 40 and 45 °C treatments, indicating that their proline accumulation may have reached a saturation point under high-temperature stress.

Proline accumulation is often associated with enhanced tolerance to high temperatures, allowing plants to mitigate oxidative damage and maintain cellular function under heat stress (Raza et al. 2023; Sehar et al. 2023). The general increase in proline levels as temperatures rose from 30 to 45 °C supports the hypothesis that proline functions in the heat stress adaptation of most genotypes. However, the absence of significant proline increases between the 30 and 35 °C treatments in the majority of genotypes suggests that mild heat stress may not be sufficient to trigger a substantial proline response in these genotypes. Interestingly, genotype H33 did not show any significant increase in proline content across the 30, 35, and 40 °C treatments, suggesting a lack of proline-based heat stress adaptation, indicating alternative heat tolerance mechanisms. For example, increased synthesis of heat shock proteins (HSPs) could stabilise and refold denatured proteins, maintaining cellular integrity under heat stress (Wang et al. 2004; Mondal et al. 2023). Additionally, altered membrane lipid compositions, such as higher levels of saturated fatty acids or antioxidant systems, could enhance membrane stability and functionality at elevated temperatures (Upchurch 2008; Naliwajski and Skłodowska 2021). Traits like increased transpiration or anatomical changes in canopy structure could also help dissipate heat and maintain optimal leaf temperatures in lieu of proline accumulation (Buckley and Mott 2013).

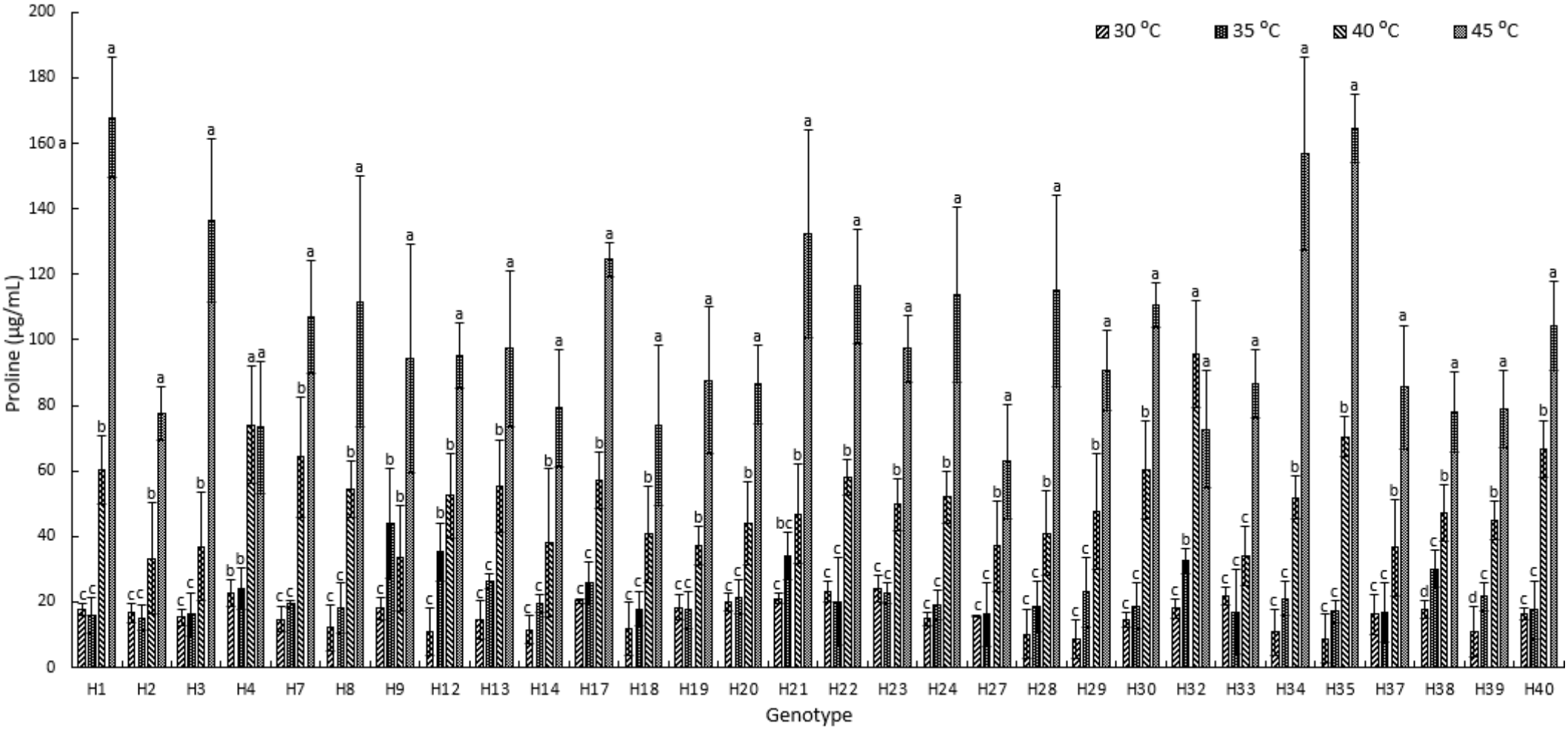


Figure 3.11: Proline content in the leaves of heat-tolerant clonal genotypes propagated through cuttings (n = 3) of *A. dubius* undergoing 30 (control), 35, 40, and 45 °C treatments. Different letters indicate statistically significant differences for a genotype (Kruskal-Wallis test; $p < 0.05$), mean ± SD.

3.3.6 Protein content

The bars in ***Figure 3.12*** illustrate the protein content in the leaves of selected *A. dubius* clonal genotypes propagated via cuttings undergoing variable watering schedules. Generally, protein concentration decreased as watering frequency decreased, except for genotypes A3, G3, and T8, which demonstrated similar or increased protein content as watering frequency decreased. Furthermore, genotype G8 showed an increase in protein content between the 4- and 8-day regimens, followed by decreases during the 12- and 16-day regimens. Additionally, genotypes A9, T10, V3, and V6 maintained consistent protein concentrations throughout the 4-, 8-, and 12-day watering schedules, and A10 demonstrated similar protein content throughout the 8-, 12-, and 16-day schedules. Genotype A10 also had similar protein content between the 4- and 8-day regimens. Genotypes A2, A6, and G7 showed similar protein content between the 4- and 8-day regimens and also demonstrated similarity between the 12- and 16-day regimens.

Moreover, genotypes G1, G3, G5, T5, and W2 had similar protein content between the 4- and 8-day watering schedules, and V4 and W7 demonstrated similarity between the 12- and 16-day schedules. Notably, genotypes T8 (3.4 ± 0.1 µg/mL) and G7 (3.3 ± 0.2 µg/mL) maintained the highest amount of protein following the 16-day watering schedule, emphasising the ability of these genotypes to prioritise protein synthesis and minimise protein degradation during extended periods of water-deficit, regulating metabolism, maintaining cell structure, and activating stress-responsive pathways to mitigate the adverse effects of water-deficit stress on cellular function.

The observed variations in protein content among genotypes are indicative of the intricate physiology of *A. dubius*, demonstrating genotype-specific responses to water-deficit stress. Genotypes maintaining consistent protein levels between the 4- and 8-day regimens demonstrated stability or limited responsiveness to moderate changes in water availability. Genotypes retaining similar protein levels across both shorter (4-day) and longer (16-day) watering schedules indicated a capacity to maintain protein content under increasing water-deficit stress, suggesting the utilisation of adaptive mechanisms which promoted protein synthesis and retention. The culmination of these mechanisms was typified by genotypes G7 and T8, which also maintained Ψ_{Leaf} as water stress increased.

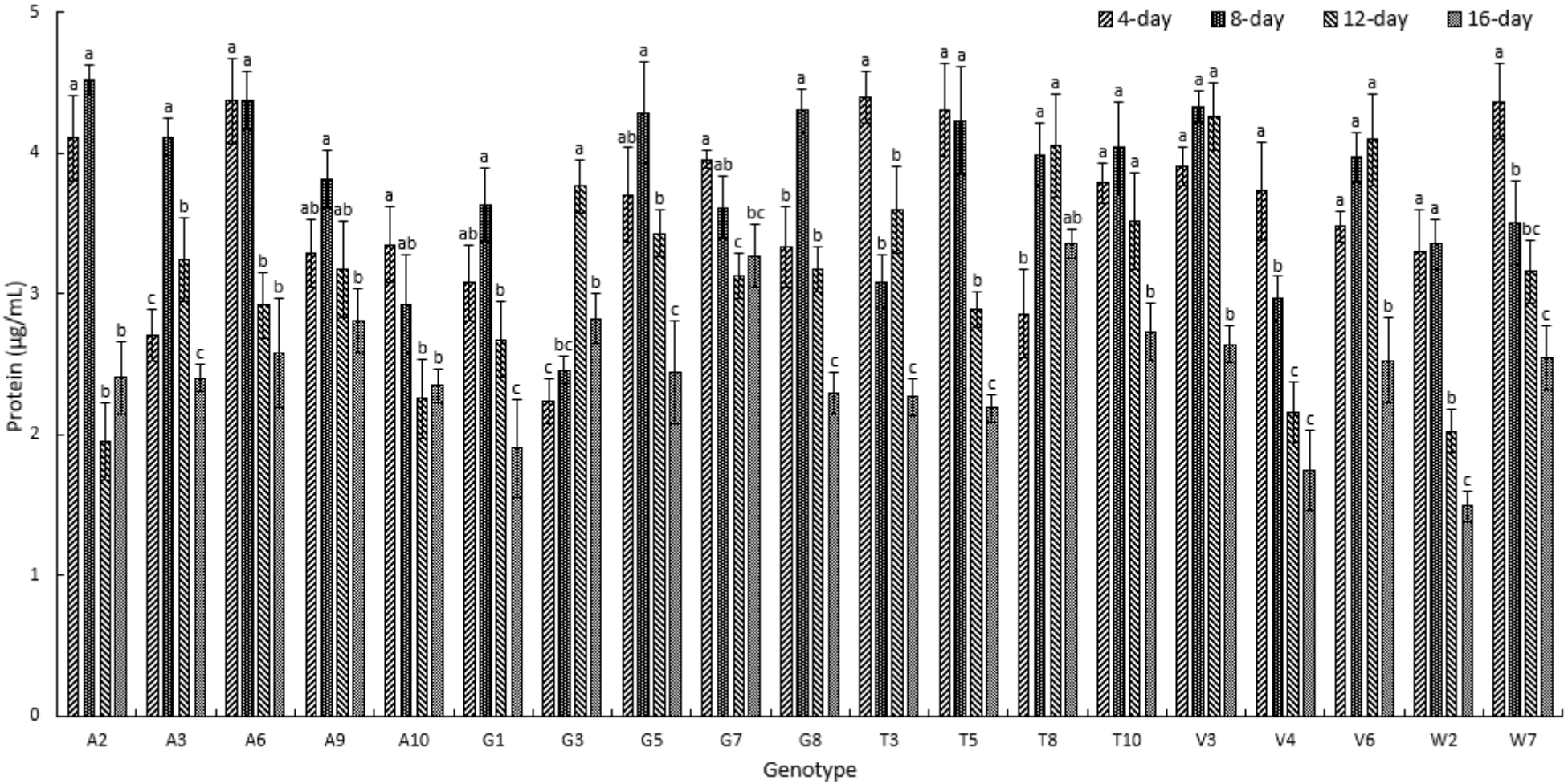


Figure 3.12: Protein concentration of leaves from water-deficit tolerant *A. dubius* genotypes propagated through cuttings (n = 3) exposed to variable watering regimens (4-, 8-, 12-, 16-day). Different letters indicate statistically significant differences for a genotype (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD.

On a molecular scale, the present results may be due to the activation of genes involved in the generation of plant hormones like abscisic acid (ABA), ethylene, and salicylic acid, which coordinate signalling cascades responsible for adaptive responses to water stress (Rabara et al. 2017; Anwar et al. 2022). Specifically, TFs, such as DREB/CBF, are pivotal in regulating the expression of stress-responsive genes, including those encoding protective proteins that aid in protein stability under water-deficit stress conditions (Amrutha et al. 2019). These TFs recognise the C-repeat (CRT)/dehydration-responsive element (DRE) DNA regulatory region present in the promoters of stress-responsive genes (Xiong and Fei 2006; Zandkarimi et al. 2015) and are also involved in the ABA-independent stress-responsive pathway (Marcolino-Gomes et al. 2013).

The observed results could also be due to stress-inducible genes encoding enzymes for osmolyte biosynthesis and detoxification to facilitate water-deficit stress tolerance through the accumulation of osmolytes such as proline and glycine betaine which promote water retention, reducing the damaging effects of water-deficit stress (Yamaguchi-Shinozaki and Shinozaki 2006; Saud et al. 2020). Furthermore, osmolytes and antioxidant enzymes such as superoxide dismutase (SOD), catalase, and peroxidases protect proteins from oxidative damage, preventing aggregation and preserving their structural integrity (Konrad and Bar-Zvi 2008). The regulation of protein content under water-deficit stress involves a complex interplay of hormonal, enzymatic, and metabolic responses. Cytokinins, jasmonic acid, salicylic acid, proline, and antioxidant enzymes have been implicated in modulating protein abundance (Merewitz et al. 2011; Tayyab et al. 2020).

Further avenues for future research include investigating the activity of cytokinins, which coordinate the regulation of carbon and nitrogen assimilation, leading to the preservation of protein content under stressful conditions (Reguera et al. 2013). Moreover, the upregulation of antioxidant and hydrolytic enzymes has been linked to significant increases in protein content under water-deficit stress (Hameed et al. 2021). Conversely, severe water-deficit stress has been shown to reduce chlorophyll and total soluble protein content, evidenced in the present study in less resilient genotypes, leading to metabolic limitations under extreme drought conditions (Ribeiro et al. 2013; Mohammadi et al. 2022). The reduction in photosynthetic pigments and the increase in biochemical components such as proline, leaf protein, and carbohydrates have been observed in response to water stress, highlighting dynamic adjustments in protein content under adverse environmental conditions.

Additionally, the activation of the unfolded protein response leads to the upregulation of molecular chaperones localised in the endoplasmic reticulum, further contributing to protein stabilisation and protection against dehydration-induced damage (Mogk et al. 2003). It can be hypothesised that *A. dubius* genotypes in the present study demonstrated synergy between chaperone activity and osmolytes, such as glycine-betaine, contributing to protein protection against dehydration-induced damage (Hoffman et al. 2018). Additional mechanisms enabling the observed protein stabilisation under water-deficit stress in some *A. dubius* genotypes include post-translational modifications (PTMs), such as nitric oxide (NO) mediated S-nitrosylation, metal nitrosylation, carbonylation, and tyrosine nitration (Santisree et al. 2015). Protein phosphorylation also aids in stabilising proteins under stressful conditions, including water-deficit stress (Luo et al. 2018). Furthermore, protein turnover processes, including ubiquitination and SUMOylation, are involved in the readjustment of protein expression under water-deficit stress conditions (Roux et al. 2020; Tang et al. 2021), resulting in the observations of the present study and necessitating further research. Proteomic analysis of grapes (*Vitis vinifera*) confirmed that numerous proteins are protected by PTMs in response to water-deficit stress (Cramer et al. 2013). The regulation, interaction, and response of these elements is largely understudied in *A. dubius* and necessitate further research.

The protein content in the leaves of selected clonal genotypes of *A. dubius* propagated via cuttings exposed to 30 (control), 35, 40, and 45 °C is illustrated in ***Figure 3.13***. Generally, protein content decreased as temperatures rose. However, during the 35 and 40 °C treatments, genotypes H3, H9, H23, H29, and H32 yielded higher protein content than the control, genotypes H18 and H22 contained more protein at 45 °C than the control, and H3, H8, H18, H19, H23, H27, H29, H32, and H37 maintained similar protein content at 45 °C as the control. Notably, genotype H12 yielded higher protein content at 35, 40, and 45 °C than at 30 °C. Interestingly, genotypes H8, H18, H19, H22, and H37 had the lowest protein when exposed to 40 °C, contrasted with significant increases during the 45 °C treatment. Among all genotypes, H8 (3.6 ± 0.1 µg/mL) and H27 (3.5 ± 0.1 µg/mL) had the highest amount of protein while growing at 45 °C.

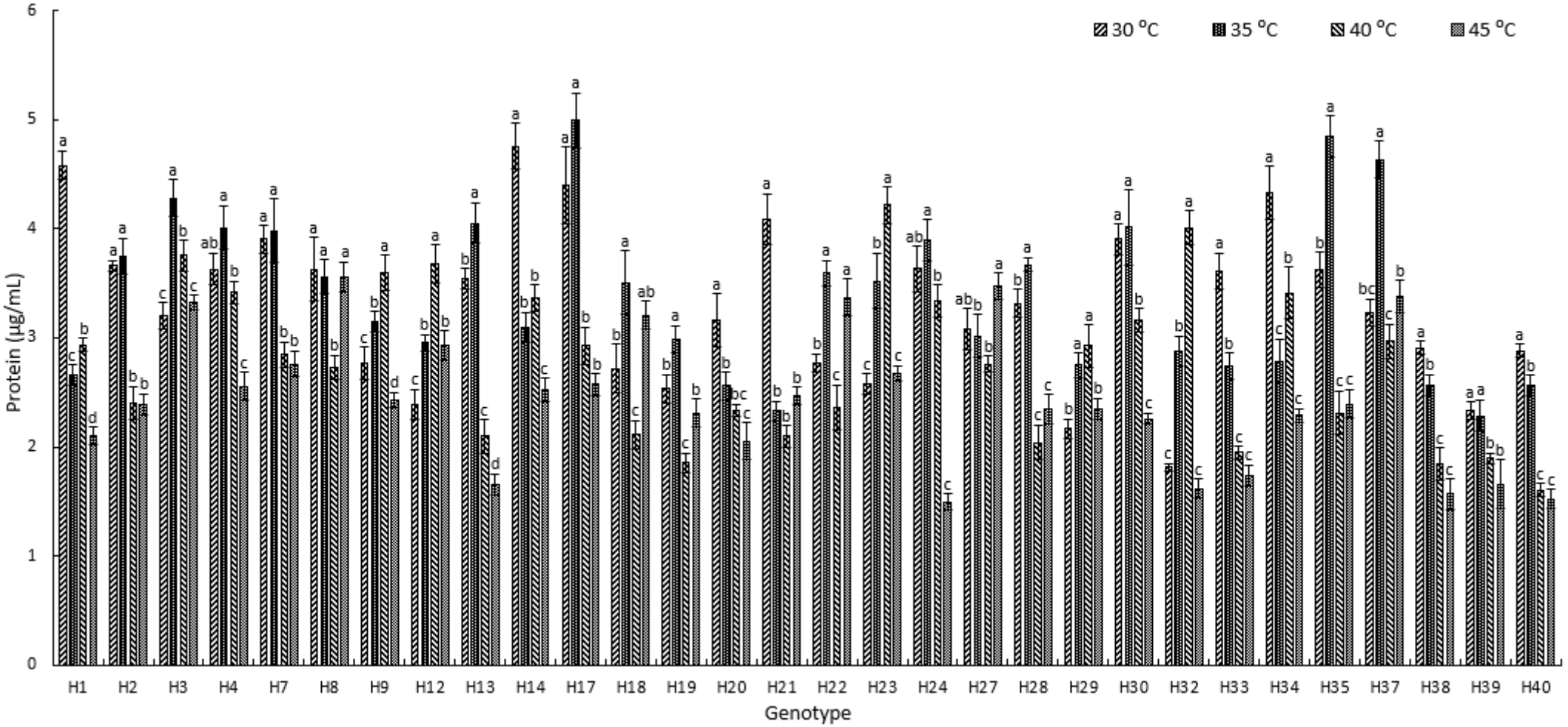


Figure 3.13: Protein content in the leaves of selected genotypes of *A. dubius* propagated via cuttings growing under varying levels of heat treatments (30 (control), 35, 40, and 45 °C). Different letters indicate statistically significant differences for a genotype (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, n = 3.

Notably, genotypes H3, H9, H12, H18, H22, H23, H29, and H32 displayed increased protein content compared to the control, suggesting potential adaptive mechanisms within these genotypes, which enhanced protein synthesis or mitigated protein degradation under heat stress. Furthermore, the exceptional performance of genotypes H8, H12, and H27 in maintaining protein content at 45 °C warrants closer examination to elucidate unique pathways, genetic attributes, and molecular mechanisms enabling this response. In *A. thaliana*, the expression of tall fescue (*Festuca arundinacea*) heat-responsive genes, including HSPs, and abscisic acid (ABA)-synthesising genes, have been shown to be upregulated in heat-tolerant plants undergoing heat stress (Wang et al. 2017). The upregulation of HSPs by ABA contributed to improved heat tolerance in *A. thaliana*, spinach (*Spinacia oleracea*), and mustard (*Brassica campestris*) accompanied by the accumulation of enzymatic antioxidants and osmolytes, mitigating heat-induced damage and contributing to the total protein content under heat stress (Wu et al. 2017; Zhao et al. 2018; Zhu et al. 2018).

3.3.7 Micropropagation

Multiple micropropagation parameters and agricultural output (foliar FM) of the identified water-deficit tolerant *A. dubius* clonal genotypes are listed in ***Table 3.3***. For each genotype, there was an average of 3.8 ± 1.0 new shoots per explant on multiplication medium, and the average shoot and root elongation rates were 2.0 ± 0.4 and 1.1 ± 0.4 cm/week, respectively. The number of new shoots per explant were higher in comparison to the micropropagation of *A. dubius* by Shaik et al. (2022), wherein each explant yielded an average of 2 new shoots. This outcome is likely due to genotype-specific variability unintentionally selected for in earlier stages of the present study which identified stress-tolerant genotypes, indicating a correlation between stress tolerance and shoot production *in vitro*. Genotypes that experienced water-deficit stress during the earlier screening stages may retain a "stress memory", enabling a more efficient response during subsequent micropropagation, manifesting in increased shoot production (Ding et al. 2012; Sun et al. 2021).

Statistical analysis revealed similarity among genotypes A2, A6, G1, G7, T5, V3, and V6, yielding equally high foliar FM (48.4 ± 4.2 g), which was similar to the foliar FM of the 4-day control group (49.6 ± 7.2 g) from the initial assay over the same 2-month culture period. Genotypes A2, A3, A10, G1, G7, G8, T3, T8, V3, V4, W2, and W7 produced the most shoots on the shoot multiplication medium, and A10, G5, T5, and W7 demonstrated the fastest shoot elongation rates on the shoot elongation medium.

Genotypes A2, A3, A9, G3, G5, G7, T5, T8, T10, V3, V4, W2, and W7 showed the fastest root elongation rates after being transferred to the rooting medium. Overall, genotypes A2, G7, T5, and V3 performed the best throughout micropropagation and subsequent acclimatisation, with A2, G7, and V3 producing more shoots than T5, but T5 demonstrating faster shoot elongation than A2, G7, and V3.

There was an antagonistic relationship observed between shoot multiplication and shoot elongation, except for genotypes A10 and W7, which produced the most shoots and also demonstrated the fastest shoot elongation rates. However, following acclimatisation, these genotypes ultimately yielded significantly lower foliar FM. This observation highlighted a critical aspect of plant development post-acclimatisation, where seemingly advantageous characteristics demonstrated in the initial stages of micropropagation did not translate into enhanced foliar biomass during subsequent growth phases. This divergence in expected outcomes underscores the complexity of post-micropropagation development, necessitating more comprehensive investigations into the mechanisms governing the transition from *in vitro* culture to *ex vitro* conditions. A plausible explanation for the disparity in foliar biomass post-acclimatisation could be linked to variations in physiological adaptations during the transition from relatively controlled *in vitro* conditions to the more dynamic *ex vitro* environment. Despite exhibiting favourable traits during the early stages of micropropagation, these genotypes demonstrated a trade-off between shoot proliferation and foliar biomass allocation, suggesting a potential resource allocation strategy where rapid multiplication and elongation is realised at the expense of final biomass accumulation.

These results prompt the consideration of specific metabolic pathways or hormonal regulation that might be differentially activated or suppressed during the acclimatisation phase within these genotypes, including the interplay of phytohormones, such as cytokinins and auxins, which may dictate shoot proliferation, elongation, and subsequent acclimatisation – the elucidation of which is necessary for optimising micropropagation techniques (Vanneste and Friml 2009). Further investigations involving transcriptomic, metabolomic, and hormonal profiling could unravel the underlying molecular mechanisms orchestrating post-acclimatisation outcomes in *A dubius*. Integrating these multi-omics approaches with morphological and physiological analyses could offer a more comprehensive understanding of post-micropropagation development.

Table 3.3: Growth measurements from the micropropagation of selected water-deficit tolerant *A. dubius* genotypes. Different letters indicate statistically significant differences among genotypes (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, n = 10. FM = fresh mass.

Genotype	Number of New Shoots	Shoot elongation (cm/week)	Root elongation (cm/week)	Foliar FM (g)
A2	$3.9^{abc} \pm 1.4$	$1.5^{ghi} \pm 0.5$	$1.5^{ab} \pm 0.3$	$48.8^{abc} \pm 5.1$
A3	$4.1^{abc} \pm 1.7$	$1.5^{ghi} \pm 0.2$	$1.0^{abcd} \pm 0.6$	$37.2^{fghij} \pm 6.4$
A6	$3.5^{bc} \pm 1.2$	$1.5^{ghi} \pm 0.2$	$0.6^{d} \pm 0.3$	$46.8^{abcde} \pm 3.6$
A9	$3.6^{bc} \pm 1.0$	$1.7^{fghi} \pm 0.5$	$1.4^{abc} \pm 0.3$	$36.1^{ghij} \pm 3.8$
A10	$4^{abc} \pm 0.7$	$3.1^{ab} \pm 0.3$	$0.6^{d} \pm 0.5$	$39.5^{efghij} \pm 5.1$
G1	$5.4^{a} \pm 1.3$	$2.3^{cdef} \pm 0.5$	$0.6^{d} \pm 0.4$	$48.3^{abcd} \pm 3.2$
G3	$3.5^{bc} \pm 1.2$	$1.9^{efgh} \pm 0.5$	$1.6^{a} \pm 0.4$	$43.5^{bcdefg} \pm 6.8$
G5	$2.9^{bc} \pm 1.4$	$3.1^{a} \pm 0.4$	$1.1^{abcd} \pm 0.6$	$32.8^{j} \pm 5.4$
G7	$4.4^{ab} \pm 1.1$	$2.0^{defg} \pm 0.3$	$1.6^{ab} \pm 0.2$	$44.9^{abcdef} \pm 5.1$
G8	$3.7^{abc} \pm 0.5$	$2.4^{bcde} \pm 0.7$	$0.7^{cd} \pm 0.4$	$37.5^{fghij} \pm 4.7$
T3	$4.1^{abc} \pm 1.0$	$2.2^{cdef} \pm 0.4$	$0.8^{bcd} \pm 0.5$	$51.7^{a} \pm 5.0$
T5	$3.4^{bc} \pm 0.8$	$2.8^{abc} \pm 0.5$	$0.9^{abcd} \pm 0.3$	$44.8^{abcdef} \pm 3.4$
T8	$4.4^{ab} \pm 0.5$	$1.9^{efgh} \pm 0.5$	$1.4^{abc} \pm 0.5$	$41.8^{cdefgh} \pm 5.6$
T10	$2.5^{c} \pm 0.8$	$1.7^{fghi} \pm 0.2$	$1.0^{abcd} \pm 0.3$	$33.3^{ij} \pm 3.9$
V3	$4.5^{ab} \pm 0.8$	$1.3^{hi} \pm 0.4$	$1.1^{abcd} \pm 0.7$	$50.4^{ab} \pm 5.5$
V4	$4.4^{ab} \pm 0.7$	$1.0^{i} \pm 0.2$	$0.9^{abcd} \pm 0.5$	$35.7^{hij} \pm 5.3$
V6	$3.4^{bc} \pm 0.5$	$2.1^{cdefg} \pm 0.6$	$0.7^{cd} \pm 0.4$	$51.1^{ab} \pm 2.7$
W2	$4.1^{abc} \pm 1.1$	$1.7^{efghi} \pm 0.5$	$1.6^{a} \pm 0.4$	$40.9^{defghi} \pm 6.3$
W7	$4.5^{ab} \pm 1.6$	$2.7^{abcd} \pm 0.6$	$1.0^{abcd} \pm 0.3$	$35.4^{hij} \pm 3.2$
Control	$2.5^{c} \pm 0.9$	$1.4^{ghi} \pm 0.4$	$0.8^{bcd} \pm 0.3$	$39.8^{efghij} \pm 4.4$
Mean	**3.8 ± 1.0**	**2.0 ± 0.4**	**1.1 ± 0.4**	**42.1 ± 4.7**

Control = Wild-type specimens which were not exposed to stress treatments.

The superior growth exhibited by genotypes A2, G7, T5, and V3 highlights the potential for targeted cultivation and breeding efforts. Faster root elongation enhances the ability of the plant to access water and nutrients from the soil, thereby improving overall resilience to water stress (Sharp et al. 2004). Additionally, Padilla et al. (2007) demonstrated that *Genista umbellate*, *Lycium intricatum*, and *Retama sphaerocarpa* seedlings responded to lower water availability with faster root elongation rates, leading to a greater absorptive root surface and enhanced relative growth rate under reduced watering. Contrastingly, *A. thaliana* root systems under nutrient deficiencies exhibited shallower architecture due to the inhibition of primary root elongation and increased lateral root formation, further emphasising the adaptability of root elongation in response to varying environmental conditions (Gruber et al. 2013).

Additionally, tissue culture-induced genome level changes have indicated the potential for altering growth characteristics through tissue culture techniques (Neelakandan and Wang 2011). For example, the WUSCHEL gene, known for promoting vegetative-to-embryonic transition in *A. thaliana*, has been shown to be influenced by tissue culture conditions, such as hormone concentrations in the culture medium (Zuo et al. 2002). Additionally, the plant tissue culture environment has been identified as a crucial factor influencing epigenetic changes in numerous species, highlighting the necessity of understanding the mechanisms underlying growth characteristics in tissue culture for further research and breeding programs (Bednarek and Orłowska 2019). Therefore, understanding the genetic basis of growth characteristics is necessary for further research and breeding programs aimed at improving the overall performance of *A. dubius*.

Compared in ***Table 3.4*** are pre- and post-micropropagation physiological parameters (Ψ_{Leaf}, protein, chlorophyll, and proline) of the identified water-deficit tolerant genotypes, which also demonstrated superior growth throughout micropropagation (A2, G7, T5, and V3). Following acclimatisation, the genotypes were re-exposed to the same 16-day watering regimen as their parents, and then the various measurements were taken to assess true-to-type fidelity. Interestingly, none of the compared parameters yielded significant differences ($p > 0.05$), indicating that micropropagation and subsequent acclimatisation did not have a significant effect on the ability of the selected genotypes to tolerate water-deficit stress, supporting true-to-type fidelity and reliability of the protocol established by Shaik et al. (2022).

Table 3.4: Comparisons between pre- and post-micropropagation parameters (leaf water pressure potential (Ψ_{Leaf}), protein, chlorophyll, and proline, mean ± SD) of identified water-deficit tolerant *A. dubius* genotypes re-exposed to 16-day watering schedules (paired samples T-test, n = 3). * indicates similarity between before and after measurements for each parameter per genotype ($p > 0.05$).

Genotype	Ψ_{Leaf} (psi)		Protein (µg/mL)		Chlorophyll (µg/cm^2)		Proline (µg/mL)	
	Before	After	Before	After	Before	After	Before	After
A2	-20.7 ± 2.3*	-19.4 ± 0.9*	2.4 ± 0.3*	2.2 ± 0.3*	2.0 ± 0.1*	2.3 ± 0.2*	109.3 ± 8.0*	104.1 ± 9.3*
G7	-17.2 ± 3.3*	-14.0 ± 2.7*	2.9 ± 0.5*	2.9 ± 0.3*	2.5 ± 0.3*	2.5 ± 0.3*	70.3 ± 0.4*	64.8 ± 6.3*
T5	-15.6 ± 1.2*	-14.1 ± 1.6*	2.2 ± 0.1*	2.4 ± 0.5*	2.4 ± 0.2*	2.7 ± 0.6*	101.3 ± 2.0*	88.4 ± 3.8*
V3	-19.1 ± 1.8*	-19.5 ± 1.5*	2.6 ± 0.1*	2.8 ± 0.4*	2.4 ± 0.3*	2.2 ± 0.8*	83.6 ± 2.5*	82.5 ± 9.9*

Multiple micropropagation parameters and the mean agricultural output (foliar FM ± SD) of the selected *A. dubius* clonal genotypes propagated via cuttings are listed in ***Table 3.5***. Overall, there was an average of 3.7 ± 1.1 new shoots per explant on multiplication medium, and the average shoot and root elongation rates were 1.5 ± 0.3 and 1.1 ± 0.3 cm/week, respectively. The number of new shoots per explant was higher in comparison to the micropropagation of *A. dubius* by Shaik et al. (2022), wherein each explant yielded an average of 2 new shoots. This outcome is likely due to genotype-specific variability unintentionally selected for in earlier stages of the present study, wherein genotypes retained a "stress memory", enabling a more efficient response during subsequent micropropagation, manifesting in increased shoot production (Ding et al. 2012; Sun et al. 2021).

Following acclimatisation, genotypes H1, H2, H3, H12, H14, H17, H20, H21, H22, H28, H30, H37, H38, and H40 yielded the highest foliar FM (48.9 ± 5.8 g), which was higher than the average foliar FM of the initial heat stress assay control group (25.3 ± 5.4 g) and greater than or equal to the non-stressed micropropagated control (39.8 ± 4.4 g) over the same culture period. This discrepancy was most likely caused by the differing growth conditions, i.e., greenhouse acclimatisation of micropropagated clonal genotypes versus growth chamber propagation in the initial assay. Within this high-yielding group, H1, H3, H12, H17, H21, H30, H37, and H40 produced the most shoots while in the shoot multiplication medium. Genotypes H1, H12, H20, H21, H28, H30, H37, and H38 demonstrated the fastest shoot elongation rates in the elongation medium. Genotypes H1, H3, H12, H14, H17, H20, H22, H28, H30, H37, and H38 showed the fastest root elongation rates when transferred to the rooting medium. Overall, genotypes H1, H12, H30, and H37 performed the best throughout micropropagation and subsequent acclimatisation.

Table 3.5: Growth measurements from the micropropagation of selected high-temperature (H) tolerant *A. dubius* genotypes. Different letters indicate statistically significant differences among genotypes (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, n = 10. FM = fresh mass.

Genotype	Number of New Shoots	Shoot elongation (cm/week)	Root elongation (cm/week)	Foliar FM (g)
H1	$4.0^{abcdefg} \pm 1.2$	$2.0^{ab} \pm 0.2$	$1.4^{abcd} \pm 0.5$	$46.1^{abcdefgh} \pm 8.3$
H2	$2.7^{fghi} \pm 0.8$	$1.4^{bcdefgh} \pm 0.4$	$0.4^{g} \pm 0.4$	$51.0^{abcd} \pm 4.6$
H3	$4.3^{abcdef} \pm 0.8$	$1.3^{defgh} \pm 0.3$	$1.0^{abcdefg} \pm 0.4$	$47.3^{abcdefg} \pm 8.8$
H4	$3.6^{bcdefgh} \pm 1.0$	$1.6^{abcdefgh} \pm 0.2$	$1.3^{abcde} \pm 0.4$	$40.6^{cdefghijk} \pm 9.5$
H7	$2.7^{fghi} \pm 1.2$	$1.1^{fgh} \pm 0.3$	$0.8^{cdefg} \pm 0.3$	$29.8^{kl} \pm 4.3$
H8	$4.1^{abcdefg} \pm 0.7$	$1.9^{abc} \pm 0.5$	$1.4^{abc} \pm 0.2$	$34.5^{ijkl} \pm 7.0$
H9	$5.4^{a} \pm 1.3$	$1.6^{abcdefgh} \pm 0.4$	$0.8^{cdefg} \pm 0.3$	$38.5^{fghijkl} \pm 6.4$
H12	$4.8^{abc} \pm 0.6$	$1.6^{abcdefgh} \pm 0.2$	$1.4^{abc} \pm 0.3$	$46.8^{abcdefg} \pm 6.5$
H13	$4.8^{abc} \pm 0.6$	$1.4^{bcdefgh} \pm 0.5$	$0.9^{bcdefg} \pm 0.3$	$29.3^{l} \pm 7.3$
H14	$3.3^{cdefghi} \pm 1.3$	$1.2^{defgh} \pm 0.5$	$1.3^{abcde} \pm 0.3$	$52.5^{ab} \pm 8.4$
H17	$4.6^{abcd} \pm 0.8$	$1.1^{fgh} \pm 0.4$	$1.5^{a} \pm 0.3$	$47.1^{abcdefg} \pm 4.1$
H18	$1.8^{i} \pm 0.8$	$1.3^{cdefgh} \pm 0.5$	$0.8^{cdefg} \pm 0.5$	$32.5^{jkl} \pm 6.9$
H19	$5.5^{a} \pm 0.7$	$1.5^{bcdefgh} \pm 0.2$	$0.9^{bcdefg} \pm 0.3$	$39.3^{efghijkl} \pm 5.2$
H20	$3.4^{cdefghi} \pm 1.3$	$2.1^{a} \pm 0.3$	$1.5^{a} \pm 0.5$	$46.6^{abcdefg} \pm 3.1$
H21	$4.6^{abcd} \pm 0.7$	$1.8^{abcde} \pm 0.5$	$0.8^{cdefg} \pm 0.3$	$51.4^{abc} \pm 4.0$
H22	$3.1^{defghi} \pm 1.3$	$1.5^{bcdefgh} \pm 0.6$	$1.2^{abcdef} \pm 0.5$	$49.2^{abcdef} \pm 3.1$
H23	$4.5^{abcd} \pm 0.8$	$1.8^{abcd} \pm 0.3$	$1.0^{abcdefg} \pm 0.5$	$35.6^{hijkl} \pm 5.0$
H24	$2.1^{hi} \pm 1.5$	$1.1^{efgh} \pm 0.2$	$1.1^{abcdefg} \pm 0.5$	$31.6^{kl} \pm 8.9$
H27	$3.3^{cdefghi} \pm 0.7$	$1.5^{bcdefgh} \pm 0.5$	$1.6^{a} \pm 0.3$	$29.3^{l} \pm 6.7$
H28	$3.6^{bcdefgh} \pm 0.8$	$1.7^{abcdef} \pm 0.4$	$1.2^{abcdef} \pm 0.4$	$50.0^{abcde} \pm 6.4$
H29	$1.7^{ghi} \pm 1.5$	$1.3^{defgh} \pm 0.4$	$0.4^{g} \pm 0.4$	$43.1^{bcdefghij} \pm 3.5$
H30	$4.1^{abcdefg} \pm 0.7$	$1.6^{abcdefgh} \pm 0.3$	$1.3^{abcde} \pm 0.4$	$45.7^{abcdefgh} \pm 7.2$
H32	$1.8^{hi} \pm 1.0$	$1.1^{gh} \pm 0.4$	$1.5^{ab} \pm 0.3$	$32.7^{jkl} \pm 4.8$
H33	$2.0^{hi} \pm 1.2$	$1.6^{abcdefgh} \pm 0.3$	$0.9^{bcdefg} \pm 0.4$	$40.0^{defghijkl} \pm 4.3$
H34	$4.4^{abcde} \pm 0.7$	$1.2^{defgh} \pm 0.2$	$0.6^{efg} \pm 0.7$	$36.6^{ghijkl} \pm 9.8$
H35	$3.4^{cdefghi} \pm 1.2$	$2.0^{ab} \pm 0.3$	$0.6^{fg} \pm 0.2$	$30.7^{kl} \pm 2.9$
H37	$5.4^{a} \pm 1.0$	$1.5^{abcdefgh} \pm 0.4$	$1.1^{abcdef} \pm 0.4$	$54.9^{a} \pm 4.8$
H38	$2.5^{fghi} \pm 1.2$	$1.7^{abcdefg} \pm 0.4$	$1.3^{abcde} \pm 0.5$	$44.7^{abcdefghi} \pm 5.3$
H39	$5.1^{ab} \pm 0.9$	$2.0^{ab} \pm 0.2$	$1.5^{ab} \pm 0.2$	$34.4^{ijkl} \pm 10.6$
H40	$5.5^{a} \pm 1.7$	$1.1^{h} \pm 0.2$	$0.7^{defg} \pm 0.4$	$51.7^{ab} \pm 6.7$
Control	$2.5^{fghi} \pm 0.9$	$1.4^{bcdefgh} \pm 0.4$	$0.8^{cdefg} \pm 0.3$	$39.8^{defghijkl} \pm 4.4$
Mean	**3.7 ± 1.0**	**1.5 ± 0.4**	**1.1 ± 0.4**	**41.5 ± 6.1**

Wild-type specimens which were not exposed to stress treatments were used as a control.

Compared in ***Table 3.6*** are pre- and post-micropropagation physiological parameters (Ψ_{Leaf}, protein, chlorophyll, and proline) of the identified heat-tolerant *A. dubius* genotypes which demonstrated superior growth throughout micropropagation (H1, H12, H30, and H37). Following acclimatisation, the clonal genotypes were exposed to the same 45 °C day-cycle as their parents, and then the various measurements were taken to assess true-to-type fidelity. Similar to the water-deficit post-micropropagation results, none of the compared parameters yielded significant differences ($p > 0.05$), indicating that micropropagation and subsequent acclimatisation did not have a significant effect on the ability of the selected genotypes to tolerate heat stress, supporting true-to-type fidelity and reliability of the protocol established by Shaik et al. (2022).

Table 3.6: Comparisons between pre- and post-micropropagation physiological parameters (leaf water pressure potential (Ψ_{Leaf}), protein, chlorophyll, and proline, mean ± SD) of identified heat-tolerant *A. dubius* clonal genotypes exposed to 45 °C (paired samples T-test, n = 3). * indicates similarity between before and after measurements for each parameter per genotype ($p > 0.05$).

Genotype	**Ψ_{Leaf}** (psi)		**Protein** (µg/mL)		**Chlorophyll** (µg/cm^2)		**Proline** (µg/mL)	
	Before	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
H1	-4.5 ± 0.3*	-5.0 ± 0.6*	2.1 ± 0.1*	2.0 ± 0.2*	1.6 ± 0.1*	1.6 ± 0.1*	167.9 ± 18.3*	157.5 ± 10.8*
H12	-4.6 ± 0.6*	-4.8 ± 0.6*	2.9 ± 0.1*	2.5 ± 0.6*	2.2 ± 0.1*	2.0 ± 0.4*	95.3 ± 9.8*	99.7 ± 11.0*
H30	-9.0 ± 0.5*	-9.2 ± 1.6*	2.3 ± 0.1*	2.4 ± 0.3*	2.6 ± 0.2*	2.3 ± 0.2*	110.6 ± 6.7*	110.4 ± 11.5*
H37	-15.6 ± 1.4*	-15.0 ± 0.5*	3.4 ± 0.1*	3.2 ± 0.2*	2.0 ± 0.1*	1.9 ± 0.1*	85.5 ± 18.8*	81.5 ± 6.3*

The absence of significant differences in Ψ_{Leaf} implied that water status remained relatively constant, attributed to efficient water uptake, retention, and a balanced transpiration rate, highlighting the ability of the genotypes to withstand water and heat stress even after micropropagation. The consistent levels of protein, chlorophyll, and proline content further emphasise the robustness of the chosen genotypes in maintaining biochemical components necessary for cellular functions and photosynthetic processes under stress. This stability observed in the physiological parameters may be attributed to the inherent adaptability and stress tolerance mechanisms within the selected genotypes. In the context of screening for superior genotypes, these findings suggest that the selected genotypes maintain desirable traits related to water-deficit and high-temperature tolerance and stress resilience during micropropagation. This stability could serve as a valuable criterion for identifying superior genotypes with consistent physiological performance, laying the foundation for their potential utilisation in breeding programs or crop improvement strategies.

3.3.8 Protein characterisation

Mass spectrometry of crude protein extracts from selected water-deficit tolerant *A. dubius* genotypes is represented in ***Figure 3.14***. The analysis of this data using National Center for Biotechnology Information (NCBI) protein databases identified similar proteins linked to stress adaptation mechanisms across genotypes, including the RNA polymerase β subunit (accession WOK42753), ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) large subunit (accession AKG26942), ribosomal protein L16 (accession YP_009922872), and ribosomal protein S16 (accession CAB45301). Additionally, unnamed proteins were detected (accessions CAI0415553 and CAI0385149). Genotype A2 yielded the highest relative intensity (48%) for the RNA polymerase β subunit among all tested genotypes. Genotypes A2 (37%) and G7 (35%) had the highest relative intensities for the RuBisCO large subunit. The intensity of ribosomal protein L16 was equal among genotypes A2, G7, and V3 (8%), whereas the intensity of ribosomal protein S16 was highest in A2 (54%).

The detection of RNA polymerase β subunits aligns with studies on drought-tolerant barley (Harb et al. 2020) and rice (Liang et al. 2021) genotypes where enhanced transcriptional activity was documented under water-deficit stress. RNA polymerase β subunits maintain transcriptional activity during stress, ensuring the expression of stress-responsive genes, such as dehydrins, DREB proteins, and antioxidants, which act in mitigating cellular damage (Wang et al. 2024). The presence of RuBisCO large subunits suggests the maintenance of photosynthetic efficiency during water-deficit stress, consistent with findings in rice, wheat, and maize, where increased RuBisCO activity was correlated with stress tolerance (Perdomo et al. 2017). RuBisCO, the main enzyme of the Calvin cycle, not only facilitates carbon fixation but also acts as a metabolic sink to regulate the redox balance under stress (Carmo-Silva and Sharwood 2023). By maintaining RuBisCO activity, plants can continue limited photosynthesis, producing sugars required for osmoprotection and energy generation, thus sustaining cellular homeostasis during stress. The detection of ribosomal proteins L16 and S16 supports findings in *Nicotiana benthamiana* and *A. thaliana* where ribosomal protein stability promoted stress tolerance (Fakih et al. 2023). Ribosomal proteins like L16 and S16 are essential for maintaining translational fidelity under stress conditions, ensuring that proteins are synthesised efficiently (Vadivel Gnanasundram and Fåhraeus 2018). Additionally, these ribosomal proteins are often involved in

ribosome repair or reassembly, safeguarding the integrity of the protein synthesis apparatus (Vadivel Gnanasundram and Fåhraeus 2018).

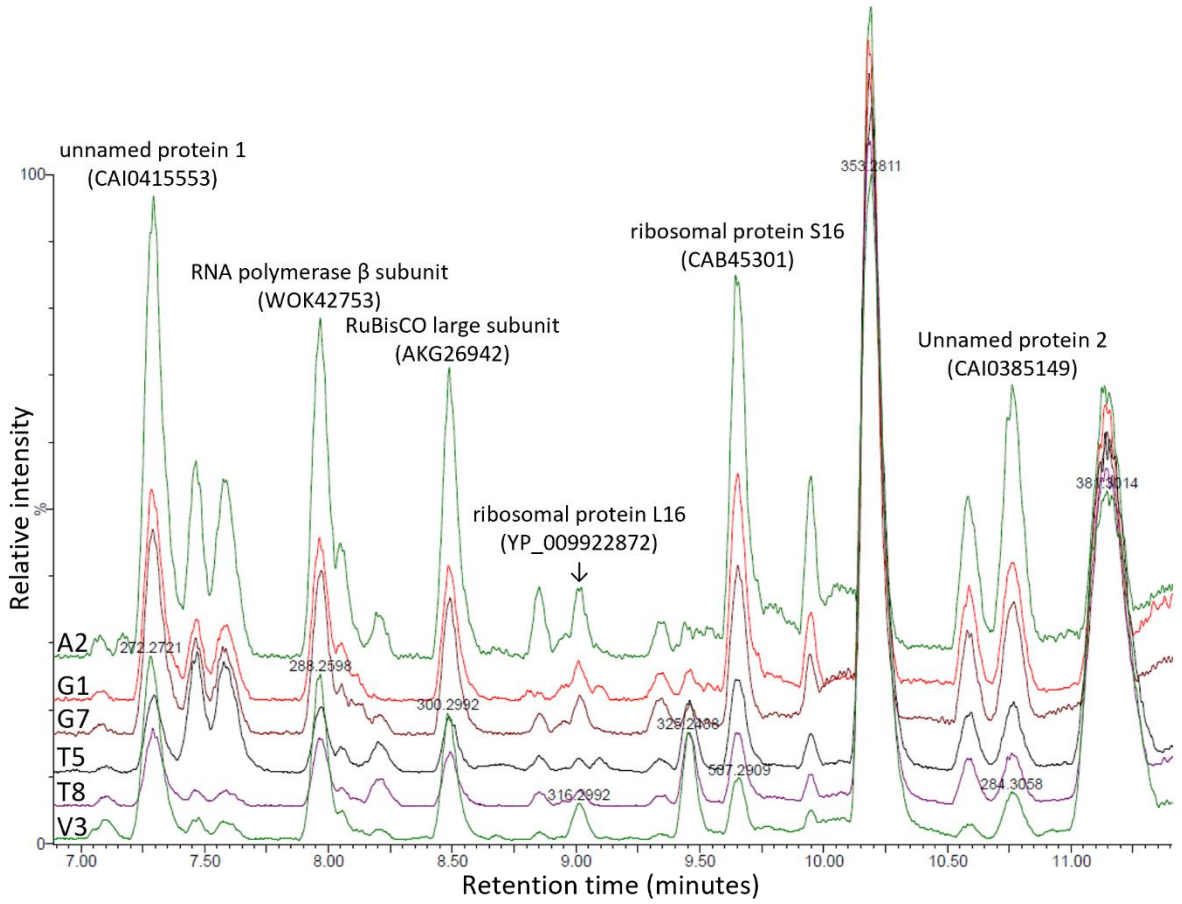


Figure 3.14: Mass spectrometry analysis with labelled base peak mass peaks of crude protein extracts from water-deficit-stressed *A. dubius* genotypes undergoing 4-, 8-, 12, and 16-day watering regimens.

The mass spectra of proteins extracted from selected heat-tolerant *A. dubius* genotypes are overlayed in ***Figure 3.15***. The identified proteins include photosystem II protein D1 (accession XIF09057), putative channel protein 1 (accession AAC03555), ribosomal protein L32 (accession XED52978), and ribosomal protein S12 (accession XCH54301). Genotype H12 showed double the relative intensity % for photosystem II protein D1 (8%) compared to the other tested genotypes (4%), suggesting greater stability or abundance of this protein in this genotype. All genotypes showed similar intensity for the channel protein (14%). Genotypes H1 and H12 demonstrated the highest relative intensity for L32 (45%), but all genotypes had comparable intensities for S12. Additionally, an unnamed protein (accession CAI0415553) was detected, which was also present in the water-deficit-stressed genotypes.

The identified proteins highlight important mechanisms underlying heat tolerance. The presence of photosystem II protein D1 reflects the role of this protein in maintaining photosynthetic efficiency under heat stress by repairing photodamage and stabilising photosystem II, as observed in other heat-adapted species (Theis and Schroda 2016). The putative channel protein 1 likely supports cellular ion homeostasis and osmotic balance because high-temperature stress leads to increased membrane fluidity and leakage of ions, which impair metabolic processes (Maurel et al. 2008). Ribosomal proteins L32 and S12 emphasise the role of translational machinery stability during heat stress. These proteins are known to protect ribosomes from thermal denaturation, ensuring protein synthesis continuity (Fakih et al. 2023). Future research should focus on functional validation of the presently identified proteins and explore the roles of unidentified proteins to fully elucidate their contributions to stress resilience.

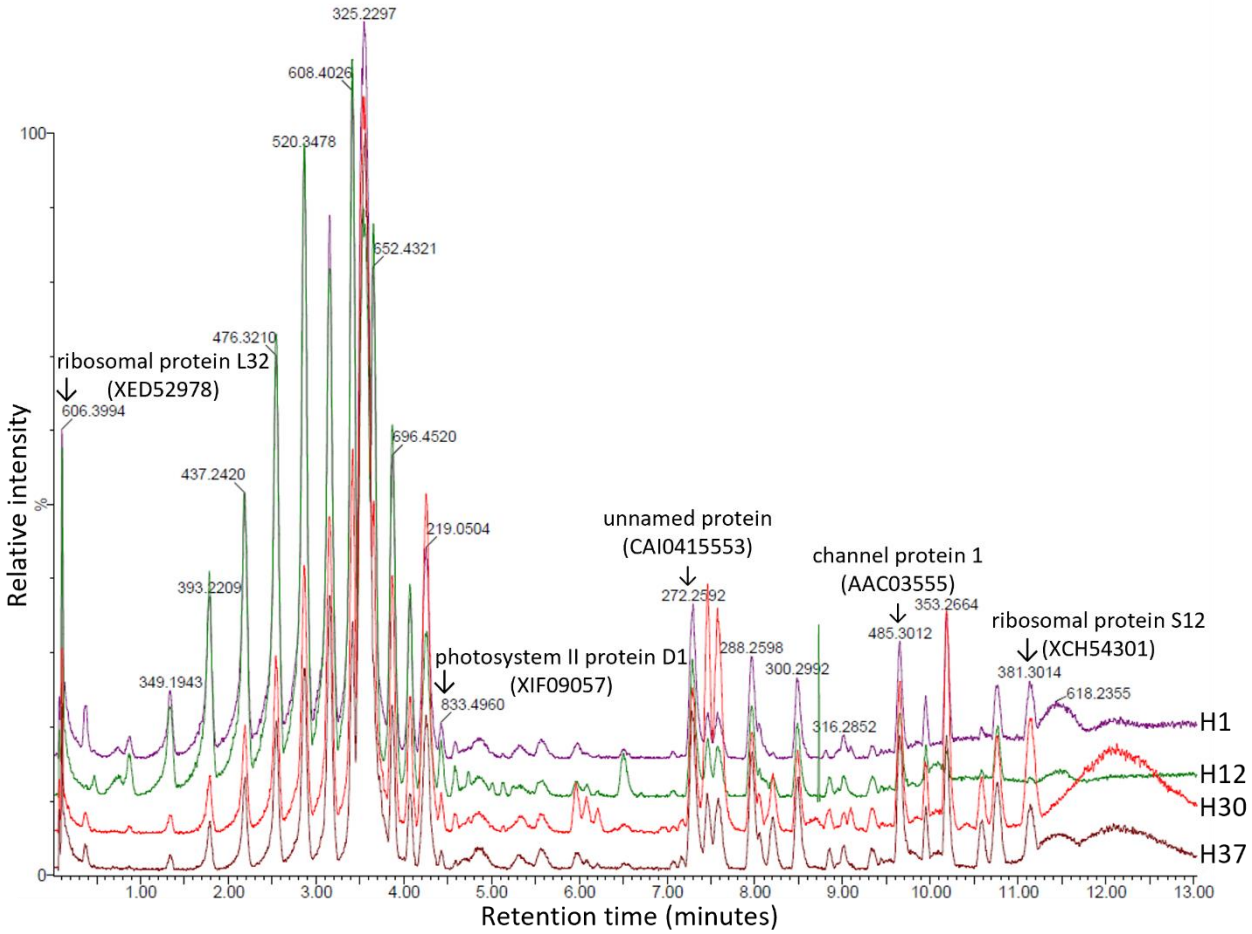


Figure 3.15: Mass spectrometry analysis with labelled base peak mass peaks of crude protein extracts from heat-stressed *A. dubius* genotypes undergoing 30, 35, 40, and 45 °C treatments.

3.3.9 Relative gene expression

RT-qPCR analysis yielded no significant amplification of putative DREB1A and annexin 1 transcripts in any of the selected water-deficit-stressed *A. dubius* genotypes propagated via cuttings. Attempts to optimise thermal cycling parameters and primer and template concentrations continued to yield no result. ***Figure 3.16*** shows the agarose gel electrophoresis of RNA extracts from water-stressed *A. dubius* leaves, wherein 2 distinct bands, representing 28S and 18S ribosomal RNA, were visualised, indicating pure and high-quality RNA samples with minimal degradation, supporting the robustness of the RNA extraction protocol. Furthermore, successful cDNA synthesis was confirmed through amplification of the internal positive control (MDH), which highlights the technical proficiency of the reverse transcription step (***Figure 3.17***), indicating complications in achieving GOI primer specificity, low GOI transcript concentrations, suboptimal PCR conditions, or the inherent genetic variations among samples contributed to the lack of amplification of GOI transcripts. The RT-qPCR experiments for water-deficit and heat stress conditions require further optimisation.

This finding, while noteworthy, prompts a comprehensive discussion connecting it to the broader experimental context, particularly the concurrent analyses of protein and chlorophyll content aimed at screening and selecting water-deficit-tolerant genotypes. The integration of protein and chlorophyll content analyses alongside a gene expression study can provide a more comprehensive understanding of the genotypic responses to water-deficit stress, enabling a more informed selection of superior genotypes for further investigation and potential agricultural applications. Protein content is a direct indicator of the ability of a plant to maintain cellular functions and adapt to stress. The absence of significant gene expression changes may not necessarily imply a lack of response to water-deficit; instead, alterations at the protein level may highlight post-transcriptional regulatory mechanisms or modifications that contribute to stress resilience (Abraham et al. 2018; Olalde-Portugal et al. 2020). The integration of physiological parameters with gene expression data allows for a more nuanced interpretation of the genotypic responses. For example, a genotype might exhibit resilience by maintaining consistent protein and chlorophyll levels even if the targeted stress-related genes show no significant change in expression (Wang et al. 2016; Santisree et al. 2017).

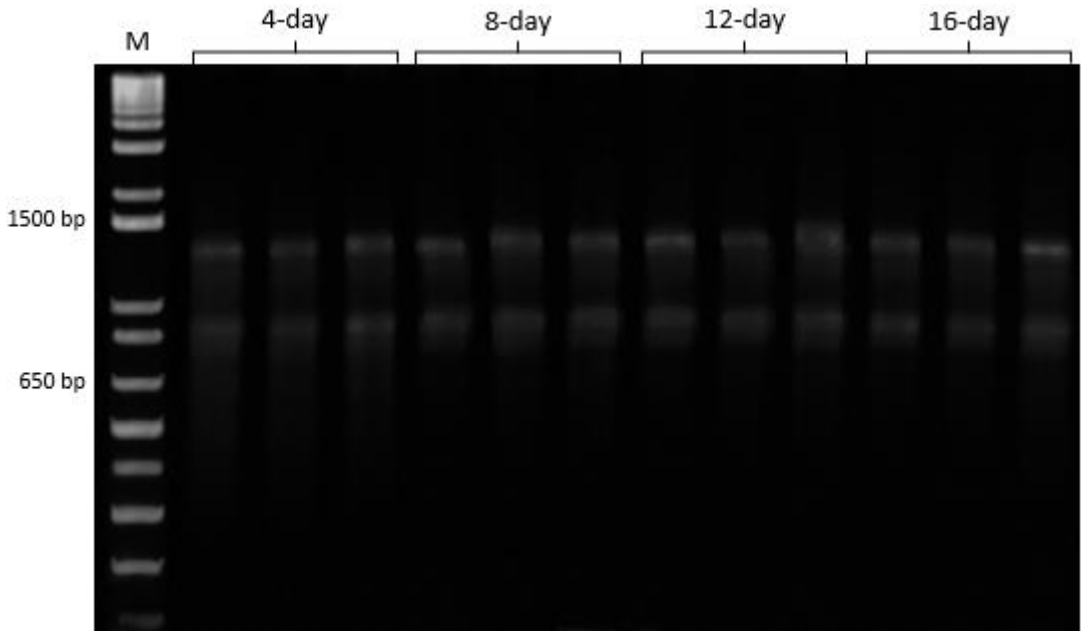


Figure 3.16: Electrophoresis of 5 µg total RNA extracts from *A. dubius* leaves exposed to variable watering regimens (4-, 8-, 12-, and 16-day intervals). M = 1 Kbp marker (Invitrogen, USA).

Considering the interplay between gene expression and physiological responses aids in the identification of potential molecular markers for water-deficit tolerance. While DREB1A and annexin 1 transcripts might not exhibit detectable changes in expression, specific trends in protein and chlorophyll content across genotypes could point towards alternative candidate genes or pathways associated with water-deficit adaptation, refining selection criteria and focusing on more reliable markers for future studies. The understanding derived from the integration of these analyses contributes to a robust foundation for selecting superior genotypes. Considering multiple facets of the response of *A dubius* genotypes to water-deficit stress enables the identification of genotypes that demonstrate not only transcriptional resilience but also maintain essential physiological processes crucial for growth and productivity, particularly pertinent for agricultural applications, where the goal is to identify genotypes with a holistic and sustainable capacity to thrive under water-limiting conditions.

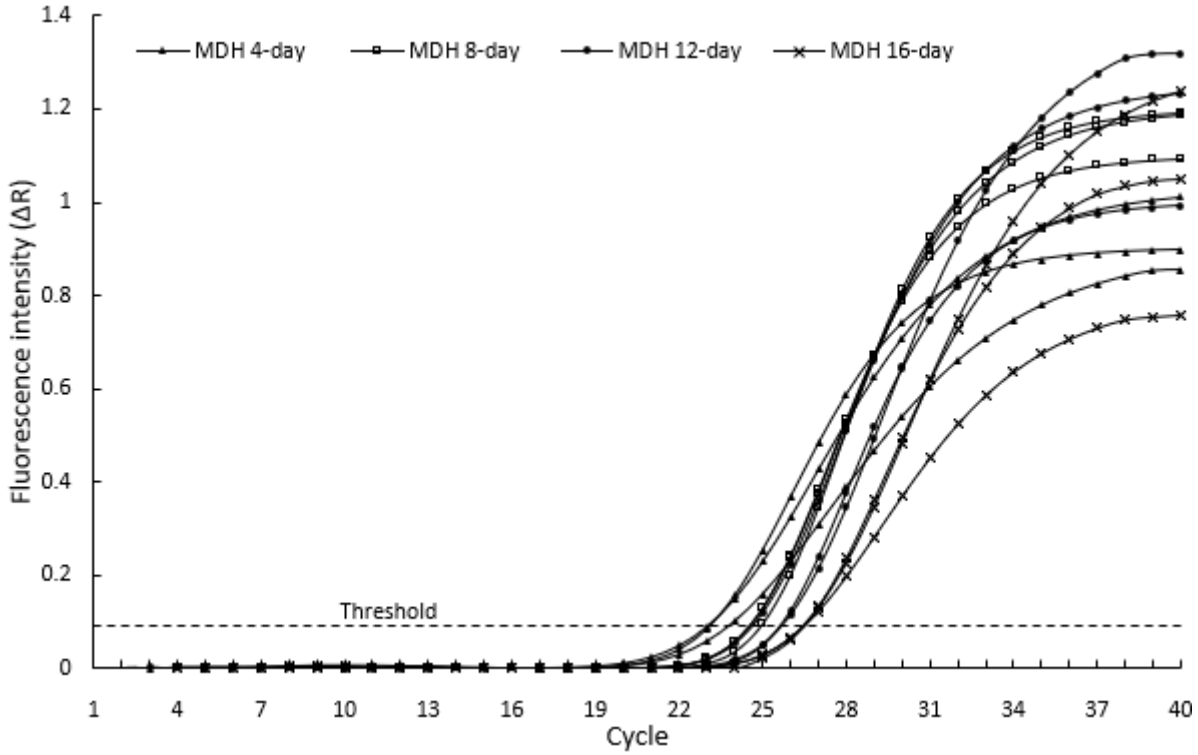


Figure 3.17: Real-time quantitative PCR (RT-qPCR) amplification plot showing expression of the malate dehydrogenase (MDH) housekeeping gene in *A. dubius* leaves subjected to varied watering intervals (4-, 8-, 12-, and 16-day).

Additionally, RT-qPCR analysis yielded no significant amplification for putative HSP70 transcripts in any of the selected heat-stressed genotypes. ***Figure 3.18*** shows the agarose gel electrophoresis of RNA extracts from *A. dubius* leaves, wherein 2 distinct bands, representing 28S and 18S ribosomal RNA, were visualised, indicating pure and high-quality RNA samples with minimal degradation. Furthermore, successful cDNA synthesis was confirmed through amplification of the internal positive control (MDH) (***Figure 3.19***). Further analysis necessitates validating the degenerate HSP70 primers by assessing their specificity across related species, reevaluating the design of the primers, and using multiple primer pairs to target different regions of this GOI. Alternatively, different quantitative methods, such as nested PCR or probe-based assays, can increase specificity, and digital PCR can absolutely quantify HSP70 expression, detecting low-abundance transcripts. Furthermore, RNA-seq can determine if the lack of amplification reported in this study reflects the actual HSP70 expression pattern, or lack thereof, in *A dubius*. However, due to the highly conserved nature of the HSP70 gene across the plant and animal kingdoms (Tsunekawa et al. 1999; Sung et al. 2001; Lai et al. 2021), it is highly unlikely that *A. dubius* does not express this protein or a similar analogue.

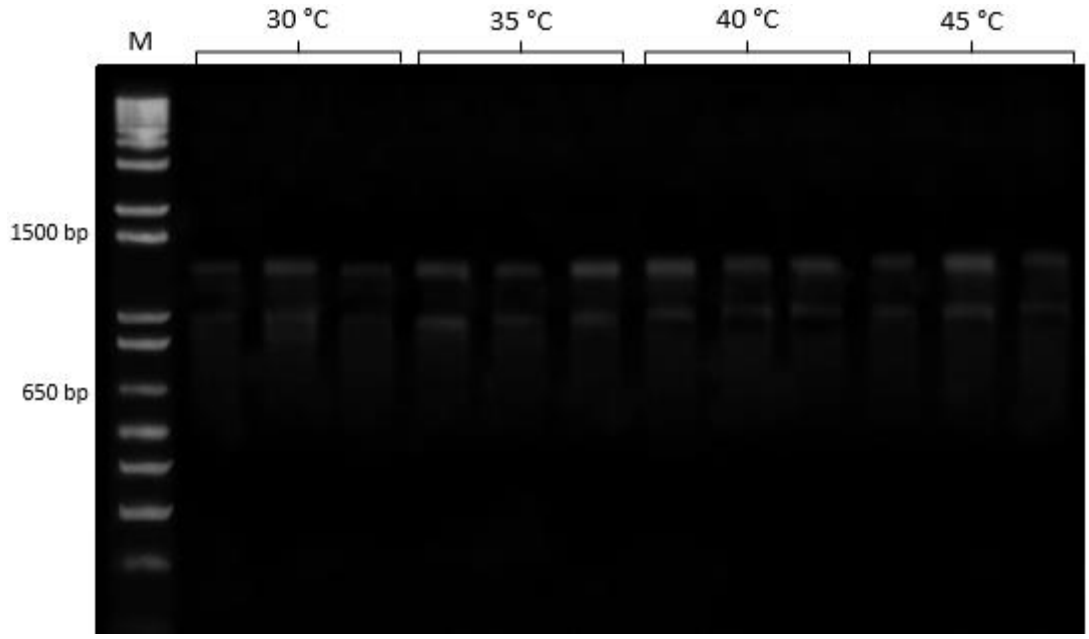


Figure 3.18: Electrophoresis of 5 µg total RNA extracted from leaves of *A. dubius* exposed to heat stress (30, 35, 40, and 45 °C). M = 1 Kbp marker (Invitrogen, USA).

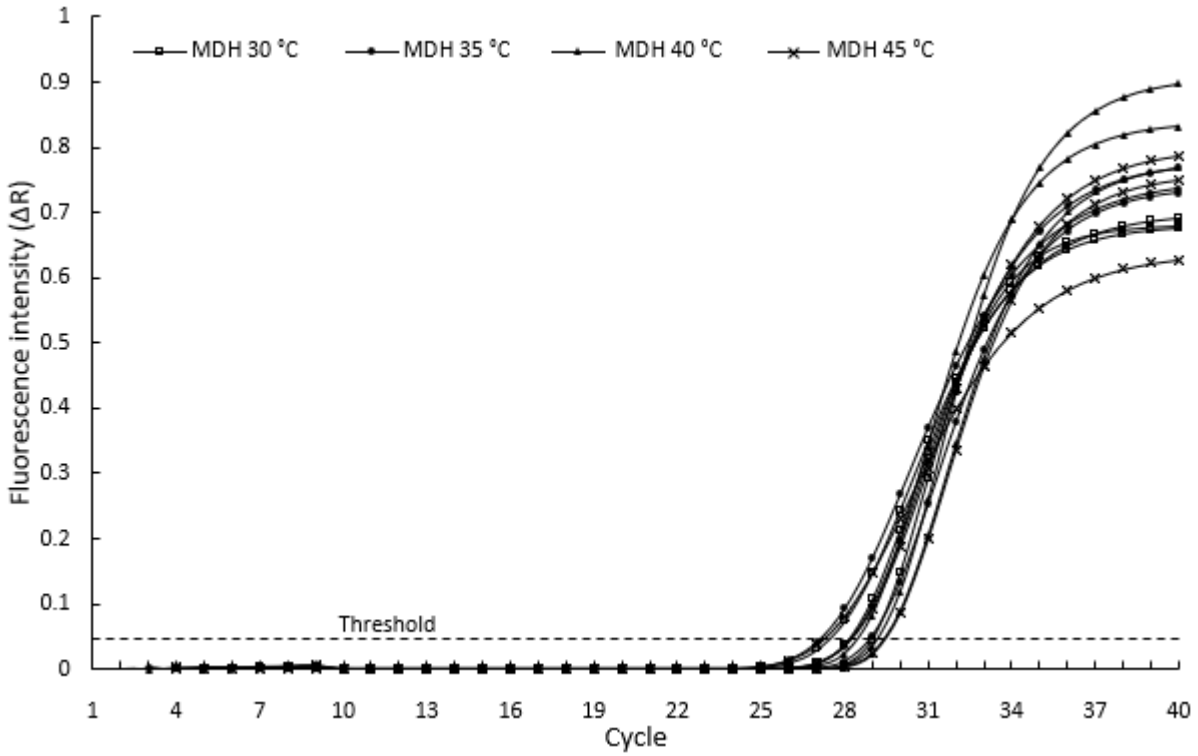


Figure 3.19: Real-time quantitative PCR (RT-qPCR) amplification plot showing expression of the malate dehydrogenase (MDH) housekeeping gene in *A. dubius* leaves subjected to heat stress (30, 35, 40, and 45 °C).

3.4 Conclusion

Across 1000 genotypes of *A. dubius* sourced from 5 different locations around KwaZulu-Natal, South Africa, the assessment of water-deficit tolerance revealed varied responses in growth parameters, Ψ_{Leaf}, protein content, and chlorophyll content, highlighting genotype-specific adaptations to varying water availability. Under severe water-deficit (16-day interval), genotypes from Genozzano and Tongaat cumulatively displayed the highest vigour ratings compared to genotypes from Amanzimtoti, Verulam, and Westville. Several genotypes maintained Ψ_{Leaf} despite decreased watering frequencies and exhibited stable Ψ_{Leaf} levels between watering intervals. Genotypes A2, G7, T5, and V3 grew the fastest and yielded the highest FM during micropropagation and subsequent acclimatisation. Furthermore, these genotypes were also able to maintain relatively stable Ψ_{Leaf}, protein, chlorophyll, and proline when exposed to variable watering regimens, and micropropagation maintained true-to-type fidelity of this stress response. Mass spectrometry analysis of tolerant genotypes detected the presence of many stress-protective proteins, including RNA polymerase β subunits (accession WOK42753), ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) subunits (accession AKG26942), and ribosomal proteins L16 (accession YP_009922872) and S16 (accession CAB45301) under water-deficit stress. Under high-temperature stress, photosystem II proteins (accession XIF09057), channel proteins (accession AAC03555), and ribosomal proteins L32 (accession XED52978) and S12 (accession XCH54301) were detected.

In the heat stress assay, among the studied *A. dubius* genotypes, H37 performed the best, demonstrating consistency in proline and chlorophyll content, protein turnover, and propagative capacity. This genotype exhibited elevated chlorophyll content under increasing temperatures, signifying an adeptness in sustaining photosynthetic pigments. Furthermore, the maintenance of leaf protein content at elevated temperatures suggests mechanisms for regulating protein synthesis and stability under heat stress, including HSPs and hormonal signalling pathways. During micropropagation and subsequent acclimatisation, genotypes H1, H12, H30, and H37 displayed superior foliar FM and rapid shoot and root elongation rates, striking a balance between shoot and root development. These genotypes also demonstrated stability in Ψ_{Leaf}, chlorophyll, protein, and proline when exposed to increasing heat stress post-micropropagation.

The multifaceted nature of the results obtained in this study provides possible explanations regarding the resilience of *A. dubius* genotypes under water-deficit and heat stress, emphasising the ability to prioritise protein synthesis and activate protective mechanisms to mitigate the adverse effects of stress on cellular function. Specific genotypes exhibited dynamic responses by modulating protein, chlorophyll, and proline content under various stress levels, demonstrating a complex interplay of hormonal, enzymatic, and metabolic adjustments to regulate protein abundance and sustain cellular functions. For those genotypes that did not show significant increases in proline, further exploration of alternative tolerance mechanisms is warranted to fully understand their potential for water-deficit and heat stress resilience. Utilising omics technologies such as transcriptomics and proteomics can further unravel molecular pathways influencing this response.

In practical agricultural applications, the insights obtained from this study guide the selection of genotypes suited to specific environmental conditions, enabling the development of crop varieties better adapted to arid regions. Furthermore, the knowledge gained aids in the implementation of targeted management practices that optimise water use efficiency and mitigate the impacts of water scarcity and high temperatures on crop productivity. The observed diversity in responses among genotypes underscores the importance of considering genetic variability in breeding for stress tolerance. These results emphasise the diversity of adaptive strategies employed by different genotypes of *A. dubius* to navigate water-deficit and heat stress, deepening the collective understanding of plant stress physiology and holding promise for the selection and future cultivation of resilient varieties, providing sustainable agriculture in dynamically changing and challenging environments.

3.5 References

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4. Relative expression of a salinity stress-responsive Na^+/H^+ exchanger (NHX) in root and leaf tissues of the African leafy vegetable, *Amaranthus dubius.*

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Abstract

Amaranthus dubius, an African leafy vegetable (ALV), is an easy-to-grow, annual shrub and a highly nutritious food source, containing elevated levels of essential nutrients in the leaves. Many ALVs, including *A. dubius,* can tolerate salinity stress, enabling their cultivation on marginal land. However, the widespread propagation of *A. dubius* as a stable food source has thus far not been realised due partially to the high frequency at which hybridisation occurs, resulting in high genotypic and phenotypic variability. Therefore, to increase the agricultural output capacity of this species on salt-affected marginal lands, it is important to screen, select and then clonally propagate the identified salinity-tolerant genotypes to ensure true-to-type fidelity in the regenerated population. It is also important, thereafter, to elucidate their underlying gene expression of the stress response. The present study exposed four-week-old *A. dubius* seedlings to 100, 200, and 400 mM NaCl to determine their degree of salt tolerance. Genotypes were then screened, selected, and clonally propagated through cuttings based on high growth rates, biomass, and salt tolerance. Generally, growth and physiological parameters decreased as substrate salinity increased. However, individual salt-stressed genotypes demonstrated similar vigour to non-stressed plants and were able to maintain total protein and chlorophyll concentrations despite increasing salinity. The relative expression of an NHX1-like transcript was quantified in 15 genotypes using degenerately primed Real Time-qPCR. The relative expression of the putative NHX1 gene was 6.7 times greater in root tissues of seedlings treated with 400 mM NaCl (10.7 ± 1.8) compared to the roots of untreated seedlings (1.6 ± 1.3) and 2.8-fold more than leaf tissues harvested from seedlings treated with 400 mM NaCl. Furthermore, the relative

electrical conductivity (EC) of root tissues was 10 times greater than the EC of leaf tissues from the same 400 mM NaCl treatment. Numerous genotypes yielded similar chlorophyll content between 200 and 400 mM NaCl treatments, with genotypes salinity-1 (S1) (3.5 ± 0.2 μg/cm^2) and S34 (4.0 ± 0.4 μg/cm^2) having the highest concentrations of chlorophyll in the 400 mM group, which was positively correlated to total protein content. Following micropropagation through direct organogenesis, selected clones maintained true-to-type traits such as similar chlorophyll, protein, and NHX1-like expression as their parent plants when exposed to 400 mM NaCl. This study revealed that some genotypes demonstrated salt stress tolerance capabilities comparable to established halophytes by regulating the constitutive or inducible expression of an NHX1-like protein in roots and leaves. The correlation between protein content and NHX1-like expression was nonlinear and nonproportional, demonstrating the complexity of this response and necessitating further exploration of specific protein families or functional groups conferring salinity tolerance in this species.

Keywords: Amaranthaceae, Salinity tolerance, Gene expression, RT-qPCR, NHX

4.1 Introduction

Soil salinisation is an indirect but undeniable consequence of the global climate crisis (Bannari and Al-Ali 2020). Rising temperatures increase evaporation in arid and semi-arid regions, concentrating salts in the soil. Unpredictable precipitation patterns compound this issue by restricting salt leeching or displacing and depositing salts from deeper layers into the surface soil. Additionally, rising ocean levels facilitate saltwater incursion into coastal and low-lying areas, contaminating freshwater and marginal land. Salinity stress is characterised by excess Na^+ ions, which disrupt the fragile ionic and osmotic equilibria required for many vital biochemical pathways (Mahajan and Tuteja 2005; Tuteja et al. 2011). Elevated soil salinity (> 40 mM NaCl) severely decreases the agricultural output capacity of many conventionally cultivated staple crops, including maize (*Zea mays*) and rice (*Oryza sativa*), by impeding water and nutrient uptake, hindering germination, respiration, and photosynthesis, resulting in wilted, stunted, and diseased crops (Machado and Serralheiro 2017; Huang et al. 2019; Ben-Asher et al. 2021). Furthermore, salinity stress generates an electropositive potential across cellular membranes, manifesting as chlorosis and necrosis (Tuteja et al. 2011).

Some plants trigger a detoxification process in response to salinity stress, including upregulating the

production of transmembrane antiporters such as Na^+/H^+ exchangers (NHX) (Yokoi et al. 2002). The NHX family of proteins utilise the inductive potential of protons to actively transport toxic Na^+ against the electrochemical gradient and away from sensitive intracellular components (Yokoi et al. 2002). Certain crucifers (*Brassica* spp.), cucurbits (*Cucumis* spp.*)*, and mallows (*Gossypium* spp.) demonstrate moderate (40-200 mM NaCl) salinity stress tolerance by regulating ion homeostasis, particularly maintaining favourable intracellular K^+ and Na^+ levels, achieved through the selective uptake and active transport of these ions, as well as compartmentalisation of Na^+ in vacuoles (Kumar et al. 2009; Akrami and Arzani 2019; Sharif et al. 2019). Extremely salt-tolerant (200-2000 mM NaCl) halophytes, such as *Kochia sieversiana* and *K. prostrata,* utilise similar mechanisms of ion sequestration but to a greater extent than their salt-sensitive glycophytic relatives (Karimi et al. 2005; Yang et al. 2007; Rahman et al. 2021). Cytosolic enzymes isolated from halophytes are similarly intolerant to salinity compared to their glycophytic counterparts, underscoring the importance of Na^+ compartmentalisation across species (Greenway and Osmond 1972; Glenn et al. 1999; Flowers and Colmer 2008).

Salinity stress has been shown to significantly affect the chlorophyll content in many plants, including tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), peanut (*Arachis hypogaea*), radish (*Raphanus sativus*), and beet (*Beta vulgaris*), culminating in reduced photosynthetic efficiency and impeding overall growth (Jamil et al. 2007; Cao et al. 2018; Tahjib-UI-Arif et al. 2019; Xie et al. 2019; Abdelaal et al. 2020). Excess soil salinity induces oxidative stress, which disrupts chlorophyll synthesis and degrades chloroplast membranes, leading to a decrease in total chlorophyll concentration (Idder et al. 2019; Abdelaal et al. 2020; Liu et al. 2023). Subsequently, excessive light energy is absorbed, further damaging the photosynthetic apparatus and decreasing carbon assimilation (Wang et al. 2021). The reduction in chlorophyll content under salinity stress has also been linked to changes in photosynthetic and metabolic pathways, modulating gene expression related to photosynthetic capacity and carbon metabolism (Kamyab et al. 2016; Lima-Melo et al. 2016; Singh et al. 2020; Li et al. 2022). Salinity stress, therefore, also significantly alters the expression of proteins, affecting total protein concentrations in plant tissues, which represents another indicator of adaptation to salinity stress (Suárez 2005; Hossain et al. 2011; Cheng et al. 2015; Attia et al. 2021).

Reduced chlorophyll and protein content under salinity stress have been correlated with increased electrolyte leakage, indicating cellular membrane damage (Klemm et al. 2002; Shahid et al. 2015; Liu et al. 2023). Therefore, electrical conductivity (EC) represents yet another parameter relevant to assessing the impact of salinity stress on plants, serving as a measure of ion leakage and compartmentalisation (Niu et al. 2017; Rao et al. 2019; Ehosioke et al. 2020). The quantification of these parameters in response to increasing levels of salinity stress provides valuable insights into plant physiology and biochemistry and has been used to identify and evaluate salinity resistant wheat (*Triticum aestivum*) (Jabeen et al. 2022), sorghum (*Sorghum bicolor*) (Bavei et al. 2011), barley (*Hordeum vulgare*) (Mohammed et al. 2021), cotton (*Gossypium hirsutum*) (Ramani et al. 2018), and thale cress (*Arabidopsis thaliana*) (Kalaji et al. 2016) varieties.

Some *Amaranthus* species, locally in South Africa and around the world, grow on marginal land and have demonstrated a high degree of resistance to salinity stress (Russell et al. 1998; Wang et al. 1999; Omamt et al. 2005; Delano-Frier et al. 2011). However, stress responses have not been genetically quantified in many indigenous amaranths, including the hardy annual shrub, *A. dubius,* which has demonstrated remarkable stress tolerance abilities and contains elevated levels of essential nutrients (Leung 1968; Ferrarotto 2003; Odhav et al. 2007; Muriuki et al. 2014; Dhanya et al. 2017; Hoang et al. 2020; Akter et al. 2022; Moyo et al. 2022). Identifying mechanisms underlying stress tolerance can facilitate selective breeding and crop improvement programs, enabling the cultivation of crops resilient to climate change-induced stresses, ensuring food security. Furthermore, elucidating the unique adaptive strategies of naturalised species encourages their conservation for future transformative and sustainable agricultural strategies, aiding both scientific understanding and practical applications in agriculture and food systems.

The only known allotetraploid in the genus, *A. dubius,* emerged via interspecific hybridisation (Sauer 1967; Waselkov et al. 2018). Increased ploidy is correlated with significantly greater genotypic variability, facilitating hybridisation and contributing to a multitude of morphological and physiological adaptations that influence the capacity of this species to thrive in harsh environments (Omamt et al. 2005; Khaing et al. 2013). Resilience in stressful environments combined with elevated nutritional contents reinforces the practicality of *A. dubius* as a crop that can alleviate malnutrition, improve resource-use efficiency, and offer economic potential in impoverished and marginalised regions,

directly addressing the global climate and food security crises. Nevertheless, indigenous foods are underutilised due to the popularisation and commercialisation of excessively processed and more conveniently supplied 'Westernised' diets, which often rely on resource-intensive agricultural practices (Dweba and Mearns 2011). In *A. dubius*, intraspecific variability and interspecific hybridisability present obstacles to extensive cultivation due to inherently unpredictable field growth characteristics (Wulff 1988; Sindhu 2002; Stetter and Schmid 2017; Viljoen et al. 2018; Areington et al. 2022).

Identification and clonal propagation of superior *A. dubius* genotypes merge traditional knowledge with modern agricultural practices, promoting sustainable, consistent, and economically viable food systems. This targeted approach minimises undesirable genetic variation, thereby enhancing consistency and uniformity for efficient scaling of genotypes with superior traits, such as high biomass and stress tolerance. Therefore, developing biotechnological techniques to quantify stress responses and screen for these superior genotypes is needed. A better understanding of the salinity tolerance strategies employed by *A. dubius* is necessary to improve and promote the widespread cultivation of this species in salt-affected regions. It is hypothesised that *A. dubius* specimens growing on marginal land express orthologous NHX proteins to aid salinity detoxification and Na^+-compartmentalisation. Additionally, quantifying the expression of NHX transcripts provides a genotypic basis for identifying salinity-tolerant cultivars.

The genome of *A. dubius* has not been sequenced, posing an obstacle to quantifying putative NHX expression levels because there are no known sequences from which to create polymerase chain reaction (PCR) primers. Therefore, a method for developing degenerate primers by aligning multiple known amino acid sequences of highly conserved NHX proteins from other well-studied members of the same class (eudicots) as *A. dubius* is required. This study investigated the ability of *A. dubius* to tolerate salinity stress by quantifying the underlying genetic expression and regulation of this stress response in root and leaf tissues to screen for salinity-tolerant genotypes. The aim was to adapt RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR) protocols to quantify the expression of potential NHX antiporters synthesised by *A. dubius* in response to saline environmental conditions. Furthermore, micropropagation of tolerant genotypes through direct organogenesis was

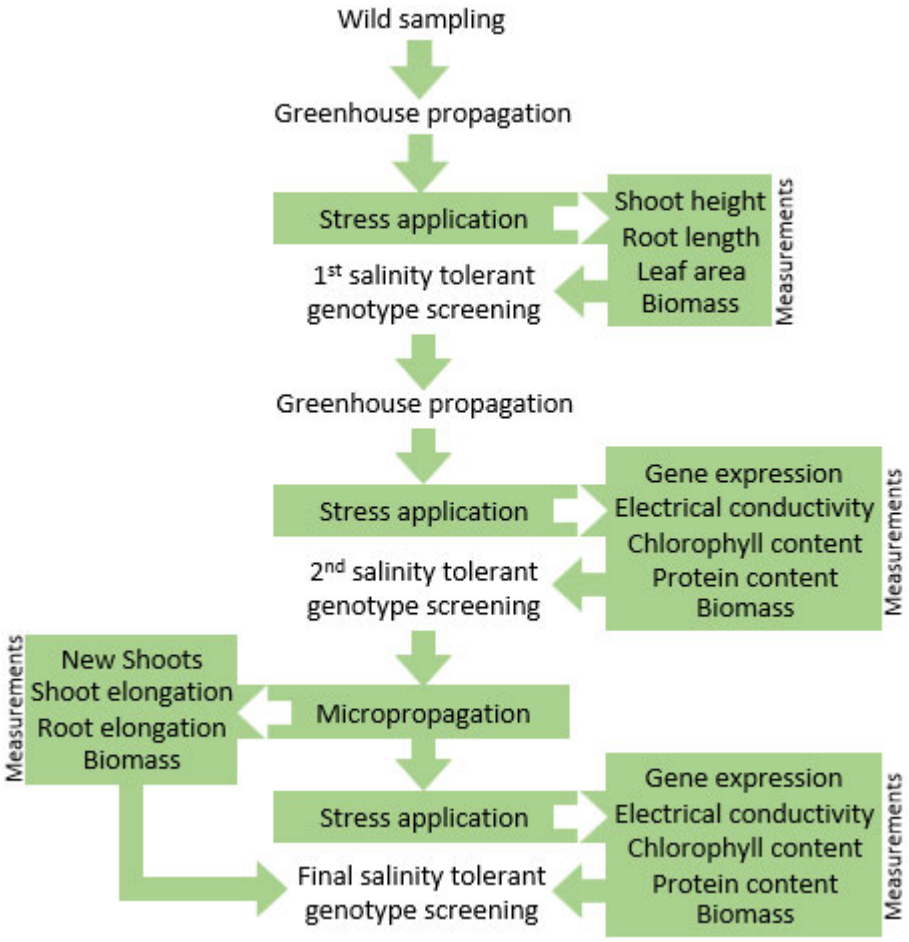
explored because this approach allows for year-round, rapid multiplication of contaminant-free, true-to-type clones.

4.2 Materials and methods

Scheme 4.1 summarises the experimental design for this study, outlining the sequence of growth, physiology, and gene expression analyses and measurements used to screen and select salinity-tolerant genotypes of *A. dubius* from the wild population. There were three screening steps following salinity stress treatments wherein the best-performing genotypes were selected at each step for further experiments. The first two screening steps were carried out in a greenhouse, while the final screening occurred following micropropagation and subsequent greenhouse acclimatisation of the identified salinity-tolerant clonal genotypes.

4.2.1 Plant material

Over 200 seeds obtained from wild *A. dubius* populations in Desainagar, South Africa (29°36'47.1"S 31°09'08.8"E) were individually sown into seedling tray inserts (37 mm width x 60 mm height) containing a 1:1 mixture of coco peat (Grovida, South Africa) and seedling mix (Grovida, South Africa). For species verification, a specimen was deposited to the Bews Herbarium (Pietermaritzburg, University of KwaZulu-Natal, accession: NU0094621). The trays were placed inside mist tents within the greenhouse at the School of Life Sciences (Westville campus, University of KwaZulu-Natal) and watered daily at 4 pm for 5 min with municipal water using an automatic system. Each insert of the seedling tray received an average of 15 mL of water per day. The minimum and maximum average temperatures were 16 °C (night) and 31 °C (day), respectively, and the average relative humidity was 60%. After one week, following cotyledon emergence, the seedlings were individually transferred to plastic flowerpots (90 mm diameter x 100 mm depth) with the same substrate as the seedling trays but with added vermiculite (10% v/v) (Grovida, South Africa) to improve aeration, water retention, and nutrient exchange (Rajkumar et al. 2017; Xu et al. 2021). Dr Fisher's Classic Multifeed® (AECI, South Africa) solution (1.3 g/L) was applied to this substrate once every seven days at a volume of 10 mL per seedling.



Scheme 4.1: Flow diagram of the experimental design to assess the salinity stress response of *A. dubius* genotypes.

4.2.2 Salinity stress application

There were 50 individual seedlings (representing 50 genotypes from 50 seeds) arranged randomly for each of the four treatments: 0 (control), 100, 200, and 400 mM NaCl (Saarchem, South Africa). To mitigate salt shock, salinity stress was gradually applied in increments of 50 mM NaCl/day (50 mL) up to the required concentrations following seedling establishment, indicated by the emergence of the fifth or sixth true leaves approximately four weeks post-germination. Thereafter, the pots were watered with 50 mL of each treatment solution at 9 AM inside a mist tent for five days. Additionally, the seedlings were misted with municipal water for 5 min in the evening at a rate of ~50 mL per pot to prevent NaCl hyperaccumulation within the substrate. Salinity stress was induced only in the soil to mimic the real-world scenario of cultivation on marginal land afflicted by soil salinisation due to

climate change and unsustainable irrigation. Applying salt to the substrate simulated the response of *A. dubius* under conditions reflective of the intended cultivation environment.

4.2.3 Seedling growth

Before starting the salinity stress treatments, baseline data was obtained for seedling height, measured using a 1000 mm stainless steel ruler (Tork Craft, South Africa). One week after continuous salinity stress application, seedling height, root length, as well as foliar and root fresh masses (FM) were measured (n = 50). A one-week experimental duration enabled efficient observations of the acute response of *A. dubius* seedlings to salt stress to assess the impact of salinity on growth, physiology, and genetic expression. These responses may occur rapidly and reach a plateau after a certain period, justifying a week-long experimental duration to capture the critical time frame during which significant changes in physiology and molecular regulation are expected to occur (Sottosanto et al. 2007; Kollist et al. 2019). Moreover, a shorter experimental period might not capture the full spectrum of responses, and prolonged exposure to high levels of salinity can lead to seedling death, even in tolerant genotypes. Limiting the experimental duration to one week allows evaluation of the sublethal effects of salt stress, providing insights into the early stages of plant responses without compromising the viability of tolerant seedlings.

Total leaf area was determined by version 4.0 of the generative pre-trained transformer (GPT-4) architecture developed by OpenAI (USA), which was prompted to identify and calculate the area of leaves in an image against an internal reference scale, adapted from Minervini et al. (2014) and Hariadi et al. (2018). Sample images were converted into hue, saturation, and value colour space components and then segmented to create a binary representation, highlighting green-coloured areas. Morphological operations such as erosion and dilation were applied to clean up the binary image and remove noise, facilitating the identification of contours to outline the boundaries of the green area. Finally, the area of all contours was summed in GPT-4 using the OpenCV function cv2.contourArea (Open Source Computer Vision, Russia) to obtain the total leaf area (n = 50).

4.2.4 Genotype tracking of salinity-tolerant clones

A seedling vigour (V) index rating system (***Equation 4.1***) was developed to calculate the stress tolerance ability of salt-treated seedlings as a function of the untreated control.

Equation 4.1: Seedling vigour index (V):

$$V = \frac{a}{\bar{a}^c} + \frac{b}{\bar{b}^c} + \frac{c}{\bar{c}^c} + \frac{d}{\bar{d}^c} + \frac{e}{\bar{e}^c}$$

Where: a = height, b = root length, c = leaf area, d = foliar FM, e = root FM, and: $\bar{a}^c$, $\bar{b}^c$, $\bar{c}^c$, $\bar{d}^c$, $\bar{e}^c$ = mean values of corresponding measurements for untreated (control) seedlings.

This novel seedling growth index equation was necessary to combine multiple stress-affected growth parameters into a single rating (V) to comprehensively compare and identify the best-performing genotypes at the optimal harvestable age and condition for consumption – young and unblemished leaves and stems. Moreover, most stress tolerance and vigour rating indices incorporate a single parameter, typically foliar FM, and assume linearity between stressors and growth, inadequately quantifying and oversimplifying potentially nonlinear and complex stress responses (Ranal and Santana 2006; Castan et al. 2018).

Shoot cuttings (5-10 cm in length, diagonally cut below the node) from the 10 best-performing genotypes from each salinity stress treatment, identified by ***Equation 4.1***, were individually maintained in 100 mL beakers filled with 80 mL distilled water supplemented with 1% indole-3- butyric acid (IBA) (PBR Trading International, South Africa) for approximately eight days under ambient greenhouse conditions until adventitious root emergence and growth to at least 1 cm. Thereafter, whole plants (roots and shoots) were transferred to plastic flowerpots (120 mm diameter x 150 mm depth) containing seedling mix with added vermiculite (10% v/v) (Grovida, South Africa). These selected genotypes were then returned to the mist tents and watered daily with municipal water for 3 min in the evenings at a rate of ~80 mL per pot. Dr Fisher's Classic Multifeed® (AECI, South Africa) solution (1.3 g/L) was applied to the substrate once every seven days at a volume of 15 mL per genotype. Clonal cuttings from the selected genotypes were allowed to regrow in mist tents for 4 weeks before 0 (control), 100, 200, and 400 mM NaCl treatments were reapplied over 1 week (n = 3).

4.2.5 Electrical conductivity

Following salinity treatments, soil EC values were generated by homogenising one part of the soil sample with five parts distilled water. The solution was then mechanically shaken (SPO-8 platform shaker, Labcon, South Africa) for 8 h at 100 rpm. To obtain EC values, the leachate was collected via vacuum filtration and measured with a PocketPro+® conductivity meter (Hach Lange, Germany)

(Rayment and Lyons 2010). The EC of leaf and root samples was similarly assessed – one part of the dried sample (apical leaf or root tissue) was homogenised in five parts of distilled water using a mortar and pestle, shaken for 8 h at 100 rpm, and vacuum filtered before measuring. There were 17 genotypes (n = 17) represented in 3 technical replicates (n = 3).

4.2.6 Chlorophyll content

The chlorophyll content of a 1 cm^2 leaf disc excised from newly developed, unblemished leaves of each genotype was spectrophotometrically quantified at the conclusion of the 2^{nd} and 3^{rd} screening stages (***Scheme 4.1***) (n = 3). The leaf discs were briefly rinsed in distilled water, individually transferred into 2 mL microcentrifuge tubes containing 1.5 mL 99.9% chilled ethanol (Protea Lab Services, South Africa), and stored on ice in the dark for 24 h. The samples were then centrifuged (Eppendorf 5710R®, Germany) for 5 min at 10,000 rpm and 4 °C. The resultant supernatant was then analysed by a UV-1800® spectrophotometer (Shimadzu, Japan) at wavelengths of 660 nm (chlorophyll-a) and 640 nm (chlorophyll-b). This data was used to calculate the total chlorophyll content (adapted from Ritchie 2008).

4.2.7 Protein content

The Bradford method of total protein quantification was used to assess the protein concentrations in roots and leaves of selected cutting and *in vitro* propagated genotypes (Bradford 1976). Homogenisation was achieved by grinding ~100 mg of tissue in liquid nitrogen (LN_2) (Afrox, South Africa), and proteins were extracted using TRIzol™ Reagent (Life Technologies, Netherlands) according to the manufacturer's specifications. Coomassie Brilliant Blue dye (Merck, Germany) was added to each sample (n = 3), resulting in a shift in the dye's absorption spectra, measured at 595 nm using a spectrophotometer (Shimadzu, Japan). A calibration curve was established using serial dilutions (12.5, 25, 50, 75, 100, and 150 µg/mL) of the protein standard, bovine serum albumin (BSA) (Merck, Germany). The unknown protein concentration of the samples was then determined using linear regression analysis of the measured absorbance values plotted against the standard curve.

4.2.8 RNA extraction, quantification, and qualitative control

Foliar tissue, amounting to three unblemished young leaves, and root tip tissue samples were randomly excised from three of the tallest and most vigorously elongating biological replicates from

each stress treatment. The tissue samples were rinsed with distilled water for 10 s and blotted dry with autoclaved paper towels. The samples were sectioned, weighed, and frozen in LN_2 (Afrox, South Africa) and then immediately prepared for RNA extraction or stored at -80 °C in an ultra-freezer (NuAire, USA).

Total RNA was isolated from foliar tissue for each salinity stress treatment using TRIzol™ Reagent (Life Technologies, Netherlands) according to the manufacturer's specifications. The RNA concentration was quantified using the RiboGreen® protocol for the NanoDrop™ 3300 Fluorospectrometer (Thermo Fisher Scientific, USA). RNA quality was evaluated by running 5 µg of the extract through electrophoresis on 1% Tris-Acetate-Ethylenediaminetetraacetic acid (TAE) agarose gel (Thermo Fisher Scientific, USA) supplemented with 1% NaOCl (Reckitt Benckiser, South Africa) for 30 min at 110 V (Aranda et al. 2012). All RNA samples were aliquoted and stored at -80 °C in an ultra-freezer (NuAire, USA) until needed for RT-qPCR analysis.

4.2.9 RT-qPCR parameters

RT-qPCR analysis was completed using the GoTaq® 2-Step RT-qPCR System (Promega, USA). The cDNA template was synthesised from 1 µg of total RNA per the manufacturer's instructions. The qPCR final reaction volume of 20 µl was composed of 1 µl cDNA template (equivalent to 1 ng starting quantity of RNA), 10 µl of GoTaq® qPCR Master Mix (2X), 1 µl of both primers (500 nM) (see **4.2.10**), and 7 µl of nuclease-free water. No template controls (NTCs) were performed with 8 µl of nuclease-free water to account for the lack of cDNA, thus ensuring a constant final reaction volume.

The RT-qPCR thermocycling reactions were performed with three biological and two technical replicates of each salt stress treatment using the Eco™ Real-Time PCR System (Illumina, USA). All assays were carried out under the following conditions: one cycle of 95 °C for two min, 40 cycles of 95 °C for 15 s and 68 °C for one min for the degenerate NHX1 primer pair (see **4.2.10**) and 60 °C for one min for the reference gene primer pairs. A melting curve was generated from 95 °C to 65 °C to verify amplicon specificity and identify erroneous hairpin and primer dimer formation. The size of the amplicon was estimated using gel electrophoresis (Armstrong and Schulz 2015).

4.2.10 Primer design

Degenerate NHX1 forward and reverse primers were developed using Base-By-Base with j-CODEHOP (Brodie et al. 2004; Tu et al. 2018), which identified degeneracies within aligned sequences and accounted for the inherent redundancy of amino acid and codon pairing. Multiple known NHX1 amino acid sequences expressed in salinity-tolerant eudicots obtained from National Center for Biotechnology Information protein databases were aligned using a constraint-based multiple alignment tool (COBALT) (Papadopoulos and Agarwala 2007) (Appendix, ***Table 7.3***). DNA sequence outputs that adhered to all other generic primer design parameters, such as melting temperature, length, guanine-cytosine content, and secondary structure formation, were selected for further analysis. The degenerate primers used for NHX1 quantification were:

5'-ATGTCCCACTACACCTGGCAYAA-3' (forward)

5'-GTCGGTCACGAACCTCCATTTC-3' (reverse)

The reference gene used for RT-qPCR normalisation was malate dehydrogenase (MDH). Primers for this reference gene of an ancestral species were developed by González-Rodríguez et al. (2019) with the following sequences:

5'-TGCTCCCAACTGCAAGGTTC-3' (forward)

5'-ACCAAGTGCCCTGTTGTGAT-3' (reverse)

4.2.11 RT-qPCR Analysis

LinRegPCR (Ruijter et al. 2009) was used to determine the fluorescence threshold and the mean RT-qPCR efficiency per amplicon. The generated quantitation cycle (Cq) values and RT-qPCR efficiencies (E) of the NHX GOI and reference gene (MDH) were used to calculate the relative quantification (RQ) for each salt stress treatment as described by ***Equation 4.2*** (Pfaffl et al. 2004). These data were then expressed as relative fold changes per salt stress treatment. BestKeeper was used to assess the stability of the reference gene by calculating the standard deviation (SD) and coefficient of variation (CV%) (Pfaffl et al. 2004).

Equation 4.2: Pfaffl method of relative quantification:

$$RQ = \frac{(E_{unknown})^{\Delta Cq_{unknown}}}{(E_{control})^{\Delta Cq_{control}}}$$

Where: $\Delta Cq_{unknown}$ = Cq of NHX1 in control treatment – Cq of NHX1 in unknown treatment, and: $\Delta Cq_{control}$ = Cq of MDH in control treatment – Cq of MDH in unknown treatment.

The relative quantification (RQ) of the GOI was calculated by determining the RT-qPCR efficiencies (E) and differences in quantitation cycles (ΔCq) between test samples (100, 200, and 400 mM NaCl) and the control (0 mM NaCl).

4.2.12 *In vitro* propagation of selected genotypes

Nodal explants (n = 6) harvested from the cuttings of the selected genotypes of *A. dubius* were clonally propagated *in vitro* using the protocol established for this species by Shaik et al. (2022). The explants were decontaminated in 1% (v/v) sodium hypochlorite (NaOCl) (Reckitt Benckiser, South Africa) with two drops of Tween 20® (Bayer, South Africa) for 10 min and then transferred to semi-solid shoot multiplication medium (half-strength Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962), 30 g/L sucrose, 2 mg/L 6-benzylaminopurine (BAP), 0.5 mg/L indole-3-acetic acid (IAA), pH 5.7) for three weeks and then subcultured onto shoot elongation medium (half-strength MS, 30 g/L sucrose, 0.1 mg/L BAP, 0.1 mg/L IAA, pH 5.7) for two weeks, before finally being transferred to rooting medium (half-strength MS, 30 g/L sucrose, 0.1 mg/L IAA, pH 5.7) for a further two weeks. All media were steam sterilised in an HL-341 autoclave (Gemmy Industrial Corporation, Taiwan) at 121 °C and 15 psi for 20 min.

The number of emergent shoots from individual explants was recorded weekly throughout the shoot multiplication period. Thereafter, the shoot elongation and root proliferation rates were measured during growth on the respective medium. Acclimatisation of rooted plantlets was performed by potting in soil (**4.2.1**) and covering with clear plastic bags for two weeks in a grow room. Thereafter, the plastic bags were removed, and the pots were transferred to a greenhouse mist tent for 4 weeks (Shaik et al. 2022), following which the yield of individual explants was recorded. Wild-type specimens which were not exposed to stress treatments were used as a control. Once the acclimatised clones grew to the equivalent vegetative stage (5-6 leaves) of their parents in the initial stress treatment

assay, they were subjected to the same salinity stress treatments and subsequent processing, extraction, and transcript quantification (section **4.2.2**).

4.2.13 Data analyses

Physiological growth, EC, protein concentration, chlorophyll content, and gene expression data were analysed using the Statistical Package for the Social Sciences (SPSS®) version 29.0 (IBM, USA). The data sets were assessed for normality (Shapiro-Wilk test) and metavariance (Levene's test) to satisfy the assumptions of the post hoc test. Significant interactions among and between treatments were identified by univariate or multivariate ANOVAs followed by Tukey post hoc testing. A probability of $p < 0.05$ was used as the threshold for statistical significance. A paired samples T-test was used to determine differences between pre- and post-micropropagation parameters.

Hierarchical cluster analysis using Ward's method with Euclidean distances as the metric for NHX1 activity and chlorophyll and protein content in root tissues of selected clones enabled the grouping of genotypes with similar salinity stress responses. Principal component analysis (PCA) of these same parameters was used to reduce dimensionality and identify components that accounted for the most variance within each dataset (Mehraj and Shimasaki 2017).

4.3 Results and discussion

4.3.1 Growth characteristics under salinity stress

The median initial height of all seedlings at the start of the salinity stress treatments was 8.5 ± 0.8 cm. ***Figure 4.1*** depicts the representative range of genotypically unique seedling growth forms following exposure to salinity stress for each of the tested treatments for 1 week. Some seedlings from the 100, 200, and 400 mM NaCl treatment groups exhibited stunted growth with leaf and stem chlorosis (***Figure 4.1*** B2, C2, D2). However, other specimens undergoing the same treatments showed vigorous growth (***Figure 4.1*** B1, C1, D1), indicating genotype-specific variation. Seedlings from the untreated control group demonstrated typical *A. dubius* growth characteristics and appearance, i.e., vibrantly green, simple, alternate, and elliptic-ovate-shaped leaves (***Figure 4.1*** A).

Table 4.1 lists the median ± standard deviation (SD) growth characteristics of *A. dubius* seedlings following each salinity stress application. Generally, growth parameters decreased with increasing

NaCl concentrations, except for height in the 100 mM NaCl group (26.7 ± 4.6 cm), which was not significantly different compared to the untreated control (26.5 ± 4.45 cm). Additionally, in the 100 mM NaCl treatment, the number of leaves (17.5 ± 3.9) and foliar FM (52.9 ± 6.2 g) did not differ from the control (15.7 ± 3.5, 56.9 ± 4.4 g, respectively). Interestingly, the number of leaves on specimens from the 400 mM NaCl group (15.3 ± 3.9) were also similar to the untreated control and shared similarity with the 200 mM NaCl group (14.8 ± 3.8), demonstrating genotypic stability of this parameter. However, the leaves of many specimens treated with NaCl were smaller, exhibiting irregular, furled and rounded morphology (***Figure 4.1*** B2, C2, D2). These observations were quantitatively reflected in the leaf area measurements of samples in the 100 (184.6 ± 10.8 cm^2), 200 (150.9 ± 11.4 cm^2), and 400 (119.8 ± 11.6 cm^2) mM NaCl groups in comparison to the untreated control (202.8 ± 8.5 cm^2), demonstrating significant decline with each increasing NaCl treatment.

Figure 4.1: Representative one-month old genotypically unique *A. dubius* seedlings after undergoing salinity stress treatments for 1 week. A: 0 (control), B: 100, C: 200, and D: 400 mM NaCl.

Additional parameters sharing similarity among the salinity treatment groups included root length and root FM, which were similar between the 100 (50.7 ± 7.7 cm, 21.3 ± 5.7 g) and 200 (46.9 ± 6.5 cm, 19.1 ± 5.7 g), and 200 and 400 (45.1 ± 8.9 cm, 16.3 ± 7.9 g) mM NaCl treatments. Furthermore, the SDs of root-related parameters were relatively more stable than the SDs of other parameters, demonstrating limited variability across treatments and potentially indicating a degree of robustness and resilience, especially in conjunction with the observed similarities in root length and FM across salinity treatments. Moreover, foliar FM was similar between the 100 and 200 (48.2 ± 7.9 g) mm NaCl groups but demonstrated increasing SD as salinity increased. Similarly, the SD of other growth parameters increased as salinity increased (with the exception of the number of leaves), indicating variability of plant height, foliar FM, and leaf area within the species in response to salinity stress, and further

demonstrating the high degree of intraspecific variability reported in previous studies on *A. dubius* (Potluri and Persad 1998; Sindhu 2002; Celine et al. 2007; Arti et al. 2018; Nyonje et al. 2021; Areington et al. 2022; Shaik et al. 2022; Amma and Rajalakshmi 2023).

The 10 most vigorously growing genotypes from each salinity treatment were determined by applying ***Equation 4.1*** (summarised in ***Table 4.1***) and selected for further assessment. The condition in salinity-stressed genotypes where $V \geq 5$ was observed in 9 genotypes treated with 100 mM NaCl and 2 genotypes treated with 200 mM NaCl, signifying cumulatively greater than or equal performance to the untreated control and indicating the absence of stress-induced growth constraints. However, achieving such equivalence was rare due to the overall detrimental impact of salinity stress on growth, evidenced by the significant decreases in vigour as salinity concentrations increased. Therefore, the top-performing genotypes (n = 10) from each salinity stress treatment were selected for further analyses, allowing for a more focused evaluation of the most promising specimens while also maintaining sample size and reducing ambiguity in the selection process.

Table 4.1: Growth parameters of one-month old *A. dubius* seedlings undergoing salinity stress treatments for 1 week. Different letters indicate statistically significant differences for each parameter (ANOVA with Tukey post hoc test; $p < 0.05$), median ± SD, n = 50. FM = fresh mass.

Treatment (mM NaCl)	**Survival** (%)	**Height** (cm)	**Root length** (cm)	**Number of leaves**	**Foliar FM** (g)	**Root FM** (g)	**Leaf area** (cm^2)	**Vigour** (V)
0 (Control)	90	$26.5^{a} \pm 4.4$	$58.7^{a} \pm 6.4$	$15.7^{ab} \pm 3.5$	$56.9^{a} \pm 4.4$	$28.4^{a} \pm 4.1$	$202.8^{a} \pm 8.5$	$5.0^{a} \pm 0.4$
100	82	$26.7^{a} \pm 4.6$	$50.7^{b} \pm 7.7$	$17.5^{a} \pm 3.9$	$52.9^{ab} \pm 6.2$	$21.3^{b} \pm 5.7$	$184.6^{b} \pm 10.8$	$4.5^{b} \pm 0.5$
200	72	$23.0^{b} \pm 5.7$	$46.9^{bc} \pm 6.5$	$14.8^{b} \pm 3.8$	$48.2^{b} \pm 7.9$	$19.1^{bc} \pm 5.7$	$150.9^{c} \pm 11.4$	$3.9^{c} \pm 0.6$
400	50	$19.4^{c} \pm 6.8$	$45.1^{c} \pm 8.9$	$15.3^{ab} \pm 3.9$	$39.0^{c} \pm 16.6$	$16.3^{c} \pm 7.9$	$119.8^{d} \pm 11.6$	$3.4^{d} \pm 0.9$

Comparatively, the number of leaves, height, and biomass of another amaranth, *A. tricolor,* significantly decreased when treated with 10 000 ppm (approximately 171 mM NaCl), demonstrating decreased protein production, cell size, and division (Siswanti and Umah 2021). Furthermore, water spinach (*Ipomoea aquatica*) genotypes undergoing 75 mM NaCl *in vitro* exhibited stunted growth and decreased seedling height, yielding inconsistent biomass (Ibrahim et al. 2019), emphasising the varied responses of different genotypes to salinity stress, which was also observed in this study. Studies have reported salinity tolerance and adaptability of spinach (*Spinacia oleracea*) (Ferreira et al. 2018; Uçgun et al. 2020). However, those results were obtained under low to moderate levels of salinity stress (<

120 mM NaCl). Moreover, the harmful effects on plant growth and yield from excess salinity are mainly caused by osmotic stress and ion toxicity, resulting in cellular dehydration and growth inhibition (Hauser and Horie 2010; Zhu et al. 2010; Mahdy and Fathi 2012; Ramadoss et al. 2013). Additionally, the overexpression of certain genes has been found to enhance salt tolerance, suggesting the potential for genetic engineering to improve growth parameters under salinity stress (Singla-Pareek et al. 2003).

The findings of the present study demonstrated that multiple growth parameters of different *A. dubius* genotypes are significantly affected by varying levels of salinity stress and can be used to preliminarily evaluate the salinity tolerance capabilities of this species to identify tolerant genotypes. However, repeated exposure to salinity stress and further investigations are needed to elucidate the physiological and molecular responses of the identified genotypes, supplementing these initial findings and further screening for salinity tolerance. Therefore, clonal cuttings from the initially identified genotypes were allowed to regrow in mist tents for 4 weeks before 0 (control), 100, 200, and 400 mM NaCl treatments were reapplied. Only 17 clonal genotypes survived re-exposure to the 400 mM NaCl treatment and were therefore labelled as salinity (S) tolerant (S1, S4, S6, S10, S11, S12, S13, S16, S17, S22, S23, S25, S28, S32, S33, S34, S40). These genotypes were selected for further gene expression, EC, protein, chlorophyll, and micropropagation analyses.

4.3.2 Relative gene expression

The electrophoresis gels for RNA extracted from *A. dubius* roots (***Figure 4.2***) and leaves (***Figure 4.3***) depict 2 distinct bands per lane, representing 28S and 18S ribosomal RNA, which are indicative of pure and high-quality RNA samples with minimal degradation. The regulation of a putative Na^+/H^+ transmembrane exchanger (NHX) expressed in 15 of the 17 selected genotypes of *A. dubius* was quantified in response to increasing levels of soil salinity stress (0 (control), 100, 200, and 400 mM NaCl) and plotted in ***Figure 4.5***. Furthermore, ***Figure 4.4*** shows the electrophoresis gel of the resultant RT-qPCR amplicons, demonstrating a single band across all samples between 100 and 200 bp. This result is congruent with the degenerate primer locations (823-975 bp) on the aligned NHX1 sequences. The relative expression of the NHX1-like gene was normalised against the expression of the housekeeping gene encoding MDH. BestKeeper inspection determined acceptable expression of this reference gene throughout all treatments (SD < 0.2, CV% < 7.0) (Pfaffl et al. 2004), and LinRegPCR

analysis determined that all samples were adequately efficient (1.8 < E < 2.2) (Ruijter et al. 2009). Finally, melt curve analysis confirmed no detectable hairpin or primer dimer constructions.

The general trend depicted in ***Figure 4.5*** shows that putative NHX1 expression in leaf and root tissues increased as more NaCl was added to the substrate. However, leaf tissue from genotypes S1, S11, S23, S25, S33, and S34 and root tissue from S16 exhibited increased GOI expression when treated with 100 or 200 mM NaCl but demonstrated significant decline when treated with 400 mM NaCl. Furthermore, genotype S4 exhibited stable leaf and root expression of the GOI throughout the control and 100 mM NaCl groups, then, during the 200 and 400 mM NaCl treatments, demonstrated downregulated leaf expression while root expression was simultaneously upregulated. Untreated leaf tissue from genotypes S1, S12, S17, S25, S32, and S34 yielded undetectable NHX1-like transcripts for RT-qPCR quantification. Similarly, untreated root tissue from genotypes S6, S10, S17, and S33 did not exhibit sufficiently detectable expression. The observed lack of transcript expression in untreated samples followed by quantifiable expression in treated samples of the same genotypes was indicative of tissue-specific inducible expression of the NHX1-like gene in response to salinity stress (Mohamed 2019; Wan et al. 2019; Yang et al. 2020). Conversely, S4, S11, S16, S22, S23, and S28 demonstrated constitutive expression of the GOI in leaf and root tissues.

Overall, in leaf tissues, the median ± SD fold change of putative NHX1 transcripts compared to the untreated control (0.5 ± 0.1) was 4.5 ± 1.7, 9.7 ± 2.2, and 8.2 ± 2.3 in the 100, 200, and 400 mM NaCl treatments, respectively. In root tissues, GOI expression was 2.5 ± 1.6, 4.5 ± 2.7, and 6.5 ± 1.8 times more than the untreated control (1.6 ± 1.3) in the presence of 100, 200, and 400 mM NaCl, respectively. Notably, the relative expression of GOI transcripts in root tissues was significantly greater than in leaf tissues of genotypes exposed to 400 mM NaCl, with S11 demonstrating the highest (31.4-fold) and S32 yielding the lowest (1.3-fold) differences in expression between the tissue types among all samples. Genotypes S13 and S40 did not yield quantifiable NHX1-like transcripts in any treatment group and were omitted from ***Figure 4.5***. However, these genotypes survived the highest tested level of salinity stress (400 mM NaCl), indicating that there are other unknown mechanisms involved in the observed salinity tolerance of these genotypes, necessitating further research. Genotypes S13 and S40 may harbour a diverse set of genes or alleles that collectively contribute to salinity tolerance. Multifunctionality and redundancy in genetic pathways could compensate for the observed lack of

NHX1-like transcripts. These genotypes may have alternative ion transporters or antiporters other than NHX1 to regulate ion homeostasis under high salinity conditions. Genotypes S13 and S40 might possess osmotic adjustment mechanisms, synthesising and accumulating compatible solutes, such as proline or glycine betaine, which are osmoprotectants, helping cells maintain turgor pressure and cellular integrity under saline conditions (Yang et al. 2017; Maqsood et al. 2020). Salinity stress often leads to the production of reactive oxygen species (Hasanuzzaman et al. 2021). Genotypes S13 and S40 may possess robust antioxidant defence mechanisms, including enzymes like superoxide dismutase or catalase, which protect against oxidative damage associated with salinity stress (Hasanuzzaman et al. 2021). Additionally, the root systems of these genotypes may exhibit specific morphological or architectural adaptations that enhance nutrient and water uptake efficiency under saline conditions, including changes in root length, branching patterns, or the development of specialised root structures (Shelden and Munns 2023).

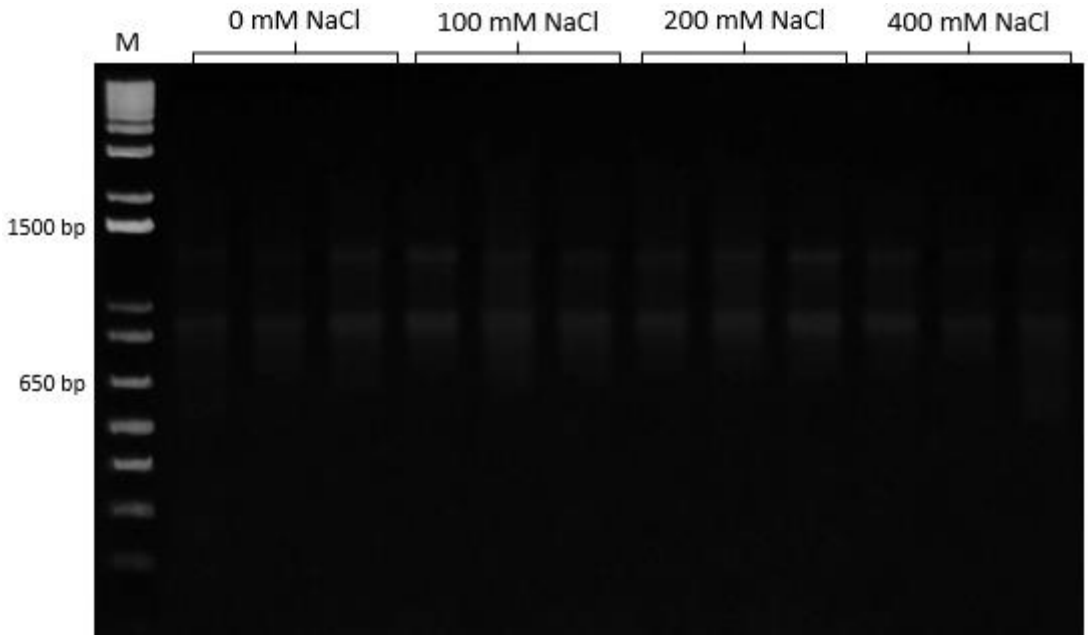


Figure 4.2: Electrophoresis of 5 µg total RNA extracts from *A. dubius* roots exposed to salinity stress (0 (control), 100, 200, and 400 mM NaCl). M = 1 Kbp marker (Invitrogen, USA).

For the majority of tested *A. dubius* genotypes, the increased function of NHX1 in roots, sequestering excess Na^+ ions, was critical for salt tolerance. Under saline conditions, plants accumulate high levels of Na^+ ions, which can be toxic to plant cells. NHX1 helps to prevent the accumulation of excess Na^+ ions in the cytosol by transporting them into vacuoles. This not only helps to maintain a low cytosolic

Na^+ concentration but also creates an osmotic gradient that helps to prevent water loss from the plant's cells. The biochemistry of salinity tolerance in *A. dubius* involves various mechanisms, including osmotic adjustment and antioxidant defence pathways (Hoang et al. 2020). The present study demonstrated the ability of *A. dubius* to regulate ion homeostasis by sequestering excess Na^+ ions through the action of the Na^+/H^+ antiporter NHX1.

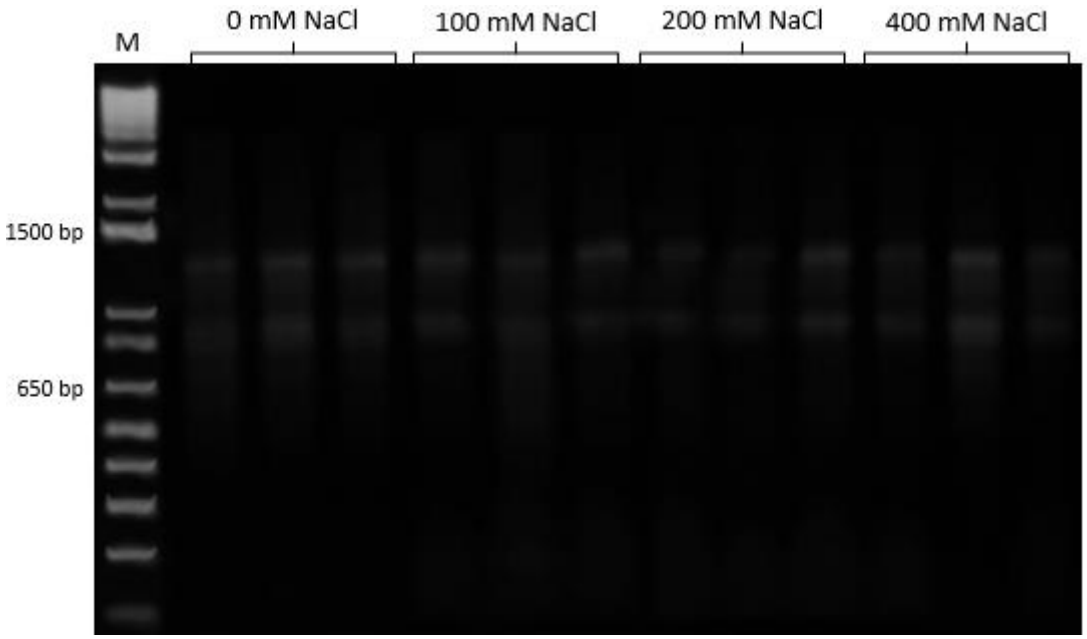


Figure 4.3: Electrophoresis of 5 µg total RNA extracts from *A. dubius* leaves exposed to salinity stress (0 (control), 100, 200, and 400 mM NaCl). M = 1 Kbp marker (Invitrogen, USA).

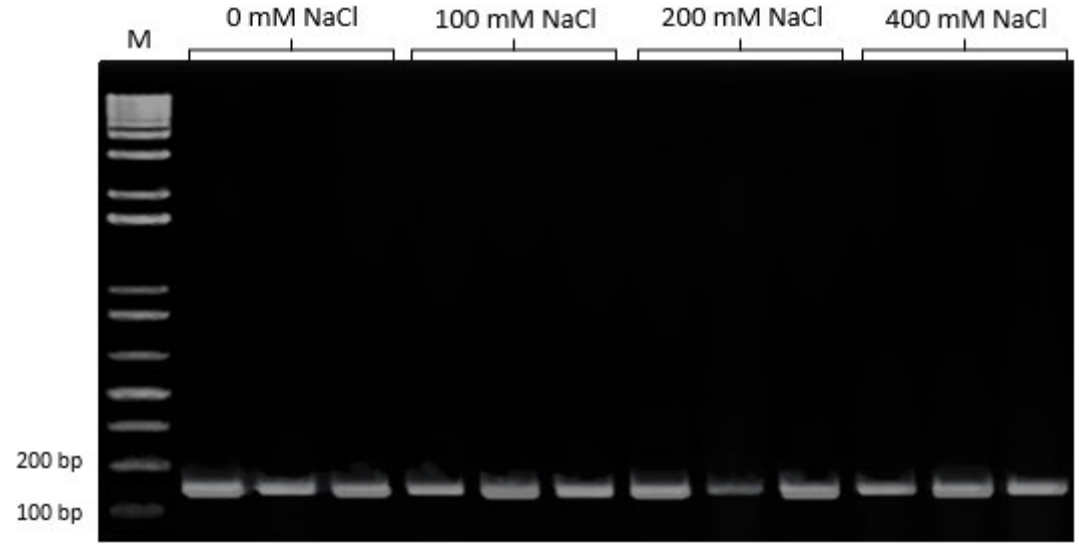


Figure 4.4: Electrophoresis of 5 µL RT-qPCR product of salinity stressed *A. dubius* specimens. M = 1 Kbp marker (Invitrogen, USA).

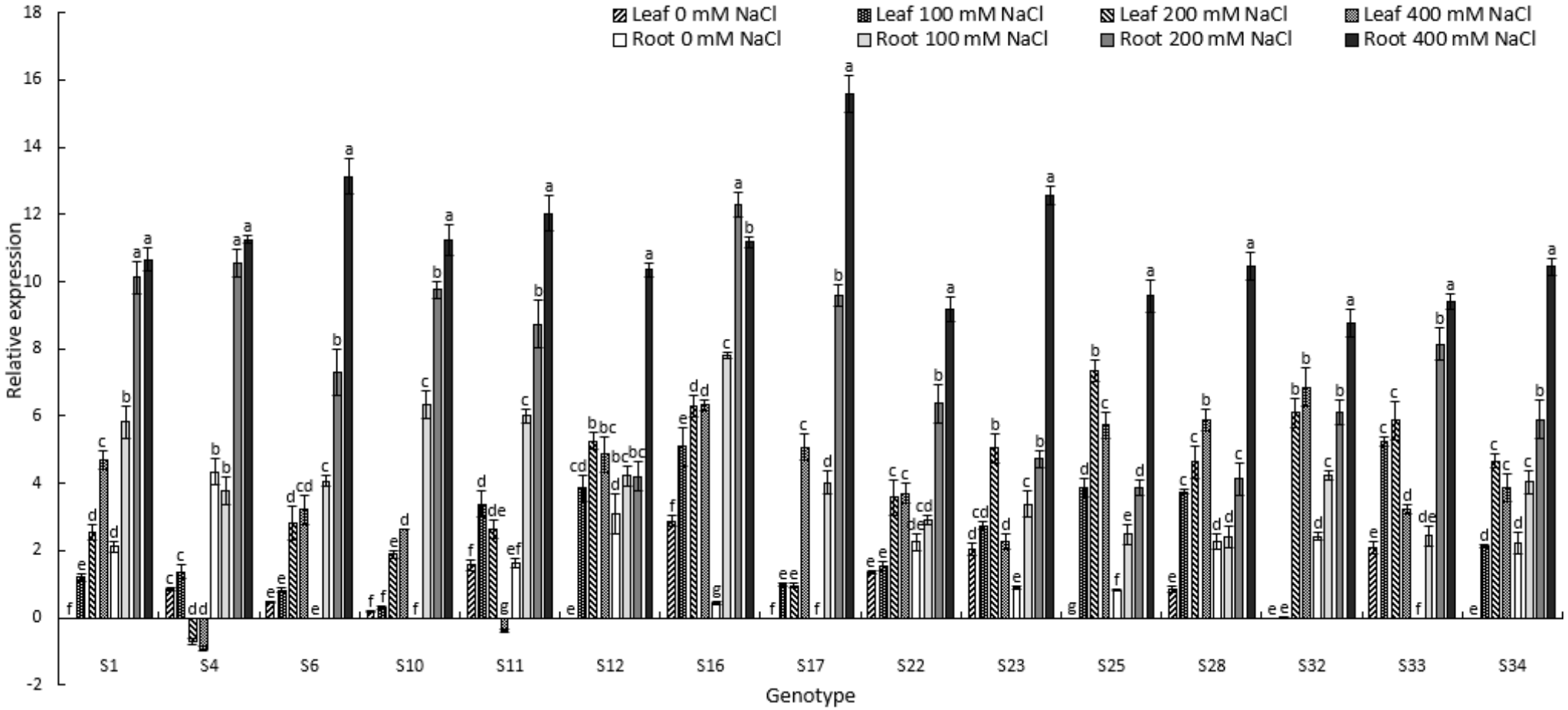


Figure 4.5: The relative expression of an NHX1-like gene in leaf and root tissues of salinity (S) tolerant *A. dubius* genotypes propagated through cuttings and exposed to 0 (control), 100, 200, and 400 mM NaCl. Different letters indicate statistically significant differences for a genotype (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, n = 3.

Table 4.2 lists the relative expression of NHX1 transcripts in leaf and root tissues of a variety of plants exposed to salinity stress. Similar to the overall findings presented in ***Figure 4.5***, osmotolerant rice (*O. sativa*) on basal salt medium supplemented with NaCl demonstrated greater NHX1 expression in roots than in leaves. Furthermore, Zhang et al. (2008) investigated the halophytic Indian walnut (*Aeluropus littoralis*) growing on perlite aerated with a culture solution containing NaCl (root application) and Moshaei et al. (2014) examined *A. littoralis* growing in sand irrigated with 500 mM NaCl (foliar application), both showing greater NHX1 expression in roots than in leaves – albeit to a lesser extent in the latter study. Contrastingly, rose gum (*Eucalyptus grandis*), silver grass (*Miscanthus sinensis*), and palisade grass (*Urochloa brizantha*) in hydroponic culture augmented with NaCl (root application), wheatgrass (*Agropyron elongatum*) in soil irrigated with an NaCl solution (foliar application), and transgenic *A. thaliana* in soil supplemented with NaCl (root application) showed greater NHX1 expression in leaves than in roots. These results demonstrated that the mode of application of NaCl does not necessarily influence tissue-specific NHX1 regulation.

The relative expression of NHX1-like transcripts in *A. dubius* leaves (3.9 ± 2.3) was comparable to the levels of NHX1 reported in leaves of wheat (*T. aestivum*) (3.5), *M. sinensis* (5.5), transgenic tobacco (*Nicotiana tabacum*) (4.1), and the halophytes, goatgrass (*Aegilops tauschii*) (5.7) and nitre bush (*Nitraria sibirica*) (5.4). Additionally, relative root NHX1 expression in *A. littoralis* (8) and sprangletop (*Leptochloa fusca*) (8.5) were similar to the expression of putative NHX1 transcripts in *A. dubius* roots (10.7 ± 1.8). The selected *A. dubius* genotypes endured similar levels of salinity stress (400 mM NaCl) as the typical halophytes *N. sibirica* (400 mM NaCl) and *A. littoralis* (400 mM NaCl). Moreover, salinity-tolerant *A. dubius* genotypes survived higher concentrations of NaCl than transgenic varieties of *A. thaliana* (200 mM NaCl), eggplant (*Solanum melongena*) (200 mM NaCl), and *N. tabacum* (300 mM NaCl). In comparison to the other food crops listed in ***Table 4.2***, the selected *A. dubius* genotypes in the present study were tolerant to higher levels of salinity stress than the osmotolerant *O. sativa* (150 mM NaCl), *T. aestivum* (150 mM NaCl), and soybean (*Glycine max*) (170 mM NaCl).

The expression levels of NHX1 transcripts are compared in ***Table 4.2*** between *A. dubius* and halophytes such as *N. sibirica* (Tang et al. 2021), *A. littoralis* (Zhang 2008), and *L. fusca* (Panahi et al. 2013) undergoing salinity stress at concentrations of NaCl similar to those presently tested. While some halophytes can survive higher concentrations of NaCl, they offer limited nutritional value for

human consumption compared to *A. dubius* (Petropoulos et al. 2018). Therefore, halophytes have primarily been investigated to produce transgenic varieties of popular crop species such as *Z. mays* and *N. tabacum.* However, transgenic constitutive overexpression of NHX1 has failed to yield crops that can tolerate similar levels of salinity stress to halophytic species or that demonstrated by *A. dubius* in the present study. Therefore, *A. dubius* presents an opportunity to diversify food sources, especially in salt-affected regions facing increasing environmental challenges due to climate change.

Table 4.2: Relative expression of NHX1 transcripts in leaf and root tissues of various plant species compared to *A. dubius.* ND = no data, * denotes transgenic species, ⁺ denotes halophytic species.

Species	**Common name**	**Age** (w)	**NaCl application** (mM)	**Tissue**	**Relative NHX1 expression**	**Reference**
Amaranthus dubius	Sugarcane herb	6	400 (root)	Leaf Root	3.9 ± 2.3 10.7 ± 1.8	Present study
Aegilops tauschii⁺	Goatgrass	6	150 (ND)	Leaf	5.7	Abbas et al. 2021
Aeluropus littoralis⁺	Indian walnut	5	400 (root)	Leaf Root	2 8	Zhang et al. 2008
		7	400 (foliar)	Leaf Root	1.9 2.3	Moshaei et al. 2014
Agropyron elongatum	Wheatgrass	16	120 (foliar)	Leaf Root	16 5	Sheikh-Mohamadi et al. 2022
*Arabidopsis thaliana**	Thale cress	5 2	200 (root) 50 (root)	Leaf Root	1.3 1.1	Huyen et al. 2022 Liu et al. 2023
Carthamus tinctorius	Safflower	4	200 (foliar)	Leaf	12	Shaki et al. 2018
Eucalyptus grandis	Rose gum	16	150 (root)	Leaf Root	9 5	García et al. 2019
Glycine max	Soybean	2	170 (root)	ND	9	Sun et al. 2019
Leptochloa fusca⁺	Sprangletop	6	500 (root)	Root	8.5	Panahi et al. 2013
Miscanthus sinensis	Silver grass	4	300 (root)	Leaf Root	5.5 1.6	Sun et al. 2021
Nicotiana tabacum *Nicotiana tabacum**	Tobacco	8	300 (foliar)	Leaf	1.8 4.1	Li et al. 2022
Nitraria sibirica⁺	Nitre bush	10	400 (root)	Leaf	5.4	Tang et al. 2021
Oryza sativa IR28 *Oryza sativa* DP	Rice	2	150 (root)	Leaf Root Leaf Root	16 16 19 102	Nguyen et al. 2023
Triticum aestivum	Wheat	8	150 (foliar)	Leaf	3.5	Al-Ashkar et al. 2021
Urochloa brizantha	Palisade grass	4	200 (root)	Leaf Root	17 -5	Silva et al. 2021

4.3.3 Electrical conductivity

The EC measurements for the selected clonal genotypes of *A. dubius* propagated via cuttings and exposed to 0, 100, 200, and 400 mM NaCl are depicted in ***Figure 4.6*** (n = 3). At higher concentrations of salinity (> 200 mM NaCl), all leaf ECs were significantly lower than the EC values of the corresponding treatment in roots. Leaf and root ECs generally increased with each increasing salinity stress treatment. However, there was no significant increase in leaf EC between the 0 and 100 mM NaCl treatments of genotypes S1, S4, S6, S10, S13, S16, S17, S22, S23, S32, S33, S34, and S40. Between 100 and 200 mM NaCl treatments, leaf EC were similar in genotypes S1, S4, S6, S10, S11, S12, S16, S17, S22, S23, S25, S32, and S40. Additionally, leaf EC did not significantly increase between 200 and 400 mM NaCl treatments for genotypes S4, S6, S10, S11, S13, S22, S23, S28, S33, and S34. Notably, genotypes S4 and S10 did not show a significant EC increase in leaf tissues throughout the 0, 100, 200, and 400 mM NaCl treatments, indicating adaptive mechanisms for ionic compartmentalisation away from leaf tissues to cope with increasing levels of salinity stress. Contrastingly, the root EC in genotype S12 did not increase when treated with higher NaCl concentrations (> 200 mM NaCl), demonstrating a diminished ability to compartmentalise ions in root tissues, resulting in elevated leaf EC.

The measured EC values showed a correlation with higher expression of NHX1 in roots and, thus, accumulation of Na^+. Practically, root accumulation of Na^+ is beneficial because only the leaves and young stems of *A. dubius* are consumed. This physiological response helps maintain the quality and safety of the edible plant portions, as high salt concentrations in leaves can adversely affect taste, texture, and nutritional content, rendering them less palatable and potentially harmful for consumption. Vacuolar transmembrane NHX1 antiporters actively transport Na^+ cations away from the cytosol using the electrochemical gradient generated by proton movement across the membrane (Apse et al. 1999). The compartmentalisation of toxic Na^+ from the sensitive cellular machinery in the cytosol ultimately confers salinity stress tolerance. Previous studies further support the present findings of NHX1 expression generally being higher in roots than in leaves, which is thought to be due to the greater need for salt tolerance in the roots (Yokoi et al. 2002; Mahajan and Tuteja 2005; Munns et al. 2012; Tuteja et al. 2011).

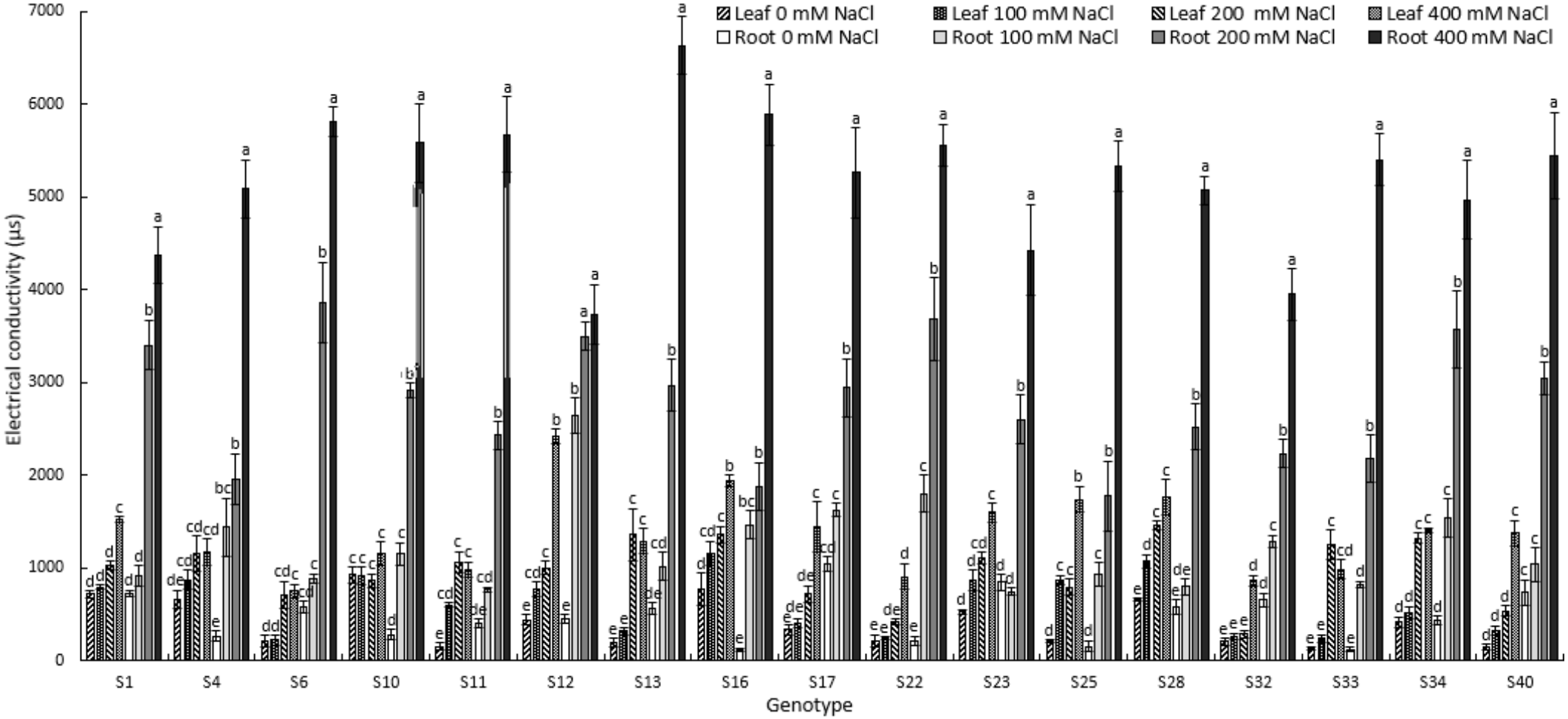


Figure 4.6: The effect of salinity treatments on the electrical conductivity (EC) of soil, roots, and leaves of *A. dubius* propagated through cuttings. Error bars denote ± SD from the mean (n = 3). Different letters indicate significant differences in EC values of leaf, root or soil samples across salinity stress treatments (0, 100, 200 or 400 mM NaCl). * Denotes similarity between 100 and 200 mM NaCl treatments and leaf tissue samples (ANOVA with Tukey post hoc test; $p < 0.05$).

Because only Na^+ was added to the substrate, EC was considered to be a reliable proxy for Na^+ concentration in specific tissues (Ramani et al. 2018; Mohammed et al. 2021; Jabeen et al. 2022). Higher EC values correlate with higher Na^+ concentrations and, coupled with the NHX1 expression data, offer a comprehensive view of the physiological response to salinity. Na-CoroNa green labelling and alternative approaches, such as the use of compartment-specific markers in conjunction with other fluorescent dyes or advanced imaging techniques like ion-specific probes coupled with confocal microscopy, could be explored in future studies. Na-CoroNa green is a useful tool for visualising Na^+ within cells, but it does not provide absolute specificity for vacuolar Na^+. The fluorescence can sometimes be observed in other compartments, leading to potential misinterpretation of results (Park et al. 2009; Wu et al. 2015; Wu et al. 2018). Therefore, ensuring the accuracy and reliability of fluorescence labelling requires rigorous optimisation and validation.

4.3.4 Chlorophyll content

The total leaf chlorophyll content in *A. dubius* clonal genotypes propagated via cuttings under different NaCl treatments is visually represented in ***Figure 4.7***. Generally, chlorophyll content decreased as salinity increased, with the exception of genotype S32, which demonstrated similar chlorophyll content throughout the 0, 100, and 200 mM NaCl groups, and S34, which maintained similar chlorophyll throughout the 100, 200, and 400 mM NaCl treatments. Furthermore, genotypes S4, S6, S10, S11, S13, S16, S28, and S33 yielded similar chlorophyll between the untreated control and 100 mM NaCl treatment groups. Moreover, genotypes S1, S4, S6, S10, S12, S16, S23, S25, S33, and S40 yielded similar chlorophyll content between 200 and 400 mM NaCl treatments. In the 400 mM NaCl group, genotypes S1 (3.5 ± 0.2 μg/cm^2) and S34 (4.0 ± 0.4 μg/cm^2) had the highest concentrations of chlorophyll.

In other studies, salinity-tolerant genotypes of various plants exhibited better growth performance and higher chlorophyll content than salinity-sensitive genotypes, indicating the importance of the NHX1 gene in quantifying salinity tolerance (Abbas et al. 2015; Su et al. 2019; Irshad et al. 2022). NHX1 expression has been shown to increase in many plants exposed to salinity stress (***Table 4.2***) and is associated with the maintenance of ion homeostasis to mitigate salinity stress (Barragán et al. 2012). Additionally, a reduction in chlorophyll content under salinity stress has been consistently observed, leading to a decline in photosynthetic activity (Gengmao et al. 2015; Khatar et al. 2017; Tufail et al.

2017; Ghorbani et al. 2018; Krishna 2018; Shaki et al. 2018; Sarkar et al. 2019; Uddin et al. 2019; Jabeen et al. 2022; Lu et al. 2022; Navyashree and Ashvathama 2022). Furthermore, the reduction in chlorophyll content has been linked to the destabilisation of chlorophyll pigments and the pigment-protein complex under salinity stress (Uddin et al. 2019). These findings collectively suggest an inverse relationship between NHX1 expression and chlorophyll content under salinity stress, indicating that NHX1 expression increases while chlorophyll content decreases in response to salinity stress. This relationship can be considered a biological index to evaluate plant tolerance to stress for genotype screening and future breeding programs (Ghorbani et al. 2018).

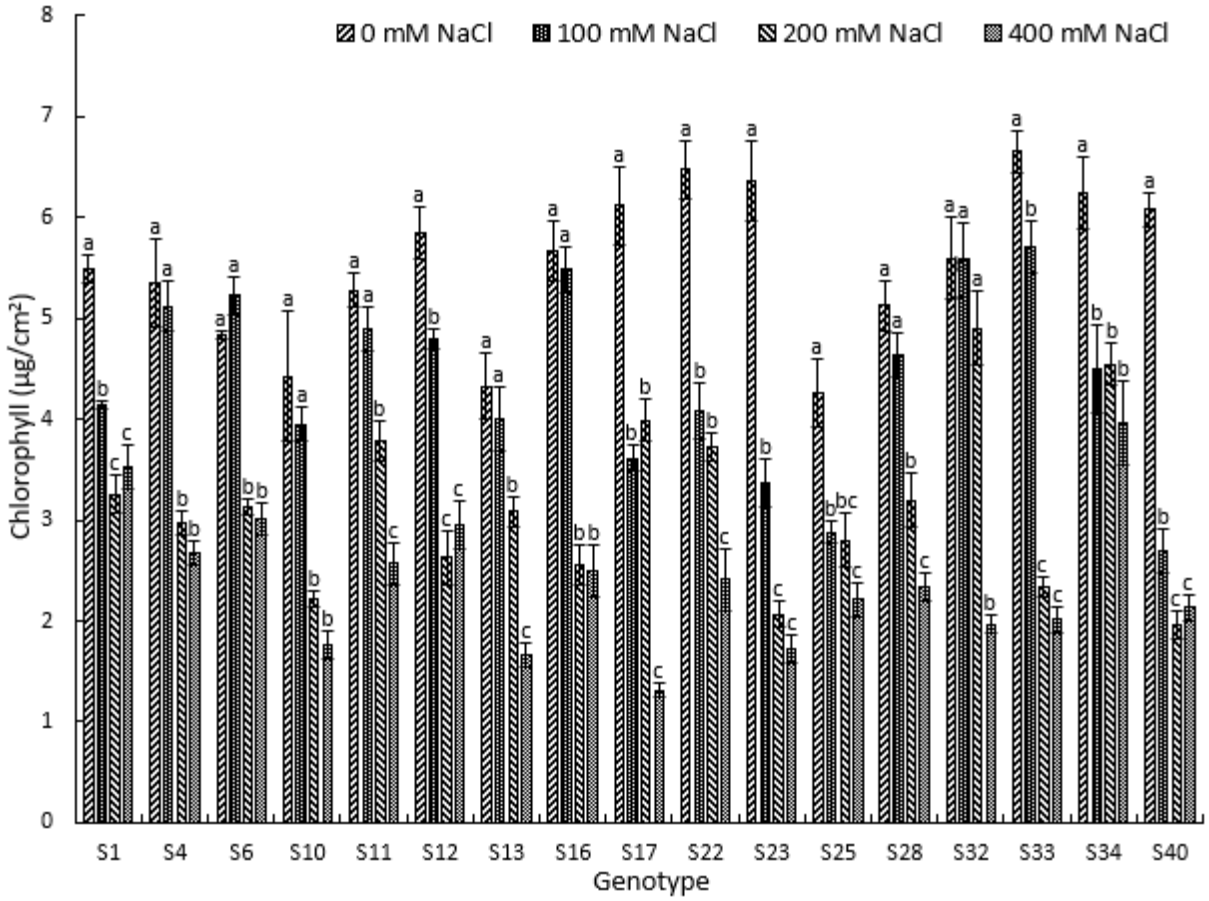


Figure 4.7: Chlorophyll content in the leaves of selected genotypes of *A. dubius* propagated through cuttings and undergoing 0 (control), 100, 200, and 400 mM NaCl soil treatments. Different letters indicate statistically significant differences for a genotype (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, n = 3.

4.3.5 Protein content

The total protein content of selected *A. dubius* clonal genotypes propagated via cuttings is represented in ***Figure 4.8***. Under the highest applied salinity treatment (400 mM NaCl), leaves from genotypes S23, S28, and S32 contained the most proteins (3.1 ± 0.2, 3.1 ± 0.1, and 3.2 ± 0.2 µg/mL, respectively). Additionally, the roots from genotype S23 contained the highest protein concentration (3.4 ± 0.3 µg/mL) when treated with 400 mM NaCl. The general trend observed among most genotypes was a decrease in leaf proteins and an increase in root proteins as higher concentrations of NaCl were applied to the substrate, with the exception of S23, S25, S28, S32, and S33, wherein leaf protein concentrations were maintained or even surpassed root proteins at higher salinity stress treatments. Leaf protein of genotypes S28 and S32 showed a positive correlation with increased leaf NHX1 expression. This result signified possible adaptive resource allocation mechanisms, prioritising the preservation of photosynthetic capacity within the leaves of these genotypes.

These findings could be the result of the accumulation of stress-responsive proteins, such as NHX1, transcription factors, antioxidants, and osmoprotectants, mediating osmotic and ionic homeostasis. In genotypes demonstrating lower leaf protein compared to roots, the observed increase in root proteins was correlated with greater NHX1 expression in S4 and S11 only. For the remaining genotypes, there was no correlation between protein content and NHX1 expression. Genotypes S1, S4, S6, S10, S11, S12, S16, S17, S22, S23, S28, and S34 demonstrated a positive correlation between root protein and root NHX1 expression between 0 and 400 mM NaCl treatments. However, the only linear correlation between root protein and root NHX1 expression throughout all treatments was observed in S10, which was not proportional.

These results suggest that the relationship between total protein content and NHX1 expression is nonlinear and nonproportional, involving changes in specific protein families or functional groups, which is not reflected in a proportional change in total protein content. These findings are congruent with similar studies examining NHX1 expression (Yokoi et al. 2002; Leidi et al. 2010; Ahmad et al. 2020; Cui et al. 2020; Long et al. 2020), wherein the correlation between NHX1 expression and protein content varied depending on the specific genotypes and cultivars, the severity and duration of the stress, and the influence of various signalling pathways regulating gene expression and protein synthesis under salinity stress**.**

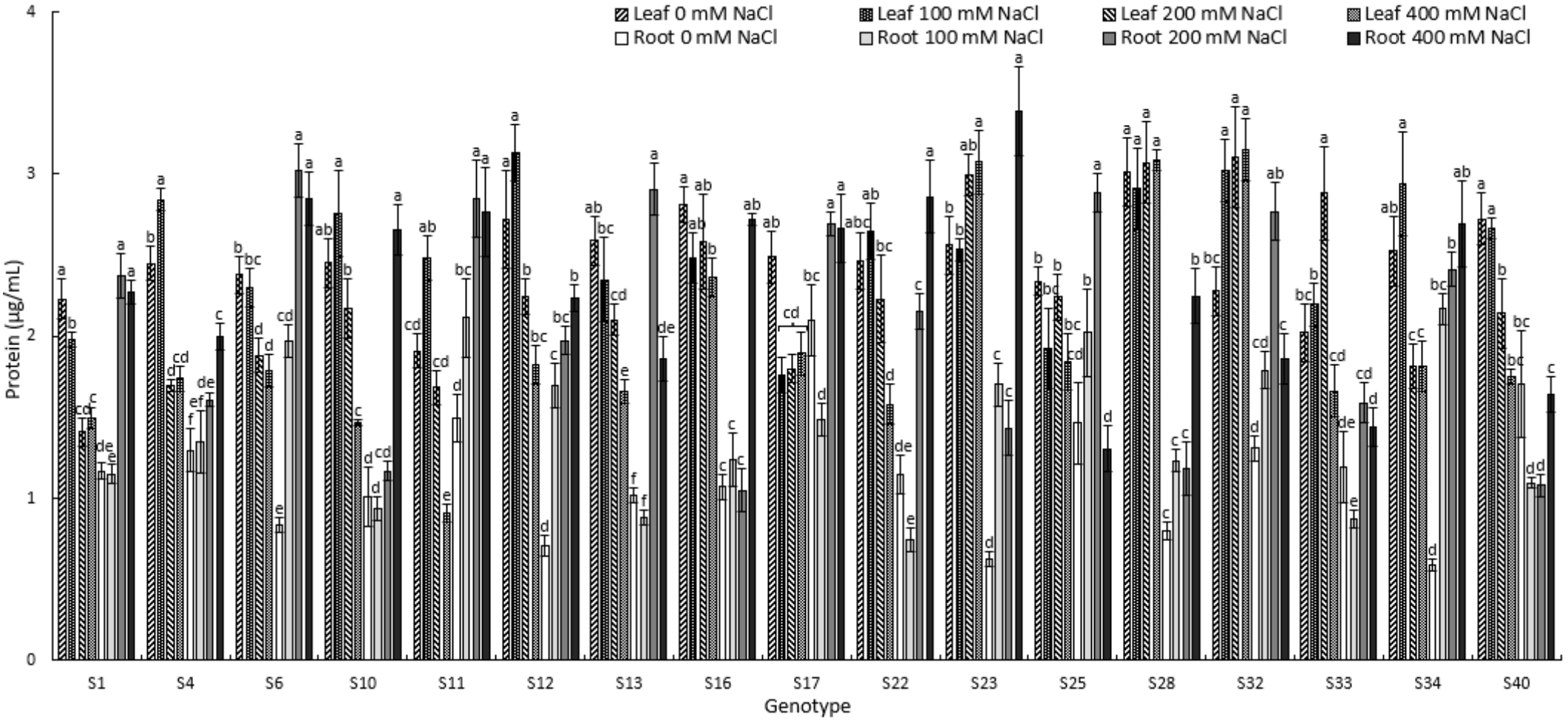


Figure 4.8: Protein concentration of leaves and roots of salinity (S) tolerant A. dubius genotypes propagated through cuttings and exposed to salinity stress (0 (control), 100, 200, and 400 mM NaCl). Different letters indicate statistically significant differences for a genotype (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, n = 3.

All tested genotypes demonstrated similar or increased root protein content as salinity stress was increased, indicating that these plants can adapt to salinity stress by enhancing or maintaining protein synthesis. In the EC and chlorophyll assays, genotypes S1, S4, S6, S10, S11, S12, S13, S16, S23, S25, S28, S32, S33, S34, and S40 maintained each parameter throughout some or all the salinity stress treatments. The stability of EC levels in the leaves of tested genotypes suggests effective osmoregulation and ion homeostasis. Consistent chlorophyll levels suggest that these genotypes can withstand salinity-induced disruptions to the photosynthetic apparatus, maintaining the energy-capturing process necessary for plant growth. The ability to maintain essential physiological parameters under salinity stress indicates that these genotypes possess efficient stress response systems. Genotypes demonstrating robust salinity tolerance physiology have potential applications in agriculture, especially in areas affected by soil salinity.

4.3.6 Cluster and principal component analyses (PCA)

The dendrogram shown in ***Figure 4.9*** obtained from the hierarchical cluster analysis revealed two major clusters. One cluster contained genotypes (S1, S4, S12, S22, S25, S28, S32, S33, and S34) that exhibited higher Na^+/H^+ exchanger (NHX) activity, leaf chlorophyll, and root protein accumulation under salinity stress, indicating more robust salinity tolerance. The other cluster comprised genotypes (S6, S10, S11, S16, S17, and S23) with relatively lower NHX activity, chlorophyll, and protein, denoting lesser salinity tolerance. PCA demonstrated a clear separation of selected genotypes along components 1 and 2, which accounted for 53% and 34% of the total variance, respectively, indicating that these two components effectively summarised the data contained in the original variables. The variables contributing most significantly to this separation were chlorophyll and protein contents.

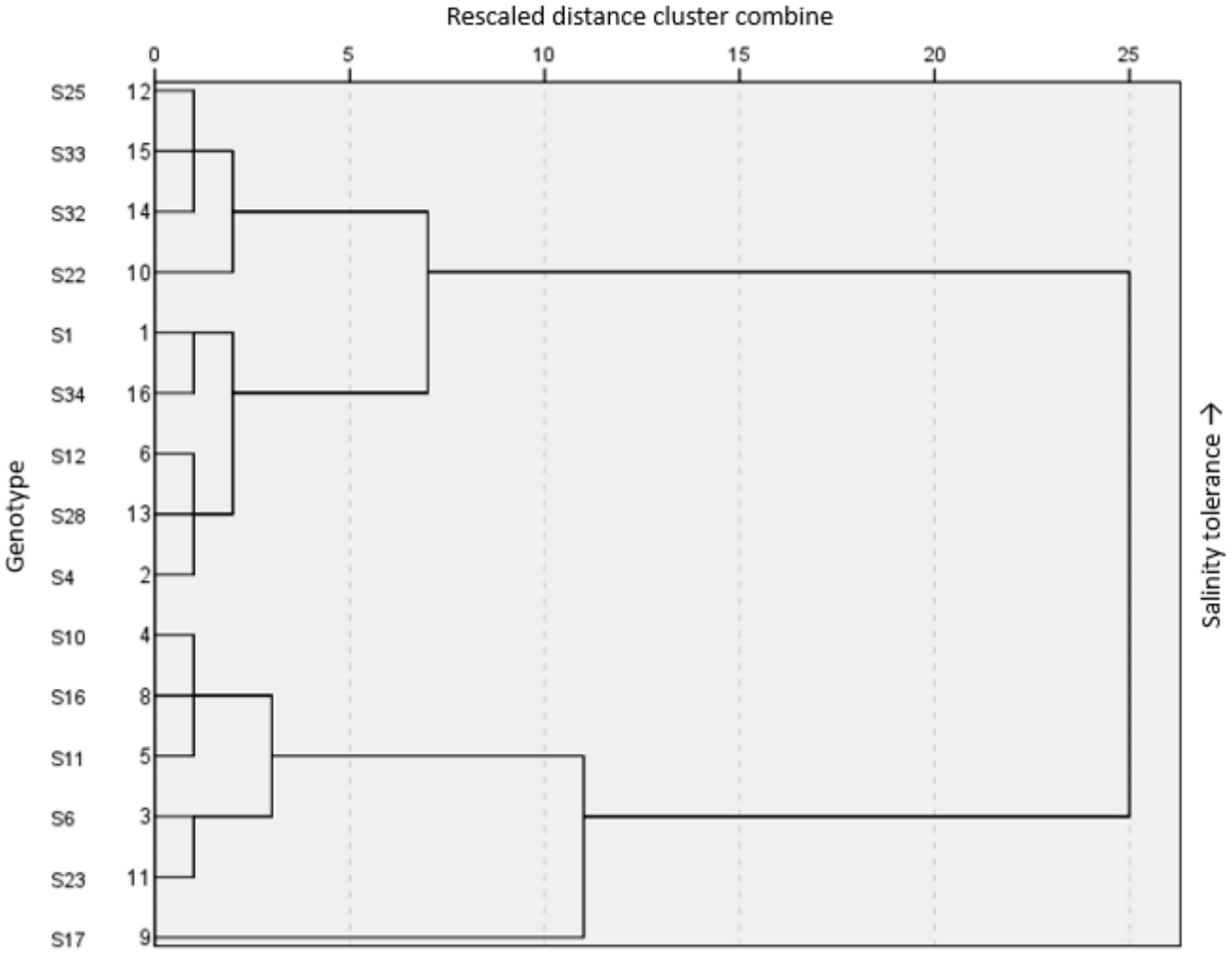


Figure 4.9: Hierarchical cluster analysis dendrogram (Ward's method with Euclidean distance) of selected genotypes responses to NHX activity and chlorophyll and protein content.

4.3.7 Micropropagation

Multiple parameters and the mean agricultural output (foliar FM ± SD) of salinity-tolerant *A. dubius* clonal genotypes following micropropagation and acclimatisation are listed in ***Table 4.3*** (n = 10). Overall, there was an average of 3.4 ± 0.6 new shoots per explant during the 3-week multiplication period, and the subsequent average shoot and root elongation rates were 2.8 ± 0.6 and 1.7 ± 0.3 cm/week, respectively. The number of new shoots per explant was higher in comparison to the micropropagation of *A. dubius* by Shaik et al. (2022), wherein each explant yielded an average of 2 new shoots. This outcome is likely due to genotype-specific variability unintentionally selected for in earlier stages of the present study, which identified stress-tolerant genotypes, indicating a correlation between stress tolerance and shoot production *in vitro*. Genotypes that experienced stress during the earlier screening stages may retain a "stress memory", enabling a more efficient response during

subsequent micropropagation, manifesting in increased shoot production (Ding et al. 2012; Sun et al. 2021).

Table 4.3: Growth measurements from the micropropagation of selected salinity (S) tolerant *A. dubius* genotypes. Different letters indicate statistically significant differences among genotypes (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, n = 6. FM = fresh mass.

Genotype	Number of New Shoots	Shoot elongation (cm/week)	Root elongation (cm/week)	Foliar FM (g)
S1	$3.3^{bcdef} \pm 0.4$	$2.1^{def} \pm 0.6$	$1.1^{h} \pm 0.2$	$50.3^{abc} \pm 3.4$
S4	$2.1^{fg} \pm 0.6$	$3.7^{ab} \pm 0.3$	$2.2^{ab} \pm 0.2$	$55.6^{a} \pm 4.9$
S6	$4.0^{abcd} \pm 0.3$	$1.9^{ef} \pm 0.8$	$1.7^{cdef} \pm 0.4$	$40.7^{d} \pm 3.2$
S10	$3.1^{cdefg} \pm 1.2$	$3.9^{a} \pm 0.4$	$1.1^{h} \pm 0.2$	$44.2^{bcd} \pm 5.3$
S11	$1.9^{g} \pm 0.6$	$3.6^{ab} \pm 0.6$	$2.3^{ab} \pm 0.3$	$56.2^{a} \pm 3.3$
S12	$4.2^{abc} \pm 0.6$	$3.6^{ab} \pm 0.6$	$1.4^{efgh} \pm 0.2$	$44.3^{bcd} \pm 2.0$
S13	$3.9^{abcde} \pm 0.6$	$2.8^{bcde} \pm 0.5$	$1.8^{bcdef} \pm 0.2$	$57.9^{a} \pm 7.2$
S16	$4.4^{ab} \pm 0.8$	$3.1^{abc} \pm 1.0$	$2.1^{abcd} \pm 0.3$	$43.5^{bcd} \pm 1.3$
S17	$2.8^{defg} \pm 0.4$	$1.5^{f} \pm 0.5$	$1.2^{gh} \pm 0.3$	$45.1^{bcd} \pm 13.3$
S22	$3.7^{abcde} \pm 0.5$	$3.0^{abcd} \pm 0.6$	$1.9^{abcde} \pm 0.1$	$57.7^{a} \pm 5.5$
S23	$2.9^{defg} \pm 0.5$	$3.5^{ab} \pm 0.4$	$2.3^{a} \pm 0.5$	$39.7^{d} \pm 5.8$
S25	$4.0^{abcd} \pm 0.5$	$1.8^{f} \pm 0.6$	$1.3^{fgh} \pm 0.5$	$55.5^{a} \pm 6.9$
S28	$3.3^{bcdef} \pm 0.7$	$2.2^{cdef} \pm 0.5$	$2.0^{abcd} \pm 0.3$	$45.4^{bcd} \pm 3.1$
S32	$2.7^{efg} \pm 0.7$	$3.3^{ab} \pm 0.5$	$1.1^{gh} \pm 0.4$	$52.1^{ab} \pm 3.6$
S33	$4.7^{a} \pm 0.9$	$1.8^{f} \pm 0.7$	$1.9^{abcd} \pm 0.4$	$41.9^{cd} \pm 5.6$
S34	$2.0^{g} \pm 0.7$	$3.3^{ab} \pm 0.7$	$1.6^{defg} \pm 0.4$	$38.0^{d} \pm 5.1$
S40	$4.5^{ab} \pm 0.7$	$1.9^{ef} \pm 0.8$	$2.2^{abc} \pm 0.3$	$58.9^{a} \pm 6.9$
Control	$2.5^{efg} \pm 0.9$	$1.4^{f} \pm 0.4$	$0.8^{h} \pm 0.3$	$39.8^{d} \pm 4.4$
Mean	**3.4 ± 0.6**	**2.8 ± 0.6**	**1.7 ± 0.3**	**48.6 ± 5.1**

Micropropagated clonal genotypes S1, S4, S11, S13, S22, S25, S32, and S40 yielded the highest foliar FM (55.5 ± 5.2 g), which was similar to the foliar FM of untreated seedlings (56.9 ± 4.4 g) from the initial salinity stress assay and significantly higher than the control (39.8 ± 4.4 g) over a similar culture duration. Genotypes S6, S12, S13, S16, S22, S25, S33, and S40 produced the most shoots on the shoot multiplication medium. Genotypes S4, S10, S11, S12, S16, S22, S23, S32, and S34 demonstrated the fastest shoot elongation rates on the elongation medium. Genotypes S4, S11, S16, S22, S23, S28, S33, and S40 showed the fastest root elongation rates when transferred to the rooting medium. Overall, genotypes S4, S11, S22, and S40 performed the best throughout micropropagation and subsequent acclimatisation, with S4 and S11 having faster shoot elongation than S40 and S40 generating more shoots than S4 and S11. Genotype S22 demonstrated the highest values for all measured parameters

throughout micropropagation.

4.3.8 Salinity stress tolerance post-micropropagation

Compared in ***Table 4.4*** are pre- and post-micropropagation physiological and genetic parameters (protein, chlorophyll, and NHX1 expression) of the identified salinity-tolerant genotypes, which also demonstrated superior growth throughout micropropagation (S4, S11, S22 and S40). Following acclimatisation, when 400 mM NaCl was re-applied to the substrate, RT-qPCR analysis revealed that the micropropagated clonal genotypes displayed putative NHX1 gene expression profiles similar to their parent genotypes, with the exception of S4, showing a marginal decrease in expression. However, none of the other compared parameters (protein and chlorophyll content) yielded significant differences ($p < 0.05$), indicating that micropropagation and subsequent acclimatisation did not have a negative effect on the ability of the selected genotypes to tolerate salinity stress. This result is indicative of true-to-type cloning, which is a consequence of the direct organogenesis pathway of morphogenesis used by Shaik et al. (2022).

The similarity in putative NHX1 gene expression profiles between micropropagated clonal genotypes and their parent genotypes suggests that the process of micropropagation has maintained genetic stability in terms of NHX1 gene expression. The decrease in NHX1 expression in S4 may be a specific response to cellular stress induced during micropropagation. The genotype might employ alternative stress response mechanisms that compensate for the reduced NHX1 expression. The regulatory networks governing NHX1 expression are complex and interconnected. Changes in other components of these networks during micropropagation could indirectly influence NHX1 expression in S4. Epigenetic modifications, such as DNA methylation or histone modifications, can influence gene expression without altering the underlying DNA sequence (Singroha et al. 2022), resulting in decreased NHX1 expression. Altered transcriptional regulation, possibly through changes in transcription factor activity or accessibility of regulatory elements, could also affect NHX1 expression by influencing the expression of transcriptional regulators (Lu et al. 2022). Additionally, post-transcriptional modifications, such as alternative splicing, could contribute to the observed decrease in NHX1 expression in genotype S4. Furthermore, microRNAs (miRNAs) can post-transcriptionally regulate gene expression (Bartel 2009). Changes in miRNA levels during micropropagation could potentially target NHX1 transcripts, leading to reduced expression. The slight deviation in relative expression

demonstrated by genotype S4 indicates a subtle variation in this response to micropropagation or acclimatisation compared to the other genotypes but ultimately did not impact the measured physiological parameters.

Table 4.4: Comparisons between pre- and post-micropropagation genetic (root NHX1-like relative expression (RE)) and physiological parameters (root protein and leaf chlorophyll content) of identified salinity-tolerant *A. dubius* genotypes re-exposed to 400 mM NaCl, mean ± SD (paired samples T-test, n = 3). * Denotes $p < 0.05$.

Genotype	Root NHX1-like (RE)		Root Protein (µg/mL)		Chlorophyll (µg/cm^2)	
	Before	**After**	**Before**	**After**	**Before**	**After**
S4	11.3* ± 0.1	10.9* ± 0.2	1.9 ± 0.1	2.0 ± 0.1	2.7 ± 0.1	2.5 ± 0.3
S11	12.0 ± 0.5	12.6 ± 0.5	2.8 ± 0.3	2.5 ± 0.3	2.6 ± 0.2	3.2 ± 0.5
S22	9.2 ± 0.4	9.1 ± 0.3	2.9 ± 0.2	2.4 ± 0.6	2.4 ± 0.3	2.0 ± 0.4
S40	10.4 ± 0.3	10.1 ± 0.1	1.6 ± 0.1	1.4 ± 0.3	2.1 ± 0.1	2.1 ± 0.1

The consistent levels of protein and chlorophyll content further underscore the robustness of the chosen genotypes in maintaining crucial biochemical components necessary for cellular functions and photosynthetic processes. This stability observed in the measured genetic and physiological parameters may be attributed to the inherent adaptability and stress tolerance mechanisms within the selected genotypes. In the context of screening for superior genotypes, these findings suggest that the selected genotypes maintain desirable traits conferring salinity tolerance and stress resilience during micropropagation. This stability could serve as a valuable criterion for identifying superior genotypes with consistent physiological performance, laying the foundation for their potential utilization in breeding programs or crop improvement strategies.

4.4 Conclusion

The observed trends in NHX1 expression, ionic compartmentalisation, chlorophyll maintenance, and protein fluctuations collectively contribute to a more holistic understanding of how different genotypes of *A. dubius* respond to salinity stress. The quantification of putative NHX1 expression in leaf and root tissues under varying NaCl concentrations revealed distinct patterns among different genotypes. Notably, tissue-specific constitutive or inducible NHX1 expression, which decreased in leaves and increased in root tissues, indicated adaptive mechanisms for the compartmentalisation of toxic ions away from sensitive leaf tissues. Additionally, the quantification of NHX1-like gene

expression in leaf and root tissues served as a molecular marker, highlighting the differential regulation of this salinity-responsive gene among roots and leaves of different genotypes.

Interestingly, the observed nonlinear and nonproportional changes in protein content underscore the complexity of the molecular response to salinity stress, emphasising the need for a more detailed exploration of specific protein families or functional groups involved in salinity tolerance mechanisms of this species. Cumulatively, this study provided a robust foundation to screen for salinity-tolerant genotypes through many salinity-affected parameters. This integrated approach enhanced the understanding of the intricate interplay between genetic factors and adaptive mechanisms, facilitating the development of resilient crop varieties tailored to thrive in saline environments.

The selection and screening of 17 salinity-tolerant clonal *A. dubius* genotypes, with S4, S11, S22, and S40 emerging as promising candidates, demonstrating exceptional performance in the ionic, chlorophyll, and protein content assays. These genotypes also demonstrated superior performance throughout micropropagation and subsequent acclimatisation. The selected genotypes could be suitable for cultivation on marginal lands affected by salinity, increasing crop productivity on otherwise underutilised lands, promoting sustainable agricultural practices.

These findings call for a better understanding of the physiology and genetics of *A. dubius* in response to salinity stress, establishing best practices to ensure that people benefit from this crop and providing a better understanding of the suitability and broader impact on the food security of this species. Further investigations are needed to quantify the elemental composition in the leaves of the presently identified salinity-tolerant clonal genotypes to determine if the demonstrated salt tolerance ability is maintained while remaining palatable and nutritious. In roots, Na^+ vacuole-specific localisation can be confirmed using compartment-specific markers in conjunction with fluorescent dyes or advanced imaging techniques like ion-specific probes coupled with confocal microscopy. The sequence of the NHX1-like transcript is of interest to molecular biologists, providing a molecular basis for taxonomic classification, aiding in future phylogenetic studies, elucidating evolutionary relationships by identifying sequence variations among species or broader taxa, and resulting in more robust and accurate reconstructions of evolutionary trees. Additionally, the maintenance of superior *A. dubius* clones *in vitro* represents an invaluable resource of disease-free stock for use in future practical and scientific explorations of this species. However, the scope of micropropagation extends beyond the

laboratory, necessitating field trials to validate the performance of clones under natural environmental conditions, providing additional insights into their adaptability, productivity, and interaction with biotic and abiotic factors in agricultural settings.

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5. Assessing cold storage protocols for preserving seeds and nodal explants of the African leafy vegetable, *Amaranthus dubius.*

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Abstract

This study investigated the response of seeds and nodal explants of the highly nutritious African leafy vegetable (ALV), *Amaranthus dubius*, to multiple cryopreservation protocols (controlled-rate, liquid nitrogen (LN_2) immersion, and LN_2 slurry) and storage temperatures (24, 4, -20, -80, -148 and -196 °C). To assess the effect of these treatments on genetic fidelity, a previously identified salinity-tolerant *A. dubius* genotype, expressing a Na^+/H^+ exchanger (NHX1) gene, was stored, and the relative expression of the NHX1 transcript was quantified in root tissues post-acclimatisation. This revealed that *A. dubius* seeds can be stored for 4 years at -20 and -80 °C without significantly decreasing germination capacity (86% and 88%, respectively) compared to the non-stored control (92%). Cooling seeds at a controlled rate was found to be optimum to maintain seed viability, irrespective of storage temperature. Freezing seeds in LN_2 slurry resulted in seedlings with the lowest number of leaves (13.5 ± 2.1), foliar fresh mass (FM) (14.8 ± 2.2 g), leaf area (133.3 ± 24.8 cm2), root length (21.7 ± 3.1 cm), and root FM (10.3 ± 2.0 g). Controlled-rate freezing was also optimum for nodal explants across storage temperatures (-20, -80, -148, and -196 °C), which, when multiplied *in vitro*, yielded 3.6 ± 0.6 shoots per explant with an 80% shoot development rate. Nodes that were cooled at a controlled rate, in LN_2 slurry, or in LN_2 maintained genetic fidelity better than those of the control, based on relative expression of the NHX1 marker gene.

Keywords: *Amaranthus*, Conservation, Cryopreservation, Micropropagation, Physiology, Salinity

5.1 Introduction

The identification of preservation-tolerant genotypes within *Amaranthus dubius* populations is important in addressing the existing challenges, such as limited starting material for research applications and the gaps in current knowledge for the conservation of this nutritionally rich, versatile, and adaptable species, providing an essential resource for addressing food security, nutritional needs, and sustainable agriculture goals. Furthermore, micropropagation of superior genotypes (through direct organogenesis) allows for year-round, rapid multiplication of contaminant-free, true-to-type clones. Gaps in the current understanding of *A. dubius* responses to cryopreservation necessitate further research, specifically determining optimal preservation protocols, long-term viability and storage stability, and post-thaw regeneration. This knowledge gap is compounded by limited research on the molecular and cellular mechanistic response to cryopreservation protocols for this species. Therefore, this study also aimed to investigate the growth and physiology of *A. dubius* in response to cryopreservation stresses and to evaluate the feasibility of multiple cold storage techniques for safeguarding seeds (-20 and -80 °C storage, pre-storage controlled-rate cooling, and LN_2 slurry immersion) and nodal explants (-20 and -80 °C storage, controlled-rate cooling, LN_2 liquid immersion, and LN_2 slurry immersion) of *A. dubius*.

Conventional germplasm storage protocols encompass a variety of methods aimed at preserving the genetic diversity of plant species for future use in breeding, research, and conservation efforts. These protocols typically involve the collection, preparation, and long-term storage of seeds, tissues, or whole plants under controlled conditions to maintain their viability and genetic integrity. Seeds are one of the most used germplasm storage materials due to their relative ease of collection, handling, and storage (Liu et al. 2005). As nature's germplasm conservation vehicle, they can be stored under low-temperature and low-moisture conditions to minimise metabolic activity and prolong viability. Common seed storage methods include drying seeds to low moisture content levels (e.g., 5-10%) and storing them in airtight containers at temperatures below freezing (e.g., -18°C or lower) (Yan 2016).

In modern plant germplasm conservation, cryopreservation has emerged as a useful approach to overcome the limitations of traditional methods of seed storage, which do not necessarily offer the same level of long-term genetic stability at minimal maintenance (Engelmann 2011). However, post-cryopreservation viability, survival, and genetic integrity of many plant species remain unknown.

Cryopreservation involves preserving biological material at ultra-low temperatures, typically within the liquid (-196 °C) or gaseous (~-150 °C) phase of nitrogen (Ren et al. 2021; Huang et al. 2022). Operating on the principle of vitrification, solidifying liquids without crystallising, thereby minimising harmful ice formation (Pegg 2014), cryostorage is a relatively cost-effective (Panis et al. 2020) and space-efficient method for preserving the genetic integrity, metabolic stability, and field performance of some clonally propagated crops (Vendrame et al. 2014; Niino and Arizaga 2015; Coelho et al. 2020; Bi et al. 2021; Vollmer et al. 2022).

Cryopreservation has also facilitated the production of virus-free plants through the conservation of regenerative meristematic tissue and the elimination or deactivation of viruses exposed to ultra-low temperature treatments, supporting sustainable development across various plant industries (Bettoni et al. 2021; Yi et al. 2014). Various cryopreservation techniques have been successfully reported across diverse plants, including *Carica papaya* (Kaity et al. 2008), *Phaseolus vulgaris* (Cejas et al. 2013), *Malus* spp. (Wang et al. 2018), *Lilium* spp. (Li et al. 2019), *Stevia rebaudiana* (Benelli et al. 2021), *Vitis* spp. (Bettoni et al. 2021), *Olea europaea* (Bradaï and Sánchez-Romero 2021), and *Musa* spp. (Roostika et al. 2022), showcasing the applicability and relevance of cryopreservation across varying plant tissues and genotypes. Moreover, cryopreservation has been acknowledged as a valuable means of conserving the biodiversity of endangered medicinal plant species (Al-Baba et al. 2015).

Despite its many demonstrated advantages, the cryopreservation process inherently imposes stress on plant cells, potentially inducing (epi)genetic modifications in cryopreserved explants and regenerated plants, leading to reduced survival and regrowth, even under optimised conditions (Azimi et al. 2005; Cejas et al. 2013; Popova et al. 2023). The molecular stability of cryopreserved plant material remains inadequately studied, highlighting gaps in our understanding of the genetic and epigenetic changes potentially occurring during cryopreservation (Bi et al. 2021; Wang et al. 2021). The limitations and concerns linked with the plethora of cryopreservation protocols underscore the necessity for further investigation, specifically to address stresses and variations induced among superior and threatened plant genotypes and species (Lee et al. 2021; Pence and Bruns 2022). The diversity of cryopreservation protocols arises from the need to address the inherent variability among individual plants and tissues to optimise preservation outcomes. While most orthodox seeds are amenable to direct cryopreservation (Hervani et al. 2018; Suzuki et al. 2018; Panis et al. 2020), this

route is not viable for recalcitrant seeds, which, being resistant to dehydration, exhibit sensitivity to cryopreservation conditions and rapidly lose viability (Berjak and Pammenter 2013). Furthermore, if the intention of cryopreservation is to conserve superior selected genotypes, then alternative explants must be sought, as, given inherent variability, whole seeds are not suitable for this purpose.

A viable alternative is the cryopreservation of nodal explants of superior selected genotypes, but it also requires stringent, sample-specific optimisation, often utilising cytotoxic cryoprotectants and demonstrating varying degrees of success across species and even among genotypes (Engelmann 2014; Paula et al. 2018). Hence, this method can be technically demanding to initiate, and cryoprotectant toxicity may pose a risk to cell viability (Pegg 2014; Coste et al. 2015). Nevertheless, numerous studies have reported successful regeneration of plantlets from vitrified cells, demonstrating normal development post-storage in liquid nitrogen (LN_2) (Uragami et al. 1989; Fukai et al. 1991; Sakai et al. 1990; Prada et al. 2015; Downey et al. 2021). To further enhance survival and post-thaw characteristics, controlled-rate cooling, representing another iteration of cryopreservation, involves precisely regulating the cooling rate in programmable freezers or cooling rate-controlled freezing containers to minimise ice crystal nucleation during freezing (Salazar et al. 2008; Kilbride et al. 2019; Wilms et al. 2020; Chang and Zhao 2021). However, implementing controlled-rate cooling systems is more complex and costly than other, simpler preservation methods like direct LN_2 immersion (Woods et al. 2007).

After preserving nodal explants, examining the effect on the genetic fidelity of recovered genotypes is necessary. Genetic markers, including simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs), amplified fragment length polymorphisms (AFLPs), and random amplified polymorphic DNA (RAPD), offer higher reliability in determining genetic fidelity compared to morphological and biochemical markers due to the direct assessment of DNA, which remains largely unaffected by environmental factors or developmental stages (Semagn et al. 2006; Mondini et al. 2009). Genetic techniques also offer higher precision than morphological and biochemical methods, which may not provide enough variability for high-resolution differentiation (Powell et al. 1996; Varshney et al. 2005). However, the above-mentioned genetic approaches lack specificity, especially pertaining to biological functionality, because only general variations are evaluated, which may not

correlate with the expression of specific genes of interest (GOI) and the activity of desirable traits, such as high yield, nutritional content, and stress tolerance.

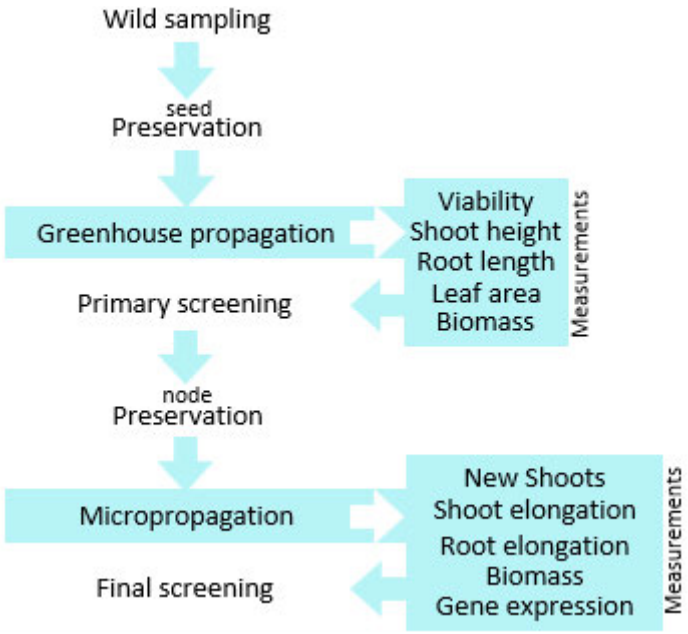
To address this limitation and gain further insights into gene-specific functionality, the present study aimed to investigate the regulation of a salinity-responsive NHX1-like gene discovered in a previously identified salinity-tolerant genotype of *Amaranthus dubius* (Haripershad et al. 2024). Salinity tolerance was explored due to its critical importance in agriculture, as soil salinisation severely limits crop productivity, especially in arid and semi-arid regions where salinity stress is a significant challenge to sustainable food production (Qadir et al. 2014). It was also hypothesised that salinity stress-tolerant genotypes might exhibit enhanced resilience to the osmotic and oxidative stresses inherent in freezing and thawing processes, as salinity-tolerant plants often possess mechanisms to improve ionic homeostasis and mitigate oxidative stress (Gupta and Huang 2013; Balasubramaniam et al. 2023), which could contribute to better survival during cryopreservation. Assessing the integrity and functionality of this gene, which is an important determinant of salinity tolerance in this species, post-cryopreservation is necessary for preserving the original (superior) genotype.

Moreover, elucidating gene expression fluctuations in response to different storage protocols can contribute to the optimisation of cryopreservation techniques, potentially ensuring minimal disruption to molecular pathways. Quantifying the relative expression of the GOI in response to stress necessitates extracting RNA from tissues and performing subsequent quantitative and qualitative assessments on the extracts. Quantifying the extracted RNA is needed to ensure that equal amounts of RNA from each sample are used in downstream analyses, as variations can affect the accuracy of gene expression measurements (Kashima et al. 2020). Qualitative control is essential to assess the integrity and purity of the RNA extracts because degraded or contaminated RNA can also lead to inaccurate gene expression data (Carvalhais et al. 2013). These analyses provide a foundation for understanding the molecular basis of stress tolerance, identifying stress-responsive genes, and developing strategies for enhancing stress resilience within the species of interest.

5.2 Materials and methods

The experimental design for this study is summarised in ***Scheme 5.1***, which outlines the sequence of growth measurements and gene expression analyses used to screen and select preservation routes

based on germination, growth, and genetic fidelity of stored seeds and nodal explants. The first step assessed germination and seedling growth parameters of multiple seed preservation protocols in a greenhouse. Then, cold storage of nodal explant was evaluated *in vitro* following greenhouse acclimatisation. Finally, the genetic fidelity of stored explants was assessed by comparing stress-responsive gene expression patterns among plantlets derived from a previously identified, salinity stress-tolerant *A. dubius* genotype exposed to the various freezing methods and storage conditions to a non-preserved, non-stored control.



Scheme 5.1: Flow diagram of the experimental design for this study to quantify storage stress.

5.2.1 Wild-type seed preservation

Amaranthus dubius seeds were obtained during September 2018 from naturally-growing populations in Desainagar, South Africa (29°36'47.1"S 31°09'08.8"E), and were hand de-chaffed, wrapped in paper towel, and then stored in an airtight glass container with silica crystals at 24 °C in the dark until experimental use. For verification, a specimen was deposited to the Bews Herbarium in Pietermaritzburg, KwaZulu-Natal (accession: NU0094621). The seeds were surface sterilised in 70% (v/v) ethanol (EtOH) (Protea Lab Services, South Africa) for 5 min prior to different cooling treatments and deep freeze storage. An untreated seed lot was wrapped in sterilised paper towel and stored in an airtight glass canister with silica crystals at 24 °C in the dark for 4 years (n = 64).

5.2.1.1 Nitrogen slurry cooling

Seeds were transferred to 2 mL round bottom cryo vials (Thermo Fisher Scientific, USA) and directly immersed in LN_2 (Afrox, South Africa) slurry under vacuum, resulting in a cooling rate of more than 500 °C/s (Echlin 2013). The seeds were then stored at -20 and -80 °C (NuAire, USA) for 4 years (n = 64).

5.2.1.2 Mr Frosty® cooling

Seeds were transferred to 2 mL round bottom cryo vials and inserted into a Mr Frosty® cooling rate-controlled freezing container (Nalgene, South Africa) containing isopropanol (IPA) (Protea Lab Services, South Africa). The seeds were cooled at a rate of 1 °C/min to -40 °C by placing Mr Frosty® into a -80 °C ultra-freezer (NuAire, USA) for 4 h. The seeds were then stored at -20 and -80 °C (NuAire, USA) for 4 years (n = 64).

5.2.1.3 Freezer cooling

Seeds were transferred to 2 mL round bottom cryo vials and stored at -20 and -80 °C (NuAire, USA) for 4 years (n = 64).

5.2.2 Thawing and seed germination

After the stipulated freezing routes, the seeds were suspended above a 65 °C water bath for 3 minutes following removal from storage after 4 years (February 2019 to February 2023). Individual seeds were then sown into seedling trays (37 mm width x 60 mm height) containing a 1:1 mixture of sterilised coco peat and seedling mix (Grovida, South Africa). The trays were placed inside growth rooms at the School of Life Sciences (University of KwaZulu-Natal, Westville campus) and monitored daily for emergence. An untreated control was established using non-stored seeds by sowing one seed lot at the beginning (n = 64) of the 4-year storage period. After approximately three weeks, seedlings that developed a second set of true leaves were transplanted into plastic flowerpots (90 mm diameter x 100 mm depth) with the same substrate as the seedling trays. However, the pots were additionally supplemented with 10% v/v vermiculite (Grovida, South Africa) to improve aeration and water and nutrient retention and exchange (Rajkumar et al. 2017; Xu et al. 2021). Dr Fisher's Classic Multifeed® (AECI, South Africa) solution (1.3 g/L) was applied to the substrate once every seven days at a volume of 10 mL per seedling. The germination rates of preserved and untreated seeds were recorded.

Furthermore, seedling height and the number of leaves and nodes were recorded before *in vitro* propagation.

5.2.3 *In vitro* propagation

Nodal explants were excised from seedlings grown from germinated cryopreserved seeds and transferred to semi-solid shoot multiplication medium (half-strength Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962), 30 g/L sucrose, 2 mg/L 6-benzylaminopurine (BAP), 0.5 mg/L indole-3-acetic acid (IAA), pH 5.7) for three weeks and then subcultured onto shoot elongation medium (half-strength MS, 30 g/L sucrose, 0.1 mg/L BAP, 0.1 mg/L IAA, pH 5.7) for two weeks (Shaik et al. 2022). All media were steam sterilised in an HL-341 autoclave (Gemmy Industrial Corporation, Taiwan) at 121 °C and 15 psi for 20 min. Thereafter, nodes were aseptically excised and cryoprotected with chilled 50% Plant Vitrification Solution 2 (PVS2) (15% glycol (Sigma, UK), 15% dimethyl sulfoxide (Sigma, UK), 0.4 M sucrose in full-strength liquid MS) for 5 min followed by 100% PVS2 for 15 min prior to cooling (Varghese et al. 2009). Nodal explants not preserved or exposed to seed storage treatments were used as a control.

5.2.4 Nodal explant preservation

5.2.4.1 Nitrogen slurry cooling

Cryoprotected nodal explants were directly submerged in LN_2 (Afrox, South Africa) slurry under vacuum, resulting in a cooling rate of more than 500 °C/s (Echlin 2013). The explants were then transferred to 2 mL round bottom cryo vials and stored at -20 and -80 °C (NuAire, USA) for 1 week each (n = 135). Additionally, cryo vials were inserted into CryoCanes and stored in LN_2 vapour (-148 °C) and LN_2 (-196 °C) for 1 week each (n = 135).

5.2.4.2 Liquid nitrogen cooling

Cryoprotected nodal explants were transferred to 2 mL round bottom cryo vials, inserted into CryoCanes, and plunged into LN_2 (Afrox, South Africa) for 2 min, resulting in a cooling rate of ~200 °C/s (Sakai 1965). The cryo vials were then stored at -20 and -80 °C (NuAire, USA) for 1 week each (n = 135). Additionally, some CryoCanes were stored in LN_2 vapour (-148 °C) and LN_2 (-196 °C) for 1 week each (n = 135).

5.2.4.3 Mr Frosty® cooling

Cryoprotected nodal explants were transferred to 2 mL round bottom cryo vials and inserted into Mr Frosty® containing IPA. The seeds were cooled at a rate of 1 °C/min to -40 °C by placing Mr Frosty® into a -80 °C ultra-freezer for 4 h. The explants were stored at -20 and -80 °C for 1 week each (n = 135). Additionally, cryo vials were inserted into and stored in LN_2 (Afrox, South Africa) vapour (-148 °C) and LN_2 (-196 °C) for 1 week each (n = 135).

5.2.4.4 Freezer cooling

Cryoprotected nodal explants were transferred to 2 mL round bottom cryo vials and stored at -20 and -80 °C (NuAire, USA) for 1 week (n = 135).

5.2.4.5 Salinity-tolerant genotype cryopreservation

Nodal explants harvested from a previously identified salinity-tolerant *A. dubius* genotype (S4) (Haripershad et al. 2024) were also subjected to cryopreservation protocols (**5.2.4.1** - **5.2.4.4**). Additionally, untreated nodal explants from S4 were micropropagated via direct organogenesis (Shaik et al. 2022) and served as a control.

5.2.5 Thawing and recovery of nodal explants

Upon retrieval from cryostorage, cryo vials were rapidly warmed in a water bath at 40 °C for 2 min, then surface sterilised with 70% EtOH and transferred to a laminar flow hood, wherein the PVS2 solution was replaced with 1.2 M sucrose and incubated at 24 °C for 20 min. The nodal explants were then rinsed with distilled water and recovered in sterile plastic Petri dishes containing full-strength MS medium supplemented with 30 g/L sucrose at 24 °C for 30 minutes. Thereafter, the samples were transferred to semi-solid shoot multiplication medium (half-strength MS, 30 g/L sucrose, 2 mg/L BAP, 0.5 mg/L IAA, pH 5.7) for three weeks and then subcultured onto shoot elongation medium (half-strength MS, 30 g/L sucrose, 0.1 mg/L BAP, 0.1 mg/L IAA, pH 5.7) for two weeks, and finally transferred to rooting medium (half-strength MS, 30 g/L sucrose, 0.1 mg/L IAA, pH 5.7) for a further two weeks (Shaik et al. 2022). The resulting rooted clones were acclimatised in plastic flowerpots (90 mm diameter x 100 mm depth) containing a 1:1 mixture of coco peat and seedling mix (Grovida, South Africa) inside greenhouse mist tents and watered daily at 4 pm with municipal water for 5 min. Before

the explants were subcultured from the multiplication medium to the rooting medium, the number of shoots, shoot length, and any callus formation were recorded. The number of root-producing explants, root number, and root length were recorded following the rooting phase. During acclimatisation, explant height and leaf number were recorded.

5.2.6 RNA extraction, quantification, and qualitative control

Following acclimatisation, salinity stress (400 mM NaCl) was incrementally applied (50 mM NaCl/day) to a subset of each S4 cryostorage group for 1 week, and then root tip tissue was randomly excised (n = 9). The tissue samples were rinsed with distilled water for 10 s and blotted dry with autoclaved paper towel. The samples were sectioned, weighed, and frozen in LN_2 (Afrox, South Africa) and then immediately prepared for RNA extraction or stored at -80 °C in an ultra-freezer (NuAire, USA) until use. Total RNA was isolated from root tissue for each treatment using TRIzol™ Reagent (Life Technologies, Netherlands) according to the manufacturer's specifications. The RNA concentration was quantified using the RiboGreen® protocol for the NanoDrop™ 3300 Fluorospectrometer (Thermo Fisher Scientific, USA). RNA quality was evaluated by running 5 µg of the extract through electrophoresis on 1% Tris-Acetate-Ethylenediaminetetraacetic acid (TAE) agarose gel (Thermo Fisher Scientific, USA) supplemented with 1% NaOCl (Reckitt Benckiser, South Africa) for 30 min at 110 V (Aranda et al. 2012). All RNA samples were aliquoted and stored at -80 °C in an ultra-freezer (NuAire, USA) until needed for RT-qPCR analysis.

5.2.7 RT-qPCR parameters

RT-qPCR analysis was completed using the GoTaq® 2-Step RT-qPCR System (Promega, USA). The cDNA template was synthesised from 1 µg of total RNA per the manufacturer's instructions. The qPCR final reaction volume of 20 µl was composed of 1 µl cDNA template (equivalent to 1 ng starting quantity of RNA), 10 µl of GoTaq® qPCR Master Mix (2X), 1 µl of both primers (500 nM) (Haripershad et al. 2024), and 7 µl of nuclease-free water. No template controls (NTCs) were performed with 8 µl of nuclease-free water to account for the lack of cDNA, thus ensuring a constant final reaction volume. The RT-qPCR thermocycling reactions were performed with three biological and two technical replicates of each treatment using the Eco™ Real-Time PCR System (Illumina, USA). All assays were carried out under the following conditions: one cycle of 95 °C for two min, 40 cycles of 95 °C for 15 s and 68 °C for

one min for the degenerate NHX1 primer pair and 60 °C for one min for the reference gene primer pairs. A melting curve was generated from 95 °C to 65 °C to verify amplicon specificity and identify erroneous hairpin and primer dimer formation. The size of the amplicon was estimated using gel electrophoresis (Armstrong and Schulz 2015).

5.2.8 RT-qPCR Analysis

LinRegPCR (Ruijter et al. 2009) was used to determine the fluorescence threshold and the mean RT-qPCR efficiency per amplicon. The generated quantitation cycle (*Cq*) values and RT-qPCR efficiencies (*E*) of the NHX1 GOI and reference gene, malate dehydrogenase (MDH), were used to calculate the relative quantification (*RQ*) for each salt stress treatment as described by ***Equation 5.1*** (Pfaffl et al. 2004). These data were then expressed as relative fold changes per salt stress treatment. BestKeeper was used to assess the stability of the reference gene by calculating the standard deviation (SD) and coefficient of variation (CV%) (Pfaffl et al. 2004).

Equation 5.1: Pfaffl method of relative quantification:

$$RQ = \frac{(E_{unknown})^{\Delta Cq_{unknown}}}{(E_{control})^{\Delta Cq_{control}}}$$

Where: $\Delta Cq_{unknown}$ = *Cq* of NHX1 in control treatment – *Cq* of NHX1 in unknown treatment, and: $\Delta Cq_{control}$ = *Cq* of MDH in control treatment – *Cq* of MDH in unknown treatment.

The relative quantification (RQ) of the GOI was calculated by determining the RT-qPCR efficiencies (E) and differences in quantitation cycles (ΔCq) between test samples (100, 200, and 400 mM NaCl) and the control (0 mM NaCl).

5.2.9 Data analyses

Germination and survival rates (Chi-square test) and growth data (ANOVA) were analysed using the Statistical Package for the Social Sciences (SPSS®) version 29.0 (IBM, USA). The growth data were assessed for normality (Shapiro-Wilk test) and metavariance (Levene's test) to satisfy the assumptions of the post hoc test. Significant interactions among and between treatments were identified by univariate ANOVAs followed by Tukey post hoc testing. In instances where the assumption of homogeneity of variances was violated, the pairwise Games-Howell post hoc test was applied. The

Kruskal-Wallis test was used to compare non-parametric data. A probability of $p < 0.05$ was used as the threshold for statistical significance.

5.3 Results and discussion

5.3.1 Seed cryopreservation

The 1-week germination of *A. dubius* seeds is plotted in ***Figure 5.1***, showing the results of various seed cooling and storage methods. No statistically significant differences existed between the control and all seeds stored at -20 and -80 °C for 4 years. However, there were significant differences between the seeds stored at 24, 4, and -20 °C and the control and LN_2 slurry and Mr Frosty® cooled seed lots stored at -20 and -80 °C. The largest statistically significant difference in germination % occurred in the samples rapidly frozen in LN_2 slurry and stored at -80 °C, yielding 94% germination compared to untreated seeds stored at 24 °C, resulting in 82% germination. Additionally, the observed lack of statistically significant differences between the control (92%) and all samples stored at -20 °C (90%) and -80 °C (90%) for 4 years indicated the effectiveness of these storage temperatures in preserving *A. dubius* seed viability for 4 years, irrespective of the cooling method. This resilience of seeds against deterioration at ultra-low temperatures suggests that storage at both -20 °C and -80 °C are suitable conditions for this species, precluding degradation, thereby maintaining seed quality comparable to unaged seeds. This is likely due to the high surface area-to-volume ratio of the minuscule seeds in conjunction with their relatively low (< 15%) water content (Omondi 2017).

Another *A. dubius* seed storage study conducted by Reddy (2022) assessed multiple cryoprotectants, cooling rates, and germination conditions, finding that LN_2 immersion resulted in the highest germination (> 80% after 24 h), in agreement with the present findings. This result is further supported by a study conducted on another amaranth, *A. cruentus,* that also resulted in better germination ability under cold storage (> 85 % germination after 300 days) (Martins et al. 2019). The effectiveness of sub-zero storage temperatures in preserving seed viability is further supported by a long-term assessment of 37 species of *Brassicaceae* (Pérez-García et al. 2007). Conversely, notable differences between seeds stored at 24, 4, and -20 °C (untreated) and those subjected to cooling methods and storage at -20 and -80 °C emphasised the impact of freezing methods on seed longevity and quality. Particularly, the significant difference in germination % observed in seeds rapidly frozen in LN_2 slurry and stored at

-80 °C (94%) underscores the potency of this technique in maintaining seed viability, surpassing traditional cold storage at 4 (84%) and -20 °C (86%). However, this method is more complex and requires more specialised equipment. The similarity in germination % between untreated seeds stored at -20 °C (86%) and the control presents a compelling case for the efficacy of this storage method in preserving *A. dubius* seed viability because it is relatively more accessible and cost-effective than other, more sophisticated methods.

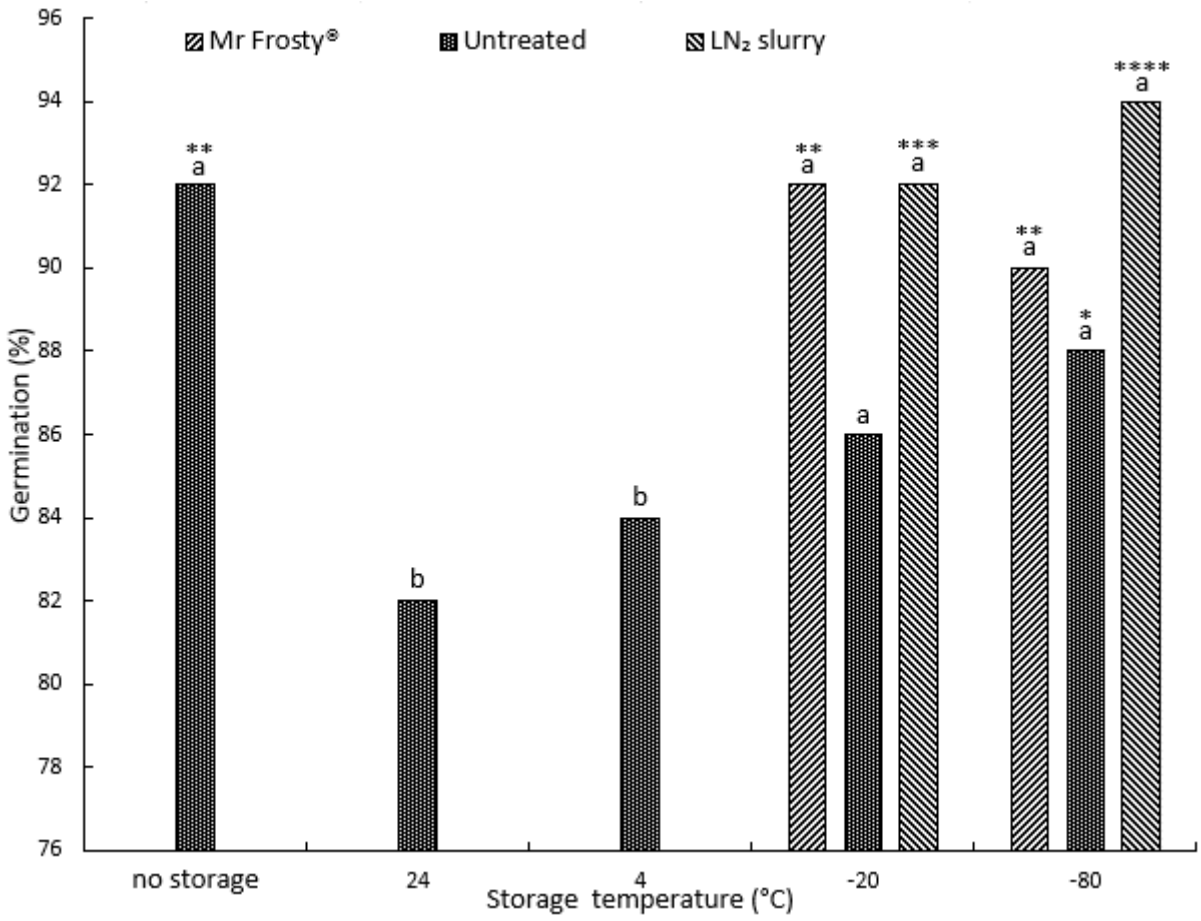


Figure 5.1: Germination % of *A. dubius* seeds after 4 years of storage compared to the control (not stored). Different letters indicate statistically significant differences from the control and increasing * denote greater differences from the seed lot stored at 24 °C (Chi-square test; $p < 0.05$), n = 64.

The rapid freezing process in LN_2 has been shown to preserve the cellular structures and biochemical integrity of seeds by minimising ice crystal formation, preventing the physical disruption of cellular membranes and thereby maintaining the integrity of the seed tissue (Zeng et al. 2024). The observed success of rapid freezing can be attributed to the resilience of the meristematic tissues within seeds to extreme cold, which facilitated successful germination. This resilience is likely controlled by several

factors, including cell membrane composition, antioxidant defence mechanisms, and gene expression. The long-term value of preserving seeds of ALVs, such as those of *A. dubius*, goes beyond the immediate considerations for maintaining seed viability.

Table 5.1 summarises the recorded growth characteristics of *A. dubius* seedlings subjected to various cooling and storage methods. The control group yielded the highest growth measurements. Mr Frosty® cooling significantly increased growth parameters in the treatment groups irrespective of storage temperature. This effect was particularly noticeable in the leaf area and root fresh mass (FM) measurements of plants grown from seeds stored at -20 and -80 °C. Seedlings from seeds stored at 4 °C had a root FM similar to the control and produced more leaves than seedlings grown from the other treated seeds. The LN_2 slurry pre-storage treatment yielded seedlings with the lowest number of leaves, foliar FM, leaf area, root length, and root FM, suggesting that this specific cooling method had a detrimental effect on growth. Interestingly, there appeared to be a contrasting relationship between the observed high germination % and low growth characteristics exhibited by seeds cooled with the LN_2 slurry. This pre-storage treatment possibly induced physiological stress on the seeds, negatively affecting growth parameters, but effectively maintained viability.

Table 5.1: Multiple growth characteristics of *A. dubius* seeds exposed to different cooling treatments and storage temperatures then germinated in seedling trays. Different letters indicate statistically significant differences among storage temperatures (lowercase letter) and cooling treatments (uppercase letters) (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, n = 50. FM = fresh mass.

Storage (°C)	**Cooling treatment**	**Plant height** (cm)	**Number of leaves**	**Foliar FM** (g)	**Leaf area** (cm^2)	**Root length** (cm)	**Root FM** (g)
Control	Control	$29.6^{aA} \pm 3.3$	$19.9^{aA} \pm 2.3$	$21.9^{aA} \pm 2.6$	$180.2^{aA} \pm 21.7$	$30.4^{aA} \pm 2.9$	$14.8^{aA} \pm 1.5$
24	None	$20.1^{cC} \pm 3.0$	$15.6^{cB} \pm 1.9$	$15.6^{dC} \pm 2.9$	$154.6^{bcB} \pm 28.5$	$19.7^{cC} \pm 2.8$	$10.3^{cB} \pm 2.3$
4	None	$21.2^{cC} \pm 2.7$	$18.1^{bB} \pm 2.2$	$18.6^{bC} \pm 2.5$	$156.3^{bcB} \pm 28.6$	$24.5^{bC} \pm 2.9$	$15.4^{aB} \pm 1.7$
-20	None	$23.1^{cC} \pm 2.8$	$17.3^{cB} \pm 1.7$	$18.8^{bcC} \pm 2.8$	$163.7^{bB} \pm 22.9$	$23.8^{bC} \pm 3.2$	$11.9^{bB} \pm 2.0$
-20	Mr Frosty®	$27.6^{bB} \pm 3.1$	$17.6^{cC} \pm 2.0$	$20.1^{bcB} \pm 2.7$	$184.6^{bA} \pm 26.6$	$27.4^{bB} \pm 2.7$	$15.2^{bA} \pm 1.6$
-20	LN_2 slurry	$22.7^{bC} \pm 3.0$	$14.8^{cD} \pm 2.0$	$14.9^{bcD} \pm 2.1$	$140.4^{bC} \pm 21.7$	$21.9^{bB} \pm 3.1$	$10.0^{bC} \pm 2.0$
-80	None	$24.7^{bB} \pm 3.1$	$15.3^{dB} \pm 2.1$	$16.2^{cdC} \pm 1.9$	$158.6^{cB} \pm 24.9$	$24.5^{bC} \pm 2.8$	$12.0^{bB} \pm 1.7$
-80	Mr Frosty®	$27.4^{bB} \pm 2.6$	$12.9^{dC} \pm 2.2$	$19.8^{cdB} \pm 2.3$	$156.6^{cA} \pm 23.3$	$26.1^{bB} \pm 3.1$	$15.7^{bA} \pm 2.2$
-80	LN_2 slurry	$23.4^{bC} \pm 2.9$	$12.1^{dD} \pm 2.2$	$14.7^{cdD} \pm 2.3$	$126.2^{cC} \pm 27.8$	$21.5^{bD} \pm 3.1$	$10.5^{bC} \pm 2.0$

Some seeds can tolerate certain stress conditions during preservation without losing their ability to germinate, i.e. viability (Sacandé et al. 1998; Sahitya et al. 2018; Gama et al. 2021). Furthermore, germination is a fundamental process that, as shown by the majority of authors for a range of species,

might be less sensitive to stress compared to subsequent growth (Galpaz and Reymond 2010; Patanè et al. 2012; Fu et al. 2019; Kong et al. 2020). Upon thawing, *A. dubius* seeds subjected to the LN_2 slurry pre-storage treatment are likely to have activated adaptive mechanisms to cope with the stress induced by the treatment, including the activation of stress-responsive genes, leading to the synthesis of stress-related proteins that repair and protect cellular structures and maintain viability (Rajjou et al. 2008; Benítez-Rodríguez et al. 2013). Moreover, rapid cooling induces oxidative stress in cells due to the production of reactive oxygen species (ROS) (Coelho et al. 2018; Bailly 2019; Jurdak et al. 2020). The treated *A. dubius* seeds may have employed repair mechanisms to address damage incurred during the rapid cooling process and activated antioxidant defence mechanisms to neutralise ROS, minimising oxidative damage. These mechanisms could enhance germination potential while compromising other aspects of subsequent growth.

The activation of stress-responsive genes and synthesis of stress-related proteins require the allocation of energy and resources. When seeds invest resources in repairing cellular structures and combating oxidative stress, there is a trade-off with other essential processes, compromising the overall vigour of subsequent growth (Paul-Victor and Turnbull 2009; Notarnicola et al. 2022). The compromised allocation of resources and energy towards growth and development may lead to seedlings that are less robust, with slower growth rates and reduced capacity to compete for resources in the early stages of development. While necessary for protecting cellular structures, activating antioxidant defence mechanisms to neutralise ROS involves complex processes that compete with metabolic pathways essential for growth, compromising the efficiency of energy utilisation for growth-related processes (Akram et al. 2017). While stress adaptations enhance short-term survival, there might be implications for the long-term survival and reproductive success of the plant, influencing its ability to establish, compete, and reproduce. Seeds of *A. dubius* that underwent stress-induced adaptations during germination may become more sensitive to these subsequent environmental challenges.

Preservation of whole seeds is preferred in cases where plant propagation and biodiversity conservation are prioritised, representing a fundamental component of plant genetic resources (Lynch et al. 2013; Moraes et al. 2019; Panis et al. 2020). Moreover, the benefits of seed storage, such as safety, minimal space requirements, and lower costs, further emphasise the significance of preserving

seeds (Figueiredo et al. 2021). Conversely, preserving nodal explants and shoot tips is necessary for conserving genetic fidelity and facilitating vegetative propagation, including those species that are threatened or have limited seed availability (Engelmann 2011; Tavazza et al. 2013; Bradaï and Sánchez-Romero 2021; Popova et al. 2023).

5.3.2 Node cryopreservation

The % of preserved *A. dubius* nodal explants developing at least 1 shoot (shoot development %) and the total number of new shoots produced per explant are shown in ***Figure 5.2***. Cooling explants with Mr Frosty®, followed by storage at -196 °C, was the only method that yielded a similar shoot development % to the control nodes, which were not cold stored. Furthermore, only direct LN_2 immersion followed by -20 and -148 °C storage yielded significantly lower shoot numbers than the control. Interestingly, there appeared to be a correlation between shoot development % and the number of shoots in all treatments, except for the explants stored at -20 °C, which showed high deviation and low shoot development %. More new shoots often correlated with enhanced shoot development %, indicating a positive influence on shoot regeneration capacity.

The successful application of controlled-rate cooling, like Mr Frosty®, for the conservation of vegetative plant germplasm has also been documented in *Rubus* spp. (Uchendu et al. 2010), *Hypericum richeri* (Coelho et al. 2020), *Cannabis sativa* (Downey et al. 2021), and *S. lycopersicum* (Kulus 2019). The precise and gradual reduction in temperature afforded by Mr Frosty® offers several benefits, including consistency and reliability in the preservation of biological samples (Zhou et al. 2009), as well as the gradual removal of water from cells, which prevents the formation of deleterious ice crystals at the final storage temperature (Schulte and Reski 2004; Ishizaki et al. 2023). This method also facilitates the use of lower cryoprotectant concentrations to achieve intracellular freezing, thereby minimising toxicity and further enhancing viability (Jiang et al. 2004; Naaldijk et al. 2013). This specific aspect was not explored in the current study but may be examined in future research aiming to optimise cryoprotectant concentrations for the preservation of *A. dubius* germplasm.

Table 5.2 lists additional growth parameters of micropropagated nodal explants. As expected, the control, which was not stored and did not undergo any cooling treatments, yielded the highest growth parameters throughout micropropagation and subsequent acclimatisation. Mr Frosty® cooled

explants yielded statistically similar shoot elongation rates to the control (2.4 ± 0.5 cm/week) when paired with storage at -20 (2.2 ± 0.6 cm/week), -80 (2.5 ± 0.4 cm/week), -148 (2.4 ± 0.5 cm/week), and -196 °C (2.4 ± 0.6 cm/week). Mr Frosty® cooled explants also achieved similar root elongation rates to the control (1.5 ± 0.6 cm/week) when stored at -148 (1.5 ± 0.4 cm/week) and -196 °C (1.5 ± 0.4 cm/week). None of the stored explants yielded similar foliar FM to the control (50.3 ± 5.1 g). Among the stored explants, those that produced the highest foliar FM were cooled by Mr Frosty® and LN_2 immersion then stored at -20 (45.2 ± 5.1 g), -80 (45 ± 5.9 g), -148 (45.3 ± 4.1 g), and -196 °C (45.3 ± 5.8 g), and -20 (42.3 ± 4.4 g), -148 (45.0 ± 7.0 g), and -196 °C (44.4 ± 7.6 g), respectively.

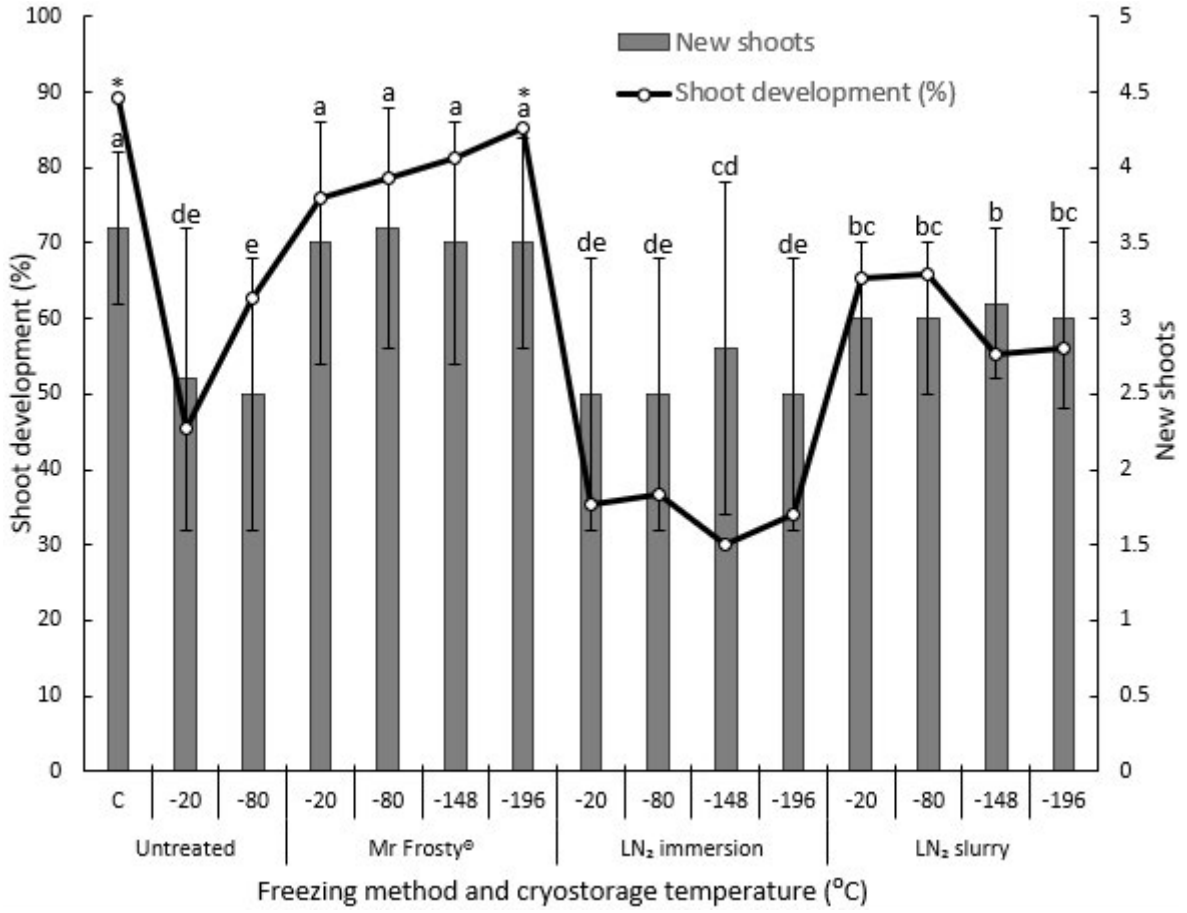


Figure 5.2: The shoot development % and number of shoots forming from *A. dubius* nodal explants exposed to various freezing and storage protocols. Different letters indicate statistically significant differences in shoot numbers to the control (C) (Kruskal-Wallis test; $p < 0.05$), mean ± SD, n = 135. * Denotes similarity in shoot development % to the control sample (Chi-square test; $p < 0.05$), n = 135.

Research into the molecular mechanisms governing shoot regeneration following cryopreservation in specific species is sparse. However, in the model plant, *A. thaliana*, this process involves a complex

interplay of transcription factors, hormonal signalling, and gene expression regulation, including the upregulation of the homeodomain transcription factor WUSCHEL (WUS) (Zhang et al. 2017) and the wound induced dedifferentiation (WIND) transcription factor, which activates the enhancer of shoot regeneration (ESR) gene (Iwase et al. 2017). Moreover, a novel chemical inhibitor of polar auxin transport has been shown to promote shoot regeneration by enhancing homeodomain-leucine zipper (HD-ZIP) transcription (Yang et al. 2022). In *Cymbidium* spp., YUCCA (YUC) mediated auxin biogenesis has been implicated in shoot regeneration (Liu et al. 2017). In the context of the present study, the insights derived from studies in model plants like *A. thaliana* can offer parallels to reference and enhance the understanding of the molecular mechanisms involved in shoot regeneration of *A. dubius*.

Table 5.2: Growth parameters of micropropagated *A. dubius* nodal explants excised from seedlings grown from preserved seeds. Different letters indicate statistically significant differences among genotypes (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, n = 135. FM = fresh mass.

Storage (°C)	**Cooling method**	**Shoot elongation** (cm/week)	**Root elongation** (cm/week)	**Foliar FM** (g)
Control	Control	2.4^{ab} ± 0.5	1.5^{a} ± 0.6	50.3^{a} ± 5.1
-20	None	1.3^{i} ± 0.7	0.7^{efg} ± 0.3	39.6^{d} ± 6.4
-80	None	1.6^{gh} ± 0.5	0.9^{cd} ± 0.4	40.8^{d} ± 7.2
-20	Mr Frosty®	2.2^{bcd} ± 0.6	1.2^{b} ± 0.6	45.2^{b} ± 5.1
-80	Mr Frosty®	2.5^{a} ± 0.4	1.1^{bc} ± 0.5	45.0^{bc} ± 5.9
-148	Mr Frosty®	2.4^{abc} ± 0.5	1.5^{a} ± 0.4	45.3^{b} ± 4.1
-196	Mr Frosty®	2.4^{abc} ± 0.6	1.5^{a} ± 0.4	45.3^{b} ± 5.8
-20	LN_2 immersion	1.0^{j} ± 0.5	0.9^{de} ± 0.4	42.3^{bcd} ± 4.4
-80	LN_2 immersion	1.4^{hi} ± 0.6	0.8^{def} ± 0.3	42.1^{cd} ± 6.0
-148	LN_2 immersion	1.5^{hi} ± 0.4	0.6^{g} ± 0.3	45.0^{bc} ± 7.0
-196	LN_2 immersion	1.4^{hi} ± 0.3	0.6^{fg} ± 0.3	44.4^{bc} ± 7.6
-20	LN_2 slurry	1.9^{ef} ± 0.5	0.9^{d} ± 0.4	39.8^{d} ± 8.1
-80	LN_2 slurry	1.8^{fg} ± 0.6	0.8^{de} ± 0.4	40.0^{d} ± 9.1

5.3.3 Genetic fidelity of the salinity stress response post-cryopreservation

The relative expression (RE) of a previously documented NHX1-like gene was used to assess the effect of the tested cooling and storage protocols on the genetic fidelity of this salinity-responsive gene in a salinity-tolerant *A. dubius* genotype (S4). ***Figure 5.3*** shows the electrophoresis gel of total RNA extracted post-acclimatisation from salt-stressed root tissue samples of S4 explants subjected to the various storage methodologies, wherein 2 distinct bands, representing 28S and 18S ribosomal RNA were visualised, indicating pure and high-quality RNA samples with minimal degradation. ***Table 5.3***

summarises the NHX1-like RE normalised against MDH and foliar FM following acclimatisation of stored S4 nodal explants. Baseline RE of the NHX1-like gene was established by the non-stored samples and an untreated salinity stress group (0 mM NaCl) compared to the stored salinity-stressed samples exposed to 400 mM NaCl.

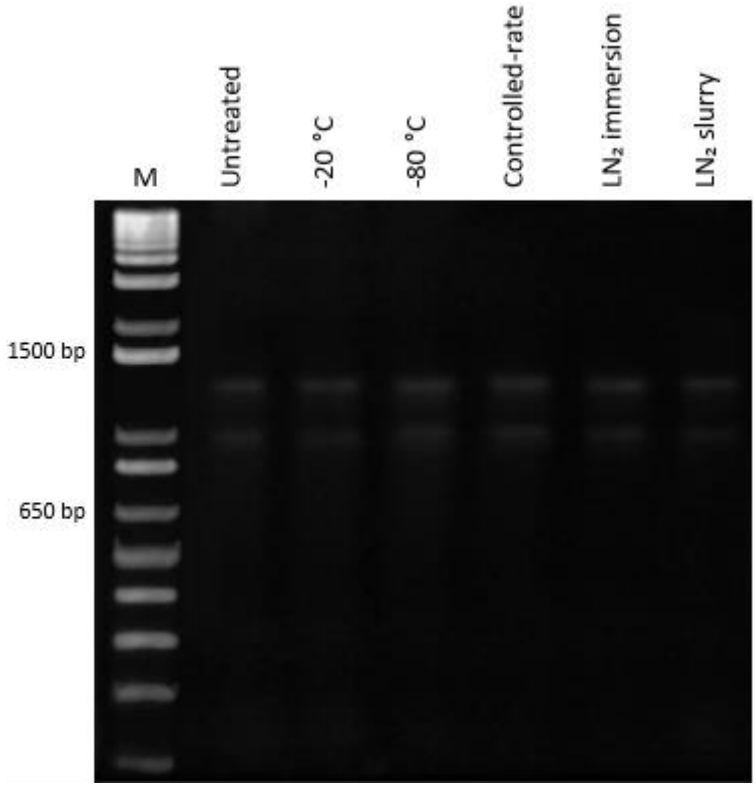


Figure 5.3: Electrophoresis of 5 µg total RNA extracts from *A. dubius* roots exposed to salinity stress (400 mM NaCl) following multiple cryopreservation conditions. M = 1 Kbp marker (Invitrogen, USA).

At 0 mM NaCl, the NHX1 RE was highest in untreated nodes stored at -20 and -80 °C, Mr Frosty® cooled nodes stored at -196 °C, LN_2 immersed nodes stored at -20 and -80 °C, and LN_2 slurry cooled nodes stored at -80 and -196 °C. These treatment groups resulted in upregulation of the NHX-like gene compared to the non-stored control group (1.7 ± 0.4) without exogenous salt application. When salt was added to the substrate (400 mM NaCl), untreated nodes stored at -20 °C, Mr Frosty® cooled nodes stored at -148 °C, and LN_2 immersed nodes stored at -148 °C yielded specimens that exceeded the RE of the non-preserved control (9.7 ± 0.6). Overall, samples which resulted in diminished NHX1-like activity compared to the non-stored control were the untreated nodes stored at -20 and -80 °C, Mr Frosty® cooled nodes stored at -196 °C, LN_2 immersed nodes stored at -20 and -80 °C, and LN_2 slurry cooled nodes stored at -80 and -196 °C, indicating that these cooling and storage conditions reduced

the genetic fidelity of the NHX1-like gene. Contrastingly, Mr Frosty® cooled nodes stored at -20, -80, and -148 °C, LN_2 immersed nodes stored at -148 and -196 °C, and LN_2 slurry cooled nodes stored at -20 and -148 °C yielded plants that maintained or surpassed the RE of the non-preserved control, which suggest that these storage conditions upheld the genetic fidelity of the GOI in genotype S4.

Table 5.3: The NHX1-like gene relative expression (RE) and foliar fresh mass (FM) of *A. dubius* following acclimatisation in response to multiple cooling and storage methods. Different letters indicate statistically significant differences (ANOVA with Tukey post hoc test; $p < 0.05$), mean ± SD, n = 9.

Cooling treatment	**Storage** (°C)	**NHX1** 0 mM NaCl (RE)	**NHX1** 400 mM NaCl (RE)	**Foliar FM** (g)
Control	Control	1.7^{bcd} ± 0.4	9.7^{bcd} ± 0.6	51.9^{a} ± 6.6
None	-20	2.7^{a} ± 0.5	10.4^{ab} ± 0.6	45.3^{abcd} ± 5.3
None	-80	2.1^{abc} ± 0.5	8.4^{defg} ± 0.7	46.4^{abcd} ± 2.6
Controlled-rate	-20	0.7^{e} ± 0.4	7.2^{gh} ± 0.8	51.7^{ab} ± 5.7
Controlled-rate	-80	1.0^{de} ± 0.6	6.9^{h} ± 0.5	50.4^{abcd} ± 2.9
Controlled-rate	-148	1.7^{bcd} ± 0.2	10.2^{abc} ± 1.3	46.0^{abcd} ± 3.4
Controlled-rate	-196	1.8^{abcd} ± 0.5	8.9^{cdef} ± 1.2	48.0^{abcd} ± 4.3
LN_2 immersion	-20	2.3^{ab} ± 0.7	9.5^{bcde} ± 0.7	43.1^{d} ± 2.9
LN_2 immersion	-80	2.7^{a} ± 0.4	8.3^{efg} ± 0.6	48.1^{abcd} ± 4.8
LN_2 immersion	-148	1.1^{de} ± 0.6	11.4^{a} ± 0.9	44.5^{bcd} ± 4.9
LN_2 immersion	-196	1.2^{cde} ± 0.5	7.8^{fgh} ± 0.8	50.2^{abcd} ± 3.1
LN_2 slurry	-20	1.1^{de} ± 0.4	9.3^{bcde} ± 1.0	46.8^{abcd} ± 5.3
LN_2 slurry	-80	2.4^{ab} ± 0.9	9.1^{bcde} ± 0.8	43.4^{d} ± 4.7
LN_2 slurry	-148	1.5^{bcde} ± 0.6	9.1^{bcde} ± 0.7	43.8^{cd} ± 4.6
LN_2 slurry	-196	2.4^{ab} ± 0.7	8.3^{efg} ± 0.7	51.1^{abc} ± 3.8

These results are further supported by the foliar FM of salinity-stressed micropropagated nodes which were cooled by Mr Frosty® then stored at -20, -80, and -148 °C, immersed in LN_2 then stored at-148 and -196 °C, and cooled in LN_2 slurry then stored at -20 and -148 °C, yielding similar FM to the non-preserved control (51.9 ± 6.6 g). Interestingly, the following treatments also yielded similar foliar FM to the non-preserved control despite exhibiting decreased NHX1 activity: direct storage at -20 and -80 °C, Mr Frosty® cooling followed by storage at -196 °C, LN_2 immersion followed by storage at -80 °C, and LN_2 slurry cooling and storage at -196 °C. This result coincides with the elevated RE observed in samples not exposed to salt stress (0 mM NaCl), suggesting that, in addition to salinity tolerance, the putative NHX1 gene also influences *A. dubius* growth in response to storage-related stresses. The elevated NHX1 RE and consistency in foliar FM across diverse preservation treatments suggest that this gene mitigates storage-related stresses during plantlet regrowth – a result that has not been

previously documented. Furthermore, the connection between elevated RE in samples which were not exposed to salt stress (0 mM NaCl) and the observed growth response further indicated that NHX1 may act as a modulator of growth in stressful environments.

The NHX1 gene, known for its role in ion transport and cellular homeostasis (Barragán et al. 2012; Chanroj et el. 2012), may influence plantlet vigour post-storage. However, this interaction has not been reported, presenting an avenue for future research. Ionic homeostasis is essential for osmotic regulation, ensuring that plant cells can control water uptake, thus maintaining turgor pressure and contributing to the overall cellular hydration and structural integrity of plantlets (Häussinger et al. 1996; Ritz et al. 2003). Additionally, cryopreservation procedures often subject plant tissues to various stresses, including dehydration and osmotic stress (Cantrel et al. 2011; Ren et al. 2021). NHX1, by facilitating ion transport and cellular water balance, may mitigate the impact of these stresses, contributing to the recovery of cell turgor and preventing cellular damage. Furthermore, NHX1 activity is intertwined with hormonal signalling pathways, including those associated with abscisic acid (ABA) and auxins (Sun and Zhou 2017; Sardans and Peñuelas 2021). The influence of NHX1 on these pathways post-cryopreservation may contribute to optimal hormonal balance, promoting vigorous shoot and root development. Proper ion transport facilitated by NHX1 can enhance nutrient uptake by plant cells. Improved nutrient availability contributes to the vigour of plantlets by supporting metabolic processes, energy production, and overall growth. Understanding the intricate mechanisms by which NHX1 influences *A. dubius* plantlet vigour post-storage requires further investigation. Integrating more molecular studies, including whole-transcriptome expression profiling, hormone quantification, and cellular imaging, can provide deeper insights into the specific pathways and processes through which NHX1 contributes to the recovery and vigour of plantlets following cryopreservation.

The varied effects of cryopreservation treatments on gene expression are well documented and have been shown to affect gene expression patterns in *Solanum tuberosum* (Seo et al. 2018), *S. lycopersicum* (Kyu et al. 2019), *Hosta capitata* (Pe et al. 2021), *Olea europaea* (Bradaï and Sánchez-Romero 2021), and *A. thaliana* (Li et al. 2013; Gross et al. 2016). However, it is often ambiguous if those effects depend on other factors like regeneration conditions and specific genotypes. Moreover, other studies have documented genetic changes post-cryopreservation resulting from tissue culture

conditions rather than cryopreservation (Castillo et al. 2010; Coste et al. 2015; Lee et al. 2021). Therefore, it cannot be conclusively said that the NHX1-like activity observed in the present study was solely due to preservation, micropropagation, or a combination thereof, necessitating further research.

5.4 Conclusion

The investigation into the long-term storage effects on *A. dubius* seeds yielded best-practices for preserving seed viability over extended periods (4 years). Notably, no statistically significant differences were observed between the unaged control and seeds stored at -20 and -80 °C for 4 years, suggesting the effectiveness of these methods in arresting seed deterioration and maintaining viability similar to unaged seeds, thus presenting a compelling case for the efficacy of this storage method in maintaining *A. dubius* seed viability. The findings from the various cooling and storage methods presently tested on *A. dubius* nodal explants offer practical methods for preserving germplasm with enhanced shoot regeneration capacity. Among the tested methodologies, controlled-rate cooling followed by storage at -196 °C emerged as the sole method yielding shoot development percentages akin to the non-cryostored control nodes.

The findings reported herein establish a foundation for refining preservation protocols, guiding the selection of optimal storage conditions necessary for preserving *A. dubius* seeds and nodal explants. Ultimately, this contributes to advancing preservation techniques, which are essential for the conservation and sustainable utilisation of plant genetic resources. These findings provide knowledge for maintaining *A. dubius* seed viability, ensuring the preservation of genetic diversity and opportunities for enhancing agricultural productivity, ensuring that unique, and often region-specific, genetic traits are conserved, such as potential resistance to pests, diseases, or environmental stresses (Linguya et al. 2015). Therefore, extending cold storage assessments to genotypes tolerant to other stressors, such as drought and heat, presents a future research direction. Future experiments could also build on the findings of this study by assessing how the genotypes identified herein respond to other stresses. Beyond agricultural applications, the ability to store *A. dubius* seeds has implications for ecological restoration efforts, as many ALVs have vital roles in local ecosystems, contributing to soil health and ecosystem stability (Maseko et al. 2017), thus enabling the conservation and potential

reintroduction of these species in degraded or disturbed areas, supporting ecological restoration initiatives and promoting biodiversity conservation.

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6. Conclusions and recommendations

6.1 Stress to success: *Amaranthus dubius* tolerance

This study aimed to systematically quantify the impact of variable watering schedules and salinity and heat levels on the growth parameters, physiological processes, and molecular mechanisms in the African leafy vegetable (ALV), *Amaranthus dubius*. Assessments included seedling height, root length, leaf area, biomass, as well as physiological indicators like leaf water pressure potential (Ψ_{Leaf}), electrical conductivity (EC), chlorophyll content, proline content, and protein characterisation. Additionally, molecular investigations into stress-responsive gene expression were performed. The principal goal was to identify superior genotypes resilient to these stressors and facilitate their propagation, primarily through cloning, to preserve true-to-type genetic traits. Furthermore, this study evaluated the feasibility of multiple storage techniques for preserving *A. dubius* germplasm, examining post-preservation viability, vigour, and genetic fidelity. This research provides novel information on stress responses in *A. dubius*, offering insights into potential applications for climate-resilient, sustainable agriculture and biodiversity conservation.

Across 1000 genotypes of *A. dubius* sourced from 5 different locations around KwaZulu-Natal, South Africa, the assessment of water-deficit and high-temperature tolerance revealed varied responses in growth parameters, Ψ_{Leaf}, and proline and chlorophyll content. Variable watering regimens (4-, 8-, 12-, and 16-day intervals) elicited distinct genotype-specific adaptations. Under severe water-deficit (16-day interval), genotypes from Genozzano and Tongaat cumulatively displayed the highest vigour ratings compared to genotypes from Amanzimtoti, Verulam, and Westville. This result was likely due to the geographical proximity of these locales and the high species richness observed therein, naturally selecting for adaptive traits, such as vigorous growth and enhanced seed dispersal, which culminated in better fitness to stress conditions. Several genotypes maintained Ψ_{Leaf} and proline and chlorophyll content despite decreased watering frequencies and elevated daytime temperatures, even following micropropagation. Mass spectrometry analysis identified six proteins in the water-deficit and heat-tolerant plant genotypes. Four of these proteins were linked to important stress adaptation mechanisms, such as maintaining cellular homeostasis, protein synthesis and photosynthesis, while two were uncharacterised, presenting an avenue for future research. The ability of some genotypes to tolerate temperatures up to 45 °C is of particular significance given the

increasing prevalence of extreme heat events associated with climate change. Water-deficit and heat-tolerant crop varieties are essential for maintaining food security in the face of climate change-induced stresses. By facilitating stable food production in vulnerable regions, tolerant crops, like *A. dubius*, can contribute to sustainable agricultural practices, reducing the need for water-intensive irrigation and minimising the risk of crop failure during heatwaves. Unfortunately, genetic expression analyses of dehydration (dehydration-responsive element-binding protein 1A (DREB1A) and Annexin 1) and heat responsive (heat shock protein 70 (HSP70)) proteins were inconclusive, possibly due to insufficient GOI primer specificity, low GOI transcript concentrations, suboptimal PCR conditions, or the inherent genetic variations among samples, necessitating further experimentation.

In the salinity stress experiment, the quantification of putative Na^+/H^+ exchanger (NHX1) antiporter gene expression in leaf and root tissues under varying NaCl concentrations revealed distinct patterns among different *A. dubius* genotypes. Notably, tissue-specific constitutive or salt-inducible NHX1 expression generally decreased in leaves and increased in root tissues as soil salinity increased, indicating adaptive mechanisms for the compartmentalisation of toxic ions, such as Na^+, away from sensitive leaf tissues. This mechanism was confirmed by EC measurements of root and leaf tissues, which demonstrated higher EC in roots than in leaves as salinity stress increased. Similar to the water-deficit stress assays, some genotypes demonstrated maintenance of chlorophyll and protein content despite increasing levels of stress, maintaining this ability following micropropagation, which was further correlated with NHX1 expression in some clonal genotypes. The identification and quantification of a stress-responsive gene associated with salinity tolerance in *A. dubius* represents a starting point, facilitating the discovery of more genes contributing to systemic stress tolerance. The utilisation of degenerate primers and protein characterisation heralds promising avenues for the targeted exploration of *A. dubius* expression in deciphering salinity tolerance and unveiling the intricate network of genes and proteins orchestrating the stress response. This route of investigation is likely to reveal more novel candidate genes and proteins previously unexplored in the context of stress adaptation in *A. dubius*, encompassing genetic adaptations, ion transport, and osmotic regulation with the potential to serve as targets for genetic manipulation and breeding programs aimed at enhancing overall stress resilience in this species.

Finally, investigations into the long-term storage effects on *A. dubius* germplasm provided a greater understanding of nodal explant and seed preservation over extended periods. Notably, no statistically significant differences were observed between the unaged control and seeds stored at -20 and -80 °C for a duration of 4 years, suggesting that *A. dubius* seeds are highly amenable to relatively simple and cost-effective cold storage methods, exhibiting orthodox seed behaviour. The ability of *A. dubius* seeds to maintain viability during cold storage is also demonstrative of the inherent shedding of water upon maturation prior to storage, which mitigated damage from ice crystal formation during freezing. For nodal preservation, Mr Frosty® cooling emerged as the only currently tested method that yielded *in vitro* shoot development percentages similar to the non-stored control and maintained the genetic fidelity of the NHX1-like gene in a superior, salinity-tolerant *A. dubius* clonal genotype.

The identification of stress-responsive genetic markers, particularly the exploration of stress-responsive gene expression, adds a molecular dimension to this study, elucidating an underlying mechanism governing the response of *A. dubius* to salinity- and storage-induced stresses. Additionally, this research established a link between stress factors and genotype performance following cloning through *in vitro* propagation, highlighting physiological correlations that can guide the selection of superior genotypes that are high-yielding and resilient to specific stressors. The exploration of storage methods enabled the preservation of stress-tolerant traits demonstrated in individual *A. dubius* genotypes. In contrast, seed storage maintains genetic diversity and population dynamics, preserving the documented variability of *A. dubius* populations. While seeds enable genetic recombination and long-term storage, nodal explants offer not only rapid propagation and pathogen elimination but also the preservation of superior, e.g., stress tolerant, germplasm. Integrating both storage methods can provide a comprehensive approach to germplasm conservation, ensuring the preservation of diverse and desired genetic resources and facilitating their utilisation for future research, breeding, and conservation efforts. The results discussed herein not only enhance the understanding of plant stress responses but also provide a promising avenue for safeguarding plant genetic resources in the face of environmental uncertainties and anthropogenic pressures.

6.2 Omics approaches

Future examination of *A. dubius* stress responses can benefit from the utilisation of 'omics' strategies, including genomics, transcriptomics, proteomics, and metabolomics. These highly efficient

methodologies, offering substantial throughput capacity, have transformed the examination of plant-stress interactions, providing significant insights into the complex molecular mechanisms underlying these reactions (Hirayama and Shinozaki 2010). Systematically examining various molecular elements, such as genes, transcripts, proteins, and metabolites, can clarify the intricate networks and pathways involved in the stress response of *A. dubius*, rapidly identifying multiple stress-reactive proteins and elucidating the regulatory networks and pathways implicated in stress tolerance. Developments in mass spectrometry and other analytical techniques have expedited the high-throughput evaluation of proteins within proteomics, involving isolating and classifying molecular components from specimens of interest (Hirayama and Shinozaki 2010; Rasmussen et al. 2013; Huibo et al. 2022).

By concurrently assessing multiple molecular components, 'omics' strategies empower researchers to capture the intricacy and interconnectedness of stress-responsive networks and pathways (Hirayama and Shinozaki 2010; Rasmussen et al. 2013; Huibo et al. 2022). This scalability proves vital for elucidating the genetic multiplicity of stress reactions across diverse specimens and environmental conditions. The gathering of quantifiable data can be further leveraged to differentiate the expression levels or abundance of molecular elements under distinct stress conditions among diverse *A. dubius* genotypes, streamlining the detection of crucial regulators and indicators of stress resilience.

6.3 Future frontiers

Identifying areas for prospective research pertaining to plant stress responses has centred around emergent technologies and methodologies, endeavouring to overcome limitations and increase multidisciplinary collaborations. Notably, epigenetic mechanisms, typified by DNA methylation, have emerged as influential guides of plant stress reactions (Sun et al. 2021). Understanding the role of epigenetics in stress-related genetic memory and hereditary transfer could contribute to the formulation of stress-resistant crops. Another nascent area of stress-responsive epigenetic research concerns non-coding RNAs (ncRNAs), encompassing myriad functions in transcriptional and post-transcriptional regulation (Balarezo-Cisneros et al. 2021). Particularly, deciphering post-transcriptional control of stress responses presents formidable challenges and is largely understudied in ALVs, such as *A. dubius*. However, preliminary indications support the involvement of ncRNAs in plant stress reactions (Bhogireddy et al. 2021; Jha et al. 2021; Li et al. 2023).

An additional challenge to characterising plant stress responses is that different stressors lead to specific changes in gene expression, making it difficult to distinguish individual stress impacts (Zhang and Sonnewald 2017). Stress responses are susceptible to factors, such as nutrient availability, capable of reshaping the expression of distinct genes (Farjad et al. 2018). Overcoming these challenges requires a thorough understanding of the molecular foundations governing stress responses and identifying key mechanisms that regulate these interactions. Moreover, standardisation is crucial for ensuring the credibility and comparability of plant stress assessments, with most present investigations mainly focused on individual stress treatments, making them irreproducible and insufficient for determining stress-specific or stress-independent characteristics. In future pursuits, comparing multiple stress conditions in the presently identified stress tolerant *A. dubius* genotypes is central for elucidating the response of this species to environmental stresses. A standardised framework, encompassing protocols and experimental parameters across disciplines, would enable further optimisation of plant stress response research.

Interdisciplinary collaborations between different fields of study can help gain further insight into plant stress responses, enhancing the overall understanding of how plants interact with their environment. For example, the present study investigated the synergy between plant physiology and genomics, bridging the gap between phenotype and genotype, thus fostering the development of climate-resilient crops. The confluence of genomics, phenomics, and analytical tools can generate valuable and expansive datasets, providing greater scientific understanding of stress tolerance in crops. Consultations among plant scientists and specialists from alternate spheres, like data and computer scientists, can accelerate the progression of machine learning and artificial intelligence modalities to efficiently decipher large datasets, thereby uncovering patterns, correlations, and relationships within the data, such as identifying molecular biomarkers associated with specific stress responses, useful for genetic engineering and marker-assisted selection of stress tolerant crops. Moreover, by integrating diverse datasets, including genotypic, phenotypic, and environmental data, predictive models can rapidly and iteratively forecast how different plant species or genotypes will perform under specific stress conditions *in silico* without the need for costly and time-consuming laboratory or field trials. Finally, collaboration between plant scientists and outreach practitioners can help share research findings with the general public, increasing awareness of the importance of plant

stress responses in addressing global challenges like food security, resource scarcity, and climate change.

6.4 References

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7. Appendix

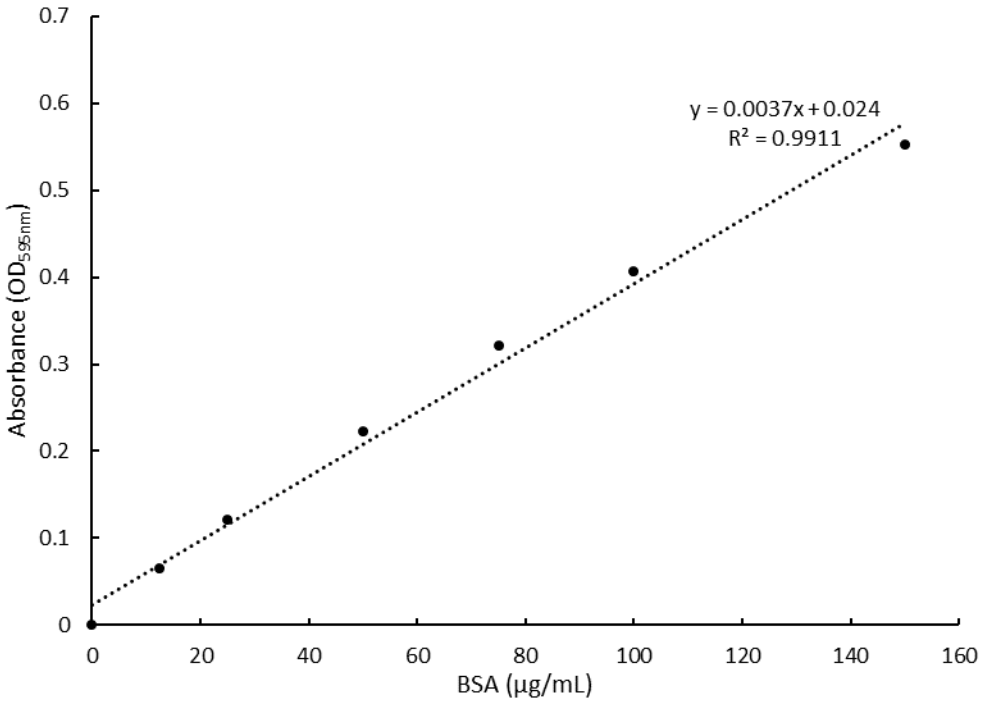


Figure 7.1: Standard curve of serially diluted bovine serum albumin (BSA) (µg/mL) absorbance (OD_{595nm}) for the Bradford protein quantification assay.

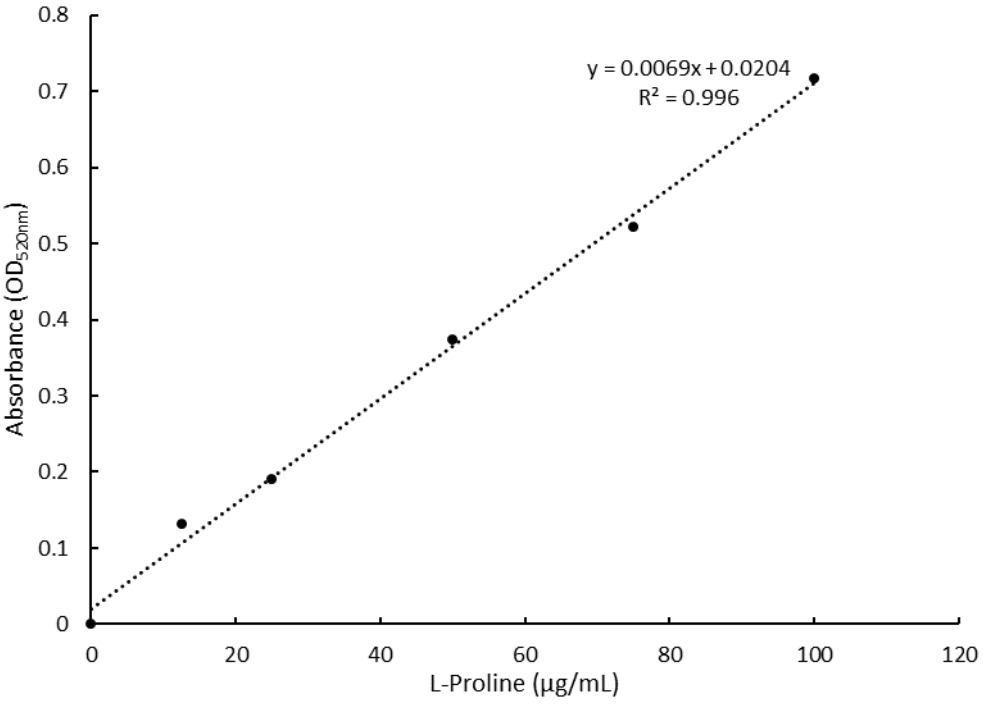


Figure 7.2: Standard curve of serially diluted L-Proline (µg/mL) absorbance (OD_{520nm}) for the proline quantification assay.

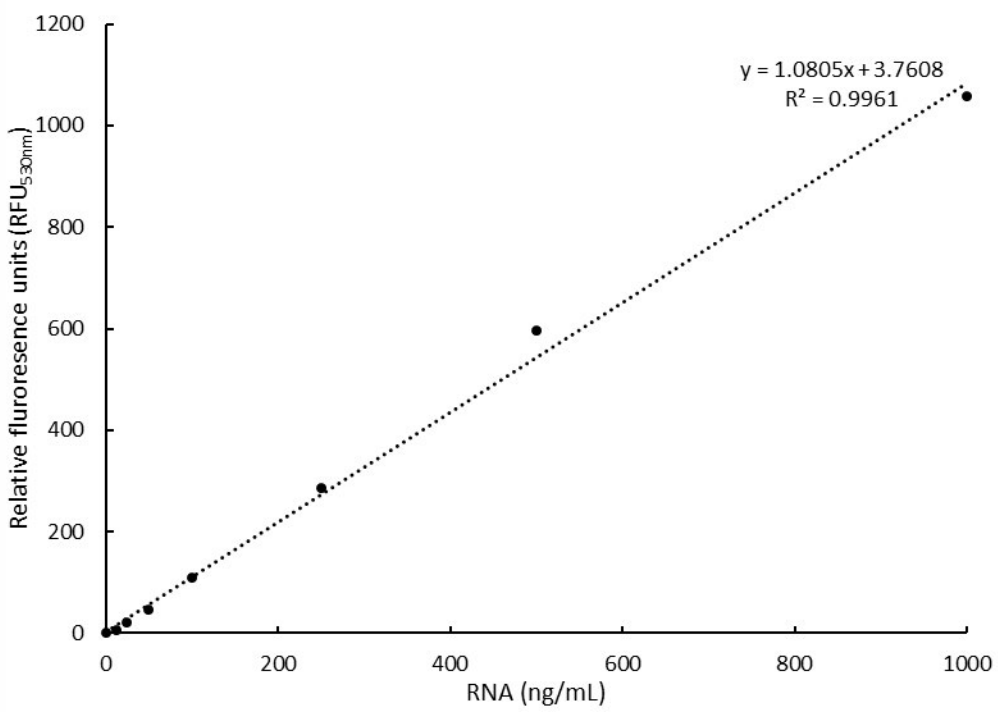


Figure 7.3: Standard curve of serially diluted RNA (ng/mL) fluorescence (RFU_{530nm}) using the RiboGreen® assay for RNA quantitation.

Table 7.1: Multiple Dicotyledon DREB1A and annexin 1 amino acid (aa) sequences aligned using a constraint-based multiple alignment tool (National Library of Medicine, USA). Red denotes highly conserved sequences, blue denotes less conserved sequences, and grey denotes variable regions.

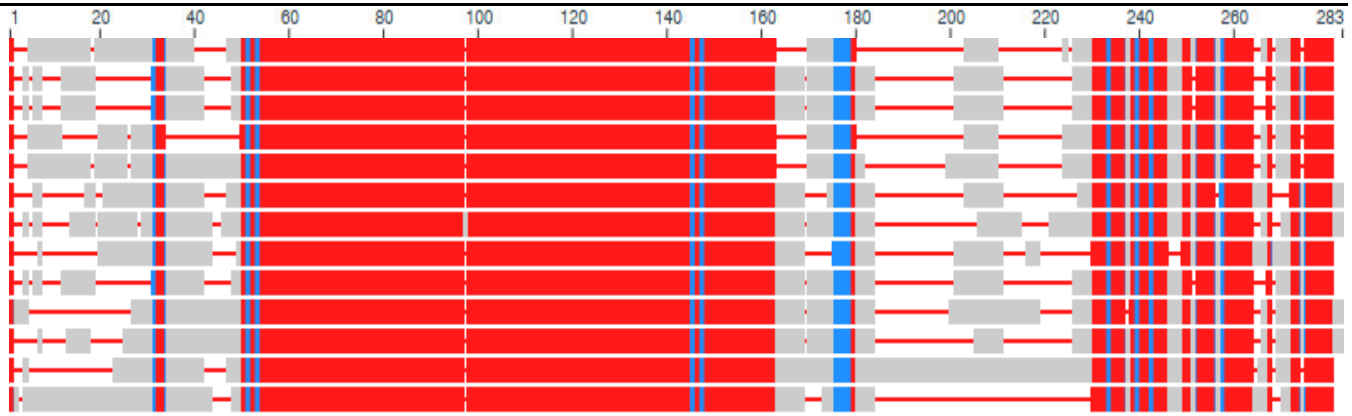
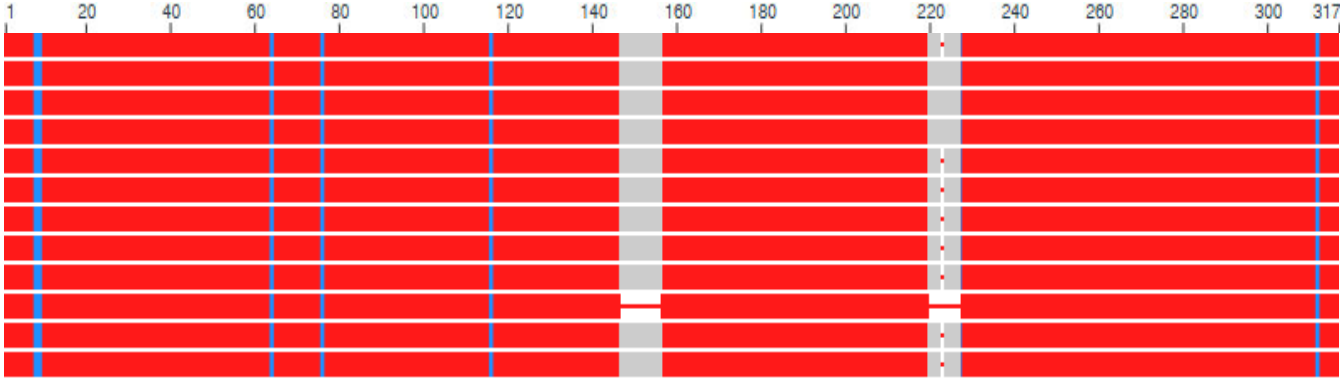
	Species	Ascension	**Alignment** (aa)
DREB1A	*Ageratina adenophora*	ADE62311.1	
	Arabidopsis thaliana	BAA33434.1	
	Capsella bursa-pastoris	ABM21468.1	
	Chrysanthemum morifolium	ABD90468.1	
	Cichorium intybus	AHI59150.1	
	Gossypium hirsutum	AAP83936.3	
	Malus sieversii	AFU52631.1	
	Medicago truncatula	ABG75914.1	
	Nicotiana tabacum	ABD65969.1	
	Petunia axillaris	QJF54155.1	
	Phaseolus vulgaris	APG23582.1	
	Populus nigra	AHN65502.1	
	Rosa hybrid	ACI42859.1	
Annexin 1	*Actinidia rufa*	GFS46779.1	
	Arabidopsis thaliana	NP174810.1	
	Brassica napus	ADK13090.1	
	Brassica rapa	AHN63213.1	
	Cyamopsis tetragonoloba	QKG27631.1	
	Gossypium hirsutum	NP1313875.1	
	Handroanthus impetiginosus	PIN21783.1	
	Hibiscus trionum	GMI82852.1	
	Populus tomentosa	AFZ78531.1	
	Prunus dulcis	BBG95743.1	
	Theobroma cacao	EOY16019.1	
	Trifolium pratense	PNY12606.1	

Table 7.2: Multiple Dicotyledon HSP70 amino acid (aa) sequences aligned using a constraint-based multiple alignment tool (National Library of Medicine, USA). Red denotes highly conserved sequences, blue denotes less conserved sequences, and grey denotes variable regions.

	Species	Ascension	Alignment (aa)
HSP70	*Arabidopsis thaliana*	OAP03566.1	
	Chrysanthemum morifolium	BAP90755.1	
	Cucumis sativus	CAA52149.1	
	Cyclamen persicum	ABP35942.1	
	Daucus carota	CAA42685.1	
	Dianthus spiculifolius	WHP37884.1	
	Glycine max	CAA44620.1	
	Hevea brasiliensis	AON76433.1	
	Ipomoea batatas	AFU34350.1	
	Kandelia obovata	AKJ21650.1	
	Kosteletzkya pentacarpos	AOR81481.1	
	Malus hupehensis	ADZ46371.1	
	Medicago ruthenica	AHX83821.1	
	Medicago sativa	AIA99450.1	
	Nicotiana benthamiana	ARH56399.1	
	Nicotiana tabacum	AAP04522.1	
	Oenanthe javanica	AXM42390.1	
	Paeonia lactiflora	WJK72279.1	
	Paeonia suffruticosa	AFA51946.1	
	Pisum sativum	CAA38536.1	
	Solanum lycopersicum	ABW76421.1	
	Trifolium repens	WJX96779.1	

Table 7.3: Multiple Dicotyledon NHX1 amino acid (aa) sequences aligned using a constraint-based multiple alignment tool (National Library of Medicine, USA). Red denotes highly conserved sequences, blue denotes less conserved sequences, and grey denotes variable regions.

	Species	Ascension	**Alignment** (aa)
NHX1	*Arabidopsis thaliana*	OAO93496.1	
	Atalantia buxifolia	WFU92747.1	
	Atriplex dimorphostegia	AAO48271.1	
	Atriplex gmelinii	BAB11940.1	
	Atriplex patens	AHC54576.1	
	Casuarina glauca	UTD53586.1	
	Citrus reticulata	AAT36679.1	
	Citrus trifoliata	AAT36678.1	
	Fagopyrum esculentum	AVG70975.1	
	Gentiana rigescens	AIA82923.1	
	Glycine max	XP4869015.1	
	Helianthus annuus	QHB80364.1	
	Ipomoea tricolor	BAP28595.1	
	Karelinia caspia	ABC46405.1	
	Lonicera japonica	UFP37803.1	
	Lotus tenuis	ACE78322.1	
	Malus zumi	ADB80440.1	
	Medicago sativa	ADB27460.1	
	Morus notabilis	AIL23819.1	
	Nicotiana benthamiana	AGS56985.1	
	Nitraria sibirica	WFD52747.1	
	Salicornia europaea	AAN08157.1	
	Solanum lycopersicum	NP1233916.1	
	Tetragonia tetragonoides	AAQ08988.1	
	Trifolium repens	WJX13144.1	
	Turnera subulata	KAJ4846215.1	
	Vitis vinifera	XP10649604.1	