

**Morphophysiological and biochemical recovery response
of lettuce (*Lactuca sativa* L. var. Crispa) to multistress and
the application of a phytohormone-based biostimulant**

by

SANELISIWE SATSHI

Dissertation submitted in fulfilment of the requirements for the degree of

MASTER OF AGRICULTURE

in the

Department of Agriculture
Faculty of Health and Environmental Sciences

at the

Central University of Technology, Free State

Supervisor: Dr Z.P. Khetsha (D.Tech. Agriculture, CUT)
Co-supervisor: Dr M.M. Masowa (PhD. Agronomy, NWU)
Co-supervisor: Dr E. Van Der Watt (PhD. Horticulture & Agronomy, UFS)

Bloemfontein

2025

DECLARATION OF OWN WORK

I, **Sanelisiwe Satshi**, declare that this dissertation, “**Morphophysiological and biochemical recovery response of lettuce (*Lactuca sativa* L. var. Crispa) to multistress and the application of a phytohormone-based biostimulant**”, submitted to the Central University of Technology, Free State, in fulfilment of the requirements of the degree **Master of Agriculture** is my independent work and has not been submitted to any other institution or organisation for any reason whatsoever. Where other people’s work has been used (either from a printed source, internet, or any other source), this has been properly acknowledged and referenced accordingly.

Signature: _____

Date: 01 April 2025

DEDICATION

This work is dedicated to my son Ngomso, my aunt Mrs N. Mthithala, my mother Ms L.P. Satshi, and my uncle Mr L. Satshi.

ACKNOWLEDGEMENTS

I wish to offer my sincere gratitude to the following people and institutions who contributed towards the completion of this thesis:

- I express my gratitude and indebtedness to my supervisors, Dr Zenzile Peter Khetsha, Dr Manare Maxson Masowa and Dr Elmarie Van Der Watt, for constant guidance, constructive commentary, support, encouragement, kindness and inspirational words throughout my research period; I learned a lot from your supervision.
- I sincerely acknowledge the National Research Foundation (NRF) scholarship, NRF - Thuthuka PostDoc Track (Dr Z.P. Khetsha – Principal Investigator), the Central University of Technology, Free State, grants, and the University of the Free State for financial assistance in this research work.
- My appreciation and heartfelt respect to Mr B. Tshabang and Ms D. Makara from the Department of Soil, Crop and Climate Sciences, University of Free State; Mr M. Taylor from the Central Analytical Facilities, Stellenbosch University, and Mr E. Mathebula from Biometry, Agricultural Research Council, for their tremendous assistance in this study.
- I thank my colleagues, Mr L. Legodi, Mr K. Moyo, Mr L.C. Sadiki, and Mr K. Motshumi, for their help and advice during the study.
- I am grateful to my lovely peers, Ms X. Dyasi and Ms O. Ndiye, for their support and assistance during the greenhouse experiments.
- Special thanks to my aunt, Mrs N. Mthithala, my mother, Ms L.P Satshi, and my uncle, Mr L. Satshi, for their ongoing moral support, mega care, and eternal love. To my entire family, I am grateful to have them all in my life.
- I am in dearth of words to articulate my heartfelt thanks to the father of my son, Zwilihle, and our son, Ngomso. I truly appreciate the love, patience, and unwavering support.
- Above all, thanks and glory to the Almighty God for granting me good health and courage to complete this study successfully.

ABSTRACT

Abiotic and biotic environmental stress factors are a threat to global food production. Abiotic stresses are the primary and leading factors constraining agricultural production, with an estimated annual reduction in crop yield of 70–80% globally. While the world population is expected to reach 9.7 billion by 2025, the agricultural sector continuously seeks creative approaches to boost production and resource efficiency to improve and maintain sustainable agricultural production to meet the food demands of the world population. In South Africa, lettuce (*Lactuca sativa* L.) is an economically important horticultural commodity with a diverse group of cultivars comprising different polymorphic characteristics and varying compositions of phytochemicals. It is consumed for its health benefits, such as minimising the risk of cardiovascular diseases, oxidative stress disorders, and colorectal cancer due to its high essential nutritional value and low calories and fats. Lettuce plants encounter complex interactions from multi-abiotic stresses. Two greenhouse experimental research trials were conducted using curly lettuce var. Crispa, during the October 2023 and June/July 2024 growing seasons at the Central University of Technology, Free State. Firstly, the study examined the effects of multi-abiotic stress, including water deficit, plant bag sizes, and mechanical wounding, on the morphophysiological and biochemical parameters of hydroponically grown curly lettuce. Secondly, the phytohormone-based biostimulant was evaluated on the morphophysiological and biochemical recovery response of curly lettuce grown under multi-abiotic stress conditions. The results in this study were obtained from ANOVA and multivariate analysis of unsupervised or supervised models, including PCAs, HCA cluster, OPLS-DA, PLS-DA, and VIP score plots. The first trial outcomes showed that water deficit, plant bag size, and mechanical wounding stress occurring individually or combined as multistress significantly decreased morphophysiological and biochemical parameters such as plant height, number of leaves, leaf area, chlorophyll contents, stomatal conductance and leaf mineral contents excluding the phenolic content, which was increased to combat the oxidative stress on plants. On the other hand, it was revealed from the second trial that the phytohormone-based biostimulant ameliorated the detrimental effects of mild, moderate, and severe abiotic stress conditions on plants and promoted the growth of plants under control conditions. However, the responses of plants were observed to be dependent on the intensity of the multistress condition and biostimulant application dose.

Keywords: Lettuce · Horticultural plants · Abiotic stresses · Multistress · Water deficit · Plant bag size · Wounding · Biostimulants · Phytohormones

LIST OF TABLES

Table 2.1	Examples of commercial biostimulants containing phytohormones as declared on the labels.	68
Table 3.1	Nutrient solution composition	116
Table 3.2	Chemical composition of tap water and cocopeat used in the experiment	117
Table 3.3	Summary of ANOVA for the selected non-destructive curly lettuce parameters as affected by the water deficit regimes, the plant bag size, the mechanical wounding, and the weak effect.	118
Table 3.4	Effects of multistress (WDR, PBS and MW) interaction on the destructive and non-destructive curly lettuce parameters at harvest (4 th week) of curly lettuce.	119
Table 3.5	Effects of multistress (WDR, PBS and MW) interaction on dry mass of curly lettuce.	120
Table 3.6	Effects of multistress (WDR, PBS and MW) interaction on stomatal conductance of curly lettuce.	121
Table 3.7	Effects of water deficit regimes and plant bag size interaction on 28 th and 29 th DAT of curly lettuce.	123
Table 3.8	Effects of water deficit regimes and mechanical wounding interaction on stomatal conductance of curly lettuce.	124
Table 3.9	Effects of multistress (WDR, PBS and MW) interaction on the leaf minerals of the curly lettuce parameters at harvest (4 th week).	125
Table 3.10	Effects of multistress (WDR, PBS and MW) interaction on nitrogen, phosphate, calcium and manganese.	126
Table 3.11	Phenolic compounds detected from curly lettuce var. Crispa.	127
Table 4.1	Effect of multistress and foliar application of phytohormone-based biostimulant (<i>Lucky Plant</i> [®]) treatments on plant parameters at harvest of curly lettuce.	128
Table 4.2	Effect of multistress and foliar application of phytohormone-based biostimulant (<i>Lucky Plant</i> [®]) treatments on the stomatal density and stomatal conductance of curly lettuce.	129
Table 4.3	Effect of multistress and foliar application of phytohormone-based biostimulant (<i>Lucky Plant</i> [®]) treatments on the leaf minerals of curly lettuce.	158
Table 4.4	Phenolic compounds detected from curly lettuce var. Crispa.	159
Table 5.1	Reporting the null hypothesis for the study: “Morphophysiological and biochemical recovery response of lettuce (<i>Lactuca sativa</i> L. var. Crispa) to multistress and the application of a phytohormone-based biostimulant”.	160

LIST OF FIGURES

Figure 2.1	Picture of curly lettuce (<i>Lactuca sativa</i> L.) var. Crispa.	68
Figure 3.1	The irrigation setup (A) drip irrigation, (B) residential irrigation controller, (C) valves, and (D) tanks and pumps.	116
Figure 3.2	Effects of water deficit regimes and plant bag size on number of leaves.	117
Figure 3.3	Effects of water deficit regimes and plant bag size (A) and the water deficit regimes and mechanical wounding on leaf area (cm ²) (B).	118
Figure 3.4	Effect of water deficit regimes and mechanical wounding on fresh mass.	119
Figure 3.5	Effect of water deficit regimes and mechanical wounding (A) and water deficit regimes and plant bag size (B) on chlorophyll.	120
Figure 3.6	Pictorial presentation of the impact of water deficit regimes, plant bag sizes, and mechanical wounding on the morphological growth of curly lettuce.	121
Figure 3.7	Pictorial presentation of the impact of water deficit regimes (100%, 80%, 60%, 40%), plant bag size (5 L, 3.5 L, and 1.25 L), and mechanical wounding (intact and leaf wounded plants), and their interactions on the root morphology of curly lettuce.	123
Figure 3.8	PCA of analysed mineral results of lettuce grown under different water irrigation regimes (100%, 80%, 60%, and 40%) and three plant bag sizes (5 L, 3.5 L, and 1.25 L) and mechanical wounding (intact and leaf wounded plants).	124
Figure 3.9	A graphical presentation of an unsupervised Principal Component Analysis (PCA) showing scores (circles) plot of phenolics attained by UPLC-MS analysis in lettuce treated with four water deficit irrigation regimes (100%, 80%, 60%, and 40%), three plant bag sizes (5 L, 3.5 L, and 1.25 L), and mechanical wounding (intact and leaf wounded plants).	125
Figure 3.10	Heat map of unsupervised hierarchical clustering analysis (HCA) showing the phenolic compounds found in lettuce grown under different water irrigation regimes (100%, 80%, 60%, and 40%), and plant bag sizes (5 L, 3.5 L, and 1.25 L) and mechanical wounding (intact and leaf wounded plants). The coloured parts correspond to the contents of various phenolic compounds (for high red and low blue shades). Rows illustrate phenolic compounds, while columns illustrate multistress treatments.	126
Figure 3.11	Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) score plot supervised modelling displaying the phenolic compound groupings found in lettuce grown under different water irrigation regimes (100%, 80%, 60%, and 40%), and plant bag sizes (5 L, 3.5 L, and 1.25 L) and mechanical wounding (intact and leaf wounded plants).	127
Figure 3.12	Partial least square discrimination analysis (PLS-DA) score plot showing the phenolic compound groups found in lettuce subjected to different water irrigation regimes (100%, 80%, 60%, and 40%), and three plant bag sizes (5 L, 3.5 L, and 1.25 L) and mechanical wounding (intact and leaf wounded plants).	128
Figure 3.13	Variables Importance in Projection (VIP) scores in OPLS-DA assigned to phenolic compound data found in lettuce grown under different water irrigation regimes (100%, 80%, 60%, and 40%), and plant bag sizes (5 L, 3.5 L, and 1.25 L) and mechanical wounding (intact and leaf wounded plants).	129
Figure 4.1	Demonstration of the experimental layout of the study.	158
Figure 4.2	Number of leaves on the fourth week for curly lettuce treated with varying doses of <i>Lucky Plant</i> [®] (0.5, 1.0, and 1.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	159
Figure 4.3	Number of leaves at harvest for curly lettuce treated with varying doses of <i>Lucky Plant</i> [®] (0.5, 1.0, and 1.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	160
Figure 4.4	Chlorophyll content at harvest for curly lettuce treated with varying doses of <i>Lucky Plant</i> [®] (0.5, 1.0, and 1.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	161

Figure 4.5	Fresh mass at harvest for curly lettuce treated with varying doses of <i>Lucky Plant</i> [®] (0.5, 1.0, and 1.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	162
Figure 4.6	Dry mass at harvest for curly lettuce treated with varying doses of <i>Lucky Plant</i> [®] (0.5, 1.0, and 1.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	163
Figure 4.7A	SEM micrographs (10 µm) of the adaxial leaf surface demonstrating the stomatal features across varying doses of distilled water (as control) foliar application under multistress (control, mild, moderate, and severe) conditions.	164
Figure 4.7B	SEM micrographs (10 µm) of the adaxial leaf surface demonstrating the stomatal features across varying doses of <i>Lucky Plant</i> [®] (0.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	165
Figure 4.7C	SEM micrographs (10 µm) of the adaxial leaf surface demonstrating the stomatal features across varying doses of <i>Lucky Plant</i> [®] (1.0 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	166
Figure 4.7D	SEM micrographs (10 µm) of the adaxial leaf surface demonstrating the stomatal features across varying doses of <i>Lucky Plant</i> [®] (1.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	167
Figure 4.8	Schematic representation (100 µm) of the adaxial leaf surface demonstrating a trichome length capitate/asciform (Sedibe <i>et al.</i> , 2013; Khetshe <i>et al.</i> , 2023) where plants were grown under moderate and mild multistress, and treated with 0.5 and 1.0 g L ⁻¹ , respectively.	168
Figure 4.9A	Schematic representation (10 µm) of the adaxial leaf surface demonstrating the stomatal density across varying doses of distilled water (as control) foliar application under multistress (control, mild, moderate, and severe) conditions.	169
Figure 4.9B	Schematic representation (50 µm) of the adaxial leaf surface demonstrating the stomatal density across varying doses of <i>Lucky Plant</i> [®] (0.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	170
Figure 4.9C	Schematic representation (50 µm) of the adaxial leaf surface demonstrating the stomatal density across varying doses of <i>Lucky Plant</i> [®] (1.0 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	171
Figure 4.9D	Schematic representation (50 µm) of the adaxial leaf surface demonstrating the stomatal density across varying doses of <i>Lucky Plant</i> [®] (1.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	172
Figure 4.10	Leaf nitrogen content at harvest for curly lettuce treated with varying doses of <i>Lucky Plant</i> [®] (0.5, 1.0, and 1.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	172
Figure 4.11	Leaf zinc content at harvest for curly lettuce treated with varying doses of <i>Lucky Plant</i> [®] (0.5, 1.0, and 1.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	173
Figure 4.12	PCA of analysed mineral results of curly lettuce treated with varying doses of <i>Lucky Plant</i> [®] (0.5, 1.0, and 1.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	174
Figure 4.13	Principal Component Analysis (PCA) showing scores plot of phenolics attained by UPLC-MS analysis in curly lettuce treated with varying doses of <i>Lucky Plant</i> [®] (0.5, 1.0, and 1.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	175
Figure 4.14	Partial least square discrimination analysis (OPLS-DA) score plot showing the phenolic compound groups found in curly lettuce treated with varying doses of <i>Lucky Plant</i> [®] (0.5, 1.0, and 1.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.	176
Figure 4.15	Heat map of unsupervised hierarchical clustering analysis (HCA) showing the phenolic compounds found in curly lettuce treated with varying doses of <i>Lucky Plant</i> [®] (0.5, 1.0, and 1.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions. The coloured parts correspond to the content of various phenolic compounds (red shades for high and blue for low). Rows illustrate phenolic compounds, while columns illustrate treatments.	177
Figure 4.16	Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) score plot supervised modelling displaying the phenolic compounds groupings found in curly lettuce treated	178

with varying doses of *Lucky Plant*[®] (0.5, 1.0, and 1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions. The legends: red circles and green triangles indicate data groupings.

- | | | |
|--------------------|--|------------|
| Figure 4.17 | Variables Importance in Projection (VIP) scores in OPLS-DA assigned to phenolic compounds data found in curly lettuce treated with varying doses of <i>Lucky Plant</i> [®] (0.5, 1.0, and 1.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions. Mini-coloured blocks on the right represent the relative content of each of the phenolic compounds. High levels are in red and low levels are in blue. | 180 |
| Figure 4.18 | Mass spectrum of phenolic compounds data of curly lettuce treated with varying doses of <i>Lucky Plant</i> [®] (distilled water, 0.5, 1.0, and 1.5 g L ⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions. | 181 |
| Figure 4.19 | Total ions chromatogram of phenolic compound data of curly lettuce not treated with <i>Lucky Plant</i> [®] biostimulant (distilled water) foliar application under multistress (control, mild, moderate, and severe) conditions. | 182 |

TABLE OF CONTENTS

DECLARATION OF OWN WORK	i
DEDICATION	ii
ACKNOWLEDGEMENTS	iii
ABSTRACT	iv
LIST OF TABLES	v
LIST OF FIGURES	vi
CHAPTER 1	1
GENERAL INTRODUCTION	1
1.1 BACKGROUND AND MOTIVATION	2
1.2 PROBLEM STATEMENT	3
1.3 STUDY OBJECTIVE	4
1.4 HYPOTHESES	5
REFERENCES	6
CHAPTER 2	10
LITERATURE REVIEW	10
2.1 INTRODUCTION TO LETTUCE PRODUCTION	11
2.1.1 Scale of production and economic importance	12
2.1.2 Marketing and uses	12
2.1.3 Cultivation requirements	14
2.1.4 Nutrition requirements	18
2.1.5 Climate requirements	20
2.1.6 Harvesting techniques	22
2.2 EFFECTS OF MULTIPLE, COMBINED AND INDIVIDUAL ABIOTIC STRESS FACTORS AFFECTING HORTICULTURAL PLANTS	25
2.2.1 Global losses associated with multi-abiotic stress factors for horticultural plants	25
2.2.2 Combined stress factoring of the water deficit, wound, and plant bag size on horticultural plants	25
2.2.3 Effect of water deficit stress factor on horticultural plants	26
2.2.4 Wounding as a stress factor for horticultural plants	29
2.2.5 Plant bag size as a potential stress factor for horticultural plants	32
2.3 COMPREHENSIVE OVERVIEW OF PHYTOHORMONES AS ENHANCING BIOSTIMULANTS IN HORTICULTURE	34
2.3.1 Common biostimulants used in South Africa for individual, combined and multiple stress factors on horticultural plants	34
2.3.2 Phytohormone-based biostimulants as a potential abiotic stress mitigation in horticultural plants	36
2.3.2.1 Absciscic acid	37
2.3.2.2 Cytokinin	39

2.3.2.3 Salicylic acid.....	39
2.3.2.4 Jasmonic acid.....	40
2.3.2.5 Auxins.....	41
2.3.2.6 Ethylene	42
2.3.2.7 Brassinosteroids.....	43
2.3.2.8 Gibberellins	44
2.4 CONCLUSION	46
REFERENCES	47
CHAPTER 3.....	69
EFFECTS OF MULTI-ABIOTIC STRESS ON THE MORPHOPHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS OF CURLY LETTUCE (<i>Lactuca sativa</i> L. var. <i>Crispa</i>) GROWN IN HYDROPONICS SYSTEM.....	69
ABSTRACT	70
3.1 INTRODUCTION	71
3.2 MATERIAL AND METHODS	72
3.2.1 Description of trial site.....	72
3.2.2 Experimental design and treatments	73
3.2.2.1 Experiment design	73
3.2.2.2 Experimental treatments	73
3.2.3 Agronomic practices	74
3.2.3.1 Plant materials and propagation	74
3.2.3.2 Cultivation method	75
3.2.3.3 Nutrients management	75
3.2.3.4 Pest management	76
3.2.4 Data and plant measurements.....	76
3.2.4.1 Morphological measurements	76
3.2.4.2 Physiological analysis.....	77
3.2.4.3 Biochemical analysis	77
3.2.5 Statistical analysis	80
3.3 RESULTS AND DISCUSSION	80
3.3.1 Morphological and yield parameters.....	80
3.3.2 Physiological parameters	87
3.3.3 Biochemical parameters.....	89
3.4 CONCLUSION	96
REFERENCES	97
CHAPTER 4.....	130
EFFECT OF THE PHYTOHORMONE-BASED BIOSTIMULANT ON YIELD, MORPHOPHYSIOLOGICAL AND BIOCHEMICAL RECOVERY RESPONSE OF CURLY LETTUCE (<i>Lactuca sativa</i> L. Var. <i>Crispa</i>) GROWN UNDER MULTI-ABIOTIC STRESS CONDITIONS.....	130

ABSTRACT	131
4.1 INTRODUCTION	132
4.2 MATERIAL AND METHODS	133
4.2.1 Description of trial site.....	133
4.2.2 Experimental design and plant treatments	134
4.2.3 Agronomic management practices.....	135
4.2.4 Plant measurements and data collection	135
4.2.5 Statistical analysis	136
4.3 RESULTS AND DISCUSSION	136
4.3.1 The response of morphological parameters of curly lettuce to multistress and phytohormone-based biostimulant	136
4.4 CONCLUSION	147
REFERENCES	149
CHAPTER 5.....	183
SUMMARY, CONCLUSION AND RECOMMENDATIONS.....	183
5.1 SUMMARY	184
5.2 SYNTHESIS.....	184
5.2.1 Effects of multi-abiotic stress on curly lettuce yield and morphophysiological and biochemical parameters	185
5.2.2 Foliar application of phytohormone-based biostimulant in varying doses on yield and the recovery response of curly lettuce (<i>Lactuca sativa</i> L. var. Crispa) grown under extreme multi-abiotic stress ...	185
5.3 IMPLICATIONS	186
5.4 RECOMMENDATIONS	187
5.5 CONCLUSION	187
APPENDIX	190
Published part of the article extracted from Chapter 1	190
Published part of the article extracted from Chapter 2	191

CHAPTER 1

GENERAL INTRODUCTION

Publication: Khetsha, Z., Van Der Watt, E., Masowa, M., Legodi, L., Satshi, S., Sadiki, L. and Moyo, K., 2024. Phytohormone-based biostimulants as an alternative mitigating strategy for horticultural plants grown under adverse multi-stress conditions: Common South African stress factors. *Caraka Tani: Journal of Sustainable Agriculture* 39(1), 167-193.

1.1 BACKGROUND AND MOTIVATION

Abiotic and biotic environmental stress factors are a threat to global food production. The negative impacts caused by living organisms, such as weeds, viruses, bacteria, and fungi, are known as biotic stresses (Chahal *et al.*, 2022). In contrast, variations in physical or chemical factors cause abiotic stress (Tiwari *et al.*, 2020). Abiotic stresses are the primary and leading factors constraining agricultural production, with an estimated decrease in crop yield by 70–80% across the globe (Verma *et al.*, 2024). Abiotic stresses, such as water deficit, radiation, mechanical wounding, and plant bag size in hydroponics, challenge many horticultural crops, resulting in extensive crop yield failures (Turner, 2019; He *et al.*, 2022). These plant stresses occur individually or simultaneously, presenting an integrated reaction (Sehgal *et al.*, 2022).

Climate change events, such as low rainfall and precipitation, temperature extremes, and high light intensity, are increasingly limiting water availability for irrigation, especially in coastal agricultural lands and arid and semiarid regions (Ratnaparkhe *et al.*, 2022). Due to water scarcity, the probability of low crop yield is projected to be 4.5 times higher by 2030 and up to 25 times higher globally by 2050 (Caparas *et al.*, 2021). This leads to water deficit stress and increased need for implementation of different water-saving strategies, such as deficit irrigation (Fernandez *et al.*, 2023).

Horticultural producers use planting bags but have limited information about the exact requirements of bag sizes for a particular crop (Sahu *et al.*, 2024). On limited land, using small containers can grow more plants in a small space while larger pots take up more space (Rizar *et al.*, 2023; Gimeno-Páez *et al.*, 2025). However, smaller pots restrict root development (Rizar *et al.*, 2023). In addition, plant bag size, accompanied by soil bulk density, can also lead to water deficit stress as the volume of root media from which to draw water can be small compared to direct planting in the soil (He *et al.*, 2022). The relationship between the mass and volume of the root media represents a bulk density and varies based on the pot size (Turner, 2019), which affects plant growth and development during the growing period and can be extended to stresses. Creating optimal growing conditions requires appropriate choices regarding cultivation facilities, such as plant bag sizes, which are essential to achieve high water and nutrient use efficiency while maximising horticultural crop yields and quality (Massa *et al.*, 2020; Sahu *et al.*, 2024).

One of the major challenges for crop production among environmental stress factors is wounding caused by physical injury, such as mechanical damage, heavy snow, rain, radiation,

hail, or wind, and herbivore attacks (Shi *et al.*, 2022; Moyo *et al.*, 2024). Damaged tissues become potential bacterial and viral infection sites, potentially limiting quality and yield (Wielkopolan *et al.*, 2022).

According to predictions from the United Nations (2024), the population of Southern Africa is expected to reach 465 million by 2030. Therefore, the agricultural sector continuously seeks creative approaches to boost production and resource efficiency to improve and maintain sustainable agricultural production to meet food demands for the rising global population (Food and Agriculture Organisation (FAO); International Fund for Agricultural Development (IFAD); United Nations Children's Fund (UNICEF); World Food Programme (WFP); World Health Organization (WHO), 2022). Over a decade, biostimulants have been developed as environmentally friendly derivatives to elevate agricultural production sustainably (Shahid & Liu, 2022; Ahmed *et al.*, 2023). Applying plant biostimulants enhances morphological, physiological, biochemical, and molecular alterations and thus helps plants generate the ability to adapt to adverse environmental conditions and increase yield (Ramzan & Younis, 2022; Ahmed *et al.*, 2023).

Lettuce (*Lactuca sativa* L.) is a horticultural commodity widely grown as an important vegetable, a thriving and diverse group of short-cycle herbaceous plants. Lettuce leaves are commonly consumed in sandwiches and as salad mixtures and potherbs. Bioactive phytochemicals such as carotenoids, phenolics, anthocyanins, flavonoids, vitamins, minerals, proteins and amino acids, carbohydrates, fatty acids, and fibre in lettuce improve human nutrient intake and boost human health (El-Nakhel *et al.*, 2020; Venkatesh *et al.*, 2021). It is commercially produced in open fields and protected environments, for instance, under hydroponics, shade nets, and greenhouse conditions. The value of lettuce is determined by its size, texture, taste, and nutritional parameters. However, multiple abiotic stresses adversely affect the morphophysiological features and biochemical signalling, inhibiting plant development and directly leading to economic crop yield losses (Demirel *et al.*, 2020; Shabbir *et al.*, 2022). The potential impact of this research on future food security is significant, as it could provide crucial insights into enhancing crop resilience and productivity.

1.2 PROBLEM STATEMENT

Agriculture is challenged by the limited availability of water, arable land, and land degradation (Wang, 2022). To meet food demands, farmers use alternative strategies such as deficit irrigation in planting bags to grow large production quantities indoors or in fields.

However, horticultural plants like lettuce have shallow root systems and are susceptible to water deficit stress (Patra *et al.*, 2023). Besides, improper choices of plant bag sizes hamper plant growth and yield (Turner, 2019; Massa *et al.*, 2020). Moreover, plants are exposed to physical injuries such as mechanical wounding caused by agricultural practices (Kim *et al.*, 2019). To face these multi-abiotic stressors, farmers adapt strategies such as using crop varieties that are more resilient, organic and inorganic chemicals like biostimulants and agrochemicals, to improve the tolerance of horticultural crops (Jiménez-Arias *et al.*, 2022). Nonetheless, some of these approaches are associated with drawbacks, like environmental pollution and high cost, time-consuming, and labour-intensive (Wazny *et al.*, 2021). Scientific evidence endorses the effectiveness of biostimulants on crop performance and yield (Roy, 2024; Velasco-Clares *et al.*, 2025). However, more research is still needed to understand the morphophysiological and biochemical recovery response of stressed lettuce plants underlying commercial phytohormone-based biostimulant action as a mitigation strategy to intensify productivity.

1.3 STUDY OBJECTIVE

The main objective of this study was to determine the recovery response of curly lettuce to multi-abiotic stress effects using phytohormone-based biostimulants as an alternative mitigation strategy. The following specific objectives were undertaken to achieve the main objective:

- 1.3.1 To investigate the effects of multistress (water deficit regimes, plant bag sizes and mechanical wounding) conditions on lettuce yield and morphophysiological and biochemical parameters.
- 1.3.2 To evaluate the morphophysiological and biochemical recovery response of curly lettuce grown under multi-abiotic stress conditions and treated with phytohormone-based biostimulant (*Lucky Plant*[®]) under multi-abiotic stress (water deficit regimes, plant bag sizes and mechanical wounding) conditions.

1.4 HYPOTHESES

Objective 1:

H₀: Multistress factors (water deficit regimes, plant bag sizes and mechanical wounding) conditions may have a non-significant effect on lettuce yield and morphophysiological and biochemical parameters.

H_a: Multistress factors (water deficit regimes, plant bag sizes and mechanical wounding) conditions might scientifically reduce the lettuce yield and morphophysiological and biochemical parameters.

Objective 2:

H₀: The curly lettuce grown under multi-abiotic stress conditions and treated with phytohormone-based biostimulant (*Lucky Plant*[®]) will have a non-significant effect on the morphophysiological and biochemical recovery response of curly lettuce.

H₀: The curly lettuce grown under multi-abiotic stress conditions and treated with phytohormone-based biostimulant (*Lucky Plant*[®]) will significantly recover based on the improvement in the morphophysiological and biochemical parameters.

1.5 CHAPTER LAYOUT

This thesis follows an article format with five chapters. Chapter one will provide a general introduction to the research project, including the background and motivation for the study, the problem statement, the hypothesis, and the objectives. Chapter two will present the literature review on the topic, with major subtopics being a general review of lettuce production, multi-abiotic stress factors affecting horticultural plants, and the biostimulant as a stimulatory substance. Chapter three will be the first article on the effect of multi-abiotic stress on lettuce yield and morphophysiological and biochemical parameters. Chapter four will be the second article on the effects of phytohormone-based biostimulant concentrations on the recovery of lettuce grown under multi-abiotic stress. Finally, chapter five will provide the conclusion, study limitations, and recommendations for further research. Each chapter will have an introduction, a conclusion, and a list of references.

REFERENCES

- Ahmed W, Shah AN, Abbas A, Nawaz M, Qayyum A, Hassan MU, Qaisrani MM, Khan J. 2023. Role of plant biostimulants and their classification. *Biofertilizers for Sustainable Soil Management*: 65-88.
- Caparas M, Zobel Z, Castanho AD, Schwalm CR. 2021. Increasing risks of crop failure and water scarcity in global breadbaskets by 2030. *Environmental Research Letters* 16: 104013.
- Chahal GK, Ghai M, Kaur J. 2022. Stress mechanisms and adaptations in plants. *Journal of Pharmacognosy and Phytochemistry* 11: 109-114.
- Demirel U, Morris WL, Ducreux LJ, Yavuz C, Asim A, Tindas I, Campbell R, Morris JA, Verrall SR, Hedley PE, Gokce ZN. 2020. Physiological, biochemical, and transcriptional responses to single and combined abiotic stress in stress-tolerant and stress-sensitive potato genotypes. *Frontiers in Plant Science* 11: 169.
- El-Nakhel C, Petropoulos SA, Pannico A, Kyriacou MC, Giordano M, Colla G, Troise AD, Vitaglione P, De Pascale, Roupheal Y. 2020. The bioactive profile of lettuce produced in a closed soilless system as configured by combinatorial effects of genotype and macrocation supply composition. *Food Chemistry* 309: 125713.
- Food and Agriculture Organisation, International Fund for Agricultural Development, United Nations Children's Fund, World Food Programme, World Health Organization. 2022. *The State of Food Security and Nutrition in the World 2022. Repurposing food and agricultural policies to make healthy diets more affordable*. Rome, FAO. <https://doi.org/10.4060/cc0639en>.
- Fernandez O, Lemaître-Guillier C, Songy A, Robert-Siegwald G, Lebrun MH, Schmitt-Kopplin P, Larignon P, Adrian M, Fontaine F. 2023. The combination of both heat and water stresses may worsen botryosphaeria dieback symptoms in grapevine. *Plants* 12: 753.
- Gimeno-Páez E, Prohens J, Moreno-Cerveró M, de Luis-Margarit A, Díez MJ, Gramazio P. 2025. Agronomic treatments combined with embryo rescue for rapid generation advancement in tomato speed breeding. *Horticultural Plant Journal* 11: 239-250.

- He Y, Wu Z, Zhao T, Yang H, Ali W, Chen J. 2022. Different plant species exhibit contrasting root traits and penetration to variation in soil bulk density of clayey red soil. *Agronomy Journal* 114: 867-877.
- Jiménez-Arias D, Morales-Sierra S, Borges AA, Herrera AJ, Luis JC. 2022. New biostimulants screening method for crop seedlings under water deficit stress. *Agronomy* 12: 728.
- Kim HJ, Ku KM, Choi S, Cardarelli M. 2019. Vegetal-derived biostimulant enhances adventitious rooting in cuttings of basil, tomato, and chrysanthemum via brassinosteroid-mediated processes. *Agronomy* 9: 74.
- Massa D, Magán JJ, Montesano FF, Tzortzakis N. 2020. Minimizing water and nutrient losses from soilless cropping in southern Europe. *Agricultural Water Management* 241: 106395.
- Moyo K, Khetsha ZP, Masowa MM, Van Der Watt E, Moloantoa KM, Unuofin JO. 2024. Eco-organic system and silicon-based biostimulant as a strategy for vegetable production under multistress conditions in South Africa: A review. *Journal of Applied Horticulture* 26: 83-89.
- Patra SK, Sengupta S, Bhattacharya P, Bhattacharyya K. 2023. Enhancing yield of lettuce through irrigation and nitrogen management in a subtropical Inceptisol. *Israel Journal of Plant Sciences* 70: 76-90.
- Ramzan F, Younis A. 2022. Use of biostimulants in tolerance of drought stress in agricultural crops. In *Emerging Plant Growth Regulators in Agriculture* 429-446.
- Ratnaparkhe MB, Satpute GK, Kumawat G, Chandra S, Kamble VG, Kavishwar R, Singh V, Singh J, Singh AK, Ramesh S, Kumar V. 2022. Genomic designing for abiotic stress tolerant soybean. *Genomic Designing for Abiotic Stress Resistant Oilseed Crops*: 1-73.
- Rizar FF, Lakitan B, Wijaya A. 2023. Suitable substrate volumes and cultivars for enhancing growth and increasing yield of yard-long bean in urban ecosystems. *BIOVALENTIA: Biological Research Journal* 9: 110-122.
- Roy A. 2024. Plant growth regulating phytohormones: The potent targets to alleviate abiotic stress in crop plants—a research update and the way forward. *Journal of Agricultural Science* 16: 10.

- Sahu S, Asati BS, Singh J, Rastogi NK, Nishad D. 2024. Performance of tomato variety Kashi Aman under different growing containers. *International Journal of Advanced Biochemistry Research* 8: 519–522.
- Sehgal A, Reddy KR, Walne CH, Barickman TC, Brazel S, Chastain D, Gao W. 2022. Individual and interactive effects of multiple abiotic stress treatments on early-season growth and development of two brassica species. *Agriculture* 12: 453.
- Shabbir R, Singhal RK, Mishra UN, Chauhan J, Javed T, Hussain S, Kumar S, Anuragi H, Lal D, Chen P. 2022. Combined abiotic stresses: challenges and potential for crop improvement. *Agronomy* 12: 2795.
- Shahid MA, Liu G. 2022. Application of biostimulants to improve tomato yield in Florida. *Vegetable Research* 2: 1-7.
- Shi Z, Wang H, Zhang Y, Jia L, Pang H, Feng H, Wang X. 2022. The involvement of extracellular ATP in regulating the stunted growth of Arabidopsis plants by repeated wounding. *BMC Plant Biology* 22: 279.
- Tiwari RK, Lal MK, Naga KC, Kumar R, Chourasia KN, Subhash S, Kumar D, Sharma S. 2020. Emerging roles of melatonin in mitigating abiotic and biotic stresses of horticultural crops. *Scientia Horticulturae* 272: 109592.
- Turner NC. 2019. Imposing and maintaining soil water deficits in drought studies in pots. *Plant and Soil* 439: 45-55.
- United Nations, Department of Economic and Social Affairs, Population division 2024. *World Population Prospects 2024*. <https://population.un.org/wpp/>.
- Velasco-Clares D, Navarro-León E, Izquierdo-Ramos MJ, Blasco B, Ruiz JM. 2025. Enhancing drought tolerance in lettuce: The efficacy of the seaweed-derived biostimulant Cytolan® Stress applied at different growth stages. *Horticulturae* 11: 157.
- Venkatesh VP, Vani V, Nivya V, Baskaran V, Madan KP. 2021. Bioactives of *Lactuca sativa*: nutritional and clinical importance. *Advances in Health and Disease* 33: 246.
- Verma R, Singh A, Khare S, Kumar P. 2024. Alleviation of environmental stresses in crop plants by nanoparticles: recent advances and future perspectives. *Journal of Plant Biochemistry and Biotechnology*: 1-24.

- Wang X. 2022. Managing land carrying capacity: key to achieving sustainable production systems for food security. *Land* 11: 484.
- Wazny R, Rozpadek P, Jedrzejczyk RJ, Domka A, Nosek M, Kidd P, Turnau K. 2021. Phytohormone based biostimulant combined with plant growth promoting endophytic fungus enhances Ni phytoextraction of *Noccaea goesingensis*. *Science of the Total Environment* 789: 147950.
- Wielkopolan B, Frąckowiak P, Wieczorek P, Obrepalska-Stęplowska A. 2022. The impact of *oulema melanopus*—associated bacteria on the wheat defense response to the feeding of their insect hosts. *Cells* 11: 2342.

CHAPTER 2

LITERATURE REVIEW

Publication: Satshi, S., Masowa, M.M., Van Der Watt, E. and Khetsha, Z.P. (2025). Recovery response mechanisms of horticultural plants to multi-stress: plant-based biostimulants as a potential alternative strategy for lettuce (*Lactuca sativa* L.) in South Africa. *Acta Horticulturae* 1416, 463-478 (DOI: 10.17660/ActaHortic.2025.1416.60).

2.1 INTRODUCTION TO LETTUCE PRODUCTION

The genus *Lactuca sativa* L. is extensively distributed across North and Central America, Asia, Europe, and Africa (Abid & Qaiser, 2023). Lettuce belongs to the *Lactuca* spp. (Asteraceae) family with approximately 100 species distributed globally (Abid & Qaiser, 2023; Da Silva *et al.*, 2023). Its species originate from the Mediterranean and West Asia (Abid & Qaiser, 2023). They are annual plants harvested after one growing season, grown for their leaves and sometimes for stems and seeds. Lettuce is naturally self-pollinated and highly adapted to self-fertility or inbreeding.

Groups of lettuce cultivars can embrace different polymorphic characteristics in the final growth stage. Lettuce cultivars are chosen considering their wide acceptability by consumers and producers. Lettuce is grouped into four main classifications, i.e., Loose head or loose-leaf (Crispa), capitata crisphead or iceberg, and butterhead or Boston or Bibb (Capitata), and cos or romaine (Longifolia) (Medina-Lozano *et al.*, 2021). Each classification is relatively different and is explicitly used for different salad purposes. Lettuce plants vary in shape, size, texture, flavour, colouring, and leaf type. Their leaves form a loose-leaf rosette cluster or dense head and are hairless (smooth), crumpled, and curly (Figure 2.1). Moreover, their leaves are dark green or pale green, burgundy red or purple shaded.

Lettuce cultivars are also produced for their morphological traits, such as size, leaf colour, density, and growth requirements. Among the plant's quality parameters, roots are the primary organ responsible for water and nutrient uptake from soils. The capability of roots to absorb and transport water can be measured by root biomass, root volume, primary root length, root number, root depth, and root length distribution parameters (Subedi *et al.*, 2022). The lettuce taproot grows straight into the growth medium, and the rest of the roots form a small set of secondary roots that grow more copiously in a horizontal direction than a vertical one. The small secondary roots are more valuable in absorbing essential nutrients than the taproot, making it difficult for the lettuce plant to acquire sufficient water and nutrients with its shallow root system in the soil (Patra *et al.*, 2023).

The lettuce plants can have a height of 15 to 30 cm, depending on the variety and growing environmental conditions (Afroza & Rana, 2017). Lettuce has a short life cycle with the growing cycle of 55–65 days from planting to harvesting in midsummer and 65–80 days if planted in winter or late autumn, depending on the cultivar (Aires *et al.*, 2020; Coulombe *et*

al., 2020). Lettuce yields range between 20 and 60 t ha⁻¹ in open fields and 40 and 120 t ha⁻¹ in greenhouses (Fekadu *et al.*, 2021). Lettuce production yield varies depending on the variety, climate conditions, region, growing season, soil fertility, and other agricultural practices.

2.1.1 Scale of production and economic importance

Over the past decade, lettuce production has increased substantially worldwide. According to FAO data, global vegetable production was 1,148,446 tonnes in 2021, of which South Africa contributed 2,636 tonnes, including lettuce (FAO; IFAD; UNICEF; WFP; WHO, 2022). Lettuce is widely cultivated on most continents, especially subtropical regions (Abid & Qaiser, 2023). Lettuce and chicory production collectively reached 29,134,653 tons worldwide in 2019 (FAO; IFAD; UNICEF; WFP; WHO, 2022). China is the leading producer and accounts for approximately half of the world's total lettuce production, whilst the United States produces 22%, followed by Western Europe with around 13% (FAO; IFAD; UNICEF; WFP; WHO, 2022). Lettuce is considered an economically important leafy vegetable (Shatilov *et al.*, 2019). In the world around 40,000 tonnes were produced in 2019 (Volkava & Mickiewicz, 2022). In South Africa, the demand for lettuce is growing. Hence, growers aim to achieve high product quality, yield, and profitability. According to Fekadu *et al.* (2021), the production cost of greenhouse lettuce is higher than conventional lettuce.

2.1.2 Marketing and uses

Lettuce is sold based on fresh weight and appearance. Fresh-cut leaves or whole lettuce heads are the standard products on the market, and fresh mixed cuts are in demand. All lettuce products are marketed through international and national fresh produce markets, local markets, restaurants, hawkers, retailers, and chain stores. Different lettuce varieties are grown for the consumption of leaves, stems, and seeds. In some regions of the world, especially in Egypt and China, the stems of lettuce cultivars are consumed raw, cooked, and preserved instead of the leaves (Shatilov *et al.*, 2019). A particular variety of lettuce leaves is used to form cigarettes that are nicotine-free (Abid & Qaiser, 2023).

Furthermore, lettuce is the essential ingredient in fresh salads, with a higher water content of 95% compared to other leafy vegetables like kale and spinach, which have approximately 85% and 91%, respectively (Murray *et al.*, 2021; Da Silva *et al.*, 2023). Consumption of raw lettuce in salads provides humans with more nutrients than other processed or cooked leafy vegetables (Murray *et al.*, 2021). Lettuce is also a crucial source of minerals, including calcium

(Ca), magnesium (Mg), potassium (K), phosphorus (P), sulfur (S), boron (B), sodium (Na), iron (Fe), manganese (Mn), copper (Cu), molybdenum (Mo), and zinc (Zn) (Medina-Lozano *et al.*, 2021; Martínez-Ispizua *et al.*, 2022), and is rich in vitamins A, E, C, and K with moderate folate content (Dalastra *et al.*, 2020). Vitamin C is an essential antioxidant in lettuce since ascorbic acid represents 24.5% of the overall antioxidant activity (Ciriello *et al.*, 2021). Lettuce is very low in calories and fats (Ciriello *et al.*, 2021). A diverse group of lipid-soluble pigments (dark green, red, yellow, or orange) known as carotenoids (Martínez-Ispizua *et al.*, 2022), such as lutein, β -carotene, zeaxanthin, and lactucaxanthin are found in lettuce varieties. A study by de Souza *et al.* (2022) on red leafy lettuce varieties shows that total carotenoids content includes 45–48% of β -carotene, 13–20% of lutein, 11–15% of zeaxanthin, and 7–10% of lactucaxanthin depending on the growing season. Furthermore, lettuce contains dicaffeoyl tartaric acid, caffeic, and chlorogenic acids and their derivatives and phenolic compounds (Ciriello *et al.*, 2021). The total average phenolics ranged between 8.4–12.9 mg g⁻¹ dry weight in green leaf cultivars and attained 27.8 mg g⁻¹ dry weight in a red oak leaf and added up to more than 60% of the total antioxidant capacity (Shi *et al.*, 2022).

Anthocyanins found in lettuce varieties are 3-(6"-acetylglucoside), peonidin 3-O-glucoside and cyanidin 3-(6"-acetylglucoside) (Medina-Lozano *et al.*, 2021). Total anthocyanins ranged from the average of 0.50 and 127.28 mg 100 g⁻¹ dry weight in leaf tissue of red-pigmented lettuce, and there were complete zero anthocyanins in green pigment varieties (Medina-Lozano *et al.*, 2021; Yang *et al.*, 2022). The composition of these phytochemicals' changes with the lettuce variety. For example, romaine lettuce types have higher nutrient content than crisphead (Yang *et al.*, 2022). Red-pigmented lettuce contains more phenolic compounds than green leafy lettuce (Yang *et al.*, 2022). Therefore, the choice of a lettuce cultivar is vital as the vitamins, minerals, and bioactive compounds of different lettuce varieties differ due to different environmental growing conditions and other factors (Lei & Engeseth, 2021).

Lettuce contains fiber, which has been reported to decrease low-density lipoprotein cholesterol and high blood pressure (Rolnik, 2022). Hence, high intake of lettuce is recommended to minimise the risk of cardiovascular diseases by improving cholesterol metabolism and plasma antioxidant ability (Henderson, 2021; Medina-Lozano *et al.*, 2021). Lettuce can also help control blood sugar levels for people with diabetes (Medina-Lozano *et al.*, 2021). Lettuce also contains polyphenols with antioxidant properties, such as ascorbic acid,

carotenoids, and α -tocopherol (Martínez-Ispizua *et al.*, 2022). Polyphenols play a significant role in ameliorating the detrimental effects of ageing on the brain and the nervous system (Rudrapal *et al.*, 2022). Moreover, polyphenols can minimise the incidence of oxidative stress-related disorders (Rudrapal *et al.*, 2022). Lactucin in lettuce induces sleep and relaxation and blocks the excitatory signal processes of muscular and neural tissues (Rolnik, 2022). In lettuce nutrients, β -carotene and ascorbic acid can also potentially minimise the risk of colorectal cancer (Rolnik, 2022). Vitamin C compounds in lettuce speed up wound healing and boost skin collagen (Henderson, 2021; Shi *et al.*, 2022).

Furthermore, lettuce consumption can promote metabolism in the human body due to its availability of minerals like K, Fe, and Mg (Medina-Lozano *et al.*, 2021; Shi *et al.*, 2022). Eating red-leaf romaine lettuce improves the postprandial metabolic at that time through acute effects on metabolism (Henderson, 2021). This is important as consumers need to correctly metabolise their intake, as it can supply the energy required for daily routines. Therefore, long-term intake of leafy vegetables is undoubtedly advisable.

2.1.3 Cultivation requirements

2.1.3.1 Root media

Lettuce grows best on loam and well-drained soils because it warms up quickly during the daytime (Fekadu *et al.*, 2021; Martínez-Ispizua *et al.*, 2022). Osei *et al.* (2017) investigated suitable soil for lettuce using the Crispa variety on three different soil type textures namely: clay (39.9% sand, 13.3% silt, and 47.0% clay), loamy sand (75.52% sand, 20.05% silt, and 4.11% clay), and sandy loam (66.92% sand, 27.06% silt, and 6.03% clay). Sandy loam was the most suitable soil, producing the highest leaf height, fresh weight, leaf dry matter yields, and most fleshy leaves. Sandy loam is derived from the voltaian sandstone and characterised as moderately shallow to moderately deep, orange-red to reddish brown, free from stones and concretions, well-drained, and friable with adequate moisture-holding capacity, occurring on the upper and middle slopes of the catena (Osei *et al.*, 2017).

Lettuce grows well in a slightly acidic to neutral pH range of 6 to 6.5. It also prefers soil with high organic matter content of up to 35 g kg⁻¹ and silt loam textural class (11.91% sand, 12.67% clay, and 75.42% silt) (Hameed *et al.*, 2022). Based on the experience of Sarkar *et al.* (2021) with highly acidic soils, lettuce responds well to applying lime and compost mixed with the soil to a depth of 15–20 cm. Root media physical and chemical attributes are crucial for

optimal plant growth in an intensive soilless culture system. Major studies show that horticultural crop production uses substrate as a growth medium rather than soil in a protected growing environment to improve plant growth and yield (Gruda, 2019). The particle size distribution is crucial for describing the material physical characteristics and suitability for lettuce plant growth (Osei *et al.*, 2017).

Providing plants with physical support through manufactured substrates is referred to as a soilless culture, which also acts as a reservoir for plant water and nutrients. Diverse organic and inorganic substrates are widely adopted to grow lettuce in soilless culture systems (Sarkar *et al.*, 2021; Hameed *et al.*, 2022) to achieve maximum production, especially when water is a limiting factor. In soilless production, nutrients are supplied in the form of fertigation, allowing them to be immediately available to the plants, resulting in rapid growth and increased yield. Root medium is an important element that directly affects production costs. Selecting of a precise substrate material depends on use, local knowledge of use, availability, and cost-effectiveness (Dittrich *et al.*, 2022; Da Silva *et al.*, 2023). A root medium must provide the necessary physical and chemical properties for optimal plant growth and the development of specific species. Da Silva *et al.* (2023) imply that some substrate materials are blended to deliver enhanced drainage and aeration.

Organic components like peat, compost, and coir, including renewable resources from the woodworking industry such as wood fibre, wood chips, bark, and sawdust, are characterised by low water retention, uniformity, light weight, and having high cation-exchange capacity (Dittrich *et al.*, 2022). Sometimes, these materials contain phytotoxins that affect plant growth, especially during the initial cultivation phase (Gruda, 2019; Nerlich & Dannehl, 2021; Sarkar *et al.*, 2021). Woody root media are normally used once to prevent the accumulation of microorganisms caused by decomposition (Dittrich *et al.*, 2022). Wood chips and sawdust are typically utilised in a volume basis of 20–30% ratios in blends with other substrate components (Gruda, 2019; Haynes *et al.*, 2022). Lu *et al.* (2021) noted that sawdust medium used alone delayed the growth parameters of tomatoes compared to soil medium. Substrate materials are manufactured in various particle sizes to adjust air and water-holding capabilities by increasing the fraction of tiny particles. Bark media has a bulk density of 0.1–0.3 g cm⁻³ and wood fibre is approximately 70–90 kg m⁻³ with high total porosity (Dittrich *et al.*, 2022).

Perlite, volcanic rock, rockwool, enhanced clay granules, vermiculite, zeolite, pumice, sand or crushed rock grit, and other synthetic materials are used as growing media, either alone

or in combination with other materials (Purba *et al.*, 2021; Machado *et al.*, 2022; Haynes *et al.*, 2022). Clay granules are added to other substrates to increase water-holding capacity and nutrient buffering ability (Lu *et al.*, 2021; Haynes *et al.*, 2022). In compost, applying perlite substrate boosts the drainage and air content of the root media (Machado *et al.*, 2022). A study by Alu'datt *et al.* (2019) on purslane (*Portulaca oleracea* L.) grown in seven horticultural substrates involved peat moss, tuff, zeolitic tuff, and a combination of peat moss and perlite (2:1), tuff and peat moss (2:1), tuff and peat moss (1:1), and tuff, peat moss and perlite (2:1:1). In this study, the combination of tuff-peatmoss (1:1), peatmoss-perlite (2:1) and tuff obtained the highest total values of fresh yield by 26,923 g m², 17,744 g and 16,199 g respectively. The fresh yield of other substrates varied depending on the harvest cycle.

Sand, rock-crushing grit, and subsoil are added in small amounts of about 10–30% v/v, and the main ingredient for structural manufactured soils is the coarse stone aggregate (Gruda, 2019). The research undertaken by Purba *et al.* (2021) in the cultivation of lettuce with a hydroponic system comparing husk charcoal, rockwool, and coco peat media revealed that husk charcoal outperforms rockwool and coco peat media. Husk charcoal medium produced the heaviest total dry weight of 8.57 g. The husk charcoal is an organic material that contains nutrients that plants in hydroponic systems utilise. Husk charcoal is formulated from rice bran that is burned with unique features. In addition, the roasted husk has chemical compounds such as SiO₂ (52%) and C (31%) content, and small amounts of Fe₂O₃, K₂O₃, MgO₃, CaO₃, MnO₃, and Cu (Purba *et al.*, 2021). The advantages of rockwool substrates include uniformity, ease of handling, and consistency (Nerlich & Dannehl, 2021).

The material of volcanic ash has a total porosity of 71.3 and 16.8% water holding capacity with a pH ranging from 7–8, and a low cation exchange capacity (Hossain, 2021). Sphagnum peat has unique microporous characteristics and is resistant to degradation (Hossain, 2021). Peat is a limited resource with high demand, and the extraction of peat bogs has significant environmental consequences (Gruda, 2019). Finland, Ireland, Germany, Sweden, Belarus, Canada, and Russia are the leading peat-producing countries, accounting for 80% of global production (Höök, 2020). The findings of the study conducted by Kumar *et al.* (2022) on iceberg lettuce grown in different combinations of substrates reported that coco peat and perlite and vermicompost in the ratio of 2:1:1 produced a high number of leaves (20.06), plant height (23.80 cm), plant spread (30.60 cm), yield (145.60 g/plant), but the most significant root length (31.00 cm) was observed in treatment combination of cocopeat and perlite (2:1).

However, a recent study by Kumar *et al.* (2022) substantiates that the extensive use of coco peat alone or in combination is superior. This is due to its chemical and physical properties, low nutrient content, low pH, high water-holding capacity, and good drainage characteristics, as well as being free of pests and diseases (Gruda, 2019). Indeed, coco peat is widely recognised as a root medium with good and acceptable chemical properties that are used to cultivate a wide range of crop species with high quality (Sarkar *et al.*, 2021; Purba *et al.*, 2021). The major studies into root media have shown that the parameters of various substrate materials directly and indirectly affect plant growth and productivity (Isa *et al.*, 2021).

A sustainable root medium is characterised by the optimal composition of physical parameters such as texture, bulk density, and air-and-water-filled pores (aeration), which can be analysed using water-holding curves (Nerlich & Dannehl, 2021). These are essential for proper irrigation management. In contrast, chemical aspects, such as cation exchange capacity, pH, and salinity, are needed for appropriate nutrient management (Da Silva *et al.*, 2023).

2.1.3.2 Plant population

The optimum spacing efficiently utilises moisture, nutrients, and light, resulting in consistent plant growth and maximum lettuce productivity. In open-field production, lettuce seeds are typically planted 2–4 cm deep in prepared seed beds at 5–10 cm between rows and are later thinned to the final distance of 25–31 cm apart within the seed lines (Drost, 2020; Fekadu *et al.*, 2021). Three-week-old lettuce seedlings transplanted at 20 cm between rows and 20 cm between plants, providing a plant population of 250,000 plants per hectare, had the best yield in a study conducted by Petropoulou *et al.* (2023). Leaf lettuce can be grown closer together than head lettuce; for example, romaine may be as close as 13 cm apart within seed lines (Petropoulou *et al.*, 2023). Lettuce seeds are sown at 0.3–0.6 cm deep and 2.5 cm apart in the greenhouse (Fekadu *et al.*, 2021). Usually, the seeding rate of lettuce is 800 to 1000 g of seeds per hectare, and plant populations vary from about 60,000 to 100,000 plants per hectare (Drost, 2020). The space between rows is 27–60 cm, and the space between plants in the rows is 16–30 cm, depending on the lettuce variety (Drost, 2020).

The research work carried out in a greenhouse by Fekadu *et al.* (2021) on two lettuce cultivars and three levels, respectively, of inter-row (30, 40, and 50 cm) and intra-row spacing (20, 30, and 40 cm) and the researchers found that cultivars respond differently to spacing. In this trial, the results indicated that the optimal plant spacing was the combination of 40 cm x

20 cm (125,000 plants ha⁻¹) compared to other spacings, and the higher number of plants per m⁻² increased all parameters. In closed and open soilless systems, yield per unit area therefore increases as the plant spacing decreases (plant population increases). Regardless of all the recommendations, lettuce varieties vary in architecture, and soil fertility gradients also determine the number of plant densities.

2.1.4 Nutrition requirements

2.1.4.1 Nutrient solution

Fertilisers for soilless cultures mainly contain 13 essential elements for plant development (Chen *et al.*, 2020). For lettuce crops grown under open soilless culture (drain-to-waste) production systems, Combrink and Kempen (2019) prescribed a nutrient solution mixture of Fe (1.00), Mn (0.30), Zn (0.25), B (0.30), Cu (0.05), and Mo (0.05) as micronutrients concentration (mg L⁻¹) and macronutrients (meq L⁻¹) of NH₄ (0.7), K (5.5), Ca (5.8), Mg (1.0), NO₄ (10.0), H₂PO₄ (1.0), and SO₄ (2.0) with an EC of 1.30 milli-Siemens per centimetre. Electrical conductivity measurements are used to detect salinity hazards.

The recommended amount of fertiliser to make the nutrient solution is based on species and source water quality. Fertigation is the agronomic strategy to supply nutrient feed to the root zone via irrigation (Sathian *et al.*, 2021). Fertigation allows for a significant increase in nutrient use efficiency (Combrink & Kempen, 2019). An experiment by Sakamoto and Suzuki (2020) on sweet potatoes (*Ipomoea batatas* L.) examined the effects of three different nutrient solution concentrations with an EC of 0.8, 1.4, and 2.6 dS m⁻¹ on growth. They reported that the growth of shoots and storage roots was enhanced as the nutrient solution concentration increased to an EC of 2.6 dS m⁻¹. The response of plants to EC levels depends on the species, growth stage, and environmental conditions or growing technique. Hosseini *et al.* (2021) conducted an experiment on lettuce cultivar and basil (*Ocimum basilicum* L.) in automatic vertical farms using the hydroponic ebb-flow cultivation technique with five different EC values, as follows 2.0, 1.2, 0.9, 0.7, and 0.5 dS m⁻¹, to determine the effect of EC on growth and development of the two plants. High EC levels reduced lettuce yield, and the optimal EC levels for the growth and development of basil and lettuce were 1.2 and 0.9 dS m⁻¹, respectively.

2.1.4.2 Water quality

The success of irrigated crop production requires the availability of good-quality water. Hence, it is necessary to test water quality by sending a water sample of a particular water source to the laboratory to determine the available micro- and macro-nutrients before using it for irrigation. The toxicity of irrigation water occurs due to the critical concentration of certain minerals and trace elements, such as B, Al, As, Cd, Cr, Co, Cu, Fe, P, Mn, Ni, and Se content (Hajihashemi *et al.*, 2020), which can mainly cause salt stress. Saline water interferes with the uptake of nutrients by plants and hampers plant growth (Mukherjee *et al.*, 2022). Soluble salts in irrigation water can affect the irrigation system's performance by closing emitters. Sodium percentage is used to determine the quality of agricultural water (Yıldız & Karakuş, 2020). A high concentration of Na^+ reduces water penetration into the root medium (Yavuz *et al.*, 2023). Kim *et al.* (2020) assessed the effects of saline water irrigation on the growth of lettuce, and the results showed that using saline water increased the concentration of Na^+ in the lettuce leaves and decreased the lettuce yields.

Various studies report that using poor water quality for irrigation reduces crop yield and quality. For example, Al-Taey *et al.* (2019) conducted a field experiment to study the effects of irrigation water quality at two levels of salinity (1.2 and 8 dS.m^{-1}) on potato cultivars. The authors reported that fresh water (1.2 dS.m^{-1}) produced the highest chlorophyll average (36.33 SPAD units) compared to drainage water with 8 dS.m^{-1} (33.51 SPAD units). Poor water quality also hinders the uptake and distribution of K and N in spinach (*Spinacia oleracea* L.) (Yavuz *et al.*, 2022). The findings by Azarmi-Atajan and Sayyari-Zohan (2020) on lettuce (cv. Ferdos) reported that 4 and 8 dS.m^{-1} salt stress lowered fresh shoot weight by 20% and 39% , dry weight by 18% and 46% , respectively, compared to the control. The availability of water with a high salt concentration leads to a nutrient solution with an EC level that exceeds the tolerance level of many crops (Yıldız & Karakuş, 2020).

Major studies have demonstrated decreased plant growth and yield under salinity stress. On broccoli (*Brassica oleracea* L.), salt accumulation in plant leaf margins caused the burning of the edges and a significant decrease in the yield from the EC of 6 dS m^{-1} upwards (Dahlawi *et al.*, 2023). The biomass of seedlings in Chinese cabbage (*Brassica rapa* ssp.) and white cabbage (*Brassica oleracea* ssp.) was inhibited at higher salt concentrations of 100 and 200 mM NaCl in prolonged salinity stress (up to seven days) (Pavlović *et al.*, 2019). The same study by Pavlović *et al.* (2019) showed that photosystem II and photosystem I of the total

photosynthetic performance index of Chinese cabbage decreased by 26.6, 47, and 66.6% after exposure to 50, 100, and 200 millimoles (mM) NaCl, respectively. White cabbage decreased by 23.8 and 46.2% and kale by 26.7 and 41.9%, respectively, as detected after 100 and 200 mM NaCl exposure (Pavlović *et al.*, 2019). Giordano *et al.* (2021) observed a negative correlation between salinity and germination rate in cabbage.

2.1.5 Climate requirements

2.1.5.1 Moisture requirements

Lettuce has a shallow root system and is susceptible to water stress (Yavuz *et al.*, 2023). Measuring evapotranspiration enables irrigation applications to correspond to crop water requirements and avoids inadequate or excessive water applications (Million & Yeager, 2022). Excess irrigation on crops leads to a higher loss of nitrogen by leaching and causes disease outbreaks and root rot (Ten Damme *et al.*, 2022). Lettuce may be irrigated using furrow irrigation, sprinklers (Sathian *et al.*, 2021), or drip irrigation systems (Paramesha *et al.*, 2022). However, Sathian *et al.* (2021) recommend using micro-sprinklers during germination.

To initiate irrigation of lettuce during seeding and transplanting in soilless culture, 2 to 4 mm of water to prevent water deficit (Li & Liu, 2022). Based on Amare and Abebe's (2020) review, lettuce yields best when irrigated with a drip irrigation system with a two-day irrigation interval. Ors *et al.* (2021) suggest that irrigation should be conducted in the morning before the day's heat and in the evening to avoid disease establishment and excessive water evaporation. A study by Daghari *et al.* (2020) on lettuce shows that plants irrigated early in the morning or late in the afternoon have the most significant yield. Using drip irrigation is recommended to help farmers reduce costs on water by saving up to 20% of irrigation water (Paramesha *et al.*, 2022). The advantages of using drip irrigation include enhanced nutrient uptake, plant root growth, and entire soil properties (Paramesha *et al.*, 2022). The growth and development of plants require adequate soil and atmospheric moisture.

Relative air humidity is an important component in the stomatal adaptation of carbon dioxide (CO₂) (Inoue *et al.*, 2021). In a study on lettuce by Ahmed *et al.* (2022), an air temperature of $24 \pm 0.5^{\circ}\text{C}$, relative humidity in the range of 60–70%, and a photoperiod of 16 h d⁻¹ were maintained for the entire experimental period of 21 days. The results showed that the highest stomatal conductance of $0.54 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ was found at a CO₂ concentration of $1000 \mu\text{mol mol}^{-1}$ (micromoles per mole), illumination intensity of $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$, and an air

speed of 0.25 m s^{-1} . The same study revealed that under a CO_2 concentration of $500 \mu\text{mol mol}^{-1}$ and illumination intensities of 200 and $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$, the shoot fresh weight decreased as airspeed increased to 0.75 m s^{-1} . Moreover, high illumination intensity and high air speed are not recommended for lettuce seedling growth during the first days of transplant.

2.1.5.2 Temperature

Lettuce is a cool-season crop with specific temperature requirements. Temperature is the primary environmental aspect influencing plant growth and development (Pathak *et al.*, 2023). Lettuce has C3 photosynthetic metabolism and requires daily temperatures averaging between 15°C and 18°C and monthly means between 7°C and 24°C for optimum growth and development (Da Silva *et al.*, 2023). Day temperatures that range between 17°C to 27°C and between 2°C and 12°C at night are most suitable (Aires *et al.*, 2020; Abedrabboh *et al.*, 2023). Summer lettuce production, where temperatures exceed 24°C , results in physiological disorders such as ribbiness, rib discolouration, and bolting, and with high night temperatures, lettuce becomes bitter, loose, and exhibits fluffy heads (Aires *et al.*, 2020).

The best temperature for lettuce under greenhouse conditions is 22 to 26°C until 25 days after transplantation, then 20 to 24°C until harvest, with an optimal night temperature of 15 – 20°C throughout the crop cycle (Abarca *et al.*, 2022). The greenhouse study conducted by Abedrabboh *et al.* (2023) in a greenhouse showed that larger crops are produced when the constant temperature is maintained at 25°C for the entire growing season. The optimal temperature for seed germination in the soil ranges from 12°C to 18°C (Drost, 2020).

2.1.5.3 Radiation

Radiation is the primary energy source for plant photosynthesis and regulates plant development, morphogenesis, biosynthesis of cell components, and gene expression (Abedrabboh *et al.*, 2023). Lettuce production requires a daily photon accumulation of 12 to $17 \text{ MJ m}^{-2} \text{ day}^{-1}$, representing a photoperiod of 12 hours of light and a light intensity of $300 \text{ mol/m}^2/\text{s}$ for 12 dark hours (Abarca *et al.*, 2022). A photoperiod of 16 and 18 h d^{-1} is adequate for promoting plant growth and light use efficiency in greenhouse-grown lettuce (Choi *et al.*, 2022).

Abedrabboh *et al.* (2023) reported that the photosynthetically active radiation of $17 \text{ mol/m}^2 \text{ day}^{-1}$ is an optimal light source for lettuce cultivation for both natural and supplemental

light in a greenhouse. The studies by Aires *et al.* (2020) and Aires *et al.* (2021) reported that the global solar radiation between 17.6 and 19.5 MJ m² day⁻¹ is adequate for growing crisphead and iceberg lettuce. Radiation improves stomatal opening and uptake of CO₂, which stimulates photosynthetic and transpiration rates (Aires *et al.*, 2020; Aires *et al.*, 2021).

2.1.6 Harvesting techniques

2.1.6.1 Harvesting

Harvesting determines the quality and storage of products and contributes to preventing substantial losses of vegetables. Maturity is based on size and head compactness and firmness. Therefore, head lettuce should be harvested when the heads are well-formed and solid (Abedrabboh *et al.*, 2023). Leafy lettuce can be continuously harvested when the leaves are young by picking individual leaves and harvesting the entire plant before bitterness and bolting develop (Drost, 2020; Shi *et al.*, 2022). The lettuce can be harvested by hand or with sharp knives, and knives must be kept sharp and clean to prevent diseases or viruses from spreading from plant to plant (Elik *et al.*, 2019). It can also be harvested using autonomous robotic technologies (Chida *et al.*, 2020).

To fulfil commercial standards, the lettuce stem must be cut cleanly at a specific height (Chida *et al.*, 2020). The mechanical damage during harvesting and handling during and after harvesting determines the effect of browning and rotting (Meena & Kulakarni, 2022). Soiled and rotten leaves are removed during harvesting, and heads with signs of disease infection are discarded (Afroza & Rana, 2017). Lettuce should not be harvested when the heads are moist to minimise damage to wrapper leaves (Drost, 2020). Lettuce may be grown and harvested up to four seasons per year in a greenhouse (Fekadu *et al.*, 2021).

2.1.6.2 Postharvest

After harvesting, the goal is for the fresh leafy vegetables to arrive at market places and customers fresh while healthy for consumption. Therefore, managing temperature (precooling) is vital for extending shelf life and minimising post-harvest losses (Elik *et al.*, 2019). Precooling lowers field heat from fresh produce shortly after harvest to reduce respiration rate and metabolic processes and slow vegetable degradation before storage or marketing. Various horticultural production methods include evaporative cooling, room cooling, vacuum cooling, forced air cooling, hydro cooling, ice chilling, and serpentine forced cooling (Elik *et al.*, 2019;

Subedi *et al.*, 2022). The cool chain must be kept from the farm gate to the customer for optimal performance. The suggested storage and shipping temperature for lettuce is from 0 to 1°C and 95% relative humidity (Afroza & Rana, 2017).

Kongwong *et al.* (2019) conducted research to evaluate the ideal parameters for vacuum cooling as well as the effects of various commercial precooling systems, such as room cooling, forced-air cooling, and vacuum cooling, on the physicochemical qualities, bioactive compounds, and ultrastructure of baby cos lettuce during storage at 4°C. The experimental results revealed that the optimum requirements for vacuum cooling of baby cos lettuce with a high initial temperature above 20°C were at a holding pressure of 0.6 kPa with a holding time of 25 min because it could achieve the final target temperature at $4 \pm 1^\circ\text{C}$. The study findings further reported that vacuum cooling at 0.6 kPa holding pressure and 25 min holding time preserved the produce attributes significantly better than forced-air cooling at 1.4 m s^{-1} air velocity or room cooling at 0.65 m s^{-1} .

Damerum *et al.* (2020) investigated the changes in different quality indices of butterhead lettuce after storage at an optimal temperature of 0–2°C and 90–95% relative humidity. The exposure of lettuce heads to a low relative humidity of 70–72% reduced quality and shelf-life by 75%. Relative humidity of more than 85% prevents water loss due to hot or windy conditions, which causes perishability and a tendency to wilt in lettuce and thus quality loss (Damerum *et al.*, 2020). Under proper environmental storage requirements, leaf lettuce has a shelf life of 1 to 2 weeks, and head lettuce has a storage life of 2 to 3 weeks (Drost, 2020). Thus, lettuce and other leafy vegetables should be consumed as soon as possible after harvest to avoid the loss of bioactive compounds (Lei & Engeseth, 2021). Head lettuce is graded by size and compactness, and leafy types are graded by size (Chida *et al.*, 2020).

The standard quality parameters are based on biochemistry and the internal physiology of the plant. These include water content, native microflora, antioxidant capacity, chlorophyll content, and overall quality as sensory acceptability indices (appearance, texture, taste, and aroma) (Shi *et al.*, 2022; Zhang *et al.*, 2022). In addition to the mentioned quality parameters of lettuce, several researchers identified ascorbic acid, β -carotene, flavonoids, phenolic acids, and mineral concentration as nutritional quality indices (Johnson *et al.*, 2022). In general, quality refers to the level of excellence of a product for its suitability for a particular use. Assessing lettuce quality parameters is important due to factors related to the safety of vegetables, such as natural pesticides, chemical debris, heavy metals, and microbial

contamination. Fresh vegetables can be contaminated with bacteria and harmful pathogens (Deng *et al.*, 2020). Therefore, the safety of vegetable products can be increased through physical, chemical, and microbiological technologies, which are used to detect hazards (Deng *et al.*, 2020).

The firmness or texture quality index is determined by reflecting the strength and thickness of the cell wall as well as the degree of adhesion between cells (Lei & Engeseth, 2022). The colour of vegetables is a key quality indicator for consumer visual satisfaction and determines the market price (Afroza & Rana, 2017). Chlorophyll is a green pigment produced by plants and assists in photosynthesis. Parida and Parida (2021) evaluated the amount of chlorophyll content in two varieties of lettuce (green and red leaf), and outcomes showed that the red leaf chlorophyll a and b values were 23.920mg/ ml and 43. 207 mg/ml, respectively, and higher than green leaf with Chlorophyll a and b of 21.795 and 39.759 mg/ml, respectively. Chlorophyll concentration is directly related to the amount of nutrients available in plants (Awolu & Oladeji, 2021).

Lei and Engeseth (2021) piloted a study demonstrating that lettuce grown hydroponically is not the same quality as lettuce grown in soil. The findings showed that hydroponically grown lettuce had softer leaves and firmer leaf midribs than lettuce grown in the soil, meaning that hydroponically grown lettuce contained higher lignin in the cell walls compared to soil-grown lettuce. The study by Lei and Engeseth (2021) also showed that hydroponic lettuce's ascorbic acid content average was 17.84% lower than its soil-grown equivalent. Zhang *et al.* (2022) describe ascorbic acid as a natural antioxidant degrading hydrogen peroxide through the ascorbate-glutathione cycle, lowering potential cell damage. Ascorbic acid affects the flavour of vegetables and fruits by providing tartness (Zhang *et al.*, 2022). The flavour is the parameter used to evaluate the quality of fresh vegetables by taste buds in the mouth combined with aroma (Zhang *et al.*, 2022). The quantity and composition of phytochemicals in lettuce depend on the choice of cultivar, leaf colour, and maturity at harvesting (Da Silva *et al.*, 2023). Hence, understanding the nutritional composition and value of lettuce is essential. However, water deficit, plant bag size as a limiting root zone, and wounding may hamper the quality and yield of leafy vegetables.

2.2 EFFECTS OF MULTIPLE, COMBINED AND INDIVIDUAL ABIOTIC STRESS FACTORS AFFECTING HORTICULTURAL PLANTS

2.2.1 Global losses associated with multi-abiotic stress factors for horticultural plants

Global water consumption doubles every 20 years and is expected to increase by 56% by 2030 (Caparas *et al.*, 2021). Due to water scarcity caused by global warming, commercial farmers use different sources of water, such as surface water (lakes, natural and artificial ponds), groundwater, municipal tap water, rainwater, or combinations of these (Hajihashemi *et al.*, 2020; Contreras *et al.*, 2021) to meet plant water requirements. However, the scarcity of water leads to water deficit stress. Water stress is a critical agricultural problem, leading to crop losses worldwide. Besides water stress as a global concern, soil bulk density represents a significant challenge as a valuable indicator of soil compaction. Soil compaction can reduce crop yields by 25% and up to 75% when combined with water deficit stress, as compacted soils hamper roots (Huang *et al.*, 2022). When comparing the performance of crops in response to water stress, soil moisture is standardised by the highest and lowest values of pot water holding capacity (Koehler *et al.*, 2022), which differs with soil texture and bulk density.

Globally, around 20–40% of agricultural crop yield losses occur due to factors such as strong wind, radiation, mechanical damage, exposure to heavy metals, herbivores, and infection by damaging hosts like fungi, bacteria, or viruses at different stages of plant growth (Srinivasan *et al.*, 2022; Al-Khayri *et al.*, 2023).

2.2.2 Combined stress factoring of the water deficit, wound, and plant bag size on horticultural plants

Multiple abiotic stresses reduce enzymatic activity, cause degradation of chlorophyll, destruction of an organic molecule, and damage to lipid peroxidation (Lohani *et al.*, 2020). When they coincide, water deficit, plant bag size, and wounding affect critical biological processes in plants, such as photosynthesis, stomatal conduction, rate of transpiration, protein synthesis, and metabolite accumulation (Lohani *et al.*, 2020; Sehgal *et al.*, 2022; Moyo *et al.*, 2024). Morphophysiological and biochemical parameters of horticultural species are negatively affected and reduced by water deficit and wounding stress (Wielkopolan *et al.*, 2022). A plant wound is a critical feature of water deficit stress; thus, the combination of water deficit stress and mechanical wounding increases the reactive oxygen species levels (Becerra-Moreno *et al.*, 2015; Gao & Farmer, 2023). Quantitative analysis of H₂O₂ levels showed that

mechanical wounding causes a quick response, reaching the highest level at 4 hours of stress, followed by a reduction in H_2O_2 after 24 hours and 48 hours, respectively (Sabina and Jithesh, 2021). This shows that mechanical wounding contributes to water deficit stress.

Under water-deficit stress, most miniature pots dry quicker (Million & Yeager, 2022). Different metabolic and physiological responses are required when multiple stresses coexist, as they cause severe consequences to plant growth and development, productivity, and quality of horticultural species (Sehgal *et al.*, 2022; Srinivasan *et al.*, 2022). Large pots retain more substrate and hold moisture better than small containers, even when subjected to sudden temperature fluctuations and water stress (Melrose & Normandeau, 2021; Balliu *et al.*, 2021). Small pots dry out quickly due to reduced water-holding capacity; as a result, plant water status can be adversely affected (Melrose & Normandeau, 2021). Different container sizes and root restrictions significantly impact shoot growth (Melrose & Normandeau, 2021). When roots of seedlings are restricted by a polybag, roots become spiral and deformed, decreasing plant growth and leading to stress resistance (Benamirouche *et al.*, 2020). After transplanting, seedlings with spiral roots may not sufficiently anchor the plant and may have limited water and nutrient intake (Benamirouche *et al.*, 2020; Haase *et al.*, 2021). According to Bilal *et al.* (2023), physiological and biochemical processes initiated by a particular stress condition differ from those triggered by different compositions of adverse environmental stresses.

2.2.3 Effect of water deficit stress factor on horticultural plants

2.2.3.1 Extent of water deficit on plants

Water deficit is a significant abiotic stress that occurs due to the lack of soil water to maintain plant growth and respiration demand, which leads to reduced plant water potential (Dong *et al.*, 2022). Water stress can induce various morphological, physiological, and biochemical alterations in plants, which involve stomatal variation, leaf expansion, reduced plant growth, and stem elongation (Kumar *et al.*, 2020; González-Orenga *et al.*, 2023). During the critical growth stages, moisture stress decreases relative leaf water content and stomatal conductance, ultimately resulting in stunted growth (Inoue *et al.*, 2021). Under water deficit conditions, an increased transpiration rate reduces turgor pressure, and this results in wilting of leaves, which lowers light interception (Franzoni *et al.*, 2022). Water deficit hinders cell division, roots, and shoot expansion, leading to stunted plants (Kumar *et al.*, 2020). Severe water stress disturbs physiological processes such as photosynthesis, and it delays the

accumulation of dry matter due to the degradation of chlorophyll (Inoue *et al.*, 2021). Water deficit further disturbs regular metabolism, leading to plant death and reducing ultimate yield (Ahmed *et al.*, 2020).

2.3.1.2 Losses associated with water stress

Water deficit stress is characterised by the dehydration of plant cells to a degree that hampers cellular functions (Contreras *et al.*, 2021). Water deficit causes severe yield losses and affects the quality of many horticultural crops. Morphological effects of water deficit include reduced plant height and leaf area, a dense root system, and decreased shoot lengths. A study on eggplant (*Solanum melongena* L.) grown under water deficit showed an average reduction of 47.5% in total fresh weight, 18.9% in dry biomass, 16.2% in plant height, and 45.5% in leaf area, which led to partial stomatal closure (González-Orenga *et al.*, 2023). In tomatoes, Rosa *et al.* (2023) reported a decline in the number of leaves in plants watered with 40% field capacity by day 17, including diminished plant height, and an overall significant reduction of plant biomass aboveground.

Khattak *et al.* (2021) observed that on sunflowers (*Helianthus annuus* L.), severe water stress (30% field capacity) remarkably lowered plant leaf area, leaf relative water content, chlorophyll a, b and carotenoid content, head diameter, width and length of achene, 1,000 achene weight, achene oil content, and biodiesel yield. Water deficit stress significantly reduced the fresh and dry weights of shoots, roots, and leaf area of lettuce (Kumar *et al.*, 2020). Photosynthesis is thus critical to biomass accumulation and yield establishment throughout the growing season (Rosa *et al.*, 2023). In a study of lettuce heads, 30% of the lowest irrigation levels declined net photosynthesis and stomatal conductance and harmed plant growth, such as reduced fresh weight and final production (Li & Liu, 2022). In the study to investigate how the plant bag size impacts rose geraniums (*Pelargonium graveolens* L.), Khetsha & Sedibe (2015) discovered that it reduced plant height, number of branches, the fresh mass of the foliage, K tissue content, and oil yield.

2.3.1.3 Recovery mechanisms associated with water stress

Plants usually possess an inbuilt defence system to mitigate stresses, but to a threshold tolerance level (Dahlawi *et al.*, 2023). Plants respond to water deficit depending on the length and severity of the water deficiency and the plant species and cultivar, age, developmental stage, and cultivation practices (Aslani *et al.*, 2023). The responds to water stress is achieved

through biochemical, physiological, and morphological alterations to cope with water loss and sustain hydric status (Turner, 2019; Hoseini *et al.*, 2020). Morphological adaptations include the longer root system, early flowering, increased root and high tillering, and leaf folding (Sako *et al.*, 2020; Rosa *et al.*, 2023).

Jan *et al.* (2022) consider Reactive oxygen species (ROS) as by-products of plant oxidative metabolism derived in several cells, such as chloroplasts, mitochondria, and peroxisome. Higher ROS production, compared to average concentrations, can be toxic to plant cells. ROS chemical compounds can cause DNA damage and cell death (Jiménez-Arias *et al.*, 2022). These include free radicals such as superoxide (O_2^-), hydroxyl ($OH^\cdot$), and perhydroxyl ($O_2H^\cdot$) and other signalling molecules such as hydrogen peroxide (H_2O_2) or ozone (O_3) radicals (Wei *et al.*, 2023). Accumulation of antioxidant systems such as glutathione peroxidase, polyphenol oxidase, and malondialdehyde in plants under severe water stress provides an effective ROS-removal mechanism for defending plants against oxidative harm (Imran *et al.*, 2021). Jan *et al.* (2022) determined oxidative stress via glutathione peroxidase and lipid peroxidation. They visualised it via trypan and diaminobenzidine histochemical staining. The results showed that glutathione peroxidase activity was lower in transgenic-treated rice plants exposed to UV radiation only (OxF3H+UV) by 72.5% and transgenic-treated rice plants exposed to the combination of drought and UV radiation (OxF3H+D+UV) by 72.4% compared to non-transgenic-treated rice plants exposed to UV radiation only (Wt+UV) and non-transgenic-treated rice plants exposed to the combination of drought and UV radiation (Wt+D+UV), indicating that the transgenics reduced oxidative stress.

Osmotic stress induces proline synthesis and accumulation in plants under water stress to maintain turgor pressure and detoxicate ROS (Li & Liu, 2022). In a study by Aslani *et al.* (2023), the proline content in control plants ranged from 10–38 $\mu\text{mol g}^{-1}$ of dry weight among species and raised in all cases in response to the stress treatment, achieving values between 81 and 208 $\mu\text{mol g}^{-1}$ DW. To counteract stress imposed by the water deficit, plants need to adjust their osmotic balance to cope with osmotic pressure, and osmotic adjustment is the physiological adaptation mechanism by which plants subjected to water deficit accumulate cell-compatible solutes to overcome its adverse effects (Aslani *et al.*, 2023).

Osmotically active solutes act as antioxidants by contributing to osmotic adjustment to protect enzymes and macromolecules in cells from the harmful impacts of ROS (Jiménez-Arias *et al.*, 2022). These solutes include amino acids (proline, glycine betaine), ammonium

compounds, soluble sugars, and plant hormones such as polyamines (Jiménez-Arias *et al.*, 2022). Osmotic adjustment raises the content of osmotically active solutes in the cell while lowering the osmotic potential, enhancing cell hydration, and maintaining leaf turgor in active metabolic cells (Zhang *et al.*, 2022). Studies show a minor negative impact on photosynthesis under water deficiency due to limited gas exchange (Rosa *et al.*, 2023). Flavonoids stimulate maize seedling water stress tolerance by decreasing drought-induced oxidative impairment and regulating stomatal movement (Li *et al.*, 2020). Water stress applied on wounded carrots wielded a synergistic effect on the wound response at the gene expression level (Becerra-Moreno *et al.*, 2015).

2.2.4 Wounding as a stress factor for horticultural plants

2.2.4.1 Extent of insects and artificial wounding on plants

Herbivorous insects are a major biotic stress threat to attacking the plant (Al-Khayri *et al.*, 2023). Lettuce is susceptible to attacks by slugs or snails, especially during moist weather (Wielkopolan *et al.*, 2022). It may also be attacked by various insect pests, such as locusts and grasshoppers, American bollworm, *Plusia* looper, beetles, thrips, cutworms, springtails, and whiteflies (Wielkopolan *et al.*, 2022). Mechanical wounding is a procedure used to study the effects of mechanical stimulation in plants. It is also known as simulated herbivory because researchers use tools such as razors, needles, hemostats, punchers, and fine-grained sandpaper for wounding leaves to generate damage patterns in plant tissues similar to those caused by insect species (Cunha *et al.*, 2023). For example, Sabina and Jithesh (2021) performed a wounding experiment on a tomato to mimic the attack of sap-sucking and herbivorous insects that damage the fields' leaves or stems. They wounded the leaf lamina when plants were four weeks old by puncturing the apical compound leaf on the lamina surface using a hemostat of about six punctures with a 1 cm gap, leaving the midrib untouched (Sabina & Jithesh, 2021).

Mechanical wounding stimuli are the most effective indicators of potential herbivore assailants (Cunha *et al.*, 2023), as tissue lesions caused by pests and pathogens may induce similar enduring and destructive responses to growth as the ones observed when subjecting plants to artificial wounding (Cunha *et al.*, 2023). However, several studies revealed differences between natural and artificial herbivory because of the recognition of insect salivary compounds (Wielkopolan *et al.*, 2022; Guan *et al.*, 2022). Guan *et al.* (2022) reported that there are similarities between the insect wounding and artificial wounding responses by

identifying identical genes involved in plant responses to both types of wounding stresses, which include putative reverse transcriptase, ribulose biphosphate carboxylase small chain clone 512, and putative oxygen-evolving complex precursor.

Mechanical wounding consequently causes cell wall integrity loss, nutrient loss, and dehydration (Chen *et al.*, 2020) and lowers the quality and yields. In plant leaves, wounding induces the synthesis and accumulation of anti-microbial compounds (Al-Khayri *et al.*, 2023). The plant midrib is the key vein necessary for water transport to leaves and its injury leads to secondary water desiccation stress, different from wounding stress in leaves (Wielkopolan *et al.*, 2022). For example, plants exposed to local midrib injury had greater damage, considering the reduction of relative water content due to damage to the vascular bundles, than leaf lamina injury (Sabina & Jithesh, 2021). Immediately after the herbivore attack, plant cell plasma encounters several changes. Damaged tissues are potential bacterial sites and significantly impact plant signalling pathways, specifically at the sites where the insect host is feeding. For example, Wielkopolan *et al.* (2022) reported that wheat plants exposed to the cereal leaf beetle larvae with natural bacterial flora suppressed most genes linked with phytohormones such as salicylic acid (SA), abscisic acid (ABA), ethylene (ET), jasmonic acid (JA), cytokinin, and auxin.

2.2.4.2 Losses associated with wounding

A study conducted on *Arabidopsis* seedlings by Shi *et al.* (2022) showed that fresh weight, dry weight, and root length were significantly lower on the 10th day after wounding than in the unwounded seedlings. Cunha *et al.* (2023) investigated the impact of mechanically wounding on tomatoes and found that the leaves and root size were significantly reduced by 35.6% compared to control plants. Mechanical stimuli represent natural wounding events, such as, for example, plants inflicted by herbivorous chewing insects. Turgor pressure on plants is directly impacted by the disturbance of the plant vasculature caused by wounding and can lead directly to a loss of plant water content (Wielkopolan *et al.*, 2022). Cunha *et al.* (2023) also documented that wounded plants yielded tomato fruits of reduced size.

2.2.4.3 Recovery mechanisms associated with wounding

Plant responses to wounding are divided into local and systemic and include molecules that simulate regeneration, participate in signalling pathways, and changes in gene expression (Al-Khayri *et al.*, 2023). Under biotic stresses, plants respond through inducible basal defence

networks stimulated in response to correlated pathogen-associated molecular patterns (Al-Khayri *et al.*, 2023). Meents *et al.* (2019) wounded sweet potato (*Ipomoea batatas* Lam.) plants to mimic herbivore feeding without its elicitors in the oral secretion, using the mechanical caterpillar (MecWorm28) to inflict continuous mechanical wounding to plants. In this study, the mechanical damage for 18 hours displayed an emission of additional compounds such as (Z)-hex-3-enyl acetate and (Z)-jasnone or nerolidol. This increase can be clarified by combining mechanical wounding with the contribution provided by oral secretion (Meents *et al.*, 2019). In wounded plants, ROS are primarily produced by the activity of nicotinamide adenine dinucleotide phosphate oxidases that can be activated by Ca^{2+} ions (Mostafa *et al.*, 2022). A few minutes after wounding, the reactive oxygen species are synthesised, and the cytoplasmic Ca pool increases (Shi *et al.*, 2022). The 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) scavenging rate is measured to study the antioxidant activity of plant extracts, thus an increase in DPPH indicates the ability of the antioxidant to combat ROS levels (Sabina & Jithesh., 2021). Furthermore, Sabina & Jithesh. (2021) reported that plants with midrib and lamina injury demonstrated a substantial increase in DPPH scavenging rate compared to the control plants. Plant leaves with midrib injury also produced higher levels of DPPH scavenging rate by 46.6% at 48 hours compared with leaf lamina injury and control (Sabina & Jithesh, 2021). In the specific case of damage caused by herbivores, volatile metabolites are released to deter pests, attract their predators, and parasitoids (Khetsha *et al.*, 2022). Plants can recognise molecular attacks through cell surface receptors and stimulate pattern-triggered immunity (Sun & Zhang, 2021).

Transmembrane pattern recognition receptors and polymorphic nucleotide-binding leucine-rich-repeat protein products in R genes utilised by plants to detect aphid feeding (Wielkopolan *et al.*, 2022). Guan *et al.* (2022) investigated the responses of commercial wheat (*Triticum spp.*) to grain aphid (*Sitobion avenae*) infestation and mechanical wounding by mechanically wounding wheat plants with a sharp wheel roller mimicking chewing insect feeding wounds. In this study, the authors observed that aphid-feeding plants upregulated 22 putative genes and 29 upregulated after mechanical wounding. Mostafa *et al.* (2022) noted that six prolyl 4-hydroxylases in Arabidopsis were upregulated, suggesting involvement in this stress response. As several cell wall proteins, such as extensions and extension-like receptor kinases, are expressed in tissues under wounding stress (Mostafa *et al.*, 2022).

2.2.5 Plant bag size as a potential stress factor for horticultural plants

2.2.5.1 Plant bag size as limiting the root zone

In greenhouse and nursery production, bags, containers, pots, and trays hold the root media. These are made from plastics and fibreglass, cast concrete, clay, polyurethane, foam, and wood materials and all differ in size, shape, and colour (Haase *et al.*, 2021; Melrose & Normandeau, 2021). Planting bags are used to grow seedlings and plants. Usually, seeds are germinated in trays and transplanted into plant bags, containers, or pots. Selecting a plant bag for lettuce plants is crucial. The root system shape, length of the growing season, seed size, and target morphology determine the pot size required (Haase *et al.*, 2021).

Polypropylene pots are lightweight, inexpensive, and have better crop handling material (Sondhi, 2023). Polybags are used for one growing season. Clay pots are porous, unlike plant bags, and root media in clay pots are well-aerated but less likely to acquire root rot diseases (Melrose & Normandeau, 2021). Modern containers are expensive compared to polybags and can be reused for 5–12 years, depending on the material (Haase *et al.*, 2021). In containers, growers can plant more than one plant in a container tray, while they grow a single plant in a plant bag (Sondhi, 2023). Containers are obtainable in separate cells, free-standing, and aggregate blocks (Haase *et al.*, 2021; Sondhi, 2023). Furthermore, containers have rigid walls with internal vertical ribs or slots to direct downward root growth, limiting root twisting while promoting good root fibrosity (Balliu *et al.*, 2021). They also incorporate bottom openings that ensure adequate drainage (Balliu *et al.*, 2021; Haase *et al.*, 2021).

Comparing fabric and plastic containers in dwarf holly revealed that plant height was unaffected by the two container types (Million and Yeager, 2022). However, fabric increased plant width by $9 \pm 7\%$, plant size by $8 \pm 7\%$ (42 vs. 39 cm) and shoot dry weight by $12 \pm 6\%$, compared with plastic. In container production, plants are transplanted with intact root systems, increasing the potential for transplanting success (Million & Yeager, 2022). Plantable and biodegradable containers, including wood pulp fibre, bamboo, recycled paper, and rice hulls, have been produced to contribute to sustainable systems because they have a smaller carbon footprint than containers manufactured entirely of petroleum-based plastic (Haase *et al.*, 2021; Sondhi, 2023).

Small pots are often used for experiments in a controlled environment due to limited space. In small pot sizes, the root system becomes dense, branched, and matted (Lu *et al.*,

2021). For example, Turner (2019) reported that small pots contain a small soil volume, leading to soil compaction and limiting root extension and proliferation. Plant roots play an essential role in the uptake of water and nutrients and in regulating the elongation of shoots via phytohormones (Raviteja *et al.*, 2021). In addition, plant roots rely on shoots for fixed carbon from photosynthesis, and shoots depend on roots for the supply of water and minerals (Stoleru *et al.*, 2020). In the soil, the lettuce roots reach a depth of only 300 mm (Stoleru *et al.*, 2020). Thus, it is necessary to confine the nutrients and moisture requirements of lettuce to this moderately small volume of soil (Gruda, 2019).

Pot size, bulk density, nutrient status, and water status of the root media influence the formation and growth of plant roots. Increasing bulk density decreases porosity and aeration space and increases substrate available water and remaining water (Yakti *et al.*, 2023). This is potentially caused by the increase in substrate particles occupying more air space due to the increase in mass, which leads to the reduction of porosity and change of pore size distribution. Low volume of substrates requires great care in irrigation management to prevent water deficit (Isa *et al.*, 2021). Lu *et al.* (2021) observed that the pot size greatly influenced the amount of water available for transpiration; the total average water transpired from the 2.3 L and 4.1 L pots was equivalent to 1.20 L and 1.24 L, respectively. The pot size can affect the formation of new roots (Yakti *et al.*, 2023). Plants exposed to higher CO₂ partial pressures of 650 microbars and larger pots formed more shoot and root biomass as root growth was less constrained by the pot size than plants exposed to lower CO₂ levels and small pot size (Million & Yeager, 2022).

2.2.5.2 Losses associated with the plant bag size

The limited rooting space in small pots stunt the growth of plant shoots because plants produced few and small tillers in small pots (Sondhi, 2023). In potatoes (*Solanum tuberosum* L.), a large pot (3.8 L) had a significantly higher number of mini-tubers and total weight per pot than medium (2.0 L) and small (1.5 L) pots sizes (Balali *et al.*, 2008). Inadequate rooting volume reduces the photosynthetic capacity of plants (Benamirouche *et al.*, 2020). When root growth is restricted by pot size, the carboxylation efficiency occurs in response to CO₂ enrichment, indicating that ribulose-1,5-bisphosphate carboxylase/oxygenase activity may be responsive to plant source-sink balance instead of CO₂ concentration as a single element (Lu *et al.*, 2021). The whole-plant physiology and developmental regulations are governed by a complex network of interactions in hormonal pathway crosstalk between the different phytohormones (Raviteja *et al.*, 2021; Wazny *et al.*, 2021).

2.3 COMPREHENSIVE OVERVIEW OF PHYTOHORMONES AS ENHANCING BIOSTIMULANTS IN HORTICULTURE

2.3.1 Common biostimulants used in South Africa for individual, combined and multiple stress factors on horticultural plants

Biostimulants are natural bioactive substances or microorganisms with organic and or inorganic compounds to improve nutrient use efficiency, biotic and abiotic stress resistance, as well as quality attributes of crops (Khetsha *et al.*, 2022). Beneficial microorganisms include bacteria that produce microbial biostimulants such as plant growth-promoting rhizobacteria (PGPR), beneficial mycorrhizal fungi and non-mycorrhizal fungi, bacterial endosymbionts, and N₂-fixing bacteria that belong to the genera *Rhizobium*, *Azotobacter*, and *Azospirillum* (Tadele & Zerssa, 2023). Bioactive substances are products manufactured with single or several biological origin components, such as seaweed extracts, animal and vegetal protein hydrolysates, and silicon, which may not be depicted entirely in the composition (Afonso *et al.*, 2022).

The nature of biostimulants is classified according to the mode of action, the origin of active components used, and their effect on the physiological variables of plants, besides their composition (Ahmed *et al.*, 2023). The main categories of biostimulants include organic compounds such as beneficial microorganisms, organic compounds (humic and fulvic acid, macroalgae seaweed extracts and botanicals, vegetal protein hydrolysates and animal), and N (nitrogen)-containing compounds (peptides, betaines, and polyamines), chitosan and biopolymers derivatives, inorganic compounds (Si, Cu, Al, Se, and Na) (Franzoni *et al.*, 2022; Afonso *et al.*, 2022; Ahmed *et al.*, 2023; Mandal *et al.*, 2023).

Beneficial bacteria and fungal biostimulants act on plant metabolism by enhancing crop nutrient uptake (Mandal *et al.*, 2023). Arbuscular mycorrhizal fungi and Trichoderma fungi are the most commonly used microbial biostimulants. These fungi produce particularly bioactive volatiles and combat phytopathogens in the rhizosphere (Mandal *et al.*, 2023).

Seaweed extracts are represented by red, green, or brown macroalgae, usually collected from the sea and oceans (Jiménez-Arias *et al.*, 2022; Arif *et al.*, 2023). Seaweed biostimulants contain macronutrients and micronutrients in certain amounts, which can influence plant nutrient acquisition from the soil or other growing substrates (Wazny *et al.*, 2021). *Ascophyllum nodosum* is commonly used in seaweed extracts as an effective biostimulant.

Protein hydrolysates (PHs) are widely used non-microbial biostimulants. Protein hydrolysates comprise amino acids and peptides (oligopeptides and polypeptides) produced through chemical or enzymatic hydrolysis of proteins emanating from plant biomass or animal sources (Malécange *et al.*, 2023). Animal PH biostimulants are derived using animal-derived materials such as bone meal, chicken feathers, animal tissue, skin collagen, and fish debris. In contrast, PH biostimulants of plant origin are made from plant material such as fruit and vegetable debris and alfalfa hay (Malécange *et al.*, 2023). Carillo *et al.* (2022) treated plants with a legume-derived PH biostimulant Trainer[®], which enhanced photosynthetic activity, increased the number of leaves, and improved the nutrient uptake of P, S, and K without phytotoxicity effects.

Humic substances are organic compounds biologically produced by microbial activity in the soil during the decomposition of dead plants, animals, and microbial fragments (Kamran *et al.*, 2023; Ahmed *et al.*, 2023). Fulvic acids are organic components with low molecular weight formed through the deterioration of plants and animal fossils (Gedeon *et al.*, 2022). Kamran *et al.* (2023) documented that humic acid and fulvic acid effectively increased the total phenols, flavonoids, antioxidant activity, and nutrient content of the leaves, nutrient availability on soil, maximised rhizobium activity, increased root size, nodulation capability, nutrient uptake, yield, and quality.

Biostimulants are used in production of almost all plants with high economic value, such as horticultural crops, grains, and oilseeds, to increase yield, promote quality, and enhance resilience under adverse environmental conditions in protected production and in the fields (Mandal *et al.*, 2023). According to Ahmed *et al.* (2023), biostimulants are used globally in agriculture, and their market size is growing daily, increasing at a 10.2% average annual rate. Biostimulants can be applied to soil, seeds, and plants and are regarded to be more environmentally and human-friendly than xenobiotic agrochemicals because biostimulants do not accumulate over time, have low toxicity and are less susceptible to some resistant strains of pests and pathogens that may attack crops (Cellini *et al.*, 2020). Applying biostimulants during the initial stages of plant development promotes root growth, allows rapid recovery after moisture stress, and promotes rapid and uniform plant establishment (Clément *et al.*, 2023).

Although most of these biostimulants are effective, some inorganic compounds like cobalt, aluminium, sodium, and silicon are not essential for all plants when used as biostimulants (Ma *et al.*, 2022). Some biostimulants are only effective in plants grown under

optimal conditions, while some only focus on ameliorating the negative effects of stressed plants. For example, a review by Cristofano *et al.* (2021) indicates that, as a biostimulant, silicon has not been proven to increase plant growth, performance, and product quality in adequate growing conditions. Concerning microorganisms, most bacterial genera cannot be used as commercial biostimulants as designing, establishing, and developing efficient commercial formulations of microbial inoculants is the key aspect that delays the implementation of beneficial microbes in agriculture (Ma *et al.*, 2022). Overall, other biostimulants have insufficient information available about their composition, which is vital in describing their molecular and biochemical mechanisms of action (Franzoni *et al.*, 2022).

On the other hand, biostimulants containing phytohormones are important regulators of plant processes related to responsive mechanisms for plant tolerance improvement while promoting plant growth (Khetsha *et al.*, 2022). For instance, seaweed extract by-products available on the market contain micro- and macro-nutrients, phytohormones, amino acids, vitamins, fatty acids, bioactive peptides and proteins, and complex polysaccharides (Mattner *et al.*, 2023; Sabatino *et al.*, 2023). Seaweed extracts promote chlorophyll synthesis while inhibiting abscisic acid (ABA) synthesis and maintaining cytokinin concentration (Jiménez-Arias *et al.*, 2022). Some beneficial microorganisms release hormonal active metabolites, for example, cytokinins and auxins, which encourage root growth (Mandal *et al.*, 2023). Besides the commercial biostimulant product composition, containing phytohormone traces, the dose determines plant-responsive outcomes (Mattner *et al.*, 2023). For example, a study on lettuce grown under optimal conditions and treated with Cytolan® Stress with salicylic acid reduced the contents of carotenoids and chlorophyll fluorescence parameters when treated with the highest dose (500 mL/hL) (Velasco-Clares *et al.*, 2024). In contrast, the chlorophyll fluorescence parameters increased at low and intermediate doses (150 and 300 mL/hL). Recently, the exogenous application of the phytohormones MeJA and ABA has been reported to recuperate plant wounds and promote yields, particularly post-hail damage biosynthesis and rose geranium essential oil yield (Khetsha *et al.*, 2024).

2.3.2 Phytohormone-based biostimulants as a potential abiotic stress mitigation in horticultural plants

Phytohormones are small organic molecules produced naturally by plants or synthesised in laboratories to induce metabolic activities and regulate local plant growth processes within plant cells at low endogenous concentrations (Khetsha *et al.*, 2022; Verma *et al.*, 2022).

Auxins, cytokinins (CKs), gibberellins (GAs), indole acetic acid (IAA), brassinosteroids (BRs), ethylene (ET), and strigolactones (SLs) are phytohormones categorised under plant growth regulators and their biological actions differ from plant growth regulators (Iqbal *et al.*, 2020; Chen *et al.*, 2020; Jiménez-Arias *et al.*, 2022). However, abscisic acids (ABA), jasmonic acid (JA), and salicylic acid (SA) are categorised as bio-inhibitors (Verma *et al.*, 2022; Wei *et al.*, 2023). Phytohormones can induce an array of physiological and biochemical processes and regulate gene expression for adaptive plant responses to abiotic and biotic stresses (Turan *et al.*, 2021; Clément *et al.*, 2023).

The artificial application of these phytohormones in stressed plants influences the accumulation of secondary metabolism and participation in defence responses (Mostafa *et al.*, 2022). For example, the foliar application of JA and SA triggered the most vigorous systemic resistance against the Tobacco Mosaic Virus in *Nicotiana benthamiana* plants (Kudoyarova, 2022). Plant hormone signalling and accumulation are altered to sustain and facilitate plant growth under environmental stresses. Nepthali *et al.* (2020) indicated that phytohormones influence plant growth and development by integrating numerous stress signalling molecules that trigger complex signalling cascades and control the transcription of stress-responsive genes, eventually leading to multiple stress tolerance. A complex network of synergistic and malicious interactions is influenced by the signalling pathways of one another (Nepthali *et al.*, 2020). The ROS, H₂O₂, NO, and plant growth regulators (JA, SA, ET, ABA, GA, and auxins, CKs, BRs, and SLs) are responsible for the plant signalling pathways that alter the expression of defensive genes, which results in modifications at the metabolic level and improves plant defence (Zhang *et al.*, 2020; Cunha *et al.*, 2023, Baharudin & Osman, 2023).

2.3.2.1 Absciscic acid

The phytohormone ABA plays an important role during plant adaptation to environmental stress (Arif *et al.*, 2023), and the same is expected under water stress, soil bulk density, and plant mechanical wounding. Absciscic acid is also vital in responding to moisture stress through morphophysiological processes such as stomatal conductance, cuticular wax accumulation, leaf senescence, bud dormancy, seed germination, osmotic regulation, root development, and growth inhibition (Chen *et al.*, 2020). The response to water stress is connected to the action of ABA, which accumulates during moisture stress and attaches to its cognate receptor family Pyrabactin Resistance1 (PYR1)/PYR1-LIKE (PYL)/Regulatory Components of ABA Receptors (RCAR) (Wielkopolan *et al.*, 2022). Huang *et al.* (2022)

measured ABA levels in rice root tip tissues on plants grown in non-compacted soil with a bulk density of 1.1 g cm^3 and compacted soil with a bulk density of 1.6 g cm^3 , which revealed that ABA levels increased threefold in root tips growing in compacted versus non-compacted soil conditions. ABA biosynthesis is thus required to encourage radial root extension in response to high bulk-density conditions (Huang *et al.*, 2022).

Protein receptors (PYR/PIL/RCAR) bind to ABA in the ABA-dependent signalling pathway by suppressing PP2C activity, which enables SnRK2 activation through autophosphorylation (González-Morales *et al.*, 2021). Then ABA-PYR/PYL complex binds to PROTEIN PHOSPHATASES 2C (PP2Cs) and consequently hinders PP2Cs, whereby, in the lack of ABA, dephosphorylate retains subclass III Sucrose Non-fermenting-1 (SNF1)-Related Protein Kinases2 (SnRK2s) are inactivated (González-Morales *et al.*, 2021). Activated SnRK2s phosphorylate transcription factors called Absciscic Acid-Responsive Element (ABRE) Binding Proteins (AREBs)/ABRE Binding factor (ABFs) regulate the manifestation of target genes to improve plant resistance to deficit stress (Li *et al.*, 2020). The ABA homologous responsive transcript-derived fragments in wheat leaves such as IA19, IA20, and IA24, and thus, IA20 encodes transcription factors WRKY20, which regulates ABA signalling and improves water stress tolerance (González-Morales *et al.*, 2021). Another signalling pathway that water and osmotic pressure activate is ABA-independent and involves Growth-Regulating Factor7 (GRF7) (Zhang *et al.*, 2022).

During water deficiency, ABA plays a primary role in the rapid response to stomatal closure governed by a complex network of signalling pathways (Yari Kamrani *et al.*, 2022). ABA, ethylene phytohormones, and H_2O_2 and calcium ions signal messengers can be involved in guard-cell signal transduction and stomatal mobilisation (Zhang *et al.*, 2020). Water deficit triggers ABA accumulation, which activates response mechanisms through diverse ABA-responsive genes, resulting in the immediate closure of stomata and synthesis of osmoprotectants. In an experiment by Jiménez-Arias *et al.* (2022), water deficit induced SINCED2 transcription in tomato seedlings after 10 hours of treatment; this is an ABA biosynthetic pathway gene that encodes 9-cis-epoxycarotenoid dioxygenase. Therefore, this caused an evident ABA accumulation in seedling leaves after 24 hours of treatment. DWARF14 (D14) and KARRIKIN INSENSITIVE 2 (KAI2) are strigolactone and karrikin receptors, which regulate water deficiency resistance entirely in *Arabidopsis thaliana* by modulating ABA responsiveness, anthocyanin proliferation, stomatal closure, cell membrane

integrity, and cuticle building up (Li *et al.*, 2020). The root restrictions in the small pots induced abscisic acid production by the roots (Turner, 2019).

2.3.2.2 Cytokinin

The CKs significantly respond to abiotic stresses such as temperature, water, osmotic, and salt stress, and biotic stress such as specific plant pathogens and herbivores (Hai *et al.*, 2020). Cytokinins regulate cell division, chloroplast greening, and other important processes and are the likely biostimulants responsible for growth enhancement (Jiménez-Arias *et al.*, 2022). Numerous organs, including plants and pathogens, obviously use adenine derivatives as signalling molecules contained by the CKs hormone (González-Morales *et al.*, 2021). Hence, CKs are an optimal molecule for communication between phytopathogens and plants (Cellini *et al.*, 2020). The CKs signalling has developed into an intercellular transmission network system that is required for crosstalk with other types of phytohormones and their regulating pathways in mediating plant response to water deficit stress (Hai *et al.*, 2020).

The CKs signalling in plants includes two classical component approaches comprising histidine kinases and histidine phosphotransfer proteins (Hai *et al.*, 2020). In *Arabidopsis thaliana*, the D14 pathway regulates cytokinin and brassinosteroid metabolism (Li *et al.*, 2020). In contrast, photosynthesis and metabolism of glucosinolates and trehalose are regulated by both D14 and KAI2 pathways in plant response to water scarcity (Li *et al.*, 2020). In tomatoes, auxin and cytokinin act simultaneously but at different positions during the healing process of grafts by acting above and below the graft junction (Cui *et al.*, 2021). The application of a biostimulant on tomato plants under water deficit helped antioxidant defence and exercised a significant hormonal consequence in leaves by raising the endogenous content of indole-3-acetic acid (IAA), *trans*-zeatin (cytokinin), and jasmonic acid (Turan *et al.*, 2021).

2.3.2.3 Salicylic acid

The SA is a signalling molecule naturally found in plants and involved in plant defence-related actions against infection by various pathogens (Cunha *et al.*, 2023). Salicylic acid is a phenolic endogenous growth regulator that controls plant physiological processes, such as regulating ion uptake by plant roots and influencing stomatal conductivity (Khattak *et al.*, 2021). Tomato plants treated with PH biostimulant showed enhanced tolerance to ROS-mediated oxidative inequality, such as collaborative action of SA, hydroxycinnamic amide signalling, carotenoids, prenyl quinone radical scavenging, and declined tetrapyrrole

biosynthesis (Hossain *et al.*, 2022). Aazami *et al.* (2023) soaked wheat seeds in 0.05 mM SA solution for three hours, and the results revealed that SA treatment ultimately prevented the accumulation of ABA and the diminishing of the concentration of IAA and CKs in seedlings induced by salinity and water deficit. This indicates that pre-sowing treatment with SA may be necessary for wheat seedlings to recover and resume proliferating under water deficit and salinity stresses.

An experiment conducted by Li *et al.* (2020) on *Arabidopsis* plants under dry conditions showed that the mechanism relating to photosynthetic processes and the metabolism of glucosinolate, Tre, CKs, and BRs are moderated particularly by SL and KAR/KL pathways in plant responses to water-deficit conditions. Overexpression of oryza sativa flavanone 3-hydroxylase (OsF3H) reduced the accumulation of SA in drought and UV-B radiation stress in rice (Jan *et al.*, 2022). The SA moderates the reaction to water stress by regulating the production of ROS and the redox balance by antagonistically depressing ABA (Khattak *et al.*, 2021). The SA-dependent signalling pathway is activated in response to herbivores and biotrophic pathogens that typically cause tissue disruption in plants, such as piercing-sucking (Wielkopolan *et al.*, 2022). Khattak *et al.* (2021) evaluated the effects of SA on the growth of sunflowers grown under water deficit stress. They reported that the foliar spray of SA at 5 mg L⁻¹ rate effectively alleviated the negative effect of water deficiency stress on the growth of sunflower varieties, achene quality, and biodiesel yield.

2.3.2.4 Jasmonic acid

Jasmonate is a lipid-derived hormone synthesised from α -linolenic acid. Herbivore insect feeding damages plant tissue and generates a wounding response interceded by JA, synthesising protective substances such as proteinase inhibitors and polyphenol oxidases within the plant (Meents *et al.*, 2019; Cunha *et al.*, 2023). The JA signalling pathway is key for the growth responses to lesions (Cunha *et al.*, 2023). The JA controls the generation and emission of herbivore-induced plant volatiles, which produce and release terpenoids or fatty acid derivatives from green leaf volatiles and attract herbivore parasitoids and predators that help to control the infestation (Meents *et al.*, 2019).

In a study on wheat by Guan *et al.* (2022), transcripts/gene products (Chlorophyll a-b binding protein 1B-21, chloroplastic/*Hordeum vulgare*) involved in metabolism and photosynthesis convey a high percentage in response to aphid feeding and mechanical

wounding, by 40% and 39%, respectively. After aphid feeding and mechanical wounding in plant leaves, a gene encoding ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCo) small subunit was routinely up-regulated (Guan *et al.*, 2022). In rice, the antisense expression of OsPLD4 and -5 lowered the expression levels of OsHI-LOX, OsMPK3, OsHPL3, and OsACS2 genes under water deficit (Jan *et al.*, 2022). Following this, OsPLD4 and OsPLD5 expression may impact the oxylipin pathway by simulating late embryogenic abundant, the typical substrate for biosynthesis of JA and green leaf volatiles (GLVs), or altering lipoxygenase activity, mitogen-activated protein kinase 3 signalling, which, in turn, controls the biosynthesis of JA and GLVs (Wielkopolan *et al.*, 2022). In the JA-dependent signalling pathway, the more elevated expression level of the genes encoding the NOVEL INTERACTOR OF JAZ protein regulators, which suppress the transcription factors that regulate the expression of JA-responsive gene, was reported in the plants damaged by the larvae with natural bacterial flora (Wielkopolan *et al.*, 2022).

2.3.2.5 Auxins

The auxins phytohormone conforms to numerous crucial processes in plant growth, development, and environmental adaptation (Jogawat *et al.*, 2021). In various physiological processes, the functions of auxins can be comprehensively regulated via three primary regulatory actions: auxin-directed transport, signal transduction, and auxin biosynthesis and inactivation (Zhang *et al.*, 2022). Auxins act as a chemical messenger that influences the expression of genes via the nuclear signalling module identified as Skp1/Cul1/F-box protein-auxin/indole acetic acid-auxin response factor (SCFTIR1/AFB-Aux/IAA-ARF) transcription (Verma *et al.*, 2022). Auxin penetration normally initiates with TIR1/AFB receptors (Jogawat *et al.*, 2021). Auxin receptor, the F-box proteins AFB1-AFB5/TIR1 (DNA ligases) activate the type of AUXIN RESPONSE FACTOR (ARF) transcription factors, as well as the auxin/indole acetic acid-auxin transcriptional repressors (Cui *et al.*, 2021).

The ARFs genes are vital in regulating the adaptative response of plants to water stress. A large group of responsive genes, including auxin-responsive, induced proteins, ARFs, small auxin-upregulated RNA (SAUR), auxin-responsive Gretchen Hagen3 (GH3), and YUCCA genes are involved in auxin signalling pathways (Jogawat *et al.*, 2021). The transcription factor WUSCHEL-related homeobox 11 (WOX11) functions largely with WOX12 to allow the transition of the local cambium cells to root founder cells within four days after the cut, which is directly triggered by the accumulation of auxin in one day at the wound site (Vega-Muñoz

et al., 2020). Auxin regulates gene transcription through Aux/IAA proteins, can interact with ARF transcription factors, and hinders auxin signalling (Jogawat *et al.*, 2021). In an experiment by Cui *et al.* (2021) with auxin response regulators, IAA was correlated with *SLARF4* as well as *SLARF10B*, ME-IAA linked with *SLARF6B* and *SLARF10B*, and ICA1d with *SLARF5* and *SLARF7B*. This reveals that the *AUX/IAA* gene family-related genes related to IAA and ME-IAA were moderately similar.

Auxin is transported by two dissimilar linked systems in higher plants, namely a non-directional flow with photoassimilates in the phloem and slowly directed intercellular polar auxin transport (PAT) (Zhang *et al.*, 2022). In addition, the polar auxin transport (PAT) creates an auxin gradient that controls vital processes in organ patterning, cell division, and elongation (Verma *et al.*, 2022). Reactivating cell division is necessary for tissue reunion after perforation or grafting to bridge the gap and permit vascular tissue reconnection (Vega-Muñoz *et al.*, 2020). Cui *et al.* (2021) reported that the polar auxin transport of *PIN1* and *PIN6* map in the drafting process was improved at 3–12 HAG, which can elucidate that the expression of snRNA-related genes is related to the accumulation of auxin above the graft junction wound in tomato plants. Auxin accumulates at the wound site and promotes the restoration of lost tissue for wound repair (Vega-Muñoz *et al.*, 2020).

2.3.2.6 Ethylene

The ET production in plants can be regulated during the occurrence of both biotic and abiotic stress. Plants produce ET during germination, leaf abscission, fruit ripening, and floral senescence developmental stages, and it can be secreted in roots, seeds, leaves, flowers, and even in fruits (Baharudin & Osman, 2023). The C_2H_4 or $H_2C=CH_2$ is a formula representing the flammable colourless gas ethylene. Plant roots use the gaseous hormone ethylene to sense soil compaction (Huang *et al.*, 2022). In a lettuce study, Aires *et al.* (2020) reported that ETHYLENE RESPONSE FACTOR1 transcription factor was absorbed under high-temperature stresses and assisted lettuce in germinating. ETHYLENE RESPONSE FACTOR1 is a transcription factor involved in the ET signalling pathway.

In lettuce, ethylene and auxin-related genes were upregulated at day 0 of postharvest cutting, by 55–60%, respectively, whereas 26% was upregulated at day 7 (Mostafa *et al.*, 2022). These observations indicate a vigorous mobilisation of auxin and ET responding pathways instantly after the cut stress. The ET and JA signalling pathways primarily defend plants against

insect herbivores and necrotrophic pathogens (Wielkopolan *et al.*, 2022). However, ET and ABA regulate plant responses to herbivores by modifying JA signalling components (Wielkopolan *et al.*, 2022).

Cui *et al.* (2021) researched the effect of the exogenous application of IAA and 6-BA on the xylem and phloem reconnection after tomato grafting. They found that both hormones initially facilitated xylem and phloem reconnection and hampered their reconnection at a later stage as the concentration increased. On faba bean (*Vicia faba* L.), Wei *et al.* (2023) observed that 45 mg l dose of IAA promotes the chlorophyll a, chlorophyll b, and carotenoid contents of leaves by 25%, 14%, and 27%, respectively compared to control. Furthermore, these authors reported that applying IAA significantly promoted chlorophyll fluorescence, stomatal conductance, net photosynthetic rate, transpiration rate, and relative water content of bean plant leaves under regular and water deficit conditions. Declined ethylene dispersal in compacted soil induces ethylene signalling to accumulate in the root tip, activating the stabilisation of ethylene-insensitive mutants (OsEIL1), which upregulates the expression of the auxin biosynthesis gene (OsYUC8) (Huang *et al.*, 2022).

2.3.2.7 Brassinosteroids

The sterol-like hormones teasterone, brassinolide, and cathasterone are the active BRs, connected to several signalling networks, including abiotic stress response, cell wall development, and lignification (Jan *et al.*, 2022; Aazami *et al.*, 2023). The BRs biosynthesis-related genes *CYP90A1* and *CYP90D1* are suppressed by water deficit, indicating that plants adapt to drought by lowering BR concentration (Li *et al.*, 2020). Brassinosteroids also interact and crosstalk with other phytohormones such as ABA, GA, JA, SA, ET, SLs, auxin, and polyamines to regulate plant developmental and physiological processes (Aazami *et al.*, 2023; González-Orenga *et al.*, 2023). Researchers identified that BRs balance ROS production, JA signal, and the ABA response and are involved in the resistance to water stress and osmotic stress-induced stomatal closure (Kumar *et al.*, 2020; González-Orenga *et al.*, 2023). In a maize study, Gerhards *et al.* (2021) discovered that BRs functioned as regulating agents involved in producing vitamin E, which indirectly secured cell membranes from free radical harm, particularly under water stress. The authors also found that BRs are involved in plant growth and development, particularly root development, through cell elongation and division. Brassinosteroid hormones regulate senescence mechanisms in fresh-cut vegetables by changing the lipid composition of cells (Li *et al.*, 2020).

In sweet potato plants, Zhang *et al.* (2022) identified a total of 175 putative *IbNAC* genes, 15 chromosomes, and numerous hormone-related cis-elements, such as ABA-responsive elements (ABRE), auxin-responsive elements (TGA-box and AuxRE), SA responsive elements (TCA-element), and GA responsive elements (P-box, GARE-motif, and TATC-box) were found in approximately 94% of the promoters. More inferior expression levels of the transcripts encoding WRKY62, NPR1-type protein 2, PR-4-like, and MAPK 3 were observed in wheat leaves within defence response to feeding of larvae not treated with antibiotics compared to the plants wounded by antibiotics-treated larvae (Wielkopolan *et al.*, 2022).

2.3.2.8 Gibberellins

The GAs hormones are found in many plant tissues and major species of fungi. Gibberellins are a diverse group of tetracyclic diterpenes containing diterpenoid tetracyclic acids (Seema *et al.*, 2023; Qin *et al.*, 2024). The GAs comprise several hormones, such as GA₁, GA₃, GA₄, GA₇, GA₁₂, GA₁₅, and GA₂₀ (Seema *et al.*, 2023; Lupepsa *et al.*, 2024). These GAs hormones improve proline biosynthesis and ROS scavenging in drought stress tolerance (Seema *et al.*, 2023; Qin *et al.*, 2024). The most abundant hormone is GA₃ (GA₃ β-hydroxylase (3-oxidase)) due to its special role of stimulating cell elongation and promoting seed germination (Seema *et al.*, 2023). The GAox (gibberellin oxidase) gene family members have important enzymes that activate GAs (Qin *et al.*, 2024).

The role of GAs is to promote the activity of enzymes such as carbonic anhydrase and nitrate reductase (Ahmad *et al.*, 2024). The GA controls important aspects of plant development and growth through its capabilities to stimulate cell growth and cell division in plants (Lupepsa *et al.*, 2024). Gibberellins acts as signalling molecules in seed germination, elongation growth, flowering, and water uptake (Chen *et al.*, 2020). In a study conducted in oregano (*Origanum vulgare* L.), Yolcu (2024) reported that the foliar applications of GA increased the seedling length, chlorophyll pigments, and essential oil content. Seema *et al.* (2023) indicated that the GAs-producing endophytic *F. proliferatum* increased the shoot length, dry weight, and chlorophyll *a* and *b*, total proline production while reducing the total phenolic content of sunflower seedlings exposed to 8% polyethylene glycol drought stress. In the same study, authors observed a decrease in the antioxidant activity of 2, 2-diphenyl-1-picrylhydrazyl, and 2-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid when treated with fungus alone.

A review by Lupepsa *et al.* (2024) provided sufficient evidence indicating a gibberellins-induced modulation of antioxidant activity and a subsequent reduction in oxidative stress induced by heavy metals, drought, salinity, extreme cold or hot temperatures. During salinity stress, the GA and nitric oxide (NO) signalling components interact antagonistically, whereby the NO-induced S-nitrosylation negatively modulates the GA signalling to maintain the balance between plant growth and plant stress (Ahmad *et al.*, 2024). Additionally, this antagonism is validated by NO-induced down-regulation of an essential GA biosynthesis enzyme encoding gene known as GA20oxidase3. Phytohormones are essential components of the biostimulants used by the plant industry (Tadele & Zeressa, 2023).

2.3.2.7 Commercial biostimulants containing phytohormones

The positive effects of plant biostimulants with traces of phytohormones on yield, fruit diameter, length, chlorophyll content, and overall functional quality have been reported on horticultural crops such as cherry tomatoes (Turan *et al.*, 2021), pepper (*Capsicum annuum* L.) and spinach (*Spinacia oleracea* L.) (Cellini *et al.*, 2020). Biostimulants have been authenticated to stimulate plant growth, development, and pathogen defence and decrease the spread and severity of some diseases (Turan *et al.*, 2021; Habib *et al.*, 2021). For example, under nutrient deficiency, drought, and weed competition, the application of ComCat[®] increased total chlorophyll by 30% and total antioxidant capacity, phenolics, and flavonoid content by 33% in spinach at an application rate of 0.1 g L⁻¹ (Gerhards *et al.*, 2021).

In addition, Bayram (2024) reported that the MC-Extra[®] biostimulant derived from *Ascophyllum nodosum*, composed of organic N (1%), K₂O (20%), organic C (20%), betaines (0.2%), mannitol (4%), and cytokinins increased stem diameter, fresh weight, dry weight, leaf area and seedling length on tomato plants and leaf chlorophyll content, root fresh weight, and photosynthesis rate on pepper plants (*Solanaceae*). Moreover, Cirillo *et al.* (2024) declared that a commercial biostimulant, Aminozone Ultra, composed of nitrogen (N), including soluble organic nitrogen, organic carbon (C), amino acids, micronutrients, vitamins, polysaccharides, and plant-origin auxins increased apricot fruit weight, production per plant, and yield efficiency by 28%, 53.80%, and 56.38%, respectively. Numerous studies demonstrated that commercial biostimulants with phytohormones have beneficial and protective functions on horticultural plants grown under abiotic stress conditions such as water deficit, drought, heat stress, salinity, herbicide application, stressed soil, and nutrient deficiency stresses (Table 2.1).

2.4 CONCLUSION

Observations from several studies revealed that the morphophysiological, biochemical, and molecular responses of plants to multiple combined stress factors are unique and cannot be extrapolated from plant response to individual stress. Plant physiology research studies therefore aim to understand plant response to multiple stress factors better and to improve plant resistance. Interestingly, biostimulants and bioactive compounds have become a progressive, adaptive, and mitigation strategy for biotic and abiotic stress.

REFERENCES

- Al Murad M, Khan AL, Muneer S. 2020. Silicon in horticultural crops: Cross-talk, signaling, and tolerance mechanism under salinity stress. *Plants* 9: 460.
- Al-Taey DK, Burhan AK. 2022. The effect of water quality, cultivar, and organic fertilizer on growth and yield of volatile oil carvone and limonene in Dill. *International Journal of Vegetable Science* 28: 342-348.
- Chowdhury M, Kiraga S, Islam MN, Ali M, Reza MN, Lee WH, Chung SO. 2021. Effects of temperature, relative humidity, and carbon dioxide concentration on growth and glucosinolate content of kale grown in a plant factory. *Foods* 10: 1524.
- Mufti S, Mushtaq F, Afroza B, Narayan S, Bhat R. 2020. High-value vegetable crops for economic development. *Division of vegetable science SKUAST - Kashmir* 33-39.
- Syeed MM, Hossain MS, Karim MR, Uddin MF, Hasan M, Khan RH. 2023. Surface water quality profiling using the water quality index, pollution index and statistical methods: A critical review. *Environmental and Sustainability Indicators* 18: 100247.
- Aazami MA, Maleki M, Rasouli F, Gohari G. 2023. Protective effects of chitosan based salicylic acid nanocomposite (CS-SA NCs) in grape (*Vitis vinifera* cv. 'Sultana') under salinity stress. *Scientific Reports* 13: 883.
- Abarca VHA, González-Sandoval DC, Martínez-Ávila GCG, Rojas R. 2022. Innovation in agriculture: A review of plant factory. *Biocontrol Systems and Plant Physiology in Modern Agriculture* 221-236.
- Abedrabbah O, Koç M, Biçer Y. 2023. Sustainable food development for societies in hot arid regions: Thermoeconomic assessment of passive-cooled soil-based and hydroponic greenhouses. *Journal of Cleaner Production* 412: 137250.
- Abid R, Qaiser M. 2023. Distribution pattern and ecology of *Lactuca* L. and its allied genera (*lactucineae–cichorieae*) in Pakistan and Kashmir. *Pakistan Journal of Botany* 55: 159-169.
- Afonso S, Oliveira I, Meyer AS, Gonçalves B. 2022. Biostimulants to improved tree physiology and fruit quality: a review with special focus on sweet cherry. *Agronomy* 12: 659.
- Afroza B, Rana MK. 2017. Lettuce. *Vegetable Crops Science* 169–182.

- Ahmad A, Ahmad I, Ferdosi MFH. 2020. Indigenous soilless substrate compositions affect growth, yield and quality of cut *Antirrhinum majus*. *Sarhad Journal of Agriculture* 36: 586-592.
- Ahmad B, Mukarram M, Choudhary S, Petřík P, Dar TA, Khan MMA. 2024. Adaptive responses of nitric oxide (NO) and its intricate dialogue with phytohormones during salinity stress. *Plant Physiology and Biochemistry* 208: 108504.
- Ahmed HA, Tong Y, Li L, Sahari SQ, Almogahed AM, Cheng R. 2022. Integrative effects of CO₂ concentration, illumination intensity and air speed on the growth, gas exchange and light use efficiency of lettuce plants grown under artificial lighting. *Horticulturae* 8: 270.
- Ahmed W, Shah AN, Abbas A, Nawaz M, Qayyum A, Hassan MU, Mansoor M. 2023. Role of plant bio-stimulants and their classification. *Biofertilizers for Sustainable Soil Management* 65-88.
- Aires ES, Aragão CA, Dantas BF, Rodrigues JD, Ono EO. 2021. Light intensity modulates the accumulation of carbohydrates, antioxidant enzymes and production of iceberg lettuce under tropical conditions. *Horticulturae* 7: 553.
- Aires ES, Aragão CA, Gomes ILS, Souza GN, Andrade IGV, Oliveira ABN, and Yuri JE. 2020. Growth and production of crisphead lettuce cultivars in protected cultivation and high temperatures. *Brazilian Journal of Agricultural Sciences* 15: 1-9.
- Al-Khayri JM, Rashmi R, Toppo V, Chole PB, Banadka A, Sudheer WN, Nagella P, Shehata WF, Al-Mssallem MQ, Alessa FM, Almaghasla MI. 2023. Plant secondary metabolites: the weapons for biotic stress management. *Metabolites* 13: 716.
- Al-Taey DK, Al-Naely IJ, Kshash BH. 2019. A study on effects of water quality, cultivars, organic and chemical fertilizers on potato (*Solanum tuberosum* L.) growth and yield to calculate the economic feasibility. *Bulgarian Journal of Agricultural Science* 25: 1239-1245.
- Ali O, Ramsubhag A, Jayaraman J. 2021. Biostimulant properties of seaweed extracts in plants: implications towards sustainable crop production. *Plants* 10: 531.
- Alu'datt MH, Rababah T, Alhamad MN, Al-Tawaha A, Al-Tawah, AR, Gammoh S, Ereifej KI, Al-Karaki G, Hamasha HR, Tranchant CC, Kubow S. 2019. Herbal yield, nutritive composition, phenolic contents and antioxidant activity of purslane (*Portulaca*

- oleracea* L.) grown in different soilless media in a closed system. *Industrial Crops and Products* 141: 111746.
- Amare DG, Abebe ZK. 2020. Review on the effect of irrigation interval on different crop production. *International Journal of Plant and Soil Science* 32: 1-13.
- Arif Y, Bajguz A, Hayat S. 2023. Moringa oleifera extract as a natural plant biostimulant. *Journal of Plant Growth Regulation* 42: 1291-1306.
- Aslani Z, Hassani A, Mandoulakani BA, Barin M, Maleki R. 2023. Effect of drought stress and inoculation treatments on nutrient uptake, essential oil and expression of genes related to monoterpenes in sage (*Salvia officinalis*). *Scientia Horticulturae* 309: 111610.
- Awolu O, Oladeji O. 2021. Natural plant pigments and derivatives in functional foods developments. *Eurasian Journal of Food Science and Technology* 5: 25-40.
- Azarmi-Atajan F, Sayyari-Zohan MH. 2020. Alleviation of salt stress in lettuce (*Lactuca sativa* L.) by plant growth-promoting rhizobacteria. *Journal of Horticulture and Postharvest Research* 3: 67-78.
- Azizi A, Bagnazari M, Mohammadi M. 2024. Seaweed and phosphate-solubilizing bacteria biofertilizers ameliorate physiochemical traits and essential oil content of *Calendula officinalis* L. under drought stress. *Scientia Horticulturae* 328: 112653.
- Baharudin NF, Osman NI. 2023. Plant development, stress responses and secondary metabolism under ethylene regulation. *Plant Stress* 7: 100146.
- Balali GR, Hadi MR, Yavari P, Bidram H, Naderi AG, Eslami A. 2008. Effect of pot size, planting date and genotype on minituber production of Marfona potato cultivar. *African Journal of Biotechnology* 7: 1265-1270.
- Balliu A, Zheng Y, Sallaku G, Fernández JA, Gruda NS, Tuzel Y. 2021. Environmental and cultivation factors affect the morphology, architecture and performance of root systems in soilless grown plants. *Horticulturae* 7: 243.
- Bayram CA. 2024. The effects of mycorrhiza and seaweed applications on seedling growth parameters: tomato and pepper. *Communications in Soil Science and Plant Analysis* 55: 1375-1389.
- Becerra-Moreno A, Redondo-Gil M, Benavides J, Nair V, Cisneros-Zevallos L, and Jacobo-Velázquez DA. 2015. Combined effect of water loss and wounding stress on gene

- activation of metabolic pathways associated with phenolic biosynthesis in carrot. *Frontiers in plant science* 6: 837.
- Benamirouche S, Chouial M, Guechi W. 2020. Radicle length and container size effects on root deformities in the Mediterranean oak *Quercus suber* L. *Bois & Forets Des Tropiques* 343: 17-26.
- Bilal S, Shahzad R, Asaf S, Imran M, Al-Harrasi A, Lee IJ. 2023. Efficacy of endophytic SB10 and glycine betaine duo in alleviating the phytotoxic impact of combined heat and salinity in *Glycine max* L. via regulation of redox homeostasis and physiological and molecular responses. *Environmental Pollution* 316: 120658.
- Caparas M, Zobel Z, Castanho AD, Schwalm CR. 2021. Increasing risks of crop failure and water scarcity in global breadbaskets by 2030. *Environmental Research Letters* 16: 104013.
- Carillo P, De Micco V, Ciriello M, Formisano L, El-Nakhel C, Giordano M, Colla G, Rouphael Y. 2022. Morpho-anatomical, physiological, and mineral composition responses induced by a vegetal-based biostimulant at three rates of foliar application in greenhouse lettuce. *Plants* 11: 2030.
- Chen K, Li GJ, Bressan RA, Song CP, Zhu JK, Zhao Y. 2020. Absciscic acid dynamics, signaling, and functions in plants. *Journal of integrative plant biology* 62: 25-54.
- Chen TW, Gomez Pineda IM, Brand AM, Stützel H. 2020. Determining ion toxicity in cucumber under salinity stress. *Agronomy* 10: 677.
- Chen YL, Fan KT, Hung SC, Chen YR. 2020. The role of peptides cleaved from protein precursors in eliciting plant stress reactions. *New Phytologist* 225: 2267-2282.
- Chida Y, Okamiya Y, Tamura M, Takahashi Y, Usui T, Yoshimura T, Fushiki T, Nakai T, Narisawa Y, Usami T, Nishizawa T. 2020. Development of an automatic lettuce harvester. In *The Proceedings of the International Conference on Motion and Vibration Control 2020* 15: 10120.
- Choi S, Colla G, Cardarelli M, Kim HJ. 2022. Effects of plant-derived protein hydrolysates on yield, quality, and nitrogen use efficiency of greenhouse grown lettuce and tomato. *Agronomy* 12: 1018.
- Ciriello M, Formisano L, Pannico A, El-Nakhel C, Fascella G, Duri LG, Cristofano F, Gentile BR, Giordano M, Rouphael Y, Fusco GM. 2021. Nutrient solution deprivation as a tool

- to improve hydroponics sustainability: yield, physiological, and qualitative response of lettuce. *Agronomy* 11: 1469.
- Cirillo A, Izzo L, Ciervo A, Ledenko I, Cepparulo M, Piscitelli A., Di Vaio C. 2024. Optimizing Apricot yield and quality with biostimulant interventions: a comprehensive analysis. *Horticulturae* 10: 447.
- Clément J, Delisle-Houde M, Nguyen TTA, Dorais M, Tweddell RJ. 2023. Effect of biostimulants on leafy vegetables (baby leaf lettuce and batavia lettuce) exposed to abiotic or biotic stress under two different growing systems. *Agronomy* 13: 879.
- Combrink NJJ, Kempen E. 2019. Nutrient solution management. Department of Agronomy Stellenbosch University.
- Contreras JI, Roldán-Cañas J, Moreno-Pérez MF, Gavilán P, Lozano D, Baeza R. 2021. Distribution uniformity in intensive horticultural systems of almería and influence of the production system and water quality. *Water* 13: 233.
- Coulombe G, Catford A, Martinez-Perez A, Buenaventura E. 2020. Outbreaks of *Escherichia coli* O157: H7 infections linked to romaine lettuce in Canada from 2008 to 2018: An analysis of food safety context. *Journal of Food Protection* 83: 1444-1462.
- Cristofano F, El-Nakhel C, Rouphael Y. 2021. Biostimulant substances for sustainable agriculture: Origin, operating mechanisms and effects on cucurbits, leafy greens, and nightshade vegetables species. *Biomolecules* 11: 1103.
- Cunha AFA, Rodrigues PHD, Anghinoni AC, de Paiva VJ., Pinheiro, DGDS, Campos ML. 2023. Mechanical wounding impacts the growth versus defense balance in tomato (*Solanum lycopersicum*). *Plant Science* 329: 111601.
- Da Silva JLT, Cavalcanti M, Barros JAS. 2023. Stability and adaptability of lettuce cultivars on different substrates. *Comunicata Scientiae* 14: 1-9.
- Daghari I, El Zarroug MR, Muanda C, Shanak N. 2020. Best irrigation scheduling way with saline water and desalinated water: field experiments. *La Houille Blanche* 4: 72-74.
- Dahlawi S, Sadiq M, Sabir M, Farooqi ZUR, Qadir AA, Faraj TK. 2023. Differential response of brassica cultivars to potentially toxic elements and their distribution in different plant parts irrigated with metal-contaminated water. *Sustainability* 15: 1966.

- Damerum A, Chapman MA, Taylor G. 2020. Innovative breeding technologies in lettuce for improved post-harvest quality. *Postharvest Biology and Technology* 168: 111266.
- de Souza ASN, de Oliveira Schmidt H, Pagno C, Rodrigues E, da Silva MAS, Flôres SH, de Oliveira Rios A. 2022. Influence of cultivar and season on carotenoids and phenolic compounds from red lettuce influence of cultivar and season on lettuce. *Food Research International* 155: 111110.
- Deng LZ, Mujumdar AS, Pan Z, Vidyarthi SK, Xu J, Zielinska M, Xiao HW. 2020. Emerging chemical and physical disinfection technologies of fruits and vegetables: a comprehensive review. *Critical reviews in food science and nutrition* 60: 2481-2508.
- Dittrich C, Pecenka R, Selge B, Ammon C, Kruggel-Emden H. 2022. Influence of processing parameters on fibre properties during twin-screw extrusion of poplar wood chips. *Horticulturae* 8: 762.
- Dong X, Tang H, Zhang Q, Zhang C, Wang Z. 2022. Transcriptomic analyses provide new insights into jujube fruit quality affected by water deficit stress. *Scientia Horticulturae* 291: 110558.
- Drost D. 2020. Lettuce in the Garden. https://digitalcommons.usu.edu/extension_curall/249/
- Elik A, Yanik DK, Istanbulu Y, Guzelsoy NA, Yavuz A, Gogus F. 2019. Strategies to reduce post-harvest losses for fruits and vegetables. *Strategies* 5: 29-39.
- Fekadu GM, Getachew T, Zebeay D, Gizachew A, Fasil TT. 2021. Effect of planting density on yield and yield components of lettuce (*Lactuca sativa* L.) at two agro-ecologies of Ethiopia. *African Journal of Agricultural Research* 17: 549–556.
- Ferreira AMO, Rodríguez VAC, da Silva Dias G, Vilas Boas LV, López ME, Bicalho EM. 2024. Strategies induced by methyl jasmonate in soybean seedlings under water restriction and mechanical wounding. *Acta Physiologiae Plantarum* 46: 66.
- Food and Agriculture Organisation, International Fund for Agricultural Development, United Nations Children's Fund, World Food Programme, World Health Organization. FAO, IFAD, UNICEF, WFP, WHO. 2022. *The State of Food Security and Nutrition in the World 2022. Repurposing food and agricultural policies to make healthy diets more affordable*. Rome, FAO. <https://doi.org/10.4060/cc0639en>.
- Franzoni G, Cocetta G, Prinsi B, Ferrante A, Espen L. 2022. Biostimulants on crops: their impact under abiotic stress conditions. *Horticulturae* 8: 189.

- Gao YQ, Farmer EE. 2023. Osmoelectric siphon models for signal and water dispersal in wounded plants. *Journal of Experimental Botany* 74: 1207-1220.
- Gedeon S, Ioannou A, Balestrini R, Fotopoulos V, Antoniou C, 2022. Application of biostimulants in tomato plants (*Solanum lycopersicum*) to enhance plant growth and salt stress tolerance. *Plants* 11: 3082.
- Gerhards R, Ouidoh FN, Adjogboto A, Avohou VAP, Dossounon BLS, Adisso AKD, Heyn A, Messelhäuser M, Santel HJ, Oebel H. 2021. Crop response to leaf and seed applications of the biostimulant ComCat® under stress conditions. *Agronomy* 11: 1161.
- Giordano M, Petropoulos SA, Rouphael Y. 2021. Response and defence mechanisms of vegetable crops against drought, heat and salinity stress. *Agriculture* 11: 463.
- González-Morales S, Solís-Gaona S, Valdés-Caballero MV, Juárez-Maldonado A, Loredó-Treviño A, Benavides-Mendoza A. 2021. Transcriptomics of biostimulation of plants under abiotic stress. *Frontiers in Genetics* 12: 583888.
- González-Orenga S, Plazas M, Ribera E, Pallotti C, Boscaiu M, Prohens J, Vicente O, Fita A. 2023. Transgressive biochemical response to water stress in interspecific eggplant hybrids. *Plants* 12: 194.
- Gruda NS. 2019. Increasing sustainability of growing media constituents and stand-alone substrates in soilless culture systems. *Agronomy* 9: 298.
- Guan W, Edwards MG, Gatehouse JA, Gatehouse AM. 2022. Responses of wheat (*Triticum aestivum*) to grain aphid (*Sitobion avenae*) infestation and mechanical wounding using a cDNA subtractive library approach. *Agricultural Sciences* 13: 715-740.
- Haase DL, Bouzza K, Emerton L, Friday JB, Lieberg B, Aldrete A. 2021. The high cost of the low-cost polybag system: A review of nursery seedling production systems. *Land* 10: 826.
- Habib S, Lwin YY, Li N. 2021. Down-regulation of *SlGRAS10* in tomato confers abiotic stress tolerance. *Genes* 12: 623.
- Hai NN, Chuong NN, Tu NHC, Kisiala A, Hoang XLT, Thao NP. 2020. Role and regulation of cytokinins in plant response to drought stress. *Plants* 9: 422.

- Hajihashemi S, Mbarki S, Skalicky M, Noedoost F, Raeisi M, Brestic M. 2020. Effect of wastewater irrigation on photosynthesis, growth, and anatomical features of two wheat cultivars (*Triticum aestivum* L.). *Water* 12: 607.
- Hameed MK, Umar W, Razzaq A, Aziz T, Maqsood MA, Wei S, Niu Q, Huang D, Chang L. 2022. Differential metabolic responses of lettuce grown in soil, substrate and hydroponic cultivation systems under $\text{NH}_4^{+}/\text{NO}_3^{-}$ application. *Metabolites* 12: 444.
- Haynes RJ, Zhou YF, Weng X. 2022. Formulation and use of manufactured soils: A major use for organic and inorganic wastes. *Critical Reviews in Environmental Science and Technology* 52: 4113-4133.
- Henderson GC. 2021. Lettuce romaine calm and manage our glycemia: adding leafy greens to a meal may improve postprandial metabolism. *Lipids in Health and Disease* 20: 1-3.
- Hernández-Figueroa KI, Sánchez E, Ramírez-Estrada CA, Anchondo-Páez JC, Ojeda-Barrios DL, Pérez-Álvarez S. 2024. Efficacy and differential physiological–biochemical response of biostimulants in green beans subjected to moderate and severe water stress. *Crops* 4: 27-42.
- Höök M. 2020. Coal and peat: Global resources and future supply. In *Fossil Energy* 309-331.
- Hoseini Z, Barzegar T, Ghahremani Z, Nikbakht J. 2020. Physiological responses of common bean (*Phaseolus vulgaris* L., cv. Sanry) to foliar spray of salicylic acid and biostimulant megafol under deficit irrigation stress. *Environmental Stresses in Crop Sciences* 13: 845-855.
- Hossain A, Pamanick B, Venugopalan VK, Ibrahimova U, Rahman MA, Siyal AL, Maitra S, Chatterjee S, Aftab T. 2022. Emerging roles of plant growth regulators for plants adaptation to abiotic stress–induced oxidative stress. In *Emerging plant growth regulators in agriculture* 1-72.
- Hosseini H, Mozafari V, Roosta HR, Shirani H, van de Vlasakker PC, Farhangi M. 2021. Nutrient use in vertical farming: optimal electrical conductivity of nutrient solution for growth of lettuce and basil in hydroponic cultivation. *Horticulturae* 7: 283.
- Huang G, Kilic A, Karady M, Zhang J, Mehra P, Song X, Sturrock CJ, Zhu W, Qin H, Hartman, S, Schneider HM. 2022. Ethylene inhibits rice root elongation in compacted soil via ABA-and auxin-mediated mechanisms. *Proceedings of the National Academy of Sciences* 119: e2201072119.

- Imran M, Latif Khan A, Shahzad R, Aaqil Khan M, Bilal S, Khan A, Kang SM, Lee IJ. 2021. Exogenous melatonin induces drought stress tolerance by promoting plant growth and antioxidant defence system of soybean plants. *AoB Plants* 13: plab026.
- Inoue T, Sunaga M, Ito M, Yuchen Q, Matsushima, Y., Sakoda, K., Yamori, W. 2021. Minimizing VPD fluctuations maintains higher stomatal conductance and photosynthesis, resulting in improvement of plant growth in lettuce. *Frontiers in Plant Science* 12: 646144.
- Iqbal MS, Singh AK, Ansari MI. 2020. Effect of drought stress on crop production. *New Frontiers in Stress Management for Durable Agriculture* 35-47.
- Isa MM, Kasim KF, Muttalib MFA, Jaafar MN. 2021. Physical and chemical properties of different media mixture as a growing medium for containerized plant. *In AIP Conference Proceedings* 2339: 020122.
- Jan R, Khan MA, Asaf S, Waqas M, Park JR, Asif S, Kim N, Lee IJ, Kim KM. 2022. Drought and UV radiation stress tolerance in rice is improved by overaccumulation of non-enzymatic antioxidant flavonoids. *Antioxidants* 11: 917.
- Jiménez-Arias D, Morales-Sierra S, Borges AA, Herrera AJ, Luis JC. 2022. New biostimulants screening method for crop seedlings under water deficit stress. *Agronomy* 12:728.
- Jogawat A, Yadav B, Lakra N, Singh AK, Narayan OP. 2021. Crosstalk between phytohormones and secondary metabolites in the drought stress tolerance of crop plants: A review. *Physiologia Plantarum* 172: 1106-1132.
- Johnson JB, Budd C, Mani JS, Brown P, Walsh KB, Naiker M. 2022. Carotenoids, ascorbic acid and total phenolic content in the root tissue from five Australian-grown sweet potato cultivars. *New Zealand Journal of Crop and Horticultural Science* 50: 32-47.
- Kamran A, Mushtaq M, Arif M, Rashid S. 2023. Role of biostimulants (ascorbic acid and fulvic acid) to synergize Rhizobium activity in pea (*Pisum sativum* L. var. Meteor). *Plant Physiology and Biochemistry* 196: 668-682.
- Khattak A, Ullah F, Shinwari ZK, Mehmood S. 2021. The effect of titanium dioxide nanoparticles and salicylic acid on growth and biodiesel production potential of sunflower (*Helianthus annuus* L.) under water stress. *Pakistan Journal of Botany* 53: 1987-1995.

- Khetsha ZP, Moloantoa K, Masowa MM, Sedibe MM. 2024. Comparative effects of two elicitors on the essential oil biosynthesis of simulated hail-damaged rose geranium (*Pelargonium graveolens* L'Her. cv. BOURBON'). *African Journal of Food, Agriculture, Nutrition and Development* 24: 26442-26463.
- Khetsha ZP, Sedibe MM, Pretorius RJ, Rathebe PC, Moloantoa K. 2022. Using biostimulants containing phytohormones to recover hail-damaged essential oil plants. In *Revisiting Plant Biostimulants*. IntechOpen.
- Khetsha ZP, Sedibe MM. 2015. Effect of potassium and potting-bag size on foliar biomass and related attributes and oil composition of rose geranium (*Pelargonium graveolens*). *South African Journal of Plant and Soil* 32: 113-115.
- Kim H, Kim S, Jeon J, Jeong H. 2020. Effects of irrigation with desalinated water on lettuce grown under greenhouse in South Korea. *Applied Sciences* 10: 2207.
- Koehler T, Moser DS, Botezatu Á, Murugesan T, Kaliamoorthy S, Zarebanadkouki M, Bienert, MD, Bienert GP, Carminati A, Kholová J, Ahmed M. 2022. Going underground: soil hydraulic properties impacting maize responsiveness to water deficit. *Plant and Soil*, 478: 43-58.
- Kongwong P, Boonyakiat D, Poonlarp P. 2019. Extending the shelf life and qualities of baby cos lettuce using commercial precooling systems. *Postharvest Biology and Technology*, 150: 60-70.
- Kudoyarova G. 2022. Phytohormones 2020. *Biomolecules* 12: 1305.
- Kumar R, Hegde AS, Sharma K, Parmar P, Srivatsan V. 2022. Microalgae as a sustainable source of edible proteins and bioactive peptides-current trends and future prospects. *Food Research International* 157: 111338.
- Kumar S, Bhushan B, Wakchaure GC, Meena KK, Kumar M, Meena NL, Rane J. 2020. Plant phenolics under water-deficit conditions: Biosynthesis, accumulation, and physiological roles in water stress alleviation. In *Plant Phenolics in Sustainable Agriculture* 1: 451-465.
- Kumar Sootahar M, Zeng X, Wang Y, Su S, Soothar P, Bai L, Kumar M, Zhang Y, Mustafa A, Ye N. 2020. The short-term effects of mineral-and plant-derived fulvic acids on some selected soil properties: improvement in the growth, yield, and mineral nutritional status of wheat (*Triticum aestivum* L.) under soils of contrasting textures. *Plants* 9: 205.

- Lei C, Engeseth NJ. 2021. Comparison of growth characteristics, functional qualities, and texture of hydroponically grown and soil-grown lettuce. *LWT* 150: 111931.
- Li S, Liu F. 2022. Vapour pressure deficit and endogenous ABA level modulate stomatal responses of tomato plants to soil water deficit. *Environmental and Experimental Botany* 199: 104889.
- Li W, Gupta A, Tian H, Nguyen KH, Tran CD, Watanabe Y, Tian C, Li K, Yang Y, Guo J, Luo Y. 2020. Different strategies of strigolactone and karrikin signals in regulating the resistance of *Arabidopsis thaliana* to water-deficit stress. *Plant Signaling and Behavior* 15: 1789321.
- Lohani N, Jain D, Singh MB, Bhalla PL. 2020. Engineering multiple abiotic stress tolerance in canola, *Brassica napus*. *Frontiers in Plant Science* 11: 3.
- Lu J, Shao G, Gao Y, Zhang K, Wei Q, Cheng J. 2021. Effects of water deficit combined with soil texture, soil bulk density and tomato variety on tomato fruit quality: A meta-analysis. *Agricultural Water Management* 243: 106427.
- Lupepsa BF, Ferrarese-Filho O, Constantin RP, Marchiosi R, dos Santos WD. 2024. Gibberellin biosynthesis, its roles in plant physiology and abiotic stress responses. *Brazilian Journal of Development* 10: e74232-e74232.
- Ma Y, Freitas H, Dias MC. 2022. Strategies and prospects for biostimulants to alleviate abiotic stress in plants. *Frontiers in Plant Science* 13: 1024243.
- Machado RM, Alves-Pereira I, Morais C, Alemão A, Ferreira R. 2022. Effects of coir-based growing medium with municipal solid waste compost or biochar on plant growth, mineral nutrition, and accumulation of phytochemicals in spinach. *Plants* 11: 1893.
- Malécange M, Sergheraert R, Teulat B, Mounier E, Lothier J, Sakr S. 2023. Biostimulant Properties of Protein Hydrolysates: Recent Advances and Future Challenges. *International Journal of Molecular Sciences* 24: 9714.
- Mandal S, Anand U, López-Bucio J, Kumar M, Lal MK, Tiwari RK, Dey A. 2023. Biostimulants and environmental stress mitigation in crops: A novel and emerging approach for agricultural sustainability under climate change. *Environmental Research* 233: 116357.
- Martínez-Ispizua E, Calatayud Á, Marsal JI, Cannata C, Basile F, Abdelkhalik A, Soler S, Valcárcel JV, Martínez-Cuenca MR. 2022. The nutritional quality potential of

- microgreens, baby leaves, and adult lettuce: An underexploited nutraceutical source. *Foods* 11: 423.
- Mattner SW, Villalta ON, McFarlane DJ, Islam MT, Arioli T, Cahill DM. 2023. The biostimulant effect of an extract from *Durvillaea potatorum* and *Ascophyllum nodosum* is associated with the priming of reactive oxygen species in strawberry in south-eastern Australia. *Journal of Applied Phycology* 35: 1789-1800.
- Medina-Lozano I, Bertolín JR, Diaz A. 2021. Nutritional value of commercial and traditional lettuce (*Lactuca sativa* L.) and wild relatives: vitamin C and anthocyanin content. *Food Chemistry* 359: 129864.
- Meena SS, Kulakarni A. 2022. Management of iceberg lettuce quality. *Asian Journal of Agricultural Extension, Economics and Sociology* 40: 593-602.
- Meents AK, Chen SP, Reichelt M, Lu HH, Bartram S, Yeh KW, Mithöfer A. 2019. Volatile DMNT systemically induces jasmonate-independent direct anti-herbivore defense in leaves of sweet potato (*Ipomoea batatas*) plants. *Scientific Reports* 9: 1-13.
- Melo GB, da Silva AG, da Costa AC, Alves da Silva A, Rosa M, Bessa LA, Rodrigues CR, Castoldi G, Vitorino LC. 2024. Foliar application of biostimulant mitigates water stress effects on soybean. *Agronomy* 14: 414.
- Melrose J, Normandeau S. 2021. *The Prairie Gardener's Go-to for Small Spaces* 4: 160.
- Million JB, Yeager TH. 2022. Fabric containers increased irrigation demand but decreased leachate loss of nitrogen and phosphorus compared with conventional plastic containers during production of dwarf burford holly. *HortScience* 57: 743-749.
- Mostafa S, Wang Y, Zeng W, Jin B. 2022. Plant responses to herbivory, wounding, and infection. *International Journal of Molecular Sciences* 23: 7031.
- Moyo K, Khetsha ZP, Masowa MM, Van Der Watt E, Moloantoa KM, Unuofin JO. 2024. Eco-organic system and silicon-based biostimulant as a strategy for vegetable production under multistress conditions in South Africa: A review. *Journal of Applied Horticulture* 26: 83-89.
- Mukherjee I, Singh UK, Chakma S. 2022. Evaluation of groundwater quality for irrigation water supply using multi-criteria decision-making techniques and GIS in an agro-economic tract of lower Ganga basin, India. *Journal of Environmental Management* 309: 114691.

- Murray JJ, Basset G, Sandoya G. 2021. Nutritional benefits of lettuce consumed at recommended portion sizes. *Edis* 3: 1-8.
- Nephali L, Piater LA, Dubery IA, Patterson V, Huyser J, Burgess K, Tugizimana F. 2020. Biostimulants for plant growth and mitigation of abiotic stresses: A metabolomics perspective. *Metabolites* 10: 505.
- Nerlich A, Dannehl D. 2021. Soilless cultivation: dynamically changing chemical properties and physical conditions of organic substrates influence the plant phenotype of lettuce. *Frontiers in Plant Science* 11: 601455.
- Niu C, Wang G, Sui J, Liu G, Ma F, Bao Z. 2022. Biostimulants alleviate temperature stress in tomato seedlings. *Scientia Horticulturae* 293: 110712.
- Osei BY, Agyiri M, Aseidu, EK, Agyarko K. 2017. Assessment of the relative suitability of three different soils for dry season lettuce production in Ghana. *International Journal of Environment, Agriculture and Biotechnology* 2: 238937.
- Paramesha V, Rajanna GA, Kumar P, Sannagoudar MS, Halli HM. 2022. Drip fertigation for enhancing crop yield, nutrient uptake, nutrient, and water use efficiency. *Sustainable Agriculture Systems and Technologies* 267-278.
- Parida K, Parida S. 2021. Nutritional analysis using XRF technique and chlorophyll estimation of two varieties of lettuce. *Plant Archives* 21: 816-820.
- Pathak M, Barik S, Dash SK, Moharana DP. 2023. Impact of climate change on leafy and salad vegetables production. In *Advances in Research on Vegetable Production Under a Changing Climate* 2: 109-126.
- Patra SK, Sengupta S, Bhattacharya P, Bhattacharyya K. 2023. Enhancing yield of lettuce through irrigation and nitrogen management in a subtropical Inceptisol. *Israel Journal of Plant Sciences* 70: 76-90.
- Pavlović I, Mlinarić S, Tarkowská D, Oklestkova J, Novák O, Lepeduš H, Bok VV, Brkanac SR, Strnad M, Salopek-Sondi B. 2019. Early Brassica crops responses to salinity stress: a comparative analysis between Chinese cabbage, white cabbage, and kale. *Frontiers in Plant Science* 10: 450.
- Petropoulou AS, van Marrewijk B, de Zwart F, Elings A, Bijlaard M, van Daalen T, Jansen G, Hemming S. 2023. Lettuce production in intelligent greenhouses—3d imaging and computer vision for plant spacing decisions. *Sensors* 23: 2929.

- PM AS, Kumar N, Sneha M, Sathian KK. 2021. Standardization of irrigation and fertigation requirement for amaranth under polyhouse. Doctoral dissertation, Department of Soil and Water Conservation Engineering, Kerala Agricultural University.
- Purba JH, Parmila IP, Dadi W. 2021. Effect of soilless media (hydroponic) on growth and yield of two varieties of lettuce. *Agricultural Science* 4: 154-165.
- Qin T, Huang Q, Li J, Ayyaz A, Farooq MA, Chen W, Zhou Y, Wu X, Ali B, Zhou W. 2024. Comprehensive characterization of gibberellin oxidase gene family in *Brassica napus* reveals *BnGA2ox15* involved in hormone signaling and response to drought stress. *International Journal of Biological Macromolecules* 282: 136822.
- Raviteja MSV, Laxman RH, Rashmi K, Kannan S, Namratha MR, Reddy KM. 2021. Effect of container size and types on the root phenotypic characters of *Capsicum*. *Journal of Horticultural Sciences* 16: 261-270.
- Rosa AP, Barão L, Chambel L, Cruz C, Santana MM. 2023. Early identification of plant drought stress responses: changes in leaf reflectance and plant growth promoting rhizobacteria selection-the case study of tomato plants. *Agronomy* 13: 183.
- Rudrapal M, Khairnar SJ, Khan J, Dukhyil AB, Ansari MA, Alomary MN, Alshabrimi FM, Palai S, Deb PK, Devi R. 2022. Dietary polyphenols and their role in oxidative stress-induced human diseases: insights into protective effects, antioxidant potentials and mechanism (s) of action. *Frontiers in Pharmacology* 13: 283.
- Sabatino L, Consentino BB, Rouphael Y, Baldassano S, De Pasquale C, Ntatsi G. 2023. *Ecklonia maxima*-derivate seaweed extract supply as mitigation strategy to alleviate drought stress in chicory plants. *Scientia Horticulturae* 312: 111856.
- Sabina S, Jithesh MN. 2021. Mechanical wounding of leaf midrib and lamina elicits differential biochemical response and mitigates salinity induced damage in tomato. *Journal of Applied Horticulture* 23: 3-10.
- Sakamoto M, Suzuki T. 2020. Effect of nutrient solution concentration on the growth of hydroponic sweet potato. *Agronomy* 10: 1708.
- Sako K, Nguyen HM, Seki M. 2020. Advances in chemical priming to enhance abiotic stress tolerance in plants. *Plant and Cell Physiology* 61: 1995-2003.

- Sangiorgio D, Cellini A, Donati I, Pastore C, Onofrietti C, Spinelli F. 2020. Facing climate change: application of microbial biostimulants to mitigate stress in horticultural crops. *Agronomy* 10: 794.
- Sarkar MD, Rahman MJ, Uddain J, Quamruzzaman M, Azad MOK, Rahman MH, Islam MJ, Rahman MS, Choi KY, Naznin MT. 2021. Estimation of yield, photosynthetic rate, biochemical, and nutritional content of red leaf lettuce (*Lactuca sativa* L.) grown in organic substrates. *Plants* 10: 1220.
- Seema N, Hamayun M, Hussain A, Shah M, Irshad M, Qadir M, Iqbal A, Alrefaei AF, Ali S. 2023. Endophytic *Fusarium proliferatum* reprogrammed phytohormone production and antioxidant system of *Oryza sativa* under drought stress. *Agronomy* 13: 873.
- Sehgal A, Reddy KR, Walne CH, Barickman TC, Brazel S, Chastain D, Gao W. 2022. Individual and interactive effects of multiple abiotic stress treatments on early-season growth and development of two Brassica species. *Agriculture* 12: 453.
- Shatilov MV, Razin AF, Ivanova MI. 2019. Analysis of the world lettuce market. In *IOP Conference Series: Earth and Environmental Science* 395: 012053.
- Shi M, Gu J, Wu H, Rauf A, Emran TB, Khan Z, Mitra S, Aljohani AS, Alhumaydhi FA, Al-Awthan YS, Bahattab O. 2022. Phytochemicals, nutrition, metabolism, bioavailability, and health benefits in lettuce—a comprehensive review. *Antioxidants* 11: 1158.
- Sogoni A, Ngcobo BL, Jimoh MO, Kambizi L, Laubscher CP. 2024. Seaweed-derived bio-stimulant (Kelpak[®]) enhanced the morphophysiological, biochemical, and nutritional quality of salt-stressed spinach (*Spinacia oleracea* L.). *Horticulturae* 10: 1340.
- Sondhi S. 2023. Applications of bioplastic in composting bags and planting pots. *Handbook of Bioplastics and Biocomposites Engineering Applications* 605-617.
- Srinivasan TS, Thankappan S, Balasubramaniam M, Bhaskar V. 2022. Impact of plant health on global food security: A holistic view. In *Agriculture, Environment and Sustainable Development: Experiences and Case Studies* 43-66.
- Stoleru V, Burlica R, Mihalache G, Dirlau D, Padureanu S, Teliban GC, Astanei D, Cojocaru A, Beniuga O, Patras A. 2020. Plant growth promotion effect of plasma activated water on *Lactuca sativa* L. cultivated in two different volumes of substrate. *Scientific Reports* 10: 20920.

- Subedi S, Kattel D, Thokar N. 2022. A Review on post-harvest precooling methods of fruits and vegetables. *Food and Agri Economics Review* 2: 96-99.
- Sun T, Zhang Y. 2021. Short-and long-distance signaling in plant defense. *The Plant Journal* 105: 505-517.
- Tadele KT, Zerssa GW. 2023. Biostimulants and phytohormones improve productivity and quality of medicinal plants under abiotic stress. In *Medicinal Plants: Their Response to Abiotic Stress* 335-362.
- Tandathu T, Kotzé E, Van Der Watt E, Khetsha ZP. 2024. Effect of biostimulants and glyphosate on morphophysiological parameters of *Zea mays* (L.) seedlings under controlled conditions. *Agronomy* 14: 2396.
- Ten Damme L, Jing S, Montcalm AM, Jepson M, Andersen MN, Hansen EM. 2022. Proper management of irrigation and nitrogen-application increases crop N-uptake efficiency and reduces nitrate leaching. *Acta Agriculturae Scandinavica, Section B-Soil & Plant Science* 72: 913-922.
- Turan M, Yildirim E, Ekinici M, Argin S. 2021. Effect of biostimulants on yield and quality of cherry tomatoes grown in fertile and stressed soils. *HortScience* 56: 414-423.
- Turner NC. 2019. Imposing and maintaining soil water deficits in drought studies in pots. *Plant and Soil* 439: 45-55.
- Vega-Muñoz I, Duran-Flores D, Fernández-Fernández ÁD, Heyman J, Ritter A, Stael S. 2020. Breaking bad news: dynamic molecular mechanisms of wound response in plants. *Frontiers in Plant Science* 11: 610445.
- Velasco-Clares D, Navarro-León E, Izquierdo-Ramos MJ, Blasco B, Ruiz JM. 2025. Enhancing drought tolerance in lettuce: The efficacy of the seaweed-derived biostimulant Cytolan® Stress applied at different growth stages. *Horticulturae* 11: 157.
- Velasco-Clares D, Navarro-León E, Atero-Calvo S, Ruiz JM, Blasco B. 2024. Is the application of bioactive anti-stress substances with a seaweed-derived biostimulant effective under adequate growth conditions? *Physiologia Plantarum* 176: e14193.
- Verma PK, Verma S, Pandey N. 2022. Root system architecture in rice: impacts of genes, phytohormones and root microbiota. *Biotech* 12: 239.

- Voko MP, Kulkarni MG, Finnie JF, Van Staden J. 2022. Seed priming with vermicompost leachate, *Ecklonia maxima* extract-Kelpak® and smoke-water induce heat stress amelioration and growth in *Vigna unguiculata* L. seedlings. *South African Journal of Botany* 147: 686-696.
- Volkava K, Mickiewicz B. 2022. Main trends of dynamic development of the vegetable products and fruits global market. *VUZF Review* 7: 78.
- Wazny R, Rozpadek P, Jedrzejczyk RJ, Domka A, Nosek M, Kidd P, Turnau K. 2021. Phytohormone based biostimulant combined with plant growth promoting endophytic fungus enhances Ni phytoextraction of *Noccaea goesingensis*. *Science of The Total Environment* 789: 147950.
- Wei X, Liao R, Zhang X, Zhao Y, Xie Z, Yang S, Su H, Wang Z, Zhang L, Tian B, Wei F. 2023. Integrative transcriptome, mirnas, degradome, and phytohormone analysis of *Brassica rapa* L. in response to *plasmodiophora brassicae*. *International Journal of Molecular Sciences* 24: 2414.
- Wielkopolan B, Frąckowiak P, Wieczorek P, Obrępańska-Stęplowska A. 2022. The impact of *oulema melanopus*—associated bacteria on the wheat defense response to the feeding of their insect hosts. *Cells* 11: 2342.
- Yakti W, Müller M, Klost M, Mewis I, Dannehl D, Ulrichs C. 2023. Physical Properties of Substrates as a Driver for *Hermetia illucens* (L.) (Diptera: *Stratiomyidae*) Larvae Growth. *Insects* 14: 266.
- Yang X, Gil MI, Yang Q, Tomás-Barberán FA. 2022. Bioactive compounds in lettuce: highlighting the benefits to human health and impacts of preharvest and postharvest practices. *Comprehensive Reviews in Food Science and Food Safety* 21: 4-45.
- Yari Kamrani Y, Shomali A, Aliniaieifard S, Lastochkina O, Moosavi-Nezhad M, Hajinajaf N, Talar U. 2022. Regulatory role of circadian clocks on ABA production and signaling, stomatal responses, and water-use efficiency under water-deficit conditions. *Cells* 11: 1154.
- Yavuz D, Kiliç E, Seymen M, Dal Y, Kayak N, Kal Ü, Yavuz N. 2022. The effect of irrigation water salinity on the morpho-physiological and biochemical properties of spinach under deficit irrigation conditions. *Scientia Horticulturae* 304: 111272.

- Yavuz D, Rashid BAR, Seymen M. 2023. The influence of NaCl salinity on evapotranspiration, yield traits, antioxidant status, and mineral composition of lettuce grown under deficit irrigation. *Scientia Horticulturae* 310: 111776.
- Yıldız S, Karakuş CB. 2020. Estimation of irrigation water quality index with development of an optimum model: a case study. *Environment, Development and Sustainability* 22: 4771-4786.
- Yolcu MS. 2024. Effects of different doses of zeatin, kinetin, and gibberellic acid biostimulants on growth and biochemical parameters during the seedling development stage of Istanbul Oregano (*Origanum vulgare* L. ssp. *hirtum*). *International Journal of Agriculture Environment and Food Sciences* 8: 681-687.
- Zhang C, Liu S, Liu D, Guo F, Yang Y, Dong T, Zhang Y, Ma C, Tang Z, Li F, Meng X. 2022. Genome-wide survey and expression analysis of GRAS transcription factor family in sweet potato provides insights into their potential roles in stress response. *BMC Plant Biology* 22: 232.
- Zhang L, Li D, Yao Y, Zhang S. 2020. H₂O₂, Ca²⁺, and K⁺ in subsidiary cells of maize leaves are involved in regulatory signaling of stomatal movement. *Plant Physiology and Biochemistry* 152: 243-251.
- Zhang Q, Gong M, Xu X, Li H, Deng W. 2022. Roles of auxin in the growth, development, and stress tolerance of horticultural plants. *Cells* 11: 2761.

Table 2.1: Examples of commercial biostimulants containing phytohormones as declared on the labels.

Scientific name	Common name	Product name	Composition	Effect of phytohormone-based biostimulant on plants grown under abiotic stress factors	Citation
<i>Cichorium intybus</i> L.	Chicory	Kepstar®	Extract of seaweed <i>Ecklonia maxima</i> contains Organic nitrogen 1%, Organic carbon 10%, Auxin 11 mg L ⁻¹ , Cytokinin 0.03 mg L ⁻¹ , Organic substances (< 50 kDa) 30% and pH 6.00.	Increased the ascorbic acid concentration in plants exposed to severe drought stress by 15.2%.	Sabatino <i>et al.</i> (2023).
<i>Solanum lycopersicum</i> L.	Tomato	Papenfuss Kelpak®	From the brown seaweed <i>Ecklonia maxima</i> contains auxins 11 mg kg ⁻¹ , cytokinins 0.03 mg kg ⁻¹ and auxin: cytokinin ratio (367:1), carbohydrates (16.9 g kg ⁻¹), amino acids (2.5 g kg ⁻¹), vitamin B1 (0.9 mg kg ⁻¹), B2 (0.1 mg kg ⁻¹), C (20 mg kg ⁻¹), and E (0.7 mg kg ⁻¹	It promoted the growth of seedlings subjected to water-deficit stress.	Jiménez-Arias <i>et al.</i> (2022).
<i>Solanum lycopersicum</i> L.	Tomato	Boosten and Megafol®	Amino acids, betaines, proteins, vitamins, auxin, GA, and cytokinins.	Promoted plant biomass accumulation under extremely low or high-temperature stress on seedlings at the four-leaf stage.	Niu <i>et al.</i> (2022).
<i>Calendula officinalis</i> L.	Marigold	SE extract of <i>Ascophyllum nodosum</i> seaweed (pure)	Contains nitrogen, phosphorus, potassium, calcium, sulfur, magnesium, iron, manganese, zinc, copper, boron, and molybdenum. Plant growth hormones: auxin, gibberellin, cytokinin; carbohydrates: mannitol, laminarin, alginic acid, and essential amino acids.	Improved plant growth parameters and biomass. Increased stomata density and the weight of 1,000 seeds.	Azizi <i>et al.</i> (2024).
<i>Vigna unguiculata</i> L.	Cowpea	Kelpak®	It is an extract of the seaweed <i>Ecklonia maxima</i> (kelp) and hormones: auxins, cytokinins, gibberellins, brassinosteroids, polyamines, and phlorotannins.	Increased peduncle diameter, shoot length, root length, and fresh and dry weight under 35°C heat stress.	Voko <i>et al.</i> (2022).
<i>Fragaria</i> spp.	Strawberry	Seasol®	Seaweed extract from <i>Durvillea potatorum</i> and <i>Ascophyllum nodosum</i> species contains 0.2 % (w/v) N, 0.02% P, 3.7 % K, 0.3 % S, 458 mg L ⁻¹ Ca, 972 mg L ⁻¹ Mg, 115 mg L ⁻¹ Fe, 2 mg L ⁻¹ Mn, 15 mg L ⁻¹ B, and 5	Decreased the rigour of post-harvest rots in fruits by up to 52-87% and enhanced concentrations of H ₂ O ₂ and peroxidase in root cells, signifying the initiation of	Mattner <i>et al.</i> (2023).



			mg L ⁻¹ Zn and copper, 7% (w/v) total laminarins, 154 µg L ⁻¹ total auxins, 36 µg L ⁻¹ total cytokinins (including zeatin, dihydrozeatin, isopentenyl-adenine, and their corresponding ribosides and glucosides) and 382 µg L ⁻¹ total betaines.	defence priming against biotic and abiotic stress.	
<i>Zea mays</i> L.	Maize	ComCat [®]	Based on the two brassinosteroids: 24-epi-secasterone and 24-epicastasterone.	Reduced phytotoxic effect of cinmethylin under drought and nutrient deficiency stresses.	Gerhards <i>et al.</i> (2021).
<i>Diplotaxis tenuifolia</i> L.	Wild rocket	Auxym [®]	Tropical plant extract comprises micronutrient complex with natural amino acids, vitamins, and cytokinins.	Improved the SPAD index and increased nitrate content in leaves under salinity stress.	Arif <i>et al.</i> (2023).
<i>Zea mays</i> L.	Maize	Brassinosteroids	Brassinosteroids (2,4-epibrassinolide).	Increased the rate of stomatal conductance and transpiration rate on seedlings under the application of glyphosate as a weed controller stress factor.	Tandathu <i>et al.</i> (2024).
<i>Glycine max</i> L.	Soybean	Sigma Aldrich [®]	MeJA	Increased the release of ethylene under mechanical wounding conditions and shoot length under 40% water deficit treatment and catalase activity on seedlings.	Ferreira <i>et al.</i> (2024).
<i>Phaseolus vulgaris</i> L.	Green bean	Salicylic acid	C ₇ H ₆ O ₃	Increased the stomatal conductance, transpiration, and soluble amino acids on green bean seeds under 50% water stress.	Hernández-Figueroa <i>et al.</i> (2024).
<i>Solanum lycopersicum</i> L.	Tomato	Biocompost	Contains a high percentage of organic carbon and organic matter, carbonates, humic, and fulvic acids, with the humic acids being in higher quantities than the fulvic acids. It also contains N, P, K, Ca, and Mg and key trace elements Fe, Mn, B, Zn, Cu, and Mo. Microorganisms: bacteria, actinobacteria, fungi and enzymes.	Increased stem width and plant fresh weight by 16% and 30%, respectively, over control and also increased the stomatal resistance and proline content of plants under salt stress.	Gedeon <i>et al.</i> (2022).



Central University of
Technology, Free State
Phytohormones: gibberellins (GAs), auxins
(IAA), and cytokinins.

<i>Lycopersicum esculentum</i> L.	Cherry tomato	Huminbio Seed®	Microsense	Content (% dry wt): Potassium humates (80% - 85%), Potassium as K ₂ O (10–12%), total organic nitrogen (1.0%, ZnO: 35%; P: 1%, others: 2.0%. Microorganisms (1 · 10 ⁹ cfu·mL ⁻¹): <i>Bacillus megaterium</i> , <i>B. subtilis</i> , <i>B. licheniformis</i> , <i>Peanibacillus polymyxa</i> , <i>Lactococcus</i> spp. Indole-3 acetic acid: 4 mM Cytokinin: 2 mM, pH: 9–10.	Improved the fruit size (width and diameter), plant height and stem size, root dry weight and vitamin C content, under stressed soil II (with high CaCO ₃ content, low organic matter content, and high salinity).	Turan <i>et al.</i> (2021).
<i>Vigna unguiculata</i> L.	Cowpeas	Vermicompost leachate		Nutrient composition of 2.26% N, 0.99% P, 0.64% K, 2.52% Ca, and 631.03 mg/kg of sodium (Na), and the crude stock had a pH of 7.82. Phytohormones: CKs, IAA, GAs, and BRs. Phytochemical: phenolic acids.	Reduced average germination time, promoted taller plants with long roots, and increased number of roots, seedling area, and seed germination grown under 30°C heat stress.	Voko <i>et al.</i> (2022).
<i>Phaseolus vulgaris</i> L. and <i>Glycine max</i> L.	Common bean and soybean	Megafof®		Contains total N (3 %), organic N (1 %), urea N (2 %), potassium oxide (K ₂ O) (8 %), total organic C of biological origin (9 %), amino acids: glycine, and glutamic acid, betaines, proteins, vitamins: B5, PP, B1, and B6, auxin, gibberellin, cytokine.	Increased the relative water content and peroxidase enzyme activity under 75 ETc% deficit irrigation. Increased in water potential, stomatal conductance, and reduced proline under water deficit stress.	Hoseini <i>et al.</i> (2020); Melo <i>et al.</i> (2024).
<i>Spinacia oleracea</i> L.	Spinach	Kelpak®		<i>Ecklonia maxima</i> origin contains 11.0 mg L ⁻¹ of auxin and 0.031 mg L ⁻¹ of cytokinin from the algae.	Promoted relative water content, chlorophyll <i>a</i> and <i>b</i> , Zn, Mg, and Fe leaf contents and lowered superoxide radicals and malondialdehyde under salt stress.	Sogoni <i>et al.</i> (2024).
<i>Lactuca sativa</i> L.	Lettuce	Cytolan® Stress		From alkaline extract of the seaweed <i>A. nodosum</i> added proline, mannitol, vitamins C and E, salicylic acid, and menadione sodium bisulfite.	Improved above-ground biomass, leaf area, relative water content, photosynthesis performance and chlorophyll content have grown under drought stress.	Velasco-Clares <i>et al.</i> (2025).

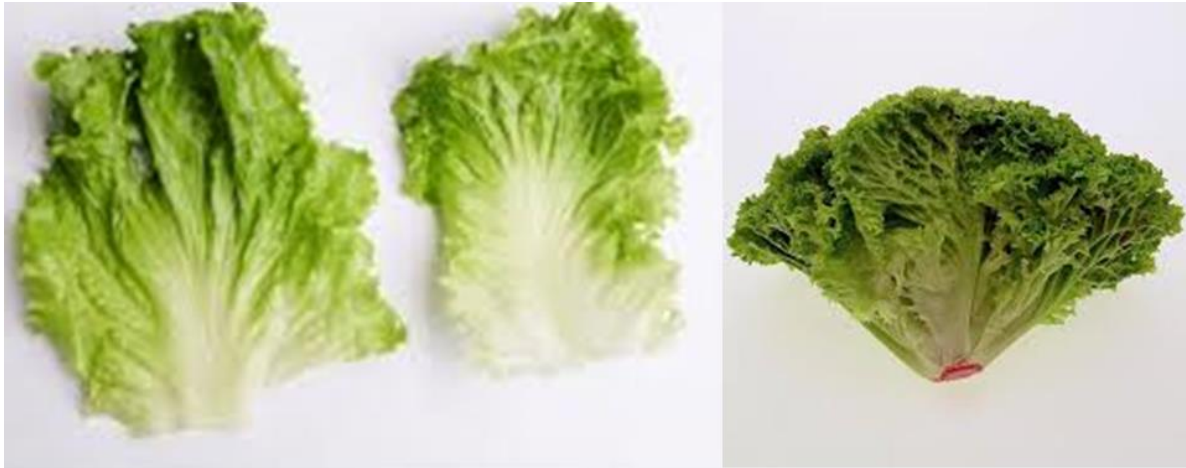


Figure 2.1: Picture of curly lettuce (*Lactuca sativa* L.) var. Crispa.

CHAPTER 3

EFFECTS OF MULTI-ABIOTIC STRESS ON THE MORPHOPHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS OF CURLY LETTUCE (*Lactuca sativa* L. var. Crispa) GROWN IN HYDROPONICS SYSTEM

ABSTRACT

The simultaneous occurrence of abiotic stresses such as water deficit, plant bag size, and mechanical wounding in hydroponics disrupts plant growth and development, leading to great yield depression worldwide. The objective of this study was to investigate the effects of multistress (water deficit regimes, plant bag sizes and mechanical wounding) on lettuce (*Lactuca sativa* L. cv. Crispa) yield and morphophysiological and biochemical parameters. A 4 x 3 x 2 Factorial Design experiment was carried out based on a randomised complete block design layout with 24 treatments and three replicates. The factors were water deficit at four irrigation regimes (100% as a control, 80%, 60%, and 40%), three plant bag sizes (5 L, 3.5 L, and 1.25 L), and two mechanical wounding treatments (intact and leaf wounded plants). The results demonstrated the most reduction in the number of leaves (22.00%), dry mass (54.50%), and stomatal conductance (70.79%) under 40% × 1.25 L, and mechanical wounding further exacerbated declines. The leaf area (507.04 cm²) decreased under 60% × 1.25 L × leaf wounded plant. The minimum leaf fresh mass (320.40 g) and stomatal conductance (155.09 mmol m⁻² s⁻¹) were recorded under 40% × wounded plants. The plant height was significantly reduced by 40% water deficit, 1.25 L plant bag size, and leaf wounding. Contrarily, chlorophyll content was enhanced under 40% × intact plants relative to control treatments. In comparison with control treatments, ANOVA analysis showed a reduction of Mg (0.38%), P (2.61%), Ca (1.15%), and Mn leaf contents observed under water deficit (60% and 40%), and plant bag sizes (3.5 L and 1.25 L) regardless of mechanical wounding treatment. Furthermore, the PCA biplot revealed a clear increase of Na, N, and Zn leaf contents found at 40% and 3.5 L or 1.25 L and leaf wounded. Multivariate analysis further revealed significantly elevated phenolic content accumulation under 60% × 1.25 L and 40% × 3.5 L, irrespective of mechanical wounding. These findings will improve the understanding of curly lettuce response to adverse effects of simultaneous abiotic stresses, contributing to improving management strategies in lettuce production.

Keywords: Curly lettuce · Multi-abiotic stress · Water deficit · Plant bag size · Mechanical wounding · Stress interactions

3.1 INTRODUCTION

Curly lettuce (*Lactuca sativa* L.) varieties like Crispa contain more water than others (Yavuz *et al.*, 2023). The simultaneous action of abiotic stresses such as water deficit, plant bag size, and mechanical wounding causes growth disruptions in physiological cell elongation and reduction of cell turgor, resulting in stunted plant height and leaf size, stopping new leaf development and yield depression (Becerra-Moreno *et al.*, 2015; Ban *et al.*, 2023; Cunha *et al.*, 2023). The primary yield of lettuce is significantly affected by the number of fresh leaves and leaf size (Tüfenkçi & Yerli, 2023). A high photosynthesis rate increases, and an increase in photosynthesis increases the number of leaves (Isa *et al.*, 2021). Leaf area enlargement improves biomass production (Inoue *et al.*, 2021). Yield is linked to biomass (fresh and dry mass) production. However, leaf expansion is hindered when lettuce is subjected to multistress conditions, decreasing fresh and dry weight (Jogawat *et al.*, 2021).

In addition, stomatal conductance is vital for gauging the extent of stomatal opening and closure, thus considering the transpiration rate in plants (Yavuz *et al.*, 2023). The closing of stomata is correlated to a tolerance mechanism against water stress (Isa *et al.*, 2021). Stomatal aperture regulates the rate of photosynthesis (Inoue *et al.*, 2021). Major studies on water deficit stress have reported that plants respond by reducing the stomatal opening period to conserve water as the growing root media dehydrate due to insufficient water supply (Hossain *et al.*, 2022; Yavuz *et al.*, 2023).

Moreover, chlorophyll content is a pigment indicator that reflects the photosynthetic enzymes' assimilation (Yavuz *et al.*, 2023). Chlorophyll promotes photosynthesis, which increases the assimilation of CO₂ in adequately watered plants (Inoue *et al.*, 2021). In lettuce plants, increasing the levels of chlorophyll affirms more water-use efficiency and lowers reactive oxygen species (ROS) imbalances, which damage leaf membrane structure (Hossain *et al.*, 2022). However, the co-occurrence of multistress degrades chlorophyll and delays the accumulation of dry biomass (Inoue *et al.*, 2021).

Leaves of lettuce are a popular source of several minerals and vitamins crucial for the healthy functioning of the human body (Yavuz *et al.*, 2023; Nițu *et al.*, 2023). However, when lettuce is grown in multistress conditions, it loses nutritional value and quality due to oxidative damage. For instance, a study by Nițu *et al.* (2023) clarifies that water deficiency decreases ascorbic acid content or vitamin C concentration in lettuce. This directly affects the quality of lettuce, which means that lettuce grown in such conditions may not provide the expected

nutritional benefits to consumers. Water deficit stress and small planting pot sizes also result in nutrient deficiencies due to insufficient water in the root zone, thereby reducing nutrient absorption (Tüfenkçi & Yerli, 2023).

Phenolic compounds are not just biomolecules but key players in a plant's defence system, formed especially in response to abiotic and biotic stress (Nephali *et al.*, 2020; Cunha *et al.*, 2023). They can scavenge and suppress lipid oxidation and free radicals, and their contents can be used to estimate the level of antioxidant activity in plant material (Utami & Damayanti, 2023; Yavuz *et al.*, 2023). During adverse abiotic stress, phenolic compounds are evoked via the phenylpropanoid pathway, which triggers the defence systems of plants (Cunha *et al.*, 2023). An earlier study by Campos-Vargas and Saltveit (2002) noted four phenolic compounds mainly accumulated in stressed and wounded lettuce: 3,5-dicaffeoylquinic (isochlorogenic acid), 5-caffeoylquinic (chlorogenic acid), caffeoyltartaric, and dicaffeoyltartaric acid. Wounds in lettuce leaves produce a signal that generates the synthesis of phenylalanine, ammonia-lyase, and the proliferation of phenolic compounds in cells 2 cm from the injury site, further causing tissue browning at the site of injury (Campos-Vargas & Saltveit, 2002; Becerra-Moreno *et al.*, 2015). By understanding these effects, researchers can potentially develop strategies to mitigate the impact of multistress on lettuce yield, offering hope for future agricultural practices. Therefore, the objective of this study was to investigate the effects of multistress (water deficit regimes, plant bag sizes and mechanical wounding) on lettuce yield and morphophysiological and biochemical parameters.

3.2 MATERIAL AND METHODS

3.2.1 Description of trial site

The study was conducted at the Central University of Technology, Free State, in Bloemfontein (29°07'16.78"S and 26°12'45.95"E, 1395 m above sea level), located in a semi-arid region in a 72 m² (12 × 6 m) polyethylene greenhouse tunnel covered with a hail net. The greenhouse tunnel was installed with an exhaust fan air extractor. Two digital wall thermometers were used to record the daily temperatures in the greenhouse tunnel. With the help of the exhaust fan air extractor, the temperature was maintained at a maximum of 26°C during the day, as recommended by Abarca *et al.* (2022). At night, temperatures ranged between 12–15°C. However, the night temperature was not regulated as it was optimal for lettuce growth (Aires *et al.*, 2020).

3.2.2 Experimental design and treatments

3.2.2.1 Experiment design

The experiment was conducted in a 4 x 3 x 2 factorial scheme, laid out in a randomised complete block design, replicated thrice. Each treatment unit comprised six plants. The treatments were four irrigation regimes as follows: 100% as a control (crop water required), 80% (of applied water of 100%), 60% (of applied water of 100%), and 40% (of applied water of 100%), three plant bag sizes: 5 L, 3.5 L, and 1.25 L and two mechanical wounding treatments: intact and leaf wounded plants.

3.2.2.2 Experimental treatments

Plant bag size

The dimensions of the three different plant bags, when filled with growth media, were as follows: a 5 L volume bag measured 24 cm in height and 17 cm in diameter, a 3.5 L volume bag measured 18 cm in height and 14.5 cm in diameter, and a 1.25 L volume bag measured 15 cm in height and 11 cm in diameter. The bags contained 970, 430, and 310 g of cocopeat, respectively, and were weighed using a digital gravimetric scale. Before weighing, the cocopeat samples were oven-dried at 105°C for 24 hours (Ilahi & Ahmad, 2017). The dry weight per unit volume was used to calculate the bulk density (BD: g cm⁻³) following the equation recommended by Kalaivani and Jawaharlal (2019):

$$\text{Bulk Density} = \frac{\text{Dry mass sample}}{\text{volume of the sample}}$$

Based on the formula, the root media bulk density in three different sizes of plant bags (5 L, 3.5 L, and 1.25 L) was 0.18, 0.14, and 0.22 g cm⁻³, respectively.

Water deficit stress

The irrigation system consisted of four 2,000 L water tanks, each equipped with a 0.75 kW water pump connected to its valve, ensuring water distribution to the drip irrigation network. This network included 15 mm diameter pipes, 4 mm drip tubes, and dripper tubing pegs, each with a flow rate of 2 L/hr, as shown in Figure 3.1. Each plant bag was allocated one dripper peg. Scheduling the water supply pump was managed automatically using the

Residential Irrigation Controller (X-CORE, Hunter, RSA) in timing mode, which distributed water at preset intervals.

Three days before the water deficit was imposed, plants were irrigated to achieve 100% pot capacity (PC) to avoid transplant shock and to promote root establishment (Qin & Leskovar, 2020). The four-week-old plants were subjected to water deficit at four irrigation regimes with different irrigation intervals. By considering 100% (control) irrigating for 8 minutes pulse after a two-day irrigation interval, 80% irrigating for 6 minutes pulse after three days irrigation interval, 60% irrigating for 4 minutes pulse after four days irrigation interval, and 40% irrigating for 2 minutes pulse after five days irrigation interval, until termination of the experiment. Therefore, plants received 460 mL, 350 mL, 230 mL, and 120 mL of water per day at 100% (control), 80%, 60%, and 40%, respectively.

Mechanical wounding stress

There were two wound treatments (intact and leaf-wounded) on plants. Plants were subjected to a wounding treatment when six weeks old, with 9 to 10 developing leaves. To simulate the mechanical wounding, the plant leaf area was cut 7 cm above the meristem using shear scissors (Agrimark, Bloemfontein, RSA) and a metric ruler to measure each plant before cutting; the plant crown was untouched. Mechanical wounding on leaves was performed within an hour on the same day.

3.2.3 Agronomic practices

3.2.3.1 Plant materials and propagation

The certified curly lettuce (*Lactuca sativa* L. var. Crispa, cv. Lollo lettuce ‘Granite’) seeds were acquired from a commercial seed supplier (Starke Ayres Pty Ltd, RSA). On the 1st of September 2023, seeds were sown in 84-cell polystyrene germination trays (Pretty Gardens Centre, RSA) filled with germination mix (Bark Unlimited Organics, Pretty Gardens Centre, RSA) at a depth of 0.1–1 cm (Adegbaju *et al.*, 2018). Sown trays followed the skotomorphogenesis programme with the purpose of encouraging the seedlings to reach light to be able to establish photosynthesis (Toman *et al.*, 2024). Seeds were considered germinated when the plumule emerged above the germination soil surface (Aires *et al.*, 2020; Nunomura *et al.*, 2020). Seedlings were irrigated twice a day with tap water, and after seven days following

seed sowing, seedlings were fertigated twice a day with a quarter-rate of the nutrients (Table 3.1) and with half strength after 14 days until the transplanting date (Yan *et al.*, 2019).

3.2.3.2 Cultivation method

Seedlings were transplanted on the 1st of October 2023, 30 days after sowing, with three to four fully developed leaves (Ahmad *et al.*, 2024) and one plant per planting bag. Three different sizes of black plastic plant bags (Pretty Gardens Centre, RSA) were filled with cocopeat (Hydroponic, RSA) and placed in clear 46 L crates (60 cm length x 40 cm width x 22 cm height) (Store & More, RSA). Some of the physical properties of cocopeat, such as particle density (0.23 g cm^{-1}), porosity (60.91%), moisture content (37.36%), and water-holding capacity (65.49%), were reported by Kalaivani and Jawaharlal (2019).

The plant bags come with bottom side holes for better drainage and aeration, and the crates were prepared with six small holes underneath using an angle drill with a 15 mm drill-bit diameter to avoid cross-contamination from the plant bag drainage (Waiba *et al.*, 2020). Each crate, regarded as a treatment unit, contained six plant bags, and crates were arranged at 30 cm between and 15 cm within rows. The plant bag spacing for 5 L, 3.5 L, and 1.25 L within each crate inter- and intra-rows was 3 x 1.5 cm, 12 x 10 cm, and 15 x 12 cm, respectively.

3.2.3.3 Nutrients management

The primary nutrient solution was modified using the Combrink and Kempen (2019) formulation, as shown in Table 3.1. The nutrient solution was prepared using tap water. The tap water and coco peat growing medium samples were sent to the laboratory for chemical composition analysis before the trial commenced. Coco peat was thoroughly rinsed with fresh water before being used to lower EC and remove potentially toxic substances such as phenol and theanine (Cahyo *et al.*, 2019). The results are presented in Table 3.2 and were considered during the nutrient solution formulation. The electrical conductivity (EC) and pH of the fertigation solution were quantified using the pre-calibrated handheld probe (GroLine Combo pH/EC/TDS/Temp tester, Hanna[®], RSA). The EC and pH of the coco peat were assessed according to a method used by Awang *et al.* (2009) by mixing the growth media with distilled water in a ratio of 1:2 in two separate 2,000 mL Schott laboratory bottles, shaken for 15 minutes and left standing for 60 minutes and 24 hours, respectively. The coco peat was filtered, and solutions were gauged using the handheld EC and pH probe.

3.2.3.4 Pest management

Curly lettuce was scouted for pests daily from the seedling to the maturity stages. Aphids and white flies were noted when plants were five weeks old, and the pesticide Malasol (Organophosphate, Efekto, RSA) was applied at a rate of 0.5 mL per 10 L of tap water, as recommended by the supplier. Due to the presence of whiteflies when plants were seven weeks old, the pesticide application was repeated 14 days later.

3.2.4 Data and plant measurements

3.2.4.1 Morphological measurements

Plant height (cm) was measured using a standard metric ruler as the vertical distance between the root media surface and the highest leaf collar in all six experimental unit plants in the first week after transplanting, at an interval of 7 days between each measurement until the end of the experiment. The number of leaves was counted manually in all plants from the first week after transplanting at an interval of 7 days between data recorded until the end of the experiment, four weeks after transplanting.

One plant was randomly selected from six plants per treatment unit, and five of its fully developed leaves were used on the day of harvest to measure the leaf area ($\text{cm}^2 \text{ plant}^{-1}$) using a LI3100 Area Meter (LI-COR[®] Biosciences, RSA). The remaining five plants per experimental unit were manually harvested on 31 October 2023, 30 days after transplanting, by cutting curly lettuce with shears serrated about 1.50 cm above the meristem. Plants were separately weighed immediately after harvesting using a digital gravimetric scale to determine the fresh weight (g plant^{-1}). The external leaves that appeared damaged or wilted were excluded. Plant dry weight (g plant^{-1}) was acquired following a procedure by Walne and Reddy (2022). After weighing fresh weight, plants were sampled into paper bags, oven-dried (ECOTerm Labotec oven, RSA) at 70°C for 48 hours and weighed for dry weight. The root systems of all treatments were gently removed from the plant bags after taking the fresh mass, soaked for two minutes in a stream of tap water, and gently washed to remove root media while keeping the roots intact and portrayed for visual appearance.

3.2.4.2 Physiological analysis

Chlorophyll content was recorded the first week after transplanting, with seven days between each measurement until the end of the experiment, using the portable non-destructive chlorophyll content meter (CCM 200 plus, Opti-sciences Inc, USA). The meter was clamped over a fully expanded young leaf in all six plants of the experimental unit. The stomatal conductance (G_s) was quantified non-destructively on day 27 after transplant, following the method provided by Michelon *et al.* (2020), clapping the upper surface of the leaf canopy exposed to sunlight on three random curly lettuce leaves per experimental unit using a handheld leaf porometer (METER SC-1, TMS, USA) twice a day at 10:00 a.m. and 2:00 p.m. for three consecutive days before harvesting. The results were expressed as $\text{mmol m}^{-2} \text{s}^{-1}$.

3.2.4.3 Biochemical analysis

Leaf mineral content analysis

Curly lettuce plants from all treatment combinations were sampled in brown paper bags and oven-dried to constant weight at 70°C for 48 hours. Dried leaf tissues used to analyse Ca, Mg, Na, K, Cu, Zn, Fe, Mn, P, and N mineral content were grounded in a CryoMill grinding ball (MM 400, Retsch, RSA) and sieved through a stainless-steel sieve with 0.5–1 mm mesh. An electric compact scale (SF-400C, Snatcher, RSA) was used to weigh approximately 2 g of samples from all treatments into a porcelain crucible for dry ashing by placing it in a cool Heraeus M 110 muffle furnace to ash at 550°C overnight. The samples were cooled, and 5 cm³ of 6 M hydrogen chloride (HCL) was added to dissolve them. Samples were then evaporated to dry in a sand bath, cooled again, 5 cm³ of nitric acid (HNO₃) added, and again heated in a sand bath and removed immediately when the solution started boiling. Samples were further filtered into a 100 cm³ volumetric flask with filter paper, diluted to volume with deionised distilled water, and melded well.

The solution obtained was transferred into 20 mm test tubes and placed in a stackable test tube rack. The solution was assayed according to Price (1972) by the atomic absorption flame spectroscopy method using an atomic absorption spectrophotometer instrument (Agilent 240FS AA, RSA) to analyse Ca, Mg, Cu, Fe, Mn, and Zn concentrations at 422.7, 285.2, 324.7, 248.3, 279.5, and 213.9 nm wavelengths, respectively. The lamp and slit width of the present diagnostic parameters were set following the manufacturer's instructions. The flame type for Mg, Cu, Zn, Mn, and Fe was air-acetylene (oxidising), and nitrous oxide-acetylene (oxidising)

was used for Ca. The aspiration of the standard solution was initiated with the lowest concentration into the flame, corresponding absorbances were read, and a graph was drawn up relating absorbance to the concentration of the element present. Leaf mineral concentrations were expressed in mg kg^{-1} . The formula below was used to calculate the concentration of the element in the sample:

$$\text{Concentration of the element in the sample (ppm)} = \frac{\text{ppm} \times 1000}{10 \times \text{sample mass(g)}}$$

In addition, P sample was prepared following the aforementioned dry ashing method. The colorimetric method was used to evaluate the P content in samples, as defined by Cavell (1955). Briefly, P concentration was measured spectrophotometrically as a phospho-vanado-molybdate complex. Initially, a 10 cm^3 sample solution was transferred into a 50 cm^3 volumetric flask, and 5 cm^3 of 5 M hydrochloric acid and 5 cm^3 of ammonium molybdate-ammonium metavanadate reagent were added to form a yellow colour. Thereafter, it was diluted with deionised water to a volume of 50 cm^3 and left to stand for 30 minutes. Next, the absorbance at 400 nm in a 10 mm optical cell was determined using a spectrophotometer (Cary 60 UV-Vis, Agilent, Germany). The P concentration (%) of leaves was determined from the standard graph curve.

Furthermore, the analytical estimation of N was carried out by the leco truspec nitrogen and carbon determinator (Leco TruSpec[®]CN, RSA) connected to an external PC with a Windows[®]-based operating system to control the data management. The instrument was calibrated according to the operator's manual using a single standard calibration. Analytical balance (CP64-0CE, Sartorius, RSA) was utilised to weigh approximately 0.10–0.20 g of the dried leaf materials that were ground to fine powder into a 502-186 Tin Foil Cup. The mass of a sample and its identifying treatment number were entered into the PC. Samples were encapsulated and placed appropriately to the correct sample number in the carousel loading head and sealed to start the process. Therefore, the thermal conductivity cell determined the N content (%), and results were revealed as weight percentages.

Profiling of phenolic compounds

Phenolic content analysis was carried out at Stellenbosch University in the Central Analytical Facilities. Curly lettuce leaves harvested from all treatment units were sampled in paper bags and oven-dried to constant weight at 70°C for 48 hours. The dry ingredients of 24 treatment combination samples were ground up in a CryoMill grinding ball (MM 400, Retsch, RSA). For each sample, a 50 mL centrifuge tube was used to add a precise amount of 1 g finely

milled plant leaf, weighed out on an electric scale (SF-400C, Snatcher, RSA) and 10 mL of 50% methanol with 1% of formic acid liquid chromatography-mass spectrometry (LCMS) grade was added. All centrifuge tubes were sealed tightly to mix thoroughly using a vortex apparatus for 60 seconds. Following that, samples were placed in an ultrasonic bath instrument for 60 minutes at room temperature to solubilise the chemical constituents from the plant material into the solvent (extraction). Of the extracts obtained, 2 mL was withdrawn into clean 50 mL centrifuge tubes and closed with a screw cap. Subsequently, the samples were centrifuged for 5 minutes at 14,000 revolutions per minute. After centrifugation, the supernatant recovered was diluted 10-fold in deionised water and transferred into 1.5 mL clear glass vials for the phenolic analysis.

Sample analysis

Phenolic analysis was performed using the LC-MS method. To detect and separate different phenolics in extracted liquid samples, a Waters Synapt G2 Quadrupole time-of-flight (QTOF) mass spectrometer (MS) linked to a Waters Acquity ultra-performance liquid chromatograph (UPLC) system (Waters, Milford, MA, USA) was utilised to perform high-resolution UPLC-MS analysis.

Electrospray ionisation was utilised in a negative ionisation mode with 15 V (cone voltage), desolvation gas at 650 L hr⁻¹, and at a temperature of 270°C. Moreover, the remaining MS settings were modified for the highest resolution and sensitivities. For MS data acquisition, samples were subjected to two mass spectrometry enhanced channel modes and m/z 150 to 1500 m/z in resolution mode scanning. MS^E modes differed by collision energy used to acquire fragmentation data; one channel was at 4 V (lowest), and the second was at an energy ramp of 40–100 V. The Leucine Enkephalin instrument was calibrated with sodium formate and used as a lock mass to determine the accurate mass.

Liquid chromatography (LC) separates phenolic compounds in the reverse phase with columns and mobile phases (López-Fernández *et al.*, 2020). Sample components were chromatographically separated using a Waters HSS T3 column (2.1 × 100 mm, 1.7 µm). The column temperature was kept at 55°C, and the flow rate was 0.3 mL min⁻¹. The injection volume used was 2 µL, and the mobile phase comprised of solvents A and B. Solvent A was 0.1% formic acid, and solvent B was the acetonitrile containing 0.1% formic acid. The gradient was initially at 100% A for 1 minute and linearly changed to 28% B for more than 22 minutes. After that, it went to 40% B over 50 seconds and followed a wash step of 1.5 minutes at 100% B, and

the next step was re-equilibration to the initial condition of 4 minutes. Finally, compounds were assessed in a relative way using a calibration curve developed by injecting a range of catechin standards, from 0.5 to 100 mg L⁻¹ catechin.

3.2.5 Statistical analysis

Statistical analyses for the analysis of variance (ANOVA) were conducted using the PROC GLIMMIX procedure in SAS 9.4 (PROC GLIMMIX, SAS Institute 2013). The data was subjected to multivariate data analysis using principal component analysis to determine any significant differences among measured values on the effects of the multistress. All data were meticulously checked for satisfaction of the normality and homogeneity of variance before the commencement of data analyses. The mean separation for least significant differences (LSD) was done according to the Tukey test at a 5% significance level.

In addition, for secondary metabolites (phenolics), multivariate data analyses were carried out using the principal component analysis (PCA) method and with the help of orthogonal partial least square discriminatory analysis (OPLS-DA), as well as hierarchical cluster analysis (HCA) using SIMCA-P (13.0, Umetrics, Sweden) software following a Pareto scaling procedure. In PCA analysis, scatter score plots were established to visualise, identify, and assess groupings, as well as strong outliers (Maree & Viljoen, 2011). The OPLS-DA was performed as a second phase analysis to separate the systematic variations in the X-matrix into two parts: (i) linearly related to the Y-matrix and (ii) unrelated to the Y-matrix, according to Maree and Viljoen (2011). The PCA is an unsupervised analysis that provides an overview of all the observations in the evaluated sample, while the OPLS-DA is a supervised pattern recognition technique (Maree & Viljoen, 2011).

3.3 RESULTS AND DISCUSSION

3.3.1 Morphological and yield parameters

Plant height

Plants respond to the abiotic effects depending on the growth stage, exposure intensity, and length of the stress occurrence. Typically, plant height is used to evaluate plant growth under optimal and stress conditions. The results of statistical analysis (summary of ANOVA) of plant height results observed for four weeks after transplanting the curly lettuce under multistress conditions are displayed in Table 3.3, and detailed results are in Table 3.4.

In general, water deficit regimes, plant bag size, and leaf wounding as individual abiotic stress factors significantly affected the plant height of curly lettuce at $p < 0.05$ in the fourth week (at harvest). The highest and the lowest plant height under water deficit stress were observed at the control (100%) and 40% treatment at 17.37 cm and 13.21 cm, respectively. The maximum plant height at harvest under 100% water-holding capacity indicates the growth effect (Tripodi *et al.*, 2024). Plants grown in the small plant bag (1.25 L) were shorter by 17.77% compared to the 5 L plant bag (Table 3.4). Plant height, therefore, increases with pot size and decreases as the size becomes smaller (Obede da Silva Aragão *et al.*, 2020; Gimeno-Páez *et al.*, 2025). A study by Zakaria *et al.* (2020) on root-restricted chilli (*Capsicum annum* L.) showed that the plant height decreased with time in the small containers of 2,392 cm³ (30.48 cm depth and 10 cm diameter) as shown by 14% reduction compared to plants in control container 9,570 cm³ (30.48 cm depth and 20 cm diameter) on day 98. In the same way, Cavalcanti *et al.* (2024) reported the reduced plant height in pepper genotypes grown in 2 dm³ and 0.75 dm³ pots, respectively, compared to 5 dm³ pots. Similarly, Magar *et al.* (2022) recorded a decrease of 24.9% and 16.3% in tomato and chilli pepper (*Capsicum frutescens* L.) seedlings grown in small pot volumes of 50 cm³ and 70 cm³, respectively, compared to large pots (90 cm³).

Mechanical wounding in plants induces a defence response to local and systemic leaves close to the wounded leaves, which consequently reduces plant performance (Sabina & Jithesh, 2021). A more recent study by Yan *et al.* (2024) revealed that the abscisic acid (ABA) and methyl jasmonate (MeJA) hormones are accumulated in response to wounding stress, but hinder cell division and elongation in improving plant resistance to stress. In this study, the plant height of leaf-wounded plants (14.55 cm) was not taller than intact plants (15.35 cm) at harvest (Table 3.4). Although it is known that plant defence mechanisms manifest a network of hormonal crosstalk to regulate growth after mechanical wounding. For instance, Yan *et al.* (2024) indicated that endogenous hormones indole-3-acetic acid (IAA), gibberellic acid (GA₃), and zeatin-riboside (ZR) are involved in growth promotion and increase to accelerate wound healing after mechanical wounding by elevating cell division and elongation. In addition, Liu *et al.* (2024) pointed out that after mechanical wounding was applied by puncturing the cucumber stem, it took approximately 15–25 hours for the wound length to be remarkably reduced. This implies that plants heal and recover fast from wounds, depending on the type of plant and wound site.

Number of leaves

The water deficit regimes, plant bag size, and leaf wounding treatments individually had a significant effect ($p < 0.05$) on the number of leaves at harvest (Table 3.4). Water deficit stress at 60% significantly reduced the number of leaves by 15.53% compared to the control treatment (100%) at harvest. An earlier study by Agami (2013) described that water deficit affected leaves by reducing midvein, blade, adaxial, and abaxial epidermis thickness, and average diameter of vessels, reducing mesophyll due to a decline in intercellular space in leaves.

The number of leaves in curly lettuce grown in a 1.25 L plant bag was reduced by 18.75% compared to the 5 L bag size. This aligned with the findings of Gimeno-Páez *et al.* (2025), who reported that two tomato varieties exhibited significantly fewer leaves in the XS (0.2 L) containers compared to XL (6 L) containers. Similarly, Cassiano *et al.* (2025) examined the effect of pot size on soybeans, and small pots (1.7 L) hindered the growth of shoots compared to large pots (7 L). The inhibition of vegetative growth imposed by small pots is attributed to the increased constriction of root systems (Cavalcanti *et al.*, 2024). Targino *et al.* (2019) also found that the maximum amount of lettuce leaves was obtained in a bigger pot volume (3.0 L).

Mechanical wounding reduced the leaf number by 9.39% at harvest compared to intact plants (Table 3.4). Shoot growth rate can be reduced as early as three days after mechanical wounding (Cunha *et al.*, 2023). Yan *et al.* (2024) observed that mechanical damage triggers the synthesis of JA antecedent and active form, namely 12-oxo phytodienoic acid (OPDA) and jasmonoyl-isoleucine (JA-Ile). Major studies in tomatoes documented that during mechanical wounding, jasmonate (JA) serves as a potent growth inhibitor as plants sense the injuries and activate its signalling pathway leading to the restriction of shoot growth (El Amerany *et al.*, 2022; Cunha *et al.*, 2023; Yan *et al.*, 2024).

The interaction between the water deficit irrigation regimes and plant bag sizes statistically impacted the number of leaves per plant ($p < 0.05$), as shown in Figure 3.2. At harvest, the interaction between 40% and 1.25 L diminished the number of leaves produced per plant by 23.41% compared to the interaction between 100% $\times$ 1.25 L treatments (Figure 3.2). These findings confirm what was presented by Rizar *et al.* (2023), who reported that the limited substrate volume coupled with water stress may cause plant stress due to restricted root growth and water availability in the root media. In contrast to the results of the present study, Rizar *et al.* (2023) found that the substrate volume did not significantly affect the number of yard-long

bean (*Vigna unguiculata* L.) leaves on the two examined pot sizes, measuring 30 cm diameter x 37 cm height (large) and 30 cm diameter x 30 cm height (small). A study by Silva *et al.* (2022) on soybeans reported that plant failure to adapt to the combination effects of water deficit stress and high bulk density resulted in irreversible leaf wilting and, eventually, plant death. Wilting is caused by the loss of turgor in the leaves, and wilting increases susceptibility to extreme light (Tripodi *et al.*, 2024). The current study supports this explanation by using representative images (Figure 3.7 D and H) of treatments. An increase in canopy light penetration depth is associated with the dropping of the leaves (Tripodi *et al.*, 2024).

Leaf area

The expansion of the leaf area is crucial for trapping and transmitting photons for photosynthesis reaction (Inoue *et al.*, 2021). In the present work, Table 3.4 shows that the maximum values of leaf area were obtained on the control treatments (100% water deficit regime, 5 L plant bag, and intact plant) with 856,22 cm², 826.99 cm², and 853.03 cm², respectively. Likewise, the lettuce grown in big pots had a significantly higher foliar area than lettuce grown in small pots (Stoleru *et al.*, 2020). Furthermore, Raviteja *et al.* (2021) reported a high leaf area in plants grown in bigger containers compared to small containers. The reduction of water potential in the growth media is relative to lower leaf water potential (Jang *et al.*, 2024). Water in plant tissue helps maintain cell turgor, improves plant synthesis processes, and facilitates the increase of leaf thickness and enlargement (Isa *et al.*, 2021).

Among the combination of abiotic stresses, the interactive 60% water deficit regimes × 1.25 L plant bag had the smallest leaf area (507.04 cm²) compared to 100% × 1.25 L interaction (822.65 cm²), as shown in Figure 3.3 A. According to Hill *et al.* (2024), small pots require more frequent irrigation due to small substrates, which can alter water retention and nutrient availability for plants, consequently affecting leaf development and photosynthesis. The lowered leaf area negatively affects photosynthesis (Spada *et al.*, 2024). When encountering water deficit, plants produce smaller leaves to lessen the transpiration rate and enhance tolerance to stress (Spada *et al.*, 2024). Nunes *et al.* (2024) reported a decrease in lettuce leaf area under 60% irrigation level of crop evapotranspiration as the water content in the substrate decreased because of evapotranspiration. Yavuz *et al.* (2023) noted that plant cell water loss caused increased membrane damage in lettuce leaves, lowering the relative water content. The restriction of roots in small containers decreased chilli leaf area by 25% at harvest compared to those in large containers (Zakaria *et al.*, 2020). Furthermore, the combination of 100% and 5 L

plant bag had the largest leaf area per plant (900.15 cm^2), followed by the interaction of $40\% \times 5 \text{ L}$ with 855.64 cm^2 (Figure 3.3 A). Due to higher water availability in large substrate volumes, the substrate effortlessly sustained its moisture content and recorded higher specific leaf area values, indicating broader leaves, which allow plants to absorb more light for photosynthesis (Rizar *et al.*, 2023).

The combination of water deficit regimes and mechanical wounding revealed that all leaf-wounded plants had a diminished leaf area per plant compared to intact plant treatments, regardless of the water deficit regime treatment (Figure 3.3 B). This phenomenon can be explained by the declined capacity of plants to grow and leaf necrosis mechanisms induced by water deficit and mechanical wounding (Ferreira *et al.*, 2024). Additionally, leaf wounds induce a reduction in the photosynthetic rate within 1 to 2 hours of mechanical injury due to the removal of the leaf area or wrecked leaf photosynthetic tissue, which affects the sink activity of the remaining wounded leaves (Konstantis *et al.*, 2022). Jiang *et al.* (2025) reported that the leaf area of defoliated rice plants was significantly reduced.

Root morphology

A healthier root system growth facilitates superior canopy growth above ground (Raviteja *et al.*, 2021). A pictorial presentation (Figure 3.7 A-D) shows the impact of the multistress on the roots of curly lettuce at harvest (30 DAT). Technically, visual differences were observed that were related to multistress factors. Water deficit stress impairs root growth by causing shorter, thinner, and fewer roots (Spada *et al.*, 2024; Araz *et al.*, 2025). Under water deficit stress, roots play a significant role by communicating with above-ground parts through signalling pathways (Raviteja *et al.*, 2021). However, the plant bag size is one of the critical factors determining plants' adaptability under water deficit stress conditions. Water deficit led to an increased root-to-shoot ratio in large pots, and these components decreased as pot size decreased (Dambreville *et al.*, 2016). In this study, visual observations showed that roots from the small plant bag size (1.25 L) had the shortest roots compared to other plant bag size treatments. In selecting the optimal pot volume for lettuce cultivation, some studies considered the pot proportion of 3 L (Targino *et al.*, 2019). In the present study, the large (5 L) and medium (3.5 L) plant bag sizes had longer plant roots with no visually significant difference. Previous research identified that plants grown in big pots exhibit a higher number of roots and root density compared to plants in small pots (Cavalcanti *et al.*, 2024). Multiple studies in horticulture consistently reported that plants grown in smaller pots usually give lower root

weight compared to larger pots, such as reported for lettuce (Stoleru *et al.*, 2020), *Capsicum* sp. genotypes (Raviteja *et al.*, 2021), peppers (Cavalcanti *et al.*, 2024), tomatoes (Gimeno-Páez *et al.*, 2025), and soybean plants (Cassiano *et al.*, 2025).

Leaf fresh mass

All individual abiotic stresses significantly affected ($p < 0.0001$) the fresh mass (g plant^{-1}) of curly lettuce (Table 3.4). The highest fresh yield of curly lettuce was recorded at 100% irrigation water regime of 867.61 g and decreased by 51.16% under a 60% water deficit regime. These results agree with those mentioned by Nunes *et al.* (2024), whereby a 60% crop evapotranspiration of water replacement level strongly reduced leaf yield plant^{-1} on the first and second cycles of lettuce crops. The variation in container size and root restriction significantly affects shoot growth (Targino *et al.*, 2019). The larger container provides more favourable conditions for plant growth due to the greater storage capacity of the substrate, which conserves more water and nutrients in order for the roots to uptake the necessary amount (Targino *et al.*, 2019). In the current study, the 5 L plant bag had a significantly higher fresh weight yield (782 g) than the smallest plant bag (1.25 L), which decreased by 40.91% (Table 3.4). Similarly, Stoleru *et al.* (2020) recorded an increase in the plant foliar weight for the bigger pot volume compared with small pots. The study further explained that the bigger pot volume allowed better radicular development, which improved nutrient uptake and, therefore, improved foliar growth.

Mechanical wounding reduced the fresh mass by 38.04% compared to intact plants with 330.41 g (Table 3.4). Moreover, the interaction of water deficit regimes and mechanical wounding stress significantly affected the fresh mass of curly lettuce at $p < 0.05$ level (Figure 3.4). The lowest fresh mass value was obtained in plants grown under the lowest water supply and leaf-wounded plants ($40\% \times$ wounded plants) with 320.40 g compared to $100\% \times$ leaf-wounded plants' interaction with 645.44 g. However, the highest fresh mass was found in control interactive treatments ($100\% \times$ intact plants) with a value of 1,089.77 g. The overall interactive effect of water deficit regimes and wounded plant treatments lowered the fresh yield of curly lettuce more than the interaction of water deficit regimes and intact plant treatments. A recent study by Ferreira *et al.* (2024) revealed that the activated mechanisms differ when water restriction and mechanical wounding are imposed on young plants. Other researchers have identified a decrease in the transpiration rate induced by mechanical wounding, an important physiological indicator of plant gas-exchange (Konstantis *et al.*, 2022). Francisco *et al.* (2023) demonstrated that the mechanical wounding of leaves affected plant growth and ultimately

reduced the fresh weight of *Brassica* plants by approximately 34%. Similarly, Jiang *et al.* (2025) noted that mechanical defoliation decreased the fresh weight of rice shoots. For the interaction of multistress treatments, the differences in the fresh mass were not statistically significant.

Leaf dry mass

The single abiotic stresses significantly affected the dry mass (g plant^{-1}) of curly lettuce (Table 3.3). The 60% water deficit, 1.25 L plant bag, and leaf wounded plants reduced the dry yield by 30.71%, 28.50%, and 35.41%, respectively, compared to control treatments (Table 3.4). These results are in line with previous studies where shoot dry mass was reduced by 60% water deficit in lettuce (Nunes *et al.*, 2024), and small containers reduced the leaf dry weight of chilli plants (Zakaria *et al.*, 2020), and mechanical wounding of leaves lowered the dry mass of broccoli, turnip greens, and rapeseed (Francisco *et al.*, 2023).

In the case of factorial treatments, the significant interactive effects of multistress (water deficit regimes $\times$ plant bag size $\times$ mechanical wounding) on the dry mass of curly lettuce are shown in Table 3.5. The dry yield produced by 40% $\times$ 1.25 L $\times$ leaf wounded plants on curly lettuce had the maximum reduction of 54.50% compared to 40% $\times$ 1.25 L $\times$ intact plants treatments. Water stress has been reported to negatively impact cell elongation and damage the cell membrane, which deteriorates the morphology of roots and shoots, causes loss of photosynthetic activity, and induces low accumulation of dry matter (Tüfenkçi & Yerli, 2023; Araz *et al.*, 2025). The amount of dry matter produced is relative to the volume of water consumed, reflecting water use efficiency. Dambreville *et al.* (2016) found that the large pot size improved the root-to-shoot ratio in rape oilseed (*Brassica napus* L.) compared to small pot sizes. A study by Magar *et al.* (2022) reported that the small pot volume constrained the vegetative growth of tomatoes and chilli peppers, as demonstrated by the decrease in dry weight. Conversely, the larger pot size with unrestricted root growth achieved greater dry weights (Magar *et al.*, 2022). The container size influences the growth of the roots, determining the dry matter distribution between above- and below-ground components (Raviteja *et al.*, 2021). According to Francisco *et al.* (2023), a large proportion of dry matter can be reduced in response to wounding stress.

3.3.2 Physiological parameters

Chlorophyll content

Chlorophyll intensifies light energy utilisation, which turns water and CO₂ into organic compounds, and promotes photosynthesis (Inoue *et al.*, 2021; Nune *et al.*, 2024). In curly lettuce plants, different responses of chlorophyll content were observed under multistress, as shown in the summary of ANOVA Table 3.3. The findings revealed that chlorophyll content was significantly impacted by the interaction of water deficit regimes $\times$ plant bag size as well as water deficit regimes $\times$ mechanical wounding (Table 3.4) at harvest. The literature provides contrasting reports about increased chlorophyll content under abiotic stresses. Like those previously reported by Jang *et al.* (2024) on Chinese cabbage seedlings and Demir *et al.* (2022) on lettuce, an increment in the content of chlorophyll pigments was evident due to increased water stress. Similar to the current study, the chlorophyll content increased under an increased water deficit regime. As shown by the correlation between the interactions of 40% $\times$ 5 L (Figure 3.5 A) and 40% $\times$ intact plant (Figure 3.5 B), with the highest chlorophyll content values. This remarkable phenomenon is likely due to the reduction in leaf relative water content inducing an increase in leaf chlorophyll content (Spada *et al.*, 2024; Araz *et al.*, 2025). On the other hand, Tüfenkçi and Yerli (2023) reported that the chlorophyll content of curly lettuce was reduced with the reduction of the irrigation water regime because of inadequate substrate moisture due to drought stress conditions causing oxidative damage to plant cells and the disruption in metabolic processes.

Furthermore, Hill *et al.* (2024) reported that the chlorophyll content was affected by the pot size and root restriction in small pots (or vice-versa) more than the unavailability of substrate water. A study by Hawash *et al.* (2024) on lettuce confirmed the potential of large substrate volume to positively affect the total chlorophyll content when obtaining the highest total chlorophyll content in 8 L rather than 6 L. It is well known that chlorophyll concentration results in increased photosynthetic activity (Araz *et al.*, 2025; Velasco-Clares *et al.*, 2025). A study by Obede da Silva Aragão *et al.* (2020) revealed that the pot size affects the process of photosynthesis in common beans (*Phaseolus vulgaris* L.) as demonstrated by the decrease of phytomass accumulation in small pots (1 kg) as compared with larger pots (3 and 5 kg).

Yan *et al.* (2024) found that among chlorophyll fluorescence related parameters, the minimum fluorescence increased significantly 12 hours after 80 compression pressure stress was applied, imitating mechanical wounding in tomato seedlings. This suggests photochemical

damage to the photosystem II reaction centre induced by the separation of the light-harvesting complex II from the photosystem II reaction centre in response to wound stress. Alternatively, diminishing the efficiency of excitation energy transfer from the chlorophyll molecules of light-harvesting complex II to the photosystem II reaction centres consequently causes physiological disorders such as a reduction of photosynthetic parameters and eventually decreases the chlorophyll content (Yan *et al.*, 2024). The same researchers discovered that the fluorescence parameters, including minimum and maximum fluorescence and variable fluorescence, were reinstituted 36 hours after mechanical wounding, stipulating that the photochemical damage to photosystem II was ameliorated (Yan *et al.*, 2024). Similar to the present study, it was evidenced by a decrease of 16.53% in the chlorophyll content due to mechanical wounding compared to the control (intact plants) in the third week (data not shown). These results are also supported by Konstantis *et al.* (2022), where authors observed that the leaf chlorophyll content of basil (*Ocimum basilicum* L.) plants in the flowering stage decreased after wounding. However, there was no significant difference between the water deficit regime and mechanical wounding interaction in the chlorophyll content values observed at harvest, and the multistress showed no significant interaction (Table 3.4).

Stomatal conductance (Gs)

Multi-abiotic stress factors induced changes in curly lettuce plant physiological gas exchange parameters. Stomatal conductance is vital for gauging the extent of stomatal opening and closure, thus related to water loss and the transpiration rate in plants (Konstantis *et al.*, 2022; Yavuz *et al.*, 2023). Stomatal conductance was significantly affected by water deficit regimes, plant bag size, and mechanical wounding as single factors for the last three consecutive days of the study (Table 3.6). Moreover, the interaction of multistress did not significantly affect the stomatal conductance.

The interaction between water deficit regime $\times$ plant bag size significantly reduced the stomatal conductance with a decrease in both factors (Table 3.7). The highest stomatal conductance value was $445.36 \text{ mmol m}^{-2} \text{ s}^{-1}$ detected in the control treatment combination of $100\% \times 5 \text{ L}$, relating to $40\% \times 1.25 \text{ L}$ interaction, stomatal conductance decreased by 70.79%. Studies by Tüfenkçi and Yerli (2023) and Chishti *et al.* (2025) reported a decrease in stomatal conductivity with an increase in water deficiency in curly lettuce and radish (*Raphanus sativus* L.). In Chinese cabbage seedlings, Jang *et al.* (2024) reported that closing stomata leaves minimises water absorption capacity, and lower stomatal conductance reduces photosynthesis.

The earlier evidence of the pot size effects on soybean plants from a study conducted by Kasai *et al.* (2012) revealed that leaf photosynthetic rate, transpiration rate, and stomatal conductance increased more in plants transplanted into larger pots (24 cm in height, 20 cm in diameter) than in plants grown in smaller pots (11.4 cm in height, 7.5 cm in diameter). Rizar *et al.* (2023) also verified that a large substrate volume has a higher moisture value, whereas a small substrate volume has reduced substrate moisture. Increased transpiration and evaporation rates accelerate water loss in the substrate (Hill *et al.*, 2024). Relating to the present study, this implies that more water in larger pots allowed necessary physiological processes to occur in an ideal manner.

Water stress and leaf wounding treatment had a significant effect on stomatal conductance (Table 3.8). The greatest value of stomatal conductance was $442.74 \text{ mmol m}^{-2} \text{ s}^{-1}$ found in 100% water deficit $\times$ intact plants, and the least was $155.09 \text{ mmol m}^{-2} \text{ s}^{-1}$ obtained in 40% and wounded plants. Numerous studies reported that as the growth medium dehydrates, lettuce plants tend to diminish the transpiration rate to minimise water loss, but this adaptation mechanism decreases stomatal conductance and disturbs the photosynthesis process (Kong *et al.*, 2023; Yavuz *et al.*, 2023; Nunes *et al.*, 2024). Do Prado Mascarenhas *et al.* (2025) demonstrated that defoliation stress and water deficit on maize diminish the available area for photosynthesis and consequently reduce photosynthates and nutrient distribution to the leaves due to the restricted irrigation, eventually decreasing the plant growth. On the other hand, it is known that leaf wounding decreases stomatal conductance (Konstantis *et al.*, 2022). Furthermore, Yan *et al.* (2024) mentioned that plants responded to mechanical wounding by closing stomata, impeding carbon dioxide (CO_2) influx, and reducing intercellular CO_2 , consequently hindering photosynthetic carbon assimilation.

3.3.3 Biochemical parameters

Leaf mineral composition

Macro and micronutrients play an essential role in the human diet by preventing metabolic disorders and providing balance (El Amerany *et al.*, 2022). The nutritional properties of curly lettuce from this study are presented in Table 3.9. Individual abiotic stresses in the current study significantly affected the N, P, Ca, and Mg macronutrients and the Na, Zn, and Mn micronutrient content of curly lettuce leaves. Regarding leaf mineral content affected by only the individual abiotic stresses, the Mg content was significantly affected ($p < 0.002$) by water

deficit regimes, where the highest and lowest Mg content, 0.51% and 0.38%, were found at 80% and 40% water deficit regimes, respectively. Likewise, Demir *et al.* (2022) and Tüfenkçi and Yerli (2023) found that when irrigation water was reduced, the Mg content of curly lettuce declined. The lowest Zn content was at 100% water deficit, 5 L plant bags, and intact plants as control treatments compared to other treatments. A high Zn content was observed in leaf-wounded curly lettuce and 1.25 L treatments. The size of the plant bag might have influenced the response of Zn content to water deficit stress.

Excessive accumulation of Na can cause toxicity by disrupting nutrient transportation and altering plant osmotic balance (Tüfenkçi & Yerli, 2023). Nevertheless, it is believed that Na content may stimulate or suppress the accumulation of K involved in osmotic adjustment (Geremew *et al.*, 2021). For instance, the accumulation of abscisic acid in plants in response to adverse abiotic stresses may be promoted, causing an imbalance of ions and leading to the leakage of K^+ from the cells surrounding the stomata and, eventually, the loss of turgor pressure on guard cells (Salem *et al.*, 2022). In this study, the lowest Na content was observed in curly lettuce receiving a 100% water supply compared to other irrigation treatments. Unlike the current study, Geremew *et al.* (2021) noted that water deficit stress reduced the Na content in the leaves of mustard.

The K element plays a significant role in plant growth by maintaining water balance and facilitating the transportation of nutrients through the xylem and phloem (Geremew *et al.*, 2021; Abd El-Fattah *et al.*, 2022). Thus, improving K nutrient uptake is beneficial for reducing the Na nutrient uptake and eliminating the competition between these two elements that negatively affect the overall nutrient uptake from the root system (Tüfenkçi & Yerli, 2023). In this study, the abiotic stresses did not significantly affect K (Table 3.7). Saia *et al.* (2019) also reported that none of the irrigation regimes significantly affected leaf K concentrations in lettuce. The Fe and Cu contents were not significantly affected by the abiotic stress in the present study.

Furthermore, N, P, Ca, and Mn concentrations were also statistically affected by the interaction between water deficit regimes $\times$ plant bag size $\times$ mechanical wounding (Table 3.10). The highest N content was obtained in 40% $\times$ 1.25 L $\times$ leaf wounded plants (5.59%), and the lowest was recorded in 60% $\times$ 5 L $\times$ intact plants (3.18%) compared to other treatments. Similarly, Hawash *et al.* (2024) recorded the lowest nutrient use efficiency value in plants grown in small substrate volumes compared to big pot sizes. According to the previously mentioned study, the lower the N content in lettuce leaves, the higher the N content use

efficiency. In this context, an increased N content in plants grown in small plant bag sizes likely contributed to the sustained chlorophyll content (Figure 3.5). However, it eventually led to the loss of lettuce production. Restricting the root zone in small pots reduced N metabolism (Cassiano *et al.*, 2025).

The increased irrigation water level reduced the P availability in the soil during the two growth seasons of lettuce as consequential leachability out of the soil substrate profile (Abd El-Fattah *et al.*, 2022). The lowest water deficit level resulted in the highest value of leaf P content, following the sequence from low to high water deficit levels (Abd El-Fattah *et al.*, 2022). In the current study, the water regime influenced the response of leaf P content as it appeared higher in $100\% \times 1.25 \text{ L} \times \text{intact}$ plants by 4.49% than in other treatments. The decrease was noted in $60\% \times 3.5 \text{ L} \times \text{intact}$ plants interaction with 2.61%, followed by 1.61% from $40\% \times 5 \text{ L} \times \text{intact}$ plants interacting treatments. Obede da Silva Aragão *et al.* (2020) reported no difference in plant development among plants receiving the same amount of nutrients in 3 kg and 5 kg pots. Similar to this study, the P content was lower in 3.5 L and 5 L. The Ca content was high in $100\% \times 1.25 \text{ L} \times \text{intact}$ plants by 1.88% and low in $100\% \times 3.5 \text{ L} \times \text{intact}$ plants with a 1.15% decrease. A recent study shows that plants in small pots (1.7 L) can accumulate high Ca content in the leaves under full irrigation conditions (Cassiano *et al.*, 2025). The maximum value of Mn content was observed in $80\% \times 1.25 \text{ L} \times \text{leaf wounded}$ plants as group *a*, and the minimum values were seen in group *h* from $40\% \times 5 \text{ L} \times \text{intact}$ plants, and all other treatments were in similar groupings. These results align with those obtained on lettuce during the two growing seasons, where Mn content was reduced by 50% water deficit and increased by the increase of water supply (Demir *et al.*, 2022). The reduction of Mn content under abiotic stress conditions is linked to a decrease in synthesising critical enzymes retaining ions, such as superoxide dismutase (Geremew *et al.*, 2021).

The principal component analysis (PCA) was further performed to display and interpret the multifactorial effects of different water deficit regimes (100%, 80%, 60%, and 40%), plant bag sizes (5 L, 3.5 L, and 1.25 L) and mechanical wounding (intact and leaf wounded plants) on curly lettuce mineral content (Figure 3.8). The PCA biplot was used to assess the variation and association between the variables (leaf mineral parameters) and the scattered multi-abiotic stress treatment in the biplots (active observations). The first principal components (F1) and second principal components (F2) had 34.00% and 18.67% variances on the X and Y axes, respectively, which explain 52.67% of the total variance in data of all analyses of leaf mineral parameters.

The correlation among Na, N, Cu, Zn, and Ca are related to the F1 biplot loading, while K, Fe, Mg, P, and Mn are assigned to the F2 biplot, as shown by vectors. The highest positive correlations were observed between Zn, Cu, and Ca on the right side of the X-axis. This correlation was influenced by the combined effects of 100% or 80% water deficit regimes, regardless of the plant bag size and wounding treatment. Moreover, Na correlated with N content in F1 loading, caused by the reduction of water supply (40% water deficit regime) and 3.5 L or 1.25 L with either intact or leaf wounded plants. The interaction of 60% $\times$ 1.25 L $\times$ leaf wounded plants or 40% $\times$ 3.5 L $\times$ leaf wounded plants induced a strong positive correlation between F2 and P concentration. In comparison, Mg and Mn showed a significant negative correlation among F2 loadings caused by 100% $\times$ 1.25 L $\times$ leaf wounded plants and 80% $\times$ 3.5 L $\times$ intact plants interactive treatments. In general, the current study demonstrated that the plant bag size and water deficit regime were more critical than leaf wounding in determining the highest nutrient use efficiency and nutrient deficiencies influencing the nutrient quality properties of curly lettuce. Logically, the results elucidate that an increase in plant bag size and the increase in the water deficit regime led to the reduction of nutrient uptake by plant roots, likely due to the insufficient nutrients in the large amounts of substrate in big plant bag sizes caused by limited fertigation as a result of reduced water supply.

Phenolic content

Phenolic compounds are secondary metabolites that exist in either soluble or non-soluble forms with the potential to scavenge harmful effects in plants caused by multiple abiotic stresses (Chauhan *et al.*, 2023; Rao & Zheng, 2025). Phenolic compounds play a vital role as antioxidants in insulating plant cells against oxidative stress, scavenging free radicals, inhibiting lipid oxidation, deactivating reactive oxygen species, and dissolving peroxides under abiotic stress to support the plant's survival (Rao & Zheng, 2025; Velasco-Clares *et al.*, 2025). Table 3.11 shows details of the phenolic compounds detected using the LCMS method. In the current study, unsupervised multivariate analysis approaches, such as PCA and HCA heat map, as shown in Figure 3.9 and Figure 3.10, respectively, were made to visualise data and detect differences between phenolic compounds. These multivariate analyses simplify the complex MS data of interaction variables with dimensionality reduction of the dataset from observations. The unsupervised PCA was carried out in curly lettuce samples to evaluate spatial patterns and screen differential phenolic compounds between multi-abiotic stress treatments. Two principal component contribution values indicate the PCA clustering. The first principal component

(PC1) explained 42.3% of the variance, while the second principal component (PC2) explained 22.4%, and both components collectively accounted for approximately 64.7% of the total variance in the phenolic compound dataset.

The score plot of PCA showed significant differences in phenolic profiles. It was revealed through intergroups that the major factors that contributed to separation were water deficit treatments followed by leaf wounding. Francisco *et al.* (2023) demonstrated how the interaction of different phenolic compounds in response to wounding affects plant biomass. The leaf-cutting significantly stimulates the synthesis of the total phenolic content, leading to increased accumulation in lettuce (Flores *et al.*, 2023). Based on plot observations, there is a clear separation between 100% and 40% water deficit regimes on the left side of PC1 and right side of PC1, respectively. These results indicate a distinction between fully and minimally irrigated curly lettuce samples regarding their phenolic composition. Phenolic compounds have previously been shown to ameliorate the adverse impacts of multi-abiotic stressors by intensifying the antioxidant system, osmoregulation, and pigment synthesis and triggering the defence signalling mechanism of plants (Chauhan *et al.*, 2023; Santander *et al.*, 2024). Treatments that cluster relatively close or overlap indicate similarities in the composition of phenolic compounds in curly lettuce samples, as shown in Figure 3.10. Overall, water deficit served as a critical factor that altered the properties of phenolic compounds rather than plant bag size and leaf wound stress factors. The phenolic contents were elevated with the intensification of water deficit stress regardless of plant bag size and leaf wound stress except for the slight stress, 80%, which showed similarities with 100%.

While the PCA provided valuable insights, it was unable to discriminate fully between compounds in different treatments. Figure 3.10 shows the HCA, which highlights the variability and interrelations among identified phenolic compounds, providing a more comprehensive understanding of their response to treatments. The class distribution and high relative abundance of the class determine the HCA clustering outcomes. The heat map outlined three distinct clustering groups of 26 compounds in curly lettuce. In this case, the allocation of clustering groups 1, 2, and 3 can be observed from the cladogram in the bottom, middle, and top positions of HCA, respectively.

Cluster 1 revealed increased concentration levels of chlorogenic acid 3CQA, similarly related to caftaric acid, and chicoric acid appeared to be closely related to chlorogenic acid 5CQA, while quercetin 3-O-glucuronide was related to 4"-O-Acetylmyricitrin. Wounding stress alone has been reported to increase chlorogenic acid by 579.8% (Chauhan *et al.*,

2023). Furthermore, 6- $\{[2-(3,4\text{-dihydroxyphenyl})\text{-}5\text{-hydroxy-}4\text{-oxo-}4\text{H-chromen-}7\text{-yl}]oxy\}$ -3,4,5-trihydroxyoxane-2-carboxylic acid was related with Quercetin 3-O-(6"-acetyl-glucoside). Lastly, Quercetin 3-O-(6"-acetyl-glucoside) is related to quercetin 3-galactoside. These concentrations increased under the interaction of $100\% \times 3.5 \text{ L} \times$ intact plants and $40\% \times 5 \text{ L} \times$ intact plants, revealed by the high abundance value and followed by $100\% \times 3.5 \text{ L} \times$ leaf wounded plants and $40\% \times 3.5 \text{ L} \times$ intact plants compared to other treatments. On the other hand, in cluster 2, compound concentrations were low, specifically in L-Tryptophan, 3-O-p-Coumaroylquinic acid, and MINEs-320976 in leaf wounded curly lettuce samples. Similarly, Francisco *et al.* (2023) noted a low 3-O-p-Coumaroylquinic acid in response to wounding across *Brassica* genotypes. Cluster 3 was formed by quinic acid-derived and unknown compounds.

In addition to the PCA and HCA, a supervised PLS-DA was applied to distinguish further and clarify the correlation between the changes in the phenolic concentration of curly lettuce and the multistress factors. Figure 3.12 shows in the PLS-DA, PC1 explains 42.2% of variances, PC2 explains 22.3%, and the cumulative percentage of both PCs is 64.5%. The overlapping quantified phenolic compounds suggest no significant difference between multistress factors. Furthermore, a supervised OPLS-DA multivariate model (Figure 3.11) was employed to more accurately distinguish groupings and provide a clear separation among investigated multifactorial treatments. Compared with unsupervised methods, the OPLS-DA employs class membership to magnify variation between groups through its improved ability to interpret the classification (Huan *et al.*, 2017).

The interactive effects of multistress showed a visible separation of two classes in curly lettuce phenolic compound samples, as illustrated in the OPLS-DA biplot (Figure 3.11). The Orthogonal T score [2] has a significantly strong effect on phenolic compounds, which explains 30.3% of the total variation in group 2. The supervised comparison revealed that the interacting treatments with a 40% water deficit, varying plant bag sizes, and mechanical wounding treatments were placed on the right of the constructed model. These results suggest that phenolic compound concentration generally increased with a decrease in the water deficit regime as a key factor. Under abiotic stress conditions, phenolic contents increase to protect plants against ROS (Santander *et al.*, 2024). The T score [1] explains 16.5% of the total variance associated with Group 1. Based on observations among phenolic compounds clustering closer to each other, it can be concluded that interactive multistress treatments had similar effects in Group 1. However, the interactive treatments allocated at the left side of PC1 had no significant

impact on phenolic compound composition. Meanwhile, 80% and 60% water deficits and 3.5 L or 1.25 L plant bag and either subjected to leaf wounding or intact clustered at the right side of positive PC1. The results obtained demonstrate that these multifactorial effects induced phenolic compounds' responsive mechanisms towards multistress.

The variables importance in projection (VIP) score method was used to identify phenolic compounds that accumulated differentially among multistress treatments based on VIP score thresholds >1.0 (Figure 3.13). The VIP plot assigned scores to variables based on their highest contribution to the discrimination observed in the OPLSD-DA model, reflecting a clear understanding of variations in the accumulation of total phenolic compounds. The VIP plot score results are in line with OPLS-DA findings. The phenolic compounds in Group 2 samples had the most significant VIP values in adverse stress conditions. In contrast, Group 1 samples ranged between mild and moderate stress levels and were less concentrated in phenolic compounds listed explicitly on the VIP score plot. Moreover, 15 phenolic compounds in the VIP plot ranked between 1.0 and 1.8. The top 5 phenolic compounds with the highest VIP scores were chicoric acid, chlorogenic acid 5CQA, caftaric acid, chlorogenic acid 3CQA and 4"-O-Acetylmyricitrin. Santander *et al.* (2024) discovered that chicoric acid, chlorogenic acid, and caffeic acid in lettuce are compounds that increase concentration in leaves in response to water deficit stress.

In general, the multistress conditions significantly changed the concentrations of documented phenolic compounds illustrated on constructed models which belong to targeted and untargeted polyphenol classes (ontology) such as phenolic glycosides, coumaric acids, and derivatives, saccharolipids, tetracarboxylic acids, and derivatives, fatty acyl glycosides of mono- and disaccharides, naphthopyrans, flavonoid-3-O-glucuronides, flavonoid-3-O-glycosides, lignan glycosides, lactones, O-glycosyl compounds, medium-chain keto acids and derivatives, long-chain fatty acids and oxanes. Coumaric acids were increased to the highest level by wounding stress (Chauhan *et al.*, 2023). In plants, the synthesis of phenolic compounds depends on the stress intensity, the nature of the stressor, and the genotype (Francisco *et al.*, 2023; Santander *et al.*, 2024). Furthermore, broccoli, turnip greens, and rapeseed wounded in the morning and evening had lower content of chlorogenic acid and sinapoyl derivatives, and the flavonoid compounds were observed, implying that the exchange between the plant growth and phenolic compounds induced by stress is contingent on the time of injury (Francisco *et al.*, 2023). Therefore, based on the above-mentioned and obtained results, this study could conclude

that plants have evolved to counteract the adverse effects of multi-abiotic stresses by employing secondary metabolites.

3.4 CONCLUSION

The research showed that the evaluated multi-abiotic stress caused a significant reduction in yield, morphophysiological, and biochemical parameters of curly lettuce. Multivariate analysis revealed a higher accumulation of phenolic contents towards the intensification of multistress, indicating induced stress tolerance mechanisms in curly lettuce. From the yield, morphophysiological, and biochemical results, it could be concluded that the mild, moderate, and severe combined abiotic stress factors were recorded in $60\% \times 1.25 \text{ L} \times \text{leaf wounding}$, $40\% \times 3.5 \text{ L} \times \text{leaf wounding}$, and $40\% \times 1.25 \text{ L} \times \text{leaf wounding}$, respectively. The current study also provides recommendations for a more efficient plant bag size to increase the growth and yield of lettuce plants. Although the 3.5 L plant bag size maintained some plant qualities, the best producing was the 5 L plant bag. There was even no remarkable difference in morphological and yield parameters in curly lettuce grown in 5 L, irrespective of the evaluated water deficit regime. In fact, it can be concluded that 40% of irrigation water and a 5 L plant bag can produce the best yield of curly lettuce when the plants encounter no mechanical wounding. These findings propose that lettuce can be cultivated effectively in areas with restricted water supply without compromising its yield and nutritional benefits.

REFERENCES

- Abd El-Fattah DA, Hashem FAE, Abd-Elrahman SH. 2022. Impact of applying organic fertilizers on nutrient content of soil and lettuce plants, yield quality and benefit-cost ratio under water stress conditions. *Asian Journal of Agriculture and Biology* 10: 202102086.
- Abarca VHA, González-Sandoval DC, Martínez-Ávila GCG, Rojas R. 2022. Innovation in agriculture: A review of plant factory. *Biocontrol Systems and Plant Physiology in Modern Agriculture* 221-236.
- Toman E, Käs MD, Taubert B, Morit M, Qi J, Bolle C. 2024. Photosynthetic and plastid performance effects of photoreceptors. *Journal of Mitochondria, Plastids and Endosymbiosis* 2: 2330972.
- Agami RA. 2013. Salicylic acid mitigates the adverse effect of water stress on lettuce (*Lactuca sativa* L.). *Journal of Applied Sciences Research* 9: 5701-5711.
- Ahmad S, Khattak AM, Farooq A, Bibi R, Habi W, Gul G, Asghar M, Ahmed M. 2024. Effect of sowing dates on lettuce yield, grown in open field and partial shade. February 16th, 2024, Preprint. *Research Square*. <https://doi.org/10.21203/rs.3.rs-3955247/v1>.
- Aires ES, Aragão CA, Gomes ILS, Souza GN, Andrade IGV, Oliveira ABN, Yuri JE. 2020. Growth and production of crisphead lettuce cultivars in protected cultivation and high temperatures. *Brazilian Journal of Agricultural Sciences* 15: 1-9.
- Araz O, Ekinici M, Yildirim E. 2025. Physiological, biochemical and molecular response of pepper genotypes exposed to water deficit. *Journal of Crop Health* 77: 46.
- Awang Y, Shaharom AS, Mohamad RB, Selamat A. 2009. Chemical and physical characteristics of cocopeat-based media mixtures and their effects on the growth and development of *Celosia cristata*. *American Journal of Agricultural and Biological Sciences* 4: 63-71.
- Ban S, Hong I, Kwack Y. 2023. Prediction of growth and quality of Chinese cabbage seedlings cultivated in different plug cell sizes via analysis of image data using multispectral camera. *Horticulturae* 9: 1288.
- Becerra-Moreno A, Redondo-Gil M, Benavides J, Nair V, Cisneros-Zevallos L, Jacobo-Velázquez DA. 2015. Combined effect of water loss and wounding stress on gene

- activation of metabolic pathways associated with phenolic biosynthesis in carrot. *Frontiers in Plant Science* 6: 837.
- Cahyo AN, Sahuri ISN, Ardika R. 2019. Cocopeat as soil substitute media for rubber (*Hevea brasiliensis* Müll. Arg.) planting material. *Journal of Tropical Crop Science* 6: 18–29.
- Campos-Vargas R, Saltveit ME. 2002. Involvement of putative chemical wound signals in the induction of phenolic metabolism in wounded lettuce. *Physiologia plantarum* 114: 73–84.
- Cassiano GH, Diniz JF, Floreste BS, Yokoyama AA, Machado AIMR, Ribeiro RV, de Andrade SAL, Mazzafera P. 2025. Small pot size limits soybean growth and affects the symbiosis with *Bradyrhizobium japonicum*. *Journal of Plant Growth Regulation* 1-14.
- Cavalcanti TF, Soares Filho ES, Araújo MDSBD, Vieira CV, Sudré CP, Bispo RB, Rodrigues, R. 2024. In-depth analysis of ornamental pepper and pot volume interaction utilizing conventional and digital tools. *Horticultura Brasileira* 42: e285735.
- Cavell AJ. 1955. The colorimetric determination of phosphorus in plant materials. *Journal of the Science of Food and Agriculture* 6: 479–480.
- Chauhan J, Kumar V, Kumar B, Indu Chand S, Anuragi H, Patel R, Singhal RK. 2023. Plant phenolics: A dynamic compound family under unfavorable environment and multiple abiotic stresses. *In Plant Phenolics in Abiotic Stress Management* 105-124.
- Chishti MS, Shahbaz M, Kaleem M, Shafi S, Mehmood A, Qingzhu Z, Mansha M, Shehzadi N, Rana S, Shahid H, Hashem A. 2025. Fertigation with alpha-tocopherol enhances morphological, physiological, and antioxidant responses in radish (*Raphanus sativus* L.) under drought stress. *BMC Plant Biology* 25: 30.
- Combrink NJJ, Kempen E. 2019. Nutrient solution management. Department of Agronomy Stellenbosch University.
- Cunha AFA, Rodrigues PHD, Anghinoni AC, de Paiva V J, Pinheiro DGDS, Campos ML. 2023. Mechanical wounding impacts the growth versus defense balance in tomato (*Solanum lycopersicum*). *Plant Science* 329: 111601.
- Dambreville A, Griolet M, Rolland G, Dauzat M, Bédiée A, Balsera C, Muller B, Vile D, Granier C. 2016. Phenotyping oilseed rape growth-related traits and their responses to water deficit: the disturbing pot size effect. *Functional Plant Biology* 44: 35-45.

- Demir H, Kaman H, Sönmez İ, Mohamoud SS, Polat E, Üçok Z. 2022. Yield, quality and plant nutrient contents of lettuce under different deficit irrigation conditions. *Acta Scientiarum Polonorum Hortorum Cultus* 21: 115-129.
- do Prado Mascarenhas SNA, Pinto BRC, dos Santos JCN, Penha HGV, Pereira IS. 2025. Defoliation and inoculation with *Bacillus aryabhattai* on maize development and productivity in Triangulo Mineiro. *Studies in Engineering and Exact Sciences* 6: e14235-e14235.
- El Amerany F, Rhazi M, Balcke G, Wahbi S, Meddich A, Taourirte M, Hause B. 2022. The effect of chitosan on plant physiology, wound response, and fruit quality of tomato. *Polymers* 14: 5006.
- Ferreira AMO, Rodríguez VAC, da Silva Dias G, Vilas Boas LV, López ME, Bicalho EM. 2024. Strategies induced by methyl jasmonate in soybean seedlings under water restriction and mechanical wounding. *Acta Physiologiae Plantarum* 46: 66.
- Flores M, Amorós A, Escalona VH. 2023. Changes in agronomic, antioxidant compounds, and morphology parameters of green and red lettuces (*Lactuca sativa* L.) by successive harvests and UV-B supplementation. *Horticulturae* 9: 677.
- Francisco M, Doghri M, Rodríguez VM. 2023. Time of day of leaf wounding determines plant biomass and affects the interplay between growth and defence in Brassica crops. *Plant Biology* 25: 785-792.
- Geremew A, Carson L, Woldeesenbet S, Carpenter C, Peace E, Weerasooriya A. 2021. Interactive effects of organic fertilizers and drought stress on growth and nutrient content of Brassica juncea at vegetative stage. *Sustainability* 13: 13948.
- Gimeno-Páez E, Prohens J, Moreno-Cerveró M, de Luis-Margarit A, Díez MJ, Gramazio P. 2025. Agronomic treatments combined with embryo rescue for rapid generation advancement in tomato speed breeding. *Horticultural Plant Journal* 11: 239-250.
- Giron HC. 1973. Atomic absorption newsletter. *Perkin Elmer Atomic Spectrophotometer* 12: 28.
- Hawash A, Abul-Soud M, Emam MSA, Maharik ZY, Mohammed MH, Ahmed SH. 2024. Effect of pot substrate volume and nitrogen concentration of the nutrient solution on lettuce production under urban conditions. *Scientific Journal of Agricultural Sciences* 6: 21-35.

- Hill D, Conte L, Nelson D, Hammond J, Bell L. 2024. Investigating the water availability hypothesis of pot binding: small pots and infrequent irrigation confound the effects of drought stress in potato (*Solanum tuberosum* L.). *Frontiers in Plant Science* 15: 1399250.
- Hossain A, Pamanick B, Venugopalan VK, Ibrahimova U, Rahman MA, Siyal AL, Maitra S, Chatterjee S, Aftab T. 2022. Emerging roles of plant growth regulators for plants adaptation to abiotic stress-induced oxidative stress. In *Emerging plant growth regulators in agriculture* 1-72.
- Huang BM, Chen TB, Xiao SY, Zha QL, Luo P, Wang YP, Cui XM, Liu L, Zhou H. 2017. A new approach for authentication of four ginseng herbs and their related products based on the simultaneous quantification of 19 ginseng saponins by UHPLC-TOF/MS coupled with OPLS-DA. *RSC Advances* 7: 46839-46851.
- Ilahi WFF, Ahmad D. 2017. A study on the physical and hydraulic characteristics of cocopeat perlite mixture as a growing media in containerized plant production. *Sains Malaysiana* 46: 975-980.
- Inoue T, Sunaga M, Ito M, Yuchen Q, Matsushima Y, Sakoda K, Yamori W. 2021. Minimizing VPD fluctuations maintains higher stomatal conductance and photosynthesis, resulting in improvement of plant growth in lettuce. *Frontiers in Plant Science* 12: 646144.
- Isa MM, Kasim KF, Muttalib MFA, Jaafar MN. 2021, May. Rapid and non-destructive leaf chlorophyll estimation of Fig (*Ficus carica* L.) cv. Iraqi grown on different root zone spatial limitation and controlled porosity level under greenhouse condition. In *IOP Conference Series: Earth and Environmental Science* 765: 012076.
- Jang Y, Kim J, Lee J, Lee S, Jung H, Park GH. 2024. Drought tolerance evaluation and growth response of Chinese cabbage seedlings to water deficit treatment. *Agronomy* 14: 279.
- Jiang C, Wang F, Tian J, Zhang W, Xie K. 2025. Two rice cultivars recruit different rhizospheric bacteria to promote aboveground regrowth after mechanical defoliation. *Microbiology Spectrum* 13: e01254-24.
- Jogawat A, Yadav B, Lakra N, Singh AK, Narayan OP. 2021. Crosstalk between phytohormones and secondary metabolites in the drought stress tolerance of crop plants: A review. *Physiologia Plantarum* 172: 1106-1132.

- Kalaivani K, Jawaharlal M. 2019. Study on physical characterization of coco peat with different proportions of organic amendments for soilless cultivation. *Journal of Pharmacognosy and Phytochemistry* 8: 2283-2286.
- Kasai M, Koide K, Ichikawa Y. 2012. Effect of pot size on various characteristics related to photosynthetic matter production in soybean plants. *International Journal of Agronomy* 2012: 751731.
- Kaya G. 2023. Ameliorative effects of foliar potassium nitrate on the growth, physiological, and stomatal properties of lettuce plants under salinity stress. *Journal of Plant Nutrition*, 46: 2882-2892.
- Konstantis N, Koskorellos K, Balou A, Paravolidaki A, Garantziotis G, Koulopoulou CE, Koulopoulos A, Zervoudakis G. 2022. The effect of leaf wounding on basil plants of different developmental stages. *Plants* 11: 2678.
- Liu Y, Wang Y, Wang B, Shi Q, Mao H. 2024. Preliminary study on the diagnosis of NK stress based on the puncture mechanical characteristics of cucumber stem. *BMC Plant Biology* 24: 26.
- López-Fernández O, Domínguez R, Pateiro M, Munekata PE, Rocchetti G, Lorenzo JM. 2020. Determination of polyphenols using liquid chromatography–tandem mass spectrometry technique (LC–MS/MS): A review. *Antioxidants* 9: 479.
- Magar AP, Nandede BM, Chilur R, Gaikwad BB, Khadatkar A. 2022. Optimization of growing media and pot size for vegetable seedlings grown in cylindrical paper pots using response surface methodology. *Journal of Plant Nutrition* 45: 1712-1721.
- Maree JE, Viljoen AM. 2011. Fourier transform near-and mid-infrared spectroscopy can distinguish between the commercially important *Pelargonium sidoides* and its close taxonomically *P. reniforme*. *Vibrational Spectroscopy* 55: 146-152.
- Michelon N, Pennisi G, Myint NO, Dall'Olio G, Batista LP, Salviano AAC, Gruda NS, Orsini F, Gianquinto G. 2020. Strategies for improved yield and water use efficiency of lettuce (*Lactuca sativa* L.) through simplified soilless cultivation under semi-arid climate. *Agronomy* 10: 1379.
- Nephali L, Piater LA, Dubery IA, Patterson V, Huyser J, Burgess K, Tugizimana F. 2020. Biostimulants for plant growth and mitigation of abiotic stresses: A metabolomics perspective. *Metabolites* 10: 505.

- Nițu I, Dănilă-guidea S, Mănilă E, Popa EE, Geicu-cristea M, Drăghici MC, Popescu PA, Popa ME, Miteluț AC. 2023. Water stress management using hydrogels and their impact on plant biochemical composition of *Lactuca sativa*. *Scientific Papers, Series B, Horticulture* 67: 528-533.
- Nunomura O, Kozai T, Shinozaki K, Oshio T. 2020. Seeding, seedling production and transplanting. In *Plant Factory* 285-297.
- Obede da Silva Aragão O, de Almeida Leite R, Araújo AP, da Conceição Jesus E. 2020. Effect of pot size on the growth of common bean in experiments with rhizobium. *Journal of Soil Science and Plant Nutrition* 20: 865-871.
- Price JW. 1972. Analytical atomic absorption spectrometry, Hayden and Son Ltd. *Analytica Chimica Acta* 65: 488-489.
- Qin K, Leskovaar DI. 2020. Humic substances improve vegetable seedling quality and post-transplant yield performance under stress conditions. *Agriculture* 10: 254.
- Rao MJ, Zheng B. 2025. The role of polyphenols in abiotic stress tolerance and their antioxidant properties to scavenge reactive oxygen species and free radicals. *Antioxidants* 14: 74.
- Raviteja MSV, Laxman RH, Rashmi K, Kannan S, Namratha MR, Reddy KM. 2021. Effect of container size and types on the root phenotypic characters of Capsicum. *Journal of Horticultural Sciences* 16: 261-270.
- Rizar FF, Lakitan B, Wijaya A. 2023. Suitable substrate volumes and cultivars for enhancing growth and in-creasing yield of yard-long bean in urban ecosystems. *BIOVALENTIA: Biological Research Journal* 9: 110-122.
- Sabina S, Jithesh MN. 2021. Mechanical wounding of leaf midrib and lamina elicits differential biochemical response and mitigates salinity induced damage in tomato. *Journal of Applied Horticulture* 23: 3-10.
- Saia S, Colla G, Raimondi G, Di Stasio E, Cardarelli M, Bonini P, Vitaglione P, De Pascale S, Rouphael Y. 2019. An endophytic fungi-based biostimulant modulated lettuce yield, physiological and functional quality responses to both moderate and severe water limitation. *Scientia horticulturae* 256: 108595.
- Salem EM, Kenawey MK, Saudy HS, Mubarak M. 2022. Influence of silicon forms on nutrients accumulation and grain yield of wheat under water deficit conditions. *Gesunde Pflanzen* 74: 539-548.

- Santander C, González F, Pérez U, Ruiz A, Aroca R, Santos C, Cornejo P, Vidal G. 2024. Enhancing water status and nutrient uptake in drought-stressed lettuce plants (*Lactuca sativa* L.) via inoculation with different *Bacillus* spp. Isolated from the Atacama Desert. *Plants* 13: 158.
- Silva JAD, Carvalho LGD, Andrade FR. 2022. Gas exchange and water stress index in soybean cultivated under water deficit and soil compaction. *Revista Ceres* 69: 218-226.
- Spada M, Marín-Sanz M, Bigini V, Quagliata G, Coppa E, Barro F, Savatin D, Ruggeri R, Sestili F, Rossini F, Astolfi S. 2024. Use of biostimulants for water stress mitigation in two durum wheat (*Triticum durum* Desf.) genotypes with different drought tolerance. *Plant Stress* 14: 100566.
- Stoleru V, Burlica R, Mihalache G, Dirlau D, Padureanu S, Teliban GC, Astanei D, Cojocaru A, Beniuga O, Patras A. 2020. Plant growth promotion effect of plasma activated water on *Lactuca sativa* L. cultivated in two different volumes of substrate. *Scientific Reports* 10: 20920.
- Targino AJDO, Oliveira FDAD, de Oliveira MK, Régis LRDL, Neta HM, Cordeiro CJ, Alves FA, Nascimento LV, Pessoa VG, de Oliveira AA, Souza MWDL. 2019. Lettuce growth in different pot volumes and irrigation frequencies under saline stress. *Journal of Agricultural Science* 11: 494.
- Tripodi P, Vincenzo C, Venezia A, Coccozza A, Pane C. 2024. Precision phenotyping of wild rocket (*diplotaxis tenuifolia*) to determine morpho-physiological responses under increasing drought stress levels using the planteye multispectral 3D system. *Horticulturae* 10: 496.
- Tüfenkçi Ş, Yerli C. 2023. Physical and physiological properties and mineral content of curly lettuce grown by applying different rates of biochar to the soil with varying irrigation water levels. *Uluslararası Tarım ve Yaban Hayatı Bilimleri Dergisi* 9: 205-217.
- Utami N, Damayanti PN. 2023. Phytochemical analysis, antioxidant activity, and FTIR spectroscopic analysis of red leaf lettuce and green leaf lettuce (*Lactuca sativa* L.). *Indian Drugs* 60: 50–56.
- Velasco-Clares D, Navarro-León E, Izquierdo-Ramos MJ, Blasco B, Ruiz JM. 2025. Enhancing drought tolerance in lettuce: The efficacy of the seaweed-derived biostimulant Cytolan® Stress applied at different growth stages. *Horticulturae* 11: 157.

- Villa e Vila V, Marques PAA, Gomes TM, Nunes AF, Montenegro VG, Wenneck GS, Franco LB. 2024. Deficit irrigation with silicon application as strategy to increase yield, photosynthesis and water productivity in lettuce crops. *Plants* 13: 1029.
- Waiba KM, Sharma P, Sharma A, Chadha S, Kaur M. 2020. Soil-less vegetable cultivation: A review. *Journal of Pharmacognosy and Phytochemistry* 9: 631-636.
- Yan H, Fu K, Li J, Li M, Li S, Dai Z, Jin X. 2024. Photosynthesis, chlorophyll fluorescence, and hormone regulation in tomato exposed to mechanical wounding. *Plants* 13: 2594.
- Yan Z, He D, Niu G, Zhai H. 2019. Evaluation of growth and quality of hydroponic lettuce at harvest as affected by the light intensity, photoperiod and light quality at seedling stage. *Scientia horticulturae* 248: 138-144.
- Yavuz D, Seymen M, Kal Ü, Atakul Z, Tanrıverdi ÖB, Türkmen Ö, Yavuz N. 2023. Agronomic and physio-biochemical responses of lettuce to exogenous sodium nitroprusside (SNP) applied under different irrigation regimes. *Agricultural Water Management* 277: 108127.
- Zakaria NI, Ismail MR, Awang Y, Megat Wahab PE, Berahim Z. 2020. Effect of root restriction on the growth, photosynthesis rate, and source and sink relationship of chilli (*Capsicum annuum* L.) grown in soilless culture. *BioMed research international* 2020: 2706937.

Table 3.1: Nutrient solution composition.

Macronutrients (meq L⁻¹)								Micronutrients (mg L⁻¹)					EC (mS cm⁻¹)	pH
NO ₄	Ca	K	SO ₄	H ₂ PO ₄	Mg	NH ₂	Fe	Mn	B	Zn	Cu	Mn		
10.0	5.8	5.5	2.0	1.0	1.0	0.7	1.0	0.3	0.3	0.25	0.05	0.05	1.3±0.5	6.5±1.0

Table 3.2: Chemical composition of tap water and cocopeat used in the experiment.

Minerals	Tap water (mg L⁻¹)	Coco peat 1 (mg kg⁻¹)	Coco peat 2 (mg kg⁻¹)
Calcium (Ca)	33.96	4425	4875
Potassium (K)	4.66	7970	8445
Magnesium (Mg)	12.79	2492	2523
Iron (Fe)	0.02	599.85	649.6
Zinc (Zn)	0.11	20.75	25.33
Manganese (Mn)	0.01	29.8	37.15
Copper (Cu)	0.02	0.75	0.75
Sodium (Na)	22.16	893.75	876.75
Phosphorus (P)	0.59	0.99%	0.12%
Nitrogen (N)	0.78	0.81%	0.31%
Boron (B)	0.04	-	-
Chlorine (Cl)	28.89	-	-
Sulfate ion (SO ₄)	18.96	-	-
Calcium carbonate (CaCO ₃)	137.44	-	-
EC (mS cm ⁻¹)	0.3	0.5	0.5
pH	6.5	6.7	6.8

Table 3.3: Summary of ANOVA for the selected non-destructive curly lettuce parameters as affected by the water deficit regimes, the plant bag size, the mechanical wounding, and the weak effect.

Source	DF	Plant height (cm)		Chlorophyll (%)		Number of leaves	
		F Value	Pr > F	F Value	Pr > F	F Value	Pr > F
WDR	3	27.14	<.0001	1.45	0.23	12.60	<.0001
PBS	2	21.38	<.0001	0.93	0.39	10.33	<.0001
MW	1	71.83	<.0001	2.83	0.09	38.22	<.0001
WDR × PBS	6	4.11	0.0007	1.62	0.14	3.92	0.001
WDR × MW	3	3.20	0.02	3.09	0.02	0.57	0.63
PBS × MW	2	2.05	0.13	0.00	0.99	1.42	0.24
WDR × PBS × MW	6	1.86	0.08	0.57	0.75	1.12	0.35
Week	3	679.52	<.0001	17.97	<.0001	1196.11	<.0001
Week × WDR	9	14.26	<.0001	1.38	0.09	7.08	<.0001
Week × PBS	9	15.67	<.0001	0.20	0.97	12.16	<.0001
Week × MW	3	45.40	<.0001	7.00	0.0002	13.00	<.0001
Week × WDR × PBS	18	1.63	0.05	1.81	0.02	1.71	0.04
Week × WDR × MW	9	1.42	0.18	1.65	0.10	0.28	0.97
Week × PBS × MW	6	0.76	0.60	0.75	0.61	0.65	0.68
Week × WDR × PBS × MW	18	0.90	0.58	1.35	0.16	0.20	0.99

Abbreviations: WDR = Water deficit regimes, PBS = Plant bag sizes, MW = Mechanical wounding.



Table 3.4: Effects of multistress (WDR, PBS and MW) interaction on the destructive and non-destructive curly lettuce parameters at harvest (4th week) of curly lettuce.

Treatment	Plant height	Chlorophyll (%)	Cent Number of leaves	Leaf area (cm ²)	Fresh mass (g)	Dry mass (g)
WDR						
100%	17.37 ± 1.67 ^a	7.15 ± 1.49 ^{ab}	16.54 ± 0.92 ^a	856.22 ± 217.34 ^a	867.61 ± 303.08 ^a	25.98 ± 80.73 ^a
80%	15.22 ± 1.77 ^b	7.67 ± 2.18 ^a	14.94 ± 1.57 ^b	763.87 ± 143.88 ^b	608.61 ± 203.28 ^b	25.07 ± 6.74 ^a
60%	14.00 ± 1.92 ^c	6.67 ± 1.39 ^b	13.97 ± 1.47 ^c	609.68 ± 149.80 ^c	423.72 ± 198.56 ^c	18.00 ± 5.61 ^b
40%	13.21 ± 2.64 ^c	7.60 ± 1.57 ^{ab}	14.16 ± 2.04 ^c	666.46 ± 227.03 ^c	465.88 ± 276.49 ^c	19.54 ± 9.56 ^b
F-value	29.22	1.83	28.95	22.66	38.24	12.23
P-value	*0.0001	0.15	*0.0001	*0.0001	*0.0001	*0.0001
LSD	0.95	0.97	0.61	65.00	92.24	3.25
PBS						
5 L	16.65 ± 2.01 ^a	7.15 ± 1.95 ^a	16.14 ± 1.26 ^a	826.99 ± 180.19 ^a	782.00 ± 268.03 ^a	26.70 ± 8.40 ^a
3.5 L	14.51 ± 2.24 ^b	7.36 ± 1.59 ^a	14.60 ± 1.58 ^b	705.49 ± 180.73 ^b	530.29 ± 267.79 ^b	20.61 ± 8.02 ^b
1.25 L	13.69 ± 2.44 ^b	7.30 ± 1.61 ^a	13.97 ± 1.91 ^c	639.69 ± 220.61 ^c	462.08 ± 274.8 ^b	19.09 ± 6.94 ^b
F-value	27.96	0.13	35.24	23.09	36.05	15.84
P-value	*0.0001	0.88	*0.0001	*0.0001	*0.0001	*0.0001
LSD	0.82	0.84	0.53	56.29	79.88	2.80
MW						
Intact plants	15.35 ± 2.85 ^a	7.29 ± 1.67 ^a	15.64 ± 1.74 ^a	852.03 ± 190.60 ^a	730.41 ± 322.97 ^a	27.56 ± 8.10 ^a
Leaf wounded	14.55 ± 2.16 ^b	7.25 ± 1.75 ^a	14.17 ± 1.63 ^b	596.09 ± 130.95 ^b	452.50 ± 197.19 ^b	17.80 ± 5.66 ^b
F-value	5.75	0.02	45.40	125.63	72.56	78.82
P-value	*0.02	0.89	*0.0001	*0.0001	*0.0001	*0.0001
LSD	0.67	0.68	0.43	45.96	65.22	2.31
WDR × PBS						
F-value	2.01	2.58	3.54	2.23	1.22	1.39
P-value	0.0836	*0.03	*0.005	*0.05	0.31	0.24
LSD	1.6	1.68	1.07	112.59	159.77	5.69
WDR × MW						
F-value	2.13	3.48	0.20	3.91	3.79	1.01
P-value	0.10	*0.02	0.89	*0.01	*0.01	0.39
LSD	1.30	1.37	0.87	91.92	130.45	4.65
PBS × MW						
F-value	0.57	0.33	0.83	2.20	0.40	0.50
P-value	0.56	0.72	0.44	0.12	0.67	0.61
LSD	1.16	1.18	0.75	79.61	112.98	159.22
WDR × PBS × MW						
F-value	0.71	0.90	0.45	1.10	0.09	2.91
P-value	0.64	0.50	0.83	0.37	0.99	*0.02
LSD	2.33	2.37	1.51	159.22	225.95	8.49

Means followed by the same letter within the column for each section are not significant $p > 0.05$. **Abbreviations:** Bold mark and asterisk * indicate $p < 0.05$ (Tukey's LSD), WDR = Water deficit regimes, PBS = Plant bag sizes, MW = Mechanical wounding.

Table 3.5: Effects of multistress (WDR, PBS and MW) interaction on the dry mass of curly lettuce.

Treatment	Dry mass (g)
WDR × PBS × MW	
100% × 3.5 L × Intact plants	40.04 ± 00.00 ^a
100% × 3.5 L × Leaf wounded	20.94 ± 5.94 ^{defgh}
100% × 5 L × Intact plants	40.60 ± 2.85 ^a
100% × 5 L × Leaf wounded	23.16 ± 1.80 ^{defg}
100% × 1.25 L × Intact plants	20.98 ± 0.70 ^{defgh}
100% × 1.25 L × Leaf wounded	21.82 ± 2.73 ^{defg}
80% × 3.5 L × Intact plants	26.98 ± 00.00 ^{bcdef}
80% × 3.5 L × Leaf wounded	21.97 ± 3.02 ^{defg}
80% × 5 L × Intact plants	34.30 ± 2.22 ^{ab}
80% × 5 L × Leaf wounded	23.37 ± 2.10 ^{cdefg}
80% × 1.25 L × Intact plants	28.86 ± 5.21 ^{bcd}
80% × 1.25 L × Leaf wounded	16.24 ± 2.80 ^{ghjki}
60% × 3.5 L × Intact plants	18.52 ± 2.28 ^{fghij}
60% × 3.5 L × Leaf wounded	12.93 ± 2.90 ^{hijk}
60% × 5 L × Intact plants	22.56 ± 00.00 ^{cdefg}
60% × 5 L × Leaf wounded	20.65 ± 5.00 ^{defghij}
60% × 1.25 L × Intact plants	23.92 ± 4.48 ^{defg}
60% × 1.25 L × Leaf wounded	12.47 ± 3.66 ^{kij}
40% × 3.5 L × Intact plants	28.37 ± 6.63 ^{bcde}
40% × 3.5 L × Leaf wounded	12.38 ± 2.40 ^{j^k}
40% × 5 L × Intact plants	30.76 ± 15.54 ^{bc}
40% × 5 L × Leaf wounded	20.26 ± 4.88 ^{efghij}
40% × 1.25 L × Intact plants	20.22 ± 3.81 ^{efghij}
40% × 1.25 L × Leaf wounded	9.20 ± 1.96 ^k
F-value	2.91
P-value	*0.02
LSD	8.49

Means followed by the same letter within the column for each section are not significant $p > 0.05$. **Abbreviations:** Bold mark and asterisk * indicate $p < 0.05$ (Tukey's LSD), WDR = Water deficit regimes, PBS = Plant bag sizes, MW = Mechanical wounding.

Table 3.6: Effects of multistress (WDR, PBS and MW) interaction on stomatal conductance of curly lettuce.

Treatment	Stomatal conductance (mmol m ⁻² s ⁻¹)		
	27 DAT	28 DAT	29 DAT
WDR			
100%	388.56 ± 127.88 ^a	387.22 ± 139.45 ^a	374.90 ± 113.60 ^a
80%	310.45 ± 73.77 ^b	281.71 ± 61.34 ^b	259.89 ± 75.05 ^b
60%	234.79 ± 82.95 ^c	242.87 ± 110.79 ^{bc}	205.02 ± 61.47 ^c
40%	224.34 ± 111.93 ^c	210.51 ± 93.18 ^c	200.72 ± 83.95 ^c
F-value	22.41	31.25	49.47
P-value	*0.0001	*0.0001	*0.0001
LSD	45.83	39.40	32.92
PBS			
5 L	349.11 ± 122.50 ^a	369.48 ± 122.47 ^a	316.07 ± 112.59 ^a
3.5 L	274.09 ± 98.12 ^b	267.05 ± 102.30 ^b	256.09 ± 101.94 ^b
1.25 L	245.41 ± 116.31 ^b	210.48 ± 88.72 ^c	208.92 ± 89.85 ^c
F-value	14.74	43.54	31.00
P-value	*0.0001	*0.0001	*0.0001
LSD	39.69	34.12	28.50
MW			
Intact plants	343.85 ± 127.31 ^a	327.27 ± 137.13 ^a	309.79 ± 116.17 ^a
Leaf wounded	235.21 ± 82.05 ^b	236.23 ± 87.73 ^b	216.53 ± 82.07 ^b
F-value	45.52	44.50	56.83
P-value	*0.0001	*0.0001	*0.0001
LSD	32.4	27.85	23.26
WDR × PBS			
F-value	1.42	2.27	2.38
P-value	0.22	*0.053	*0.04
LSD	79.39	68.31	57.32
WDR × MW			
F-value	0.03	3.98	3.32
P-value	*0.0388	*0.0135	*0.0283
LSD	64.82	55.75	46.66
PBS × MW			
F-value	0.88	2.81	2.71
P-value	0.42	0.07	0.07
LSD	45.83	43.62	62.06
WDR × PBS × MW			
F-value	0.44	0.72	0.66
P-value	0.84	0.63	0.68
LSD	112.28	96.81	82.66

Means followed by the same letter within the column for each section are not significant $p > 0.05$. **Abbreviations:** Bold mark and asterisk * indicate $p < 0.05$ (Tukey's LSD), WDR = Water deficit regimes, PBS = Plant bag sizes, MW = Mechanical wounding.

Table 3.7: Effects of water deficit regimes and plant bag size interaction on curly lettuce.

Treatment	Stomatal conductance ($\text{mmol m}^{-2} \text{s}^{-1}$)	
	28 DAT	29 DAT
WDR × PBS		
100% × 5 L	478.72 ± 163.09 ^a	445.36 ± 125.51 ^a
100% × 3.5 L	388.49 ± 94.94 ^b	398.15 ± 65.06 ^a
100% × 1.25 L	294.45 ± 92.07 ^{de}	281.20 ± 82.18 ^b
80% × 5 L	317.34 ± 39.54 ^{cd}	285.55 ± 84.55 ^b
80% × 3.5 L	283.63 ± 69.90 ^{de}	259.96 ± 59.18 ^b
80% × 1.25 L	244.17 ± 55.89 ^{ef}	234.16 ± 83.16 ^{bc}
60% × 5 L	376.83 ± 108.40 ^{bc}	256.92 ± 64.18 ^b
60% × 3.5 L	207.74 ± 47.35 ^{fg}	194.51 ± 38.11 ^{cd}
60% × 1.25 L	166.37 ± 34.51 ^{gh}	163.62 ± 43.05 ^{de}
40% × 5 L	306.26 ± 65.89 ^{de}	276.46 ± 63.57 ^b
40% × 3.5 L	188.35 ± 48.19 ^{fgh}	171.73 ± 36.00 ^{de}
40% × 1.25 L	136.91 ± 69.06 ^h	130.60 ± 81.43 ^e
F-value	2.27	2.38
P-value	*0.05	*0.04
LSD	68.31	57.32

Means followed by the same letter within the column for each section are not significant $p > 0.05$. **Abbreviations:** Bold mark and asterisk * indicate $p < 0.05$ (Tukey's LSD), WDR = Water deficit regimes, PBS = Plant bag sizes, MW = Mechanical wounding.

Table 3.8: Effects of water deficit regimes and mechanical wounding interaction on stomatal conductance of curly lettuce.

Treatment	Stomatal conductance (mmol m ⁻² s ⁻¹)		
	27 DAT	28 DAT	29 DAT
WDR × MW			
100% × Intact plants	476.53 ± 122.37 ^a	473.20 ± 133.04 ^a	442.74 ± 105.11 ^a
100% × Wounded plants	300.59 ± 48.65 ^{bc}	301.24 ± 83.63 ^{bc}	307.06 ± 77.61 ^b
80% × Intact plants	343.43 ± 70.76 ^b	304.49 ± 63.61 ^b	303.51 ± 79.38 ^{bc}
80% × Wounded plants	277.06 ± 63.63 ^c	258.94 ± 52.74 ^{bcd}	216.28 ± 37.27 ^{de}
60% × Intact plants	264.00 ± 89.90 ^{cd}	279.80 ± 130.80 ^{bc}	222.33 ± 59.84 ^{de}
60% × Wounded plants	205.59 ± 69.98 ^{de}	210.04 ± 83.69 ^{de}	187.70 ± 61.42 ^{ef}
40% × Intact plants	291.07 ± 112.84 ^{bc}	246.30 ± 95.07 ^{cd}	259.39 ± 72.85 ^{de}
40% × Wounded plants	157.61 ± 62.25 ^c	174.72 ± 80.82 ^c	155.09 ± 62.28 ^f
F-value	0.03	3.98	3.32
P-value	*0.03	*0.01	*0.02
LSD	64.826	55.752	46.667

Means followed by the same letter within the column for each section are not significant $p > 0.05$. **Abbreviations:** Bold mark and asterisk * indicate $p < 0.05$ (Tukey's LSD), WDR = Water deficit regimes, PBS = Plant bag sizes, MW = Mechanical wounding.

**Table 3.9:** Effects of multistress (WDR, PBS and MW) interaction on the leaf minerals of the curly lettuce parameters at harvest (4th week).

Treatment	N	P	Mg	K	Ca	Na	Fe	Zn	Mn	Cu
WDR										
100%	4.74 ± 0.53 ^{ab}	3.20 ± 0.88 ^a	0.44 ± 0.08 ^b	7.93 ± 1.66 ^a	1.47 ± 0.25 ^b	0.40 ± 0.08 ^c	0.05 ± 0.02 ^a	0.00 ± 0.00 ^c	0.02 ± 0.00 ^c	0.04 ± 0.01 ^{ab}
80%	4.98 ± 0.52 ^a	3.22 ± 0.49 ^{ab}	0.51 ± 0.04 ^a	7.77 ± 0.88 ^a	1.62 ± 0.20 ^a	0.50 ± 0.10 ^{ab}	0.05 ± 0.01 ^a	0.01 ± 0.00 ^{ab}	0.03 ± 0.00 ^a	0.04 ± 0.00 ^{bc}
60%	4.59 ± 0.71 ^b	2.64 ± 0.38 ^b	0.45 ± 0.07 ^b	7.54 ± 0.72 ^a	1.39 ± 0.15 ^c	0.54 ± 0.10 ^a	0.04 ± 0.01 ^a	0.00 ± 0.00 ^{bc}	0.02 ± 0.01 ^b	0.03 ± 0.01 ^c
40%	4.97 ± 0.60 ^a	2.55 ± 0.56 ^b	0.38 ± 0.07 ^c	7.80 ± 1.77 ^a	1.66 ± 0.14 ^a	0.47 ± 0.11 ^{bc}	0.04 ± 0.01 ^a	0.01 ± 0.00 ^a	0.01 ± 0.00 ^d	0.04 ± 0.01 ^a
F-value	3.23	10.55	8.35	0.26	33.99	5.79	1.16	5.22	38.65	3.24
P-value	*0.03	*0.0001	*0.0002	0.85	*0.0001	*0.0019	0.33	*0.0035	*0.0001	0.30
LSD	0.29	0.31	0.05	0.91	0.06	0.06	0.01	0.00	2.01	0.00
PBS										
5 L	4.34 ± 0.52 ^b	2.65 ± 0.65 ^b	0.44 ± 0.08 ^a	7.66 ± 1.13 ^a	1.49 ± 0.2 ^b	0.50 ± 0.10 ^a	0.05 ± 0.01 ^a	0.00 ± 0.00 ^b	0.02 ± 0.01 ^b	0.03 ± 0.01 ^b
3.5 L	5.11 ± 0.47 ^a	2.88 ± 0.49 ^b	0.46 ± 0.07 ^a	7.45 ± 1.42 ^a	1.51 ± 0.19 ^b	0.47 ± 0.13 ^a	0.04 ± 0.01 ^a	0.01 ± 0.00 ^a	0.02 ± 0.00 ^a	0.04 ± 0.00 ^a
1.25 L	5.01 ± 0.51 ^a	3.18 ± 0.75 ^a	0.45 ± 0.09 ^a	8.19 ± 1.34 ^a	1.62 ± 0.26 ^a	0.46 ± 0.09 ^a	0.04 ± 0.01 ^a	0.01 ± 0.00 ^a	0.02 ± 0.01 ^{ab}	0.04 ± 0.01 ^a
F-value	21.84	7.75	0.32	2.02	12.73	1.05	0.74	7.30	2.36	3.15
P-value	*0.0001	*0.001	0.72	0.44	*0.0001	0.35	0.48	*0.0018	0.10	0.52
LSD	0.25	0.27	0.04	0.79	0.54					
MW										
Intact plants	4.66 ± 0.61 ^b	2.74 ± 0.72 ^b	0.44 ± 0.08 ^a	7.64 ± 1.26 ^a	1.41 ± 0.20 ^b	0.49 ± 0.11 ^a	0.04 ± 0.01 ^a	0.00 ± 0.00 ^b	0.02 ± 0.00 ^a	0.04 ± 0.01 ^a
Leaf wounded	4.96 ± 0.57 ^a	3.06 ± 0.58 ^b	0.45 ± 0.09 ^a	7.89 ± 1.38 ^a	1.66 ± 0.17 ^a	0.46 ± 0.11 ^a	0.05 ± 0.01 ^a	0.01 ± 0.00 ^a	0.02 ± 0.01 ^a	0.04 ± 0.01 ^a
F-value	8.28	8.25	0.56	0.44	124.91	1.45	0.19	17.94	0.01	3.33
P-value	*0.006	*0.006	0.45	0.51	*0.0001	0.23	0.66	*0.001	0.90	0.07
LSD	0.21	0.22	0.03	0.64	0.04	0.04	0.00	0.00	0.00	0.00
WDR × PBS										
F-value	1.72	2.61	0.54	0.39	8.98	1.20	2.17	0.65	8.71	0.54
P-value	0.14	*0.02	0.77	0.87	*0.0001	0.32	0.06	0.68	*0.0001	0.77
LSD	0.51	0.54	0.08	1.58	0.10	0.11	0.02	0.00	0.00	0.01
WDR × MW										
F-value	0.55	3.74	0.78	1.61	2.92	1.97	2.21	0.17	5.51	1.44
P-value	0.64	*0.01	0.51	0.20	0.44	0.13	0.10	0.91	*0.031	0.24
LSD	0.42	0.44	0.07	1.29	0.08	0.07	0.01	0.00	0.00	0.01
PBS × MW										
F-value	0.28	1.54	0.52	1.13	0.20	1.07	1.97	1.25	2.45	0.18
P-value	0.75	0.22	0.59	0.33	0.81	0.35	0.15	0.29	0.09	0.83
LSD	0.36	0.38	0.63	1.11	0.07	0.08	0.01	0.00	0.00	0.01
WDR × PBS × MW										
F-value	2.90	2.81	1.41	1.4	9.52	0.33	1.30	0.53	5.51	0.44
P-value	*0.0188	*0.0205	0.22	0.26	*0.0001	0.91	0.27	0.78	*0.0003	0.85
LSD	0.73	0.76	0.12	2.24	0.15	0.16	0.02	0.002	0.00	0.01

Means followed by the same letter within the column for each section are not significant $p > 0.05$. **Abbreviations:** Bold mark and asterisk * indicate $p < 0.05$ (Tukey's LSD), WDR = Water deficit regimes, PBS = Plant bag sizes, MW = Mechanical wounding.

Table 3.10: Effects of multistress (WDR, PBS and MW) interaction on nitrogen, phosphate, calcium and manganese.

Treatment	N	P	Ca	Mn
WDR × PBS × MW				
100% × 5 L × Intact plants	4.38 ± 0.01 ^{efghi}	2.75 ± 0.57 ^{ef}	1.36 ± 0.07 ^{gh}	0.01 ± 0.00 ^{gh}
100% × 5 L × Leaf wounded	4.30 ± 0.57 ^{fghi}	2.51 ± 0.62 ^f	1.33 ± 0.01 ^{gh}	0.01 ± 0.00 ^{fgh}
100% × 3.5 L × Intact plants	5.08 ± 0.30 ^{abcde}	2.69 ± 0.07 ^{ef}	1.15 ± 0.11 ⁱ	0.01 ± 0.00 ^{efg}
100% × 3.5 L × Leaf wounded	5.40 ± 0.41 ^{abc}	3.57 ± 0.39 ^{bcd}	1.66 ± 0.13 ^{cd}	0.02 ± 0.00 ^{cde}
100% × 1.25 L × Intact plants	4.30 ± 0.31 ^{fghi}	4.49 ± 0.04 ^a	1.88 ± 0.00 ^a	0.02 ± 0.00 ^{cde}
100% × 1.25 L × Leaf wounded	5.19 ± 0.22 ^{abcd}	3.21 ± 0.75 ^{bcdef}	1.74 ± 0.08 ^{abc}	0.02 ± 0.00 ^{bc}
80% × 5 L × Intact plants	4.20 ± 0.33 ^{ghi}	2.69 ± 0.32 ^{ef}	1.53 ± 0.10 ^{def}	0.02 ± 0.00 ^{cde}
80% × 5 L × Leaf wounded	4.80 ± 0.42 ^{bcdefgh}	3.86 ± 0.12 ^{ab}	1.86 ± 0.02 ^a	0.02 ± 0.00 ^{bcd}
80% × 3.5 L × Intact plants	5.34 ± 0.24 ^{abc}	2.77 ± 0.31 ^{ef}	1.39 ± 0.11 ^{fgh}	0.02 ± 0.00 ^{bc}
80% × 3.5 L × Leaf wounded	5.45 ± 0.30 ^{ab}	3.29 ± 0.17 ^{bcde}	1.67 ± 0.08 ^{cd}	0.03 ± 0.00 ^{ab}
80% × 1.25 L × Intact plants	5.38 ± 0.26 ^{abc}	3.02 ± 0.07 ^{cdef}	1.45 ± 0.07 ^{efg}	0.03 ± 0.00 ^{bc}
80% × 1.25 L × Leaf wounded	4.85 ± 0.28 ^{bcdefgh}	3.68 ± 0.33 ^{bc}	1.84 ± 0.06 ^{ab}	0.03 ± 0.00 ^a
60% × 5 L × Intact plants	3.18 ± 0.55 ⁱ	2.53 ± 0.35 ^{ef}	1.28 ± 0.11 ^{hi}	0.03 ± 0.00 ^{abc}
60% × 5 L × Leaf wounded	4.08 ± 0.63 ^{hi}	2.67 ± 0.18 ^{ef}	1.45 ± 0.01 ^{efg}	0.03 ± 0.00 ^{abc}
60% × 3.5 L × Intact plants	4.41 ± 0.74 ^{efghi}	2.51 ± 0.34 ^f	1.38 ± 0.04 ^{fgh}	0.03 ± 0.00 ^{abc}
60% × 3.5 L × Leaf wounded	5.08 ± 0.20 ^{abcde}	2.70 ± 0.82 ^{ef}	1.53 ± 0.05 ^{def}	0.02 ± 0.01 ^{bcd}
60% × 1.25 L × Intact plants	5.37 ± 0.14 ^{abc}	2.55 ± 0.27 ^{ef}	1.15 ± 0.11 ⁱ	0.03 ± 0.00 ^h
60% × 1.25 L × Leaf wounded	5.04 ± 0.34 ^{abcde}	2.87 ± 0.25 ^{def}	1.55 ± 0.7 ^{de}	0.00 ± 0.00 ^{abc}
40% × 5 L × Intact plants	4.69 ± 0.14 ^{cdefgh}	1.67 ± 0.29 ^g	1.36 ± 0.10 ^{gh}	0.00 ± 0.00 ^h
40% × 5 L × Leaf wounded	4.58 ± 0.80 ^{defh}	2.53 ± 0.42 ^{ef}	1.75 ± 0.04 ^{abc}	0.01 ± 0.01 ^{def}
40% × 3.5 L × Intact plants	4.97 ± 0.59 ^{abcdef}	2.72 ± 0.36 ^{ef}	1.67 ± 0.03 ^{cd}	0.02 ± 0.00 ^{bcd}
40% × 3.5 L × Leaf wounded	5.30 ± 0.29 ^{abcd}	2.80 ± 0.54 ^{ef}	1.66 ± 0.08 ^{cd}	0.01 ± 0.00 ^{gh}
40% × 1.25 L × Intact plants	4.42 ± 0.62 ^{efghi}	2.56 ± 0.52 ^{ef}	1.69 ± 0.10 ^{bcd}	0.00 ± 0.00 ^{gh}
40% × 1.25 L × Leaf wounded	5.59 ± 0.31 ^a	3.04 ± 0.27 ^{cdef}	1.86 ± 0.22 ^a	0.00 ± 0.00 ^{gh}
F-value	2.90	2.81	9.52	5.51
P-value	*0.01	*0.02	*0.0001	*0.0003
LSD	0.73	0.76	0.15	0.00

Means followed by the same letter within the column for each section are not significant $p > 0.05$. **Abbreviations:** Bold mark and asterisk * indicate $p < 0.05$ (Tukey's LSD), WDR = Water deficit regimes, PBS = Plant bag sizes, MW = Mechanical wounding.

Table 3.11: Phenolic compounds detected from curly lettuce var. Crispa.

Alignment ID	Phenolic Compounds	Chemical formula	Retention Tim(min)	Mass to Charge (M/z)
87	Unknown amide	C ₉ H ₁₆ NO ₉	5.16	282.08438
607	Unknown	-	5.71	572.14941
89	Unknown	-	5.82	283.06821
91	Unknown	-	6.2	284.12476
150	Gentisic acid 5-O-glucoside	C ₁₃ H ₁₆ O ₉	6.91	315.07169
45	Unknown	-	7.21	218.10342
273	Unknown	-	7.47	382.1008
135	Caftaric acid	C ₁₃ H ₁₂ O ₉	7.81	311.04037
37	L-Tryptophan	-	8.11	203.08275
220	Chlorogenic acid 3CQA	C ₁₆ H ₁₈ O ₉	8.40	353.08777
232	Unknown	C ₁₁ H ₁₈ O ₁₃	10.45	357.06485
221	Chlorogenic acid 5CQA	C ₁₆ H ₁₈ O ₉	10.80	353.08786
227	Unknown	-	11.32	356.02585
190	3-O-p-Coumaroylquinic acid	C ₁₆ H ₁₈ O ₈	12.83	337.09268
375	MINEs-320976	C ₂₀ H ₃₂ O ₁₀	13.28	431.19205
302	Quinic acid deriv	C ₁₆ H ₁₇ NO ₁₁	13.53	398.07288
481	Chicoric acid	C ₂₂ H ₁₈ O ₁₂	14.94	473.07242
394	cis-3-Hexenyl b-primeveroside	C ₁₇ H ₃₀ O ₁₀	15.12	439.18195
563	Tinosposinenside C	C ₂₆ H ₃₆ O ₁₂	15.12	539.21265
676	Unknown	C ₂₆ H ₄₄ O ₁₆	15.88	611.25555
484	Quercetin 3-O-glucuronide	C ₂₁ H ₁₈ O ₁₃	16.39	477.06717
457	Quercetin 3-galactoside	C ₂₁ H ₂₀ O ₁₂	16.65	463.0878
589	Citrusin B	C ₂₇ H ₃₆ O ₁₃	16.83	567.20892
452	6- {[2-(3,4-dihydroxyphenyl)-5-hydroxy-4-oxo-4H-chromen-7-yl]oxy}-3,4,5-trihydroxyoxane-2-carboxylic acid	C ₂₁ H ₁₈ O ₁₂	16.86	461.07224
77	UNPD217745	C ₁₄ H ₂₄ O ₅	17.29	271.15494
521	Quercetin 3-O-(6"-acetyl-glucoside)	C ₂₃ H ₂₂ O ₁₃	17.44	505.09882
320	UNPD200966	C ₁₈ H ₃₀ O ₁₀	17.64	405.17725
68	Phaseolic acid	C ₁₂ H ₂₂ O ₆	17.92	261.13419
522	4"-O-Acetylmyricitrin	C ₂₃ H ₂₂ O ₁₃	17.98	505.09882
569	Unknown	-	18.26	550.08307
520	Quercetin 3-O-(6"-acetyl-glucoside)	C ₂₃ H ₂₂ O ₁₃	18.79	505.0986
84	3-Hydroxytetradecanedioic acid	C ₁₄ H ₂₆ O ₅	19.76	273.17065
374	MINEs-320976	C ₂₀ H ₃₂ O ₁₀	20.26	431.19202
376	MINEs-320976	C ₂₀ H ₃₂ O ₁₀	20.98	431.19312
114	7-Hydroxy-10-methoxydeacetyldihydrobotrydial	C ₁₆ H ₂₈ O ₅	22.09	299.18616
334	Unknown	-	22.72	414.25006



Figure 3.1: The irrigation setup (A) drip irrigation, (B) residential irrigation controller, (C) valves, and (D) tanks and pumps.

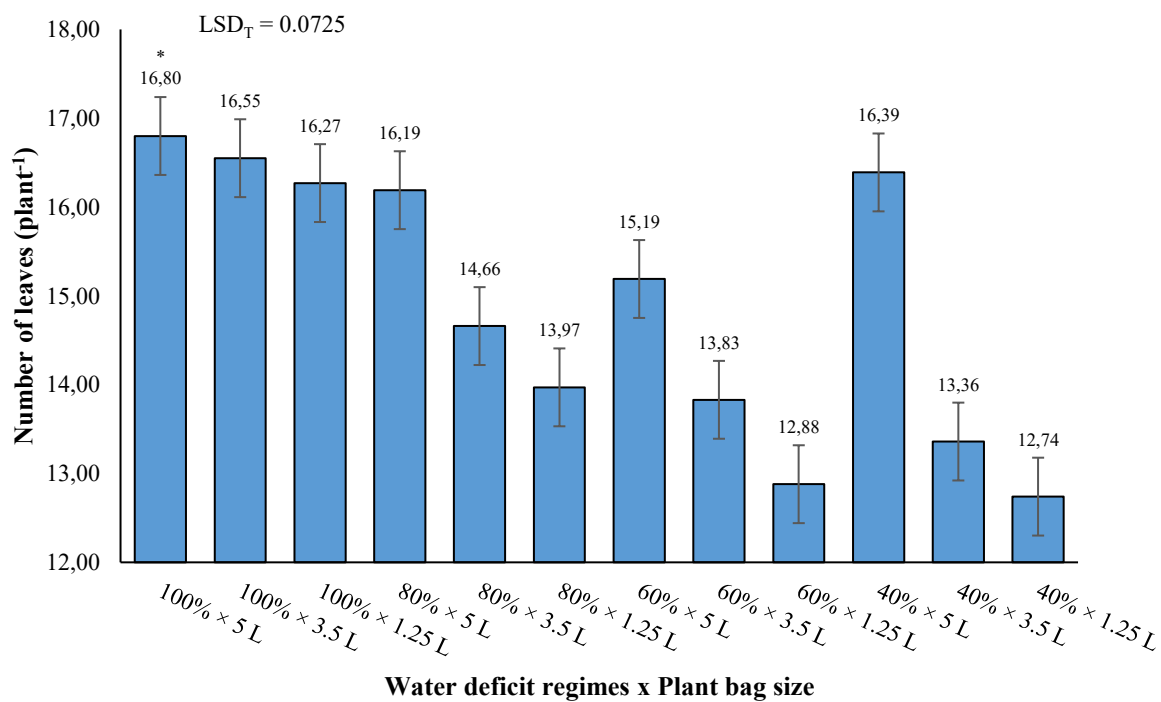


Figure 3.2: Effects of water deficit regimes and plant bag size on number of leaves. Asterisk * = Indicates the treatment level with the highest mean value.

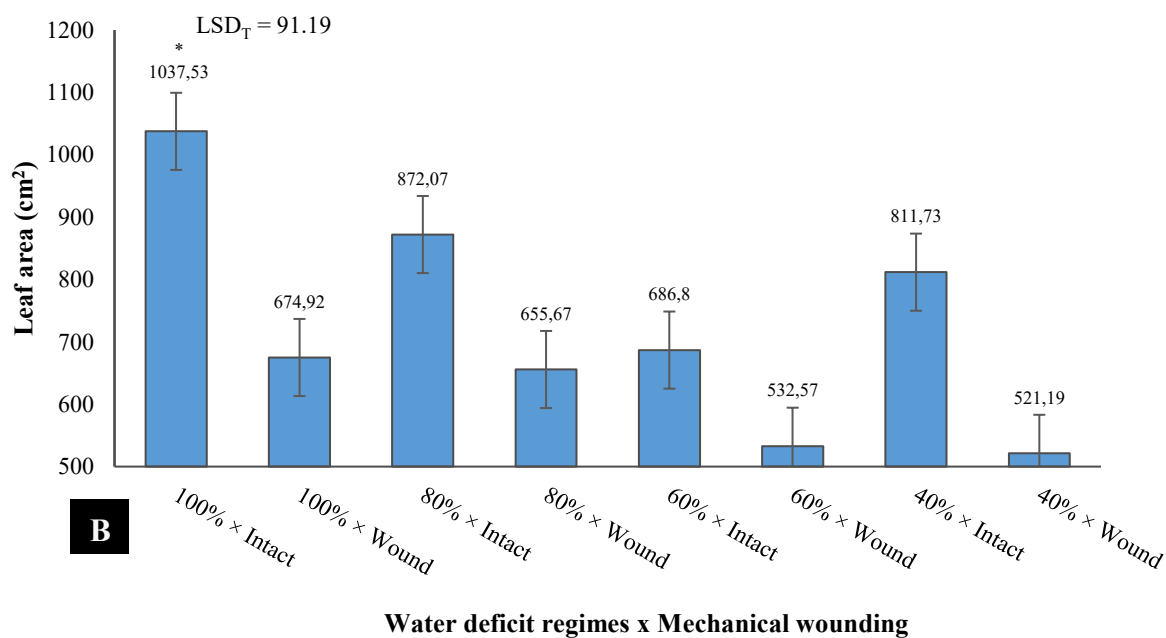
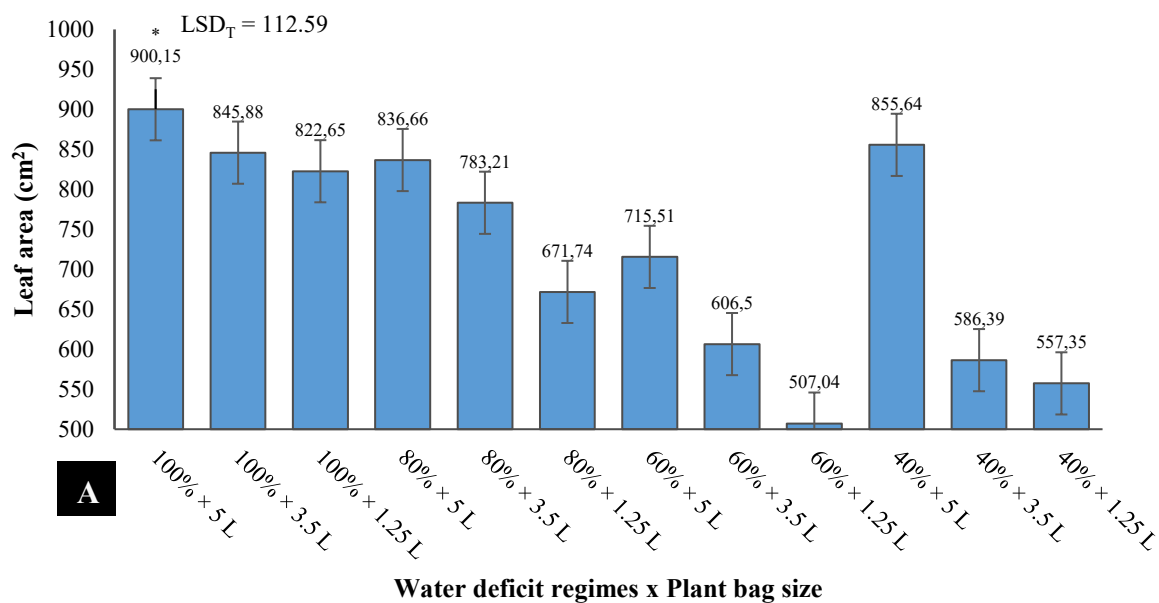


Figure 3.3: Effects of water deficit regimes and plant bag size (A) and the water deficit regimes and mechanical wounding on leaf area (cm²) (B). Asterisk * = Indicates the treatment level with the highest mean value.

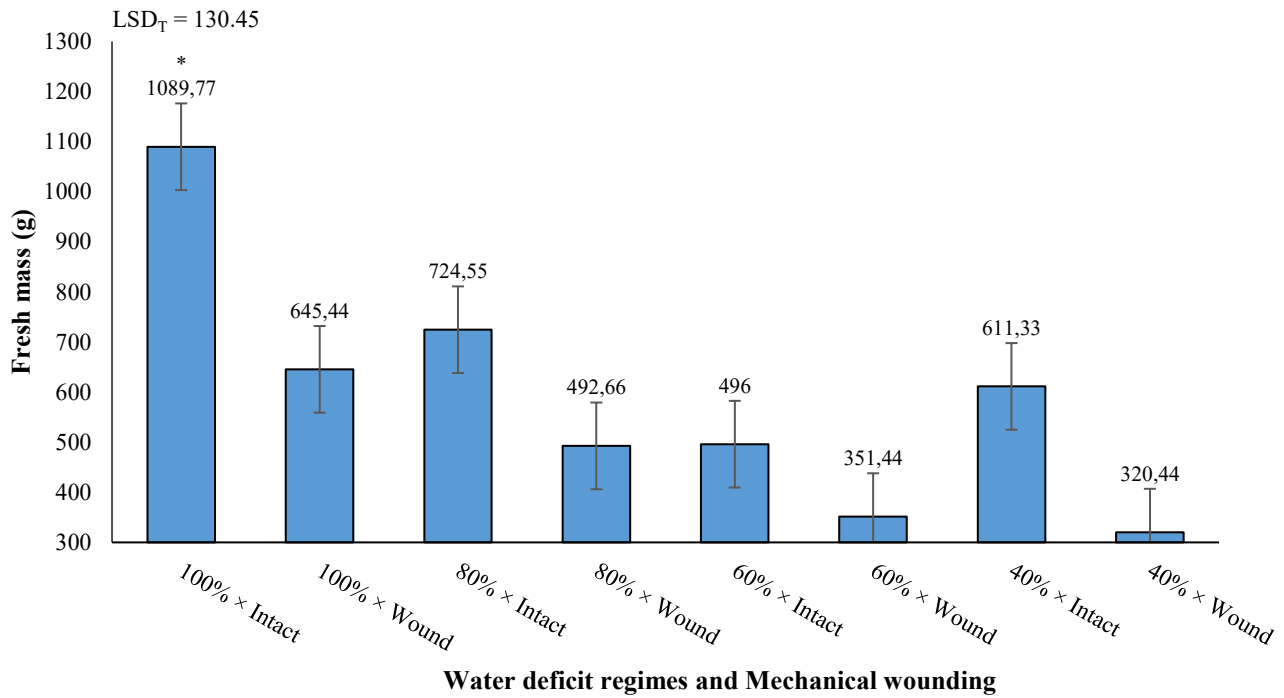


Figure 3.4: Effect of water deficit regimes and mechanical wounding on fresh mass. Asterisk * = Indicates the treatment level with the highest mean value.

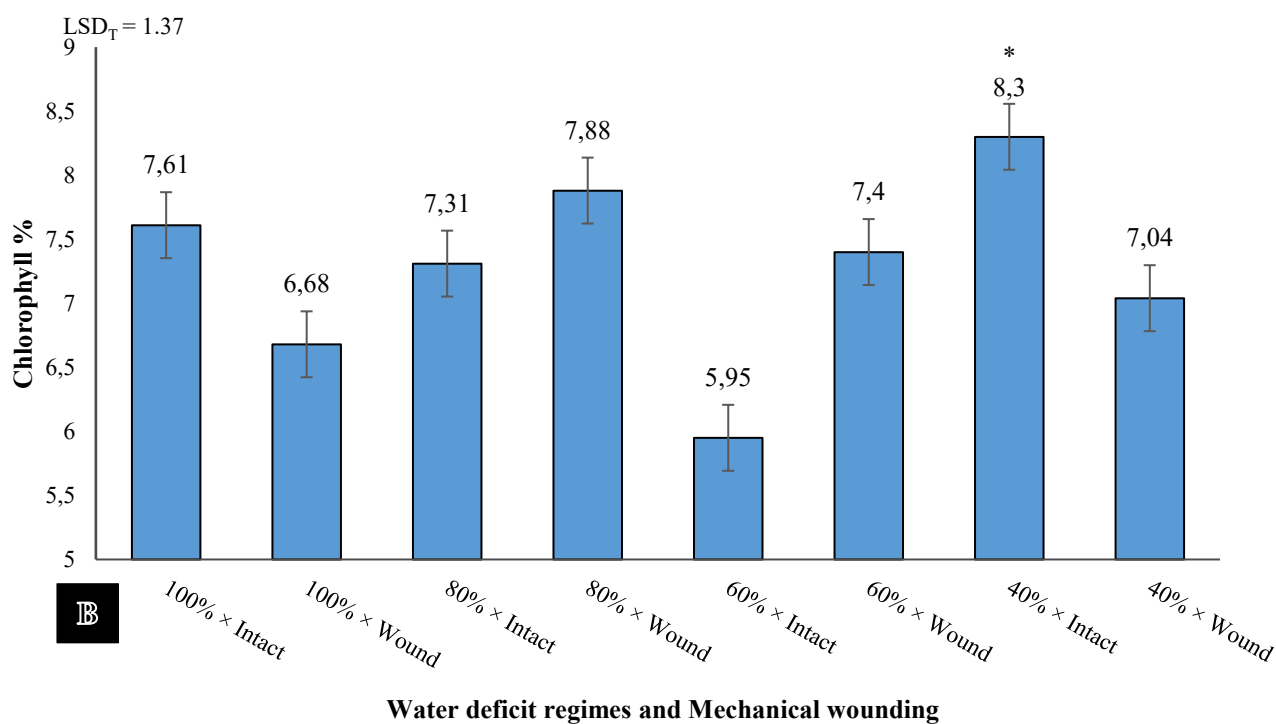
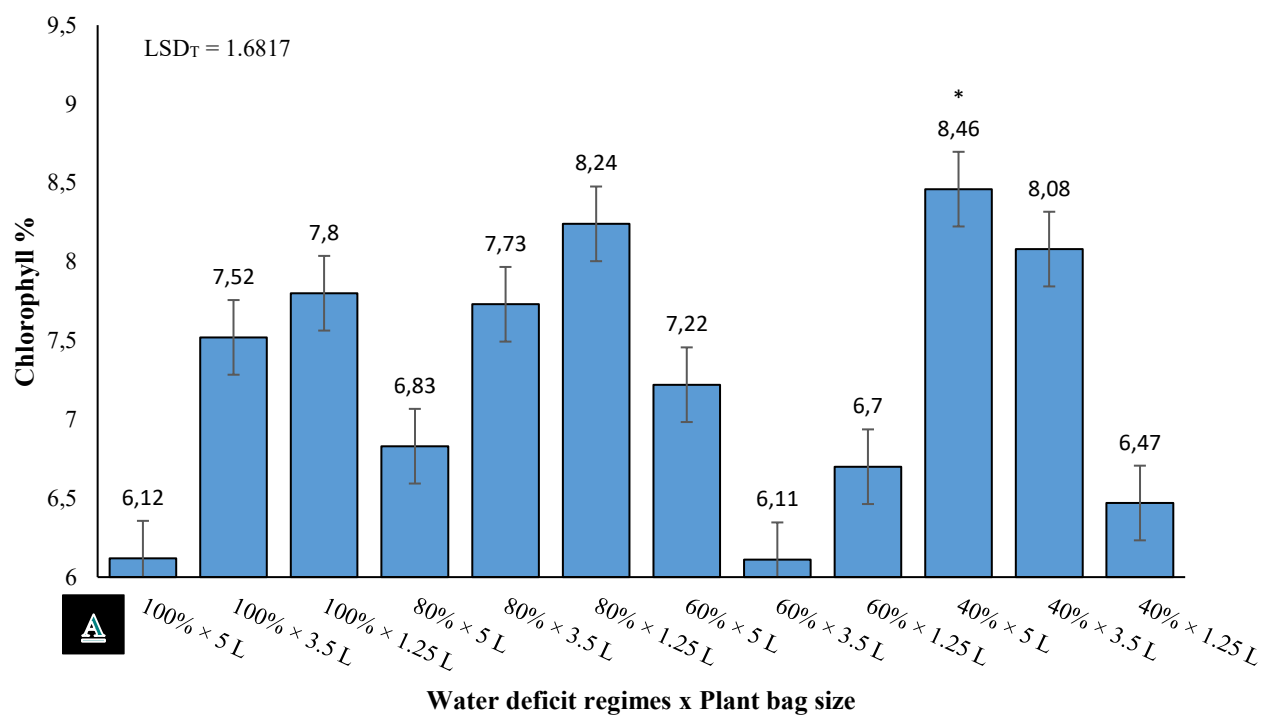


Figure 3.5: Effect of water deficit regimes and plant bag size (A) and the water deficit regimes and mechanical wounding (B) on chlorophyll. Asterisk * = Indicates the treatment level with the highest mean value.



Figure 3.6: Pictorial presentation of the impact of water deficit regimes, plant bag sizes, and mechanical wounding on the morphological growth of curly lettuce. **Abbreviations:** 100% + 5L + Int = 100% × 5 L × Intact plants; 100% + 5L + W = 100% × 5 L × Leaf wounded; 100% + 3.5 L + Int = 100% × 3.5 L × Intact plants; 100% + 3.5 L + W = 100% × 3.5 L × Leaf wounded; 100% + 1.25 L + Int = 100% × 1.25 L × Intact plants; 100% + 1.25 L + W = 100% × 1.25 L × Leaf wounded; 80% + 5L + Int = 100% × 5 L × Intact plants; 80% + 5L + W = 80% × 5 L × Leaf wounded; 80% + 3.5 L + Int = 80% × 3.5 L × Intact plants; 80% + 3.5 L + W = 80% × 3.5 L × Leaf wounded; 80% + 1.25 L + Int = 80% × 1.25 L × Intact plants; 80% + 1.25 L + W = 80% × 1.25 L × Leaf wounded; 60% + 5L + Int = 60% × 5 L × Intact plants; 60% + 5L + W = 60% × 5 L × Leaf wounded; 60% + 3.5 L + Int = 60% × 3.5 L × Intact plants; 60% + 3.5 L + W = 60% × 3.5 L × Leaf wounded; 60% + 1.25 L + Int = 60% × 1.25 L × Intact plants; 60% + 1.25 L + W = 60% × 1.25 L × Leaf wounded; 40% + 5L + Int = 40% × 5 L × Intact plants; 40% + 5L + W = 40% × 5 L × Leaf wounded; 40% + 3.5 L + Int = 40% × 3.5 L × Intact plants; 40% + 3.5 L + W = 40% × 3.5 L × Leaf wounded; 40% + 1.25 L + Int = 40% × 1.25 L × Intact plants; 40% + 1.25 L + W = 40% × 1.25 L × Leaf wounded.

+ 3.5 L + W = 80% × 3.5 L × Leaf wounded; 80% + 1.25 L + Int = 80% × 1.25 L × Intact plants; 80% + 1.25 L + W = 80% × 1.25 L × Leaf wounded; 60% + 5L + Int = 100% × 5 L × Intact plants; 60% + 5L + W = 60% × 5 L × Leaf wounded; 60% + 3.5 L + = 60% × 3.5 L × Intact plants; 60% + 3.5 L + W = 60% × 3.5 L × Leaf wounded; 60% + 1.25 L + Int = 60% × 1.25 L × Intact plants; 60% + 1.25 L + W = 60% × 1.25 L × Leaf wounded; 40% + 5L + Int = 100% × 5 L × Intact plants; 40% + 5L + W = 40% × 5 L × Leaf wounded; 40% + 3.5 L + = 40% × 3.5 L × Intact plants; 40% + 3.5 L + W = 40% × 3.5 L × Leaf wounded; 40% + 1.25 L + Int = 40% × 1.25 L × Intact plants; 40% + 1.25 L + W = 40% × 1.25 L × Leaf wounded.

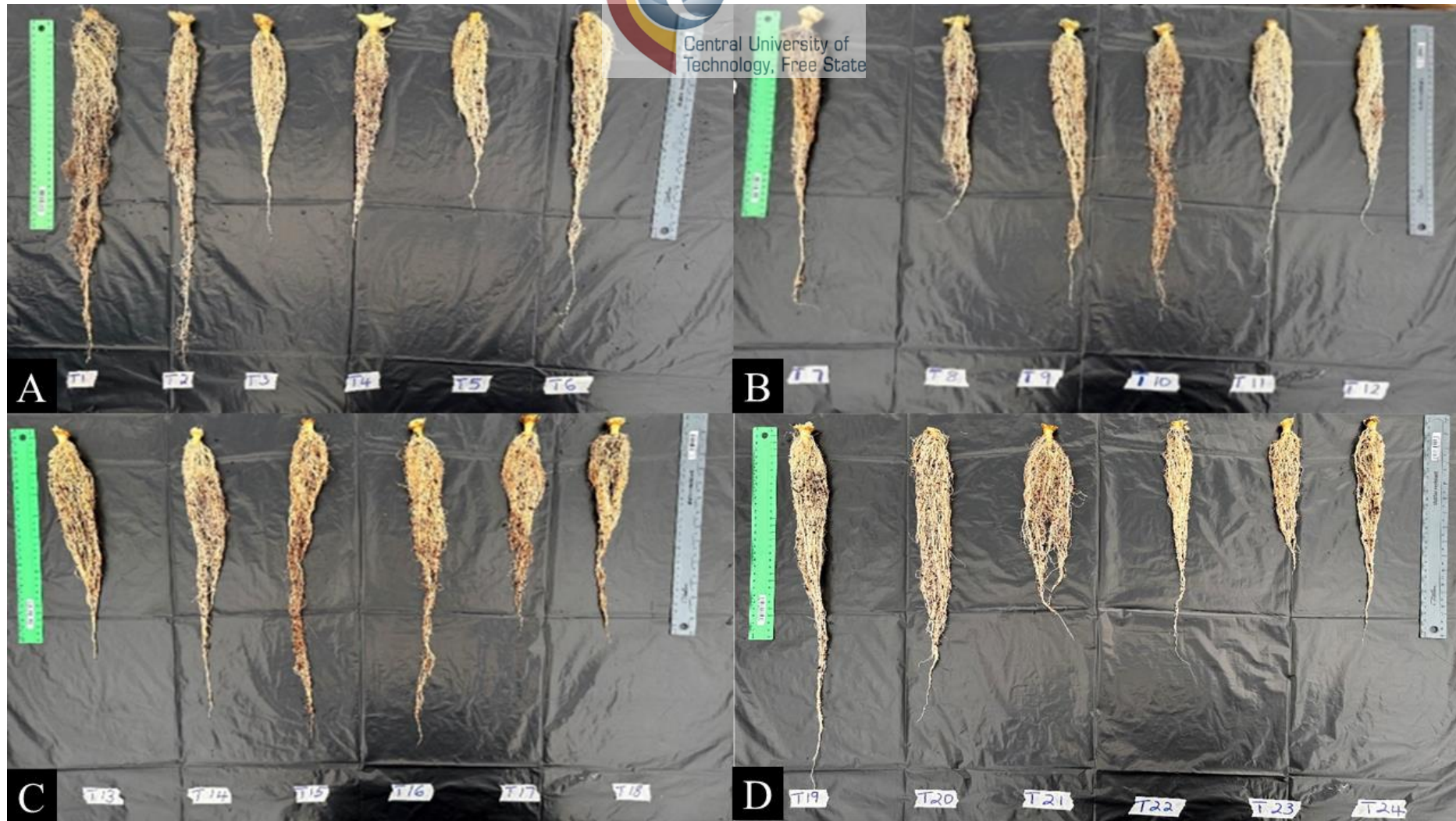


Figure 3.7: Pictorial presentation of the impact of water deficit regimes (100%, 80%, 60%, 40%) plant bag size (5 L, 3.5 L and 1.25 L), and mechanical wounding (intact and leaf wounded plants), and their interactions on the root's morphology of curly lettuce. **Abbreviation:** T1 = 100% × 5 L × Intact plants; T2 = 100% × 5 L × Leaf wounded; T3 = 100% × 3.5 L × Intact plants; T4 = 100% × 3.5 L × Leaf wounded; T5 = 100% × 1.25 L × Intact plants; T6 = 100% × 1.25 L × Leaf wounded; T7 = 80% × 5 L × Intact plants; T8 = 80% × 5 L × Leaf wounded; T9 = 80% × 3.5 L × Intact plants; T10 = 80% × 3.5 L × Leaf wounded; T11 = 80% × 1.25 L × Intact plants; T12 = 80% × 1.25 L × Leaf wounded; T13 = 60% × 5 L × Intact plants; T14 = 60% × 5 L × Leaf wounded; T15 = 60% × 3.5 L × Intact plants; T16 = 60% × 3.5 L × Leaf wounded; T17 = 60% × 1.25 L × Intact plants; T18 = 60% × 1.25 L × Leaf wounded; T19 = 40% × 5 L × Intact plants; T20 = 40% × 5 L × Leaf wounded; T21 = 40% × 3.5 L × Intact plants; T22 = 40% × 3.5 L × Leaf wounded; T23 = 40% × 1.25 L × Intact plants; T24 = 40% × 1.25 L × Leaf wounded.



Biplot (axes F1 and F2: 52.67%) of Technology, Free State

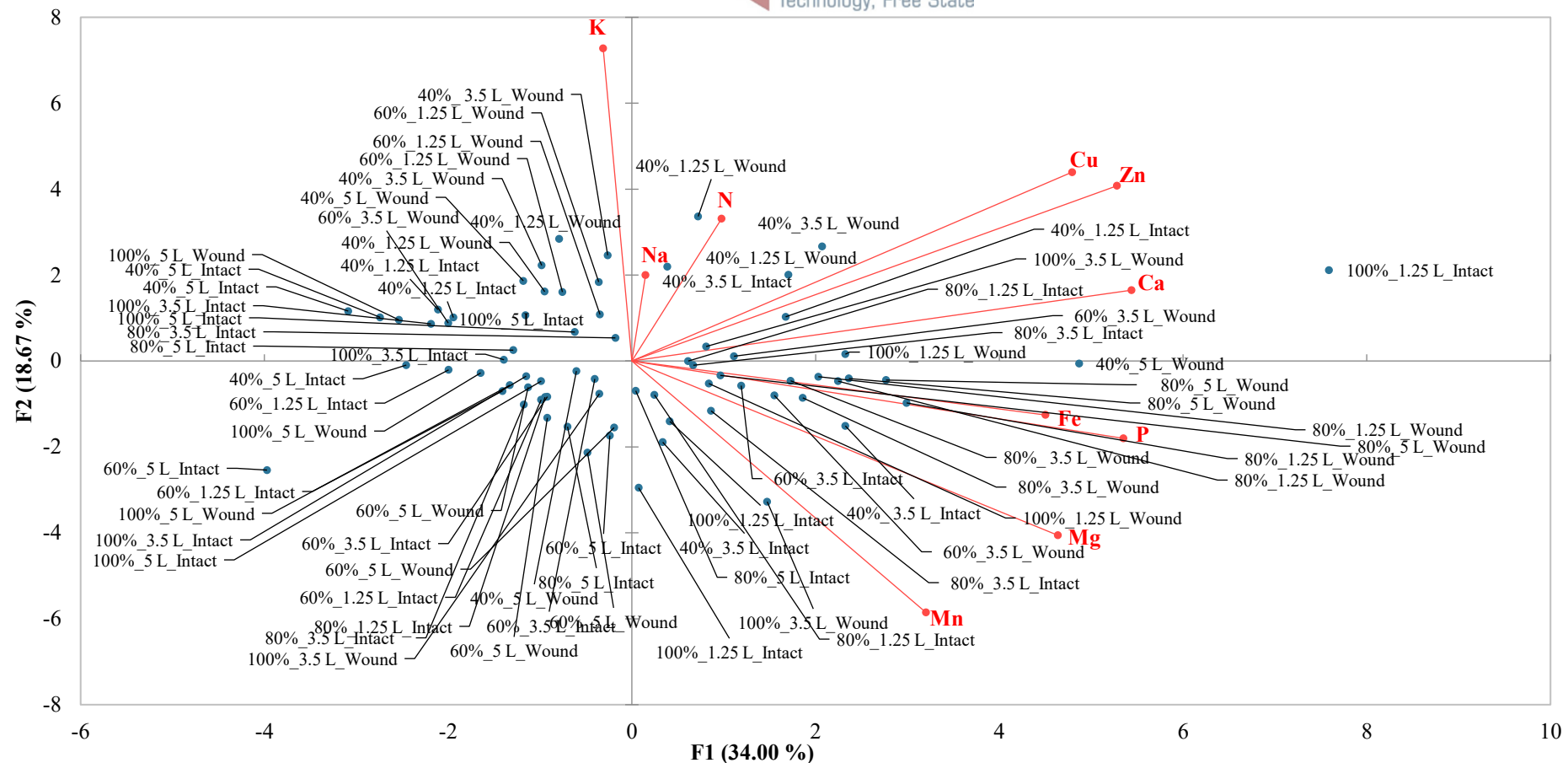


Figure 3.8: PCA of analysed mineral results of lettuce grown under different water irrigation regimes (100%, 80%, 60%, and 40%) and three plant bag sizes (5 L, 3.5 L, and 1.25 L) and mechanical wounding (intact and leaf wounded plants). **Abbreviations:** 100%_5 L_Intact plants = 100% × 5 L × Intact plants; 100%_5 L_Leaf wounded = 100% × 5 L × Leaf wounded; 100%_3.5 L_Intact plants = 100% × 3.5 L × Intact plants; 100%_3.5 L_Leaf wounded = 100% × 3.5 L × Leaf wounded; 100%_1.25 L_Intact plants = 100% × 1.25 L × Intact plants; 100%_1.25 L_Leaf wounded = 100% × 1.25 L × Leaf wounded; 80%_5 L_Intact plants = 80% × 5 L × Intact plants; 80%_5 L_Leaf wounded = 80% × 5 L × Leaf wounded; 80%_3.5 L_Intact plants = 80% × 3.5 L × Intact plants; 80%_3.5 L_Leaf wounded = 80% × 3.5 L × Leaf wounded; 80%_1.25 L_Intact plants = 80% × 1.25 L × Intact plants; 80%_1.25 L_Leaf wounded = 80% × 1.25 L × Leaf wounded; 60%_5 L_Intact plants = 60% × 5 L × Intact plants; 60%_5 L_Leaf wounded = 60% × 5 L × Leaf wounded; 60%_3.5 L_Intact plants = 60% × 3.5 L × Intact plants; 60%_3.5 L_Leaf wounded = 60% × 3.5 L × Leaf wounded; 60%_1.25 L_Intact plants = 60% × 1.25 L × Intact plants; 60%_1.25 L_Leaf wounded = 60% × 1.25 L × Leaf wounded; 40%_5 L_Intact plants = 40% × 5 L × Intact plants; 40%_5 L_Leaf wounded = 40% × 5 L × Leaf wounded; 40%_3.5 L_Intact plants = 40% × 3.5 L × Intact plants; 40%_3.5 L_Leaf wounded = 40% × 3.5 L × Leaf wounded; 40%_1.25 L_Intact plants = 40% × 1.25 L × Intact plants; 40%_1.25 L_Leaf wounded = 40% × 1.25 L × Leaf wounded.

Scores Plot

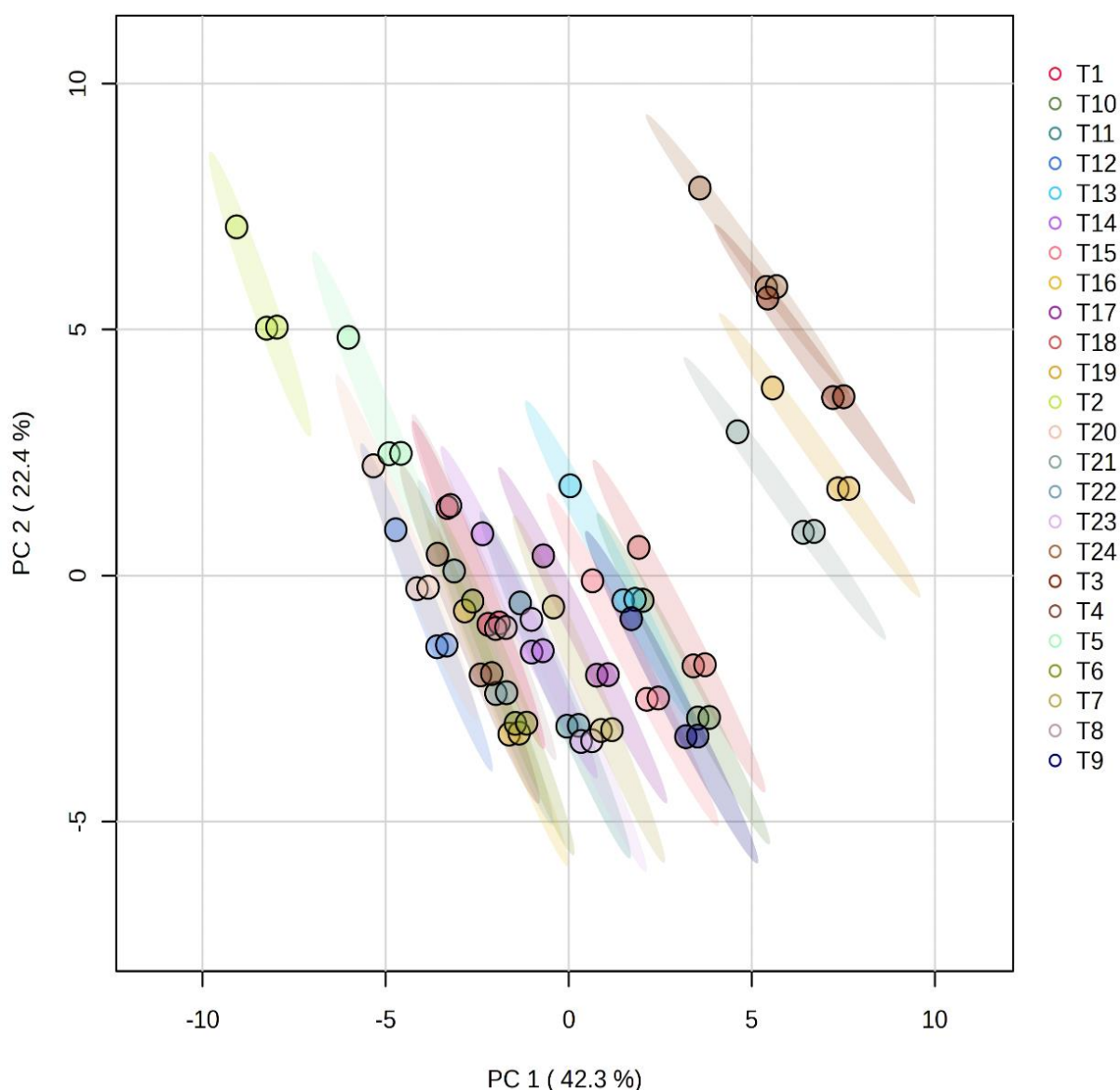


Figure 3.9: A graphical presentation of an unsupervised Principal Component Analysis (PCA) showing scores (circles) plot of phenolics attained by UPLC-MS analysis in lettuce treated with four water deficit irrigation regimes (100%, 80%, 60%, and 40%), three plant bag sizes (5 L, 3.5 L, and 1.25 L), and mechanical wounding (intact and leaf wounded plants). **Abbreviation:** T1 = 100% × 5 L × Intact plants; T2 = 100% × 5 L × Leaf wounded; T3 = 100% × 3.5 L × Intact plants; T4 = 100% × 3.5 L × Leaf wounded; T5 = 100% × 1.25 L × Intact plants; T6 = 100% × 1.25 L × Leaf wounded; T7 = 80% × 5 L × Intact plants; T8 = 80% × 5 L × Leaf wounded; T9 = 80% × 3.5 L × Intact plants; T10 = 80% × 3.5 L × Leaf wounded; T11 = 80% × 1.25 L × Intact plants; T12 = 80% × 1.25 L × Leaf wounded; T13 = 60% × 5 L × Intact plants; T14 = 60% × 5 L × Leaf wounded; T15 = 60% × 3.5 L × Intact plants; T16 = 60% × 3.5 L × Leaf wounded; T17 = 60% × 1.25 L × Intact plants; T18 = 60% × 1.25 L × Leaf wounded; T19 = 40% × 5 L × Intact plants; T20 = 40% × 5 L × Leaf wounded; T21 = 40% × 3.5 L × Intact plants; T22 = 40% × 3.5 L × Leaf wounded; T23 = 40% × 1.25 L × Intact plants; T24 = 40% × 1.25 L × Leaf wounded.

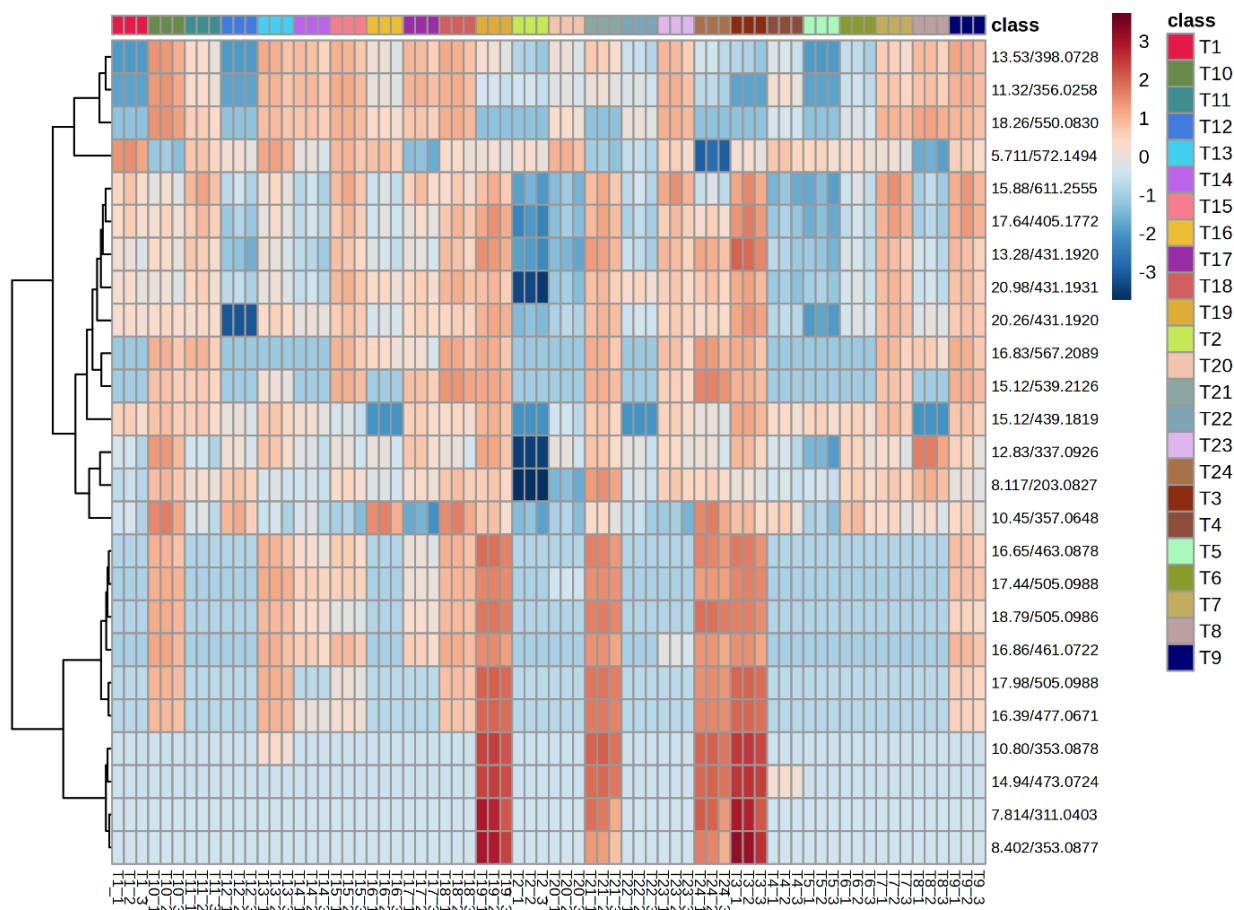


Figure 3.10: Heat map of unsupervised hierarchical clustering analysis (HCA) showing the phenolic compounds found in lettuce grown under different water irrigation regimes (100%, 80%, 60%, and 40%), and plant bag sizes (5 L, 3.5 L and 1.25 L) and mechanical wounding (intact and leaf wounded plants). The coloured parts correspond to the contents of various phenolic compounds (for high red and low blue shades). Rows illustrate phenolic compounds, while columns illustrate multistress treatments. **Abbreviation:** T1 = 100% × 5 L × Intact plants; T2 = 100% × 5 L × Leaf wounded; T3 = 100% × 3.5 L × Intact plants; T4 = 100% × 3.5 L × Leaf wounded; T5 = 100% × 1.25 L × Intact plants; T6 = 100% × 1.25 L × Leaf wounded; T7 = 80% × 5 L × Intact plants; T8 = 80% × 5 L × Leaf wounded; T9 = 80% × 3.5 L × Intact plants; T10 = 80% × 3.5 L × Leaf wounded; T11 = 80% × 1.25 L × Intact plants; T12 = 80% × 1.25 L × Leaf wounded; T13 = 60% × 5 L × Intact plants; T14 = 60% × 5 L × Leaf wounded; T15 = 60% × 3.5 L × Intact plants; T16 = 60% × 3.5 L × Leaf wounded; T17 = 60% × 1.25 L × Intact plants; T18 = 60% × 1.25 L × Leaf wounded; T19 = 40% × 5 L × Intact plants; T20 = 40% × 5 L × Leaf wounded; T21 = 40% × 3.5 L × Intact plants; T22 = 40% × 3.5 L × Leaf wounded; T23 = 40% × 1.25 L × Intact plants; T24 = 40% × 1.25 L × Leaf wounded.

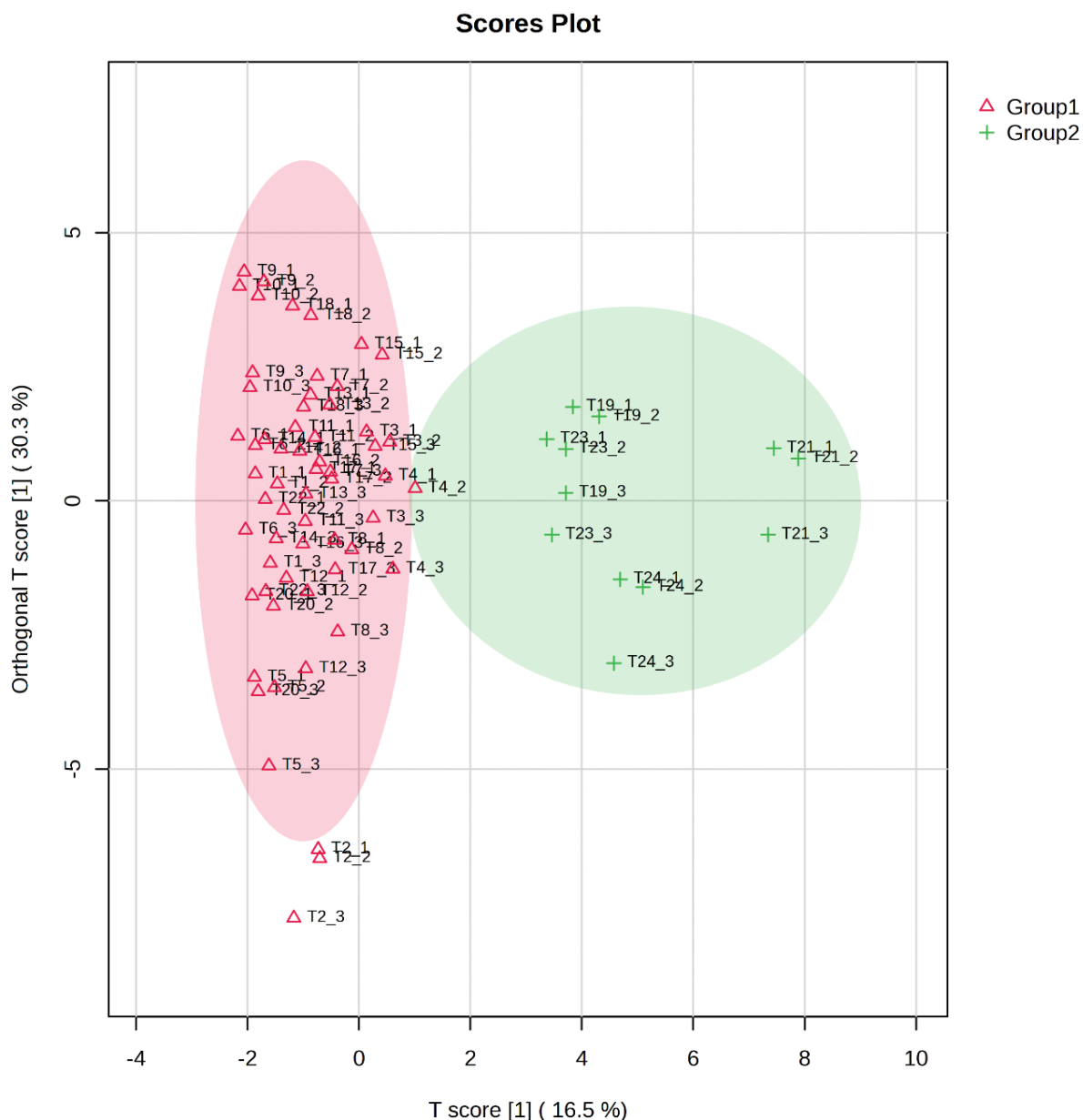


Figure 3.11: Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) score plot supervised modelling displaying the phenolic compounds groupings found in lettuce grown under different water irrigation regimes (100%, 80%, 60%, and 40%), and plant bag sizes (5 L, 3.5 L, and 1.25 L) and mechanical wounding (intact and leaf wounded plants). **The legends:** red triangles and green crosses indicate data groupings. **Abbreviation:** T1 = 100% × 5 L × Intact plants; T2 = 100% × 5 L × Leaf wounded; T3 = 100% × 3.5 L × Intact plants; T4 = 100% × 3.5 L × Leaf wounded; T5 = 100% × 1.25 L × Intact plants; T6 = 100% × 1.25 L × Leaf wounded; T7 = 80% × 5 L × Intact plants; T8 = 80% × 5 L × Leaf wounded; T9 = 80% × 3.5 L × Intact plants; T10 = 80% × 3.5 L × Leaf wounded; T11 = 80% × 1.25 L × Intact plants; T12 = 80% × 1.25 L × Leaf wounded; T13 = 60% × 5 L × Intact plants; T14 = 60% × 5 L × Leaf wounded; T15 = 60% × 3.5 L × Intact plants; T16 = 60% × 3.5 L × Leaf wounded; T17 = 60% × 1.25 L × Intact plants; T18 = 60% × 1.25 L × Leaf wounded; T19 = 40% × 5 L × Intact plants; T20 = 40% × 5 L × Leaf wounded; T21 = 40% × 3.5 L × Intact plants; T22 = 40% × 3.5 L × Leaf wounded; T23 = 40% × 1.25 L × Intact plants; T24 = 40% × 1.25 L × Leaf wounded.

Scores Plot

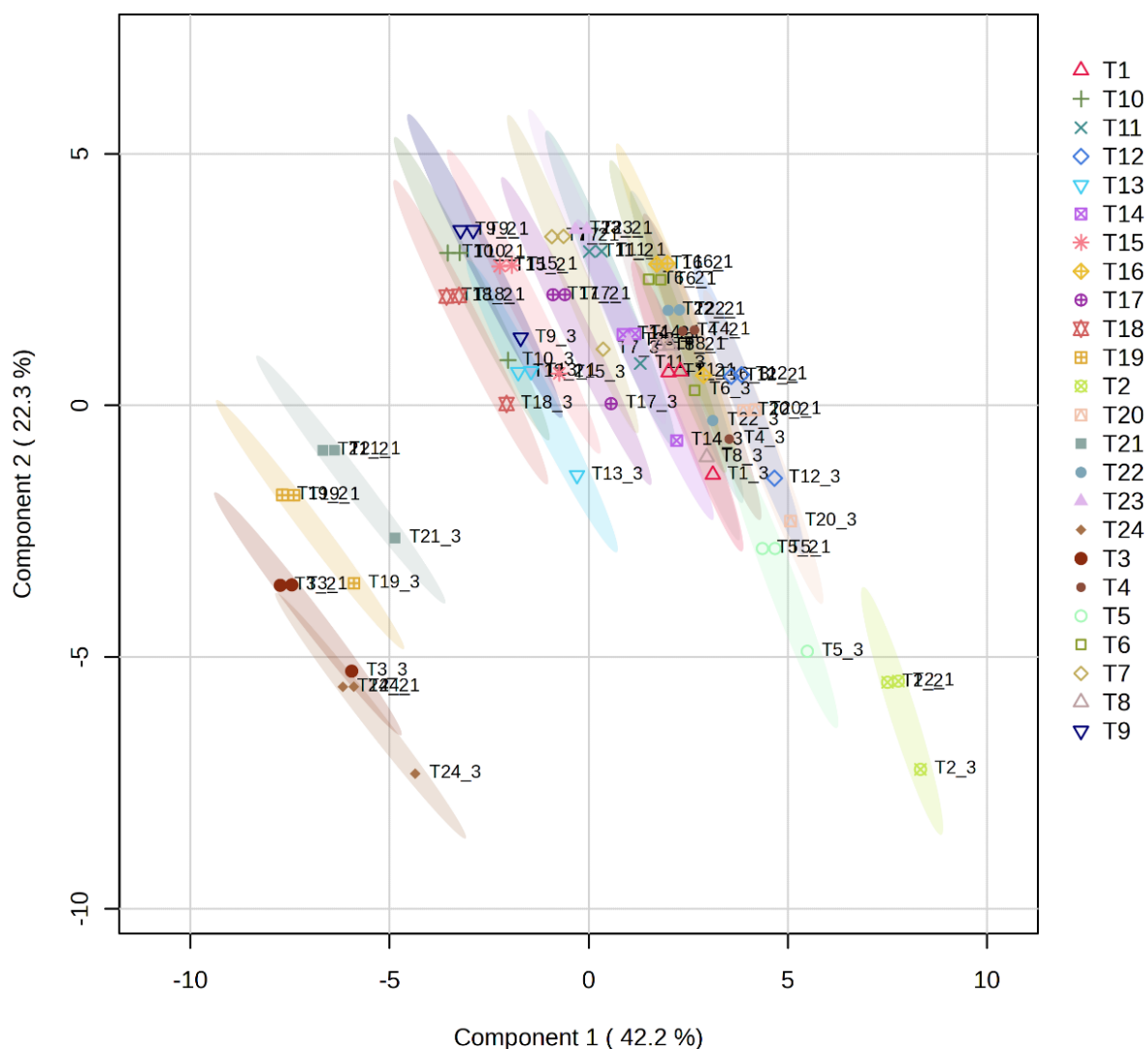


Figure 3.12: Partial least square discrimination analysis (PLS-DA) score plot showing the phenolic compounds groups found in lettuce subjected to different water irrigation regimes (100%, 80%, 60%, and 40%), and three plant bag sizes (5 L, 3.5 L and 1.25 L) and mechanical wounding (intact and leaf wounded plants). **Abbreviation:** T1 = 100% × 5 L × Intact plants; T2 = 100% × 5 L × Leaf wounded; T3 = 100% × 3.5 L × Intact plants; T4 = 100% × 3.5 L × Leaf wounded; T5 = 100% × 1.25 L × Intact plants; T6 = 100% × 1.25 L × Leaf wounded; T7 = 80% × 5 L × Intact plants; T8 = 80% × 5 L × Leaf wounded; T9 = 80% × 3.5 L × Intact plants; T10 = 80% × 3.5 L × Leaf wounded; T11 = 80% × 1.25 L × Intact plants; T12 = 80% × 1.25 L × Leaf wounded; T13 = 60% × 5 L × Intact plants; T14 = 60% × 5 L × Leaf wounded; T15 = 60% × 3.5 L × Intact plants; T16 = 60% × 3.5 L × Leaf wounded; T17 = 60% × 1.25 L × Intact plants; T18 = 60% × 1.25 L × Leaf wounded; T19 = 40% × 5 L × Intact plants; T20 = 40% × 5 L × Leaf wounded; T21 = 40% × 3.5 L × Intact plants; T22 = 40% × 3.5 L × Leaf wounded; T23 = 40% × 1.25 L × Intact plants; T24 = 40% × 1.25 L × Leaf wounded.

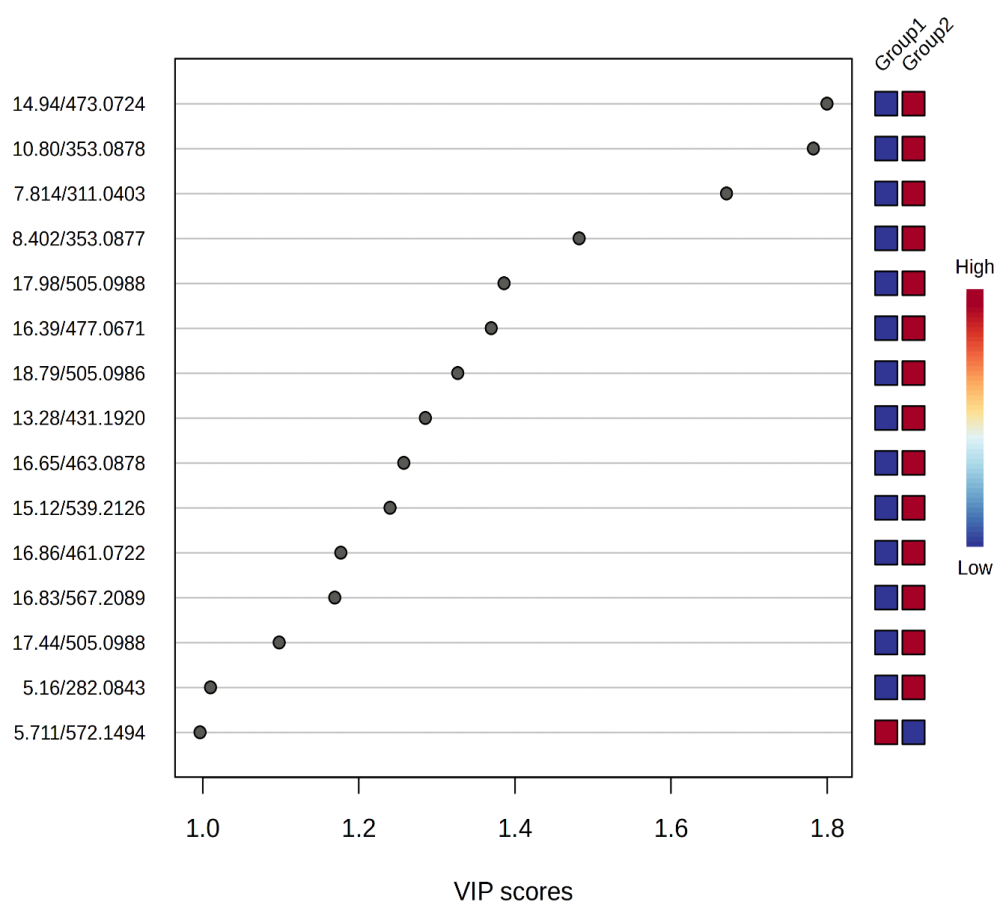


Figure 3.13: Variables Importance in Projection (VIP) scores in OPLS-DA assigned to phenolic compounds data found in lettuce grown under different water irrigation regimes (100%, 80%, 60%, and 40%), and plant bag sizes (5 L, 3.5 L, and 1.25 L) and mechanical wounding (intact and leaf wounded plants). Mini-coloured blocks on the right represent the relative content of each phenolic compound.

CHAPTER 4

EFFECT OF THE PHYTOHORMONE-BASED BIOSTIMULANT ON YIELD, MORPHOPHYSIOLOGICAL AND BIOCHEMICAL RECOVERY RESPONSE OF CURLY LETTUCE (*Lactuca sativa* L. Var. Crispa) GROWN UNDER MULTI-ABIOTIC STRESS CONDITIONS

ABSTRACT

The complex interactive effects of combined multi-abiotic stress are underrated, and not enough attention is given to the available mitigation strategies. The use of phytohormone-based biostimulants as an alternative, among others, has proven to be an innovative solution in equipping horticultural crops with abiotic tolerance by contrasting plant stresses. Therefore, the objective of this study was to evaluate the yield, morphophysiological, and biochemical recovery response of curly lettuce (*Lactuca sativa* L. var. Crispa) grown under extreme multi-abiotic stress conditions and treated with a phytohormone-based biostimulant (*Lucky Plant*[®]). The experiment was carried out in a 4 x 4 Factorial Design based on a randomised complete block design layout with three replicates. Treatments consisted of four multi-abiotic stress levels: control (100% × 5 L × intact plants), mild (60% × 1.25 L × leaf wounding), moderate (40% × 3.5 L × leaf wounding), and severe (40% × 1.25 L × leaf wounding) captured from the results of the 2023 growing season and four doses of *Lucky Plant*[®] (distilled water as control, 0.5 g L⁻¹, 1.0 g L⁻¹, and 1.5 g L⁻¹). The foliar application of *Lucky Plant*[®] was performed after three weeks of plant stress for two consecutive weeks with seven days intervals. The results showed that multi-abiotic stress, not treated with a biostimulant in the current study, reduced plant height, number of leaves, leaf area, leaf fresh and dry mass, chlorophyll content, stomatal density, stomatal conductance, nutritional quality, and produced higher phenolic content compared to the control plants. The foliar application of *Lucky Plant*[®] increased the number of leaves per plant at a dose of 1.5 g L⁻¹ under control, mild, and severe conditions. A dose of 1.5 g L⁻¹ significantly increased stomatal conductance and chlorophyll content (15.43%) under control conditions and stomatal density (57.00%) under severe stress. The highest stomatal density (n=40) was achieved with a 1.0 g L⁻¹ dose under a control conditions. PCA biplot showed the best results on the uptake of Mn, Mg, P, K, and Ca nutrients at a dose of 0.5 g L⁻¹ and Zn with 1.0 g L⁻¹ under control conditions. Notably, the N content was alleviated under mild, moderate, and severe conditions with 1.5 g L⁻¹ and 0.5 g L⁻¹ doses. Phenolic contents were most abundant under control conditions at all doses and higher under severe conditions treated with 1.5 g L⁻¹ dose. Based on these findings, this study concludes that foliar application of *Lucky Plant*[®] biostimulant at doses of 1.0 g L⁻¹ and 1.5 g L⁻¹ can be proposed on lettuce under optimal conditions.

Keywords: Curly lettuce · Multi-abiotic stress · *Lucky Plant*[®] · Gibberellic acid · Brassinosteroids · Cytokinins

4.1 INTRODUCTION

To cope with multi-abiotic stress, plants respond by making biochemical, physiological, and morphological alterations. Osmotic adjustment is one of the physiological adaptation mechanisms by which plants subjected to water deficit accumulate cell-compatible solutes to overcome their adverse effects (Aslani *et al.*, 2023). Water deficiency affects chlorophyll content by altering chloroplast membranes, causing swelling and distortion of the lamellae (Kumar *et al.*, 2020). Water stress causes stomatal closure and an interruption in water flow from the xylem to the surrounding cells, which inhibits cell elongation and affects sensitive physiological processes such as protein synthesis and transportation of nutrients (Al-Quraan *et al.*, 2021). The utilisation of appropriate growth bags is a vital cultural practice for horticultural production (Sahu *et al.*, 2024). Inadequate rooting volume causes the root system to become dense, branched, and matted and reduces the photosynthetic capacity of plants due to poor sustainability of hydric status, leading to water loss (Turner, 2019).

Plants activate numerous defence mechanisms to respond to wounding, which include strengthening the cell walls, inducing the defence-related genes, synthesising antinutritional compounds, and antimicrobial and wounding healing processes (Wielkopolan *et al.*, 2022). Plant response to wounding activate metabolic pathways that cause changes in metabolism, photosynthesis, and respiration rate (Guan *et al.*, 2022; Al-Khayri *et al.*, 2023). Sabina and Jithesh (2021) conducted a study to evaluate the biochemical response to two different types of wounding damage in the leaves of tomatoes, and the results showed that damage by wounding elicited a rapid increase in total phenolic, flavonoid and total antioxidant activity levels in midrib damage than either the lamina damage or control conditions.

When a variety of two or more environmental stress factors coincide, a severe decline in successive plant growth occurs (Lu *et al.*, 2021; Sehgal *et al.*, 2022). In horticultural plants, oxidative stress activates the biosynthesis of phenolic compounds, including flavonoids, which can counteract the impact of reactive oxygen species (ROS), which induce cellular senescence and cell damage (Kumar *et al.*, 2020; Plazas *et al.*, 2023). The total transpirable soil water for individual pot sizes corresponds closely with the volume or weight of the pot (Lu *et al.*, 2021). The substrate bulk density and potting size can affect the formation of new roots (Isa *et al.*, 2021). A higher bulk density and water deficit irrigation limit root growth and result in lower product quality due to adjusted capacity to low water matric potential and poor absorption of nutrients by roots (Lu *et al.*, 2021). Water deficit and wound stress reduce photosynthesis by

closing stomata, decreasing the efficiency of the carbon fixation process, suppressing leaf formation and expansion, and inducing the shedding of leaves on cabbage (Sehgal *et al.*, 2022).

Some biostimulants, natural substances or microorganisms that can contain bioactive cytokinins (CKs), gibberellins (GAs), and brassinosteroids (BRs) have the potential to mitigate the oxidative stress caused by multistress, which hinders regular cell metabolism, inducing the accumulation of ROS on plants (Gerhards *et al.*, 2021; Yordanova & Filipov, 2023). Phytohormone plant signalling pathways alter the expression of defensive genes, which modifies the secondary metabolite levels and improves plant tolerance (Baharudin & Osman, 2023). The BRs are linked to several signalling networks, including cell wall lignification, elongation, regulation of cell division, and defence response, and are involved in root development, which is vital for maintaining available water and nutrients during water deficit conditions (Gerhards *et al.*, 2021). The BRs hormones regulate senescence mechanisms in wounded vegetables by changing the lipid composition of cells (Jogawat *et al.*, 2021).

Plant cytokinin signalling comprises histidine kinases (Hai *et al.*, 2020). The CKs delay leaf senescence to protect photosynthesis and promote photomorphogenic cell differentiation in developing leaves during water deficiency stress (Hai *et al.*, 2020). Cytokinins are primarily involved in the formation of roots by differentiating phloem and metaxylem (Hossain *et al.*, 2022). The CK hormones are identified for stimulating the mobilisation of nutrients as per crop demand (Roy, 2024). GA signalling regulates the initiation and progression of the stalk and meristem development process and plant height increment (Hossain *et al.*, 2022). The latest review by Roy (2024) on plant growth regulating phytohormones explicitly describes the roles of ABA, BRs, and CKs in alleviating abiotic stress in crop plants. According to Jahan *et al.* (2024), the interplay between natural plant hormones and applied synthetic biostimulants within stressed plants influences the alteration of metabolic pathways, gene activity, and antioxidant systems.

4.2 MATERIAL AND METHODS

4.2.1 Description of trial site

The trial was conducted on the experimental site mentioned in Chapter 3 under materials and methods. The exhaust fan air extractor in the greenhouse tunnel was not utilised in this trial due to cool weather conditions during the winter season. Daily temperatures in the greenhouse tunnel were observed using two digital wall thermometers. A 9,000W electric space heater

(Bluetech Fans, Leroy Merlin, SA) was used to keep temperatures at 26°C during colder periods. Similarly, during the night, the space heater was used to regulate the temperature to maintain 12°C throughout the growing season. The heater with settings for heat control is suitable for heating an area of approximately 80–100 m², and the greenhouse tunnel used for the experiment was 72 m².

4.2.2 Experimental design and plant treatments

4.2.2.1 Experiment design

The second experiment was a 4 x 4 Factorial based on a randomised complete block design layout, replicated thrice. Four levels of multistress were captured from the first trial treatments: control, mild, moderate, and severe. There was four doses of phytohormone-based biostimulants (*Lucky Plant*[®]) used, namely: distilled water (control), 0.5 g L⁻¹, 1.0 g L⁻¹, and 1.5 g L⁻¹. Plants subjected to the same multistress treatment but not treated with the *Lucky Plant*[®] biostimulant, where only distilled water was administered, were considered as controls for treated plants.

4.2.2.2 Treatments

Multistress factoring

The multistress factors were selected to establish the four levels of multistress: control (100% × 5 L × Intact plants), mild (60% × 1.25 L × leaf wounding), moderate (40% × 3.5 L × leaf wounding), and severe (40% × 1.25 L × leaf wounding). Each treatment combination combined stress factors applied as described in Chapter 3 for the first experiment under materials and methods.

Biostimulants application

The multistress treatment levels were considered during the application of phytohormone-based biostimulants (*Lucky Plant*[®]). The following treatment combination was used: Control × distilled water, control × 0.5 g L⁻¹, control × 1.0 g L⁻¹, control × 1.5 g L⁻¹; mild × distilled water, mild × 0.5 g L⁻¹, mild × 1.0 g L⁻¹, mild × 1.5 g L⁻¹; moderate × distilled water, moderate × 0.5 g L⁻¹, moderate × 1.0 g L⁻¹, moderate × 1.5 g L⁻¹ and severe × distilled water, severe × 0.5 g L⁻¹, severe × 1.0 g L⁻¹, severe × 1.5 g L⁻¹.

The phytohormone-based biostimulant, registered as *Lucky Plant*[®] (Agraforum, South Africa (Pty) Ltd), is protected by patent number 4990-60. *Lucky Plant*[®] is a plant extract, and its active components are gibberellic acid (GA), brassinosteroids (BRs), and traces of cytokinins (CKs) (6-Benzylaminopurine, Kinetin, and *trans*-Zeatin). Approximately 50 mL of the biostimulant treatment solution was sprayed onto the treated plant's leaves until the solution dripped from the plants. The application rate was calculated based on the manufacturer's recommendation. The foliar application was carried out using handheld 2 L pump pressure sprayers (Red Rhino[™], SA), calibrated precision sprayers with 0.3 MPa pressure. All applications were mixed with a wetting agent (Efekto G49 wetting agent, Hydroponic, SA) at 0.01 ml L⁻¹. Plants were treated with *Lucky Plant*[®] biostimulant when they were six weeks old and visibly impacted by multistress. The foliar treatment was performed twice, 24 and 32 days after transplant (DAT), in the morning between 8:00 a.m. – 9:00 a.m. To prevent the solution from drifting onto adjacent experimental plants during spraying, 46 L clear crates (60 cm length x 40 cm width x 22 cm height) were used to cover the whole canopy and removed immediately after foliar spraying.

4.2.3 Agronomic management practices

Plant material and seed germination, cultivation and agronomic techniques, and nutrient management followed the same procedures noted in Chapter 3. However, curly lettuce seeds were sown in autumn on 7 May 2024 and transplanted in winter on 7 June 2024. In this study, no pests were scouted, and pesticides were not administered. The study was extended by one week due to the winter season. The study was terminated on the fifth week after the transplant, on 15 July 2024.

4.2.4 Plant measurements and data collection

Data was collected weekly for non-destructive measurements such as plant height, leaf number, and chlorophyll content. All parameters were quantified following the procedures mentioned in Chapter 3. In addition, the stomatal density was only measured in the second experiment. Therefore, the final data was collected at 37 DAT on the final harvest.

Measurement of stomatal density of the leaves

Samples were collected 31 days after transplant (DAT) between 10:00 am and 11:00 am, and one hour prior, plants were given foliar treatment with the phytohormone-based

biostimulant for the second and last time. Curly lettuce leaves' stomatal densities were determined with the help of a Shimadzu SSX-550 (Kyoto Japan) scanning electron microscope (SEM), as described by Khetsha *et al.* (2022). A tiny portion of leaf samples of approximately 1 cm² leaf area was cut using the convex-edged blade scissors from the leaf apex and immersed into 3% glutardialdehyde (sodium phosphate buffer 0.1 M at pH 7.0) fixative solution in 2 ml microcentrifuge tubes with a snap cap. Samples were dried in a Tousimis critical point drier apparatus (Bio-Rad E300 Rockville), pressured with CO₂ liquid at 37°C. The SEM was set at x100 µm magnification and connected to the computer on which digital images were viewed in Photoshop 7 Savvy (Sybex San Francisco, USA) files. On images captured, the number of stomata was counted in three samples for each treatment, and the mean values of the stomatal density (number of stomata) were determined using Tukey's LSD.

4.2.5 Statistical analysis

The second experiment followed the same statistical analysis procedures described in the first experiment details in Chapter 3.

4.3 RESULTS AND DISCUSSION

4.3.1 The response of morphological parameters of curly lettuce to multistress and phytohormone-based biostimulant

Plant height

Table 4.1 shows that the multistress treatments imposed had a significant effect ($p < 0.0001$) on plant height at final harvest. The severe, moderate, and mild multistress treatments decreased the plant height by 52.71%, 51.40%, and 36.70%, respectively, compared to the control treatment. In the current study, none of the *Lucky Plant*[®] doses significantly recovered the plant height from the reduction induced by multistress (Table 4.1). Likewise, Tandathu *et al.* (2024) disregarded the plant height in their study, as there were no significant differences observed under the evaluated phytohormone-based biostimulant, BR, and seaweed extract KELP on maize seedlings over four weeks after the application of treatment. In addition, the interaction between multistress and foliar-applied *Lucky Plant*[®] doses was not significant during the whole season.

Number of leaves

Multistress significantly affected the number of leaves ($p < 0.05$) at harvest (Table 4.1). The mild, moderate, and severe stress conditions reduced the number of leaves by 8.81%, 26.401%, and 30.36% respectively, compared to the control treatment. The interactions between multistress and foliar application of *Lucky Plant*[®] biostimulant significantly affected the number of leaves in the fourth week (Figure 4.2) and at harvest (Figure 4.3).

In the fourth week, the foliar application of *Lucky Plant*[®] at all doses increased the number of leaves in the control treatment, with the maximum number obtained at a dose of 1.5 g L⁻¹, and in mild conditions, 0.5 g L⁻¹ improved the number of leaves compared to distilled water. In contrast, none of the *Lucky Plant*[®] doses recovered the number of leaves under moderate and severe conditions compared to distilled water (Figure 4.2).

At harvest, the foliar application of *Lucky Plant*[®] at a dose of 1.5 g L⁻¹ produced the highest number of leaves under control and mild conditions over other foliar treatments (Figure 4.3). Similar results have been reported by El-Hameda *et al.* (2024) in tomato plants that were foliar-sprayed with a biostimulant extract enriched with gibberellic acid, ascorbate, and selenium under abiotic stress. Likewise, lettuce foliar-sprayed with Auxym[®] (Giordano *et al.*, 2022), and *Amaranthus hybridus* L. treated with Kelpak[®] (Ngoroyemoto *et al.*, 2020) under controlled growing conditions in these studies. There was no significant increase observed in any *Lucky Plant*[®] dose in the number of leaves under moderate stress conditions compared to distilled water (Figure 4.3).

Figure 4.3 also shows that all *Lucky Plant*[®] doses recovered and increased the number of leaves from severe stress over distilled water. According to El-Hameda *et al.* (2024), plants treated with a biostimulant rich in gibberellic acid phytohormone under normal and stress conditions promoted cell metabolism. This led to the enhanced concentration of plant endogenous hormones, including gibberellic acid, indole-3-acetic acid, zeatin, and cytokinin, simultaneously increasing plant stress tolerance and reducing the ABA levels, which indicate reduced plant stress response. Furthermore, improved phytohormones enable the control of plant growth through the stimulation of shoot growth or the regulation of processes that can inhibit plant growth, thus boosting plant survival and mitigating adverse effects induced by abiotic stresses.

Leaf area

Table 4.1 indicates that the multistress significantly affected ($p < 0.05$) the leaf area of curly lettuce leaves at harvest. The mild, moderate, and severe treatments reduced the leaf area by approximately 60.11%, 76.71% and 75.79% respectively, compared to the control treatment. The most typical way that plants adapt to water deficit stress is by shrinking their leaves (Jogawat *et al.*, 2021). Furthermore, the foliar application of *Lucky Plant*[®] at all doses did not significantly improve the leaf area of plants grown under multiple stresses. An increase in leaf vein and stomatal density relates to a decrease in leaf area (Hasanuzzaman *et al.*, 2023). Thereby, the elevated stomatal density under multistress following the foliar application of *Lucky Plant*[®] in this study (Table 4.2) may have initiated a decrease in leaf area as a coping mechanism to provide an adequate number of functional stomata per unit of leaf surface area and meet the required CO₂ flux to sustain the photosynthetic capacity ((Hasanuzzaman *et al.*, 2023).

Leaf fresh mass

The measurement of fresh mass showed that plants were negatively affected by multistress conditions (Table 4.1). The mild, moderate, and severe treatments decreased the fresh mass by 69.08%, 82.36% and 84.48% respectively, compared to the control. Velasco-Clares *et al.* (2025) reported a 45% reduction in the fresh mass of lettuce caused by water deficit stress only. Studies by Ngoroyemoto *et al.* (2020), Sabatino *et al.* (2023) and Consentino *et al.* (2023) reported that on *Amaranthus hybridus*, leafy green chicory and sweet basil, the use of Kelpak[®], *Ecklonia maxima* and Kelpstar[®] biostimulants, which are all derived from a seaweed extract with the presence of different phytohormones, increased the fresh mass yield under optimal and abiotic stress conditions. Moreover, the foliar application of a phytohormone-based biostimulant known as Kelpak[®] increased the fresh yield of baby lettuce significantly by 19% under optimal growing conditions (Di Mola *et al.*, 2019). The beneficial effects of the biostimulants can result from the cytokinins' effect of improving plant yield and quality in plants subjected to wounding and moisture stress (Khetsha *et al.*, 2024).

However, in the current study that there was no significant interaction between multistress levels and phytohormone-based biostimulant varying doses on the fresh mass of curly lettuce (Figure 4.5). Therefore, none of the *Lucky Plant*[®] doses applied significantly increased the fresh mass under control conditions, mild, moderate, and severely stressed plants. In addition, a dose of 0.5 g L⁻¹ and 1.0 g L⁻¹ decreased the fresh mass under control conditions compared to distilled water (Figure 4.5). Unlike this study, foliar application of BR increased

fresh mass of dry bean (*Phaseolus vulgaris* L.) and groundnut (*Arachis hypogaea* L.) seedlings compared to the control plants (Mota *et al.*, 2024). Nevertheless, the authors noted that the application of Cytolan® Stress increased the fresh mass and restored the plant growth to the same levels as the control plants.

Leaf dry mass

The mild, moderate, and severe stress conditions reduced the dry mass by 48.39%, 72.32% and 75.75% respectively, compared to the control on treatment (Table 4.1). In contrast to the current study, other studies reported an increase in dry mass after foliar application of biostimulants containing phytohormones, such as Kelpstar® in basil and Cytolan® Stress in lettuce, under abiotic and optimal growth conditions (Consentino *et al.*, 2023; Velasco-Clares *et al.*, 2024).

In the present study, the interaction between multistress and *Lucky Plant*® biostimulant treatments had no significant ($p < 0.05$) effect on the dry mass of leaves (Figure 4.6). However, a dose of 1.0 and 0.5 g L⁻¹ decreased the dry mass of leaves under control growing conditions. These results are corroborated by Giordano *et al.* (2022), who reported that with foliar spraying of Auxym®, a biostimulant containing CK traces, the dry matter content of green leafy lettuce decreased by 6.3% following treatment. The foliar application of *Lucky Plant*® was not evident on the dry mass of mild, moderate, and severely stressed plants.

4.3.2 The response of physiological parameters of curly lettuce to multistress and biostimulant

Chlorophyll content

The chlorophyll content was measured to monitor the physiological response of curly lettuce plants to the effects of multistress and foliar application of a phytohormone-based biostimulant. The statistical analysis results show that the capacity of stressed plants to attract sunlight and perform the photosynthesis process was affected by the multistress treatments, mild, moderate, and severe, which significantly decreased the chlorophyll content, compared to the control, at harvest (Table 4.1). Mild, moderate, and severe treatments at harvest reduced chlorophyll by 14.78%, 30.22%, and 32.35%, respectively, compared to the control treatment. Figure 4.4 shows that there was no significant difference observed in mild and severe conditions after the foliar application of *Lucky Plant*®.

At harvest, chlorophyll content showed a significant interaction ($p < 0.05$) between multistress and the *Lucky Plant*[®] biostimulant (Table 4.1). This interaction revealed that chlorophyll content significantly increased in plants grown under control conditions when *Lucky Plant*[®] was applied at a dose of 1.5 g L^{-1} compared to distilled water (Figure 4.4). Similar to this study, Di Mola *et al.* (2019) and Ngoroyemoto *et al.* (2020) reported that treating baby lettuce and *Amaranthus hybridus* with Kelpak[®] effectively increased the chlorophyllous pigments, including the chlorophyll content. The Kelpak[®] contains very high cytokinin content, known to positively affect the regulation of chlorophyll metabolism (Di Mola *et al.*, 2019; Roy, 2024). This shows that the phytohormone-based biostimulant stimulates an increase in the chlorophyll content of leaves. The increment of chlorophyll content is vital for the leaf colour of leafy vegetables because greenness is an important feature that attracts consumers. The highest total chlorophyll content was observed with foliar application of the BR to legume plants (Mota *et al.*, 2024).

Lucky Plant[®] induced a negligible increase in chlorophyll content in plants grown under moderate abiotic stress conditions, treated with a 1.0 g L^{-1} dose compared to distilled water (Figure 4.4). These results are in accordance with previous results on chicory, in which the chlorophyll content was reduced by exposure to abiotic stress, but the application of a biostimulant with a phytohormone restored and improved its concentration (Sabatino *et al.*, 2023). Similarly, Melo *et al.* (2024) reported that abiotic stress reduced the chlorophyll content in soybean plants. However, the foliar application of Megafol[®] biostimulant containing traces of gibberellin and cytokine increased the chlorophyll content of treated plants to values similar to the control plants. Moreover, applying a phytohormone-based biostimulant increased the total chlorophyll content of lettuce plants grown under abiotic stress compared to other stressed plants not treated (Velasco-Clares *et al.*, 2025).

Stomatal density and stomatal conductance (Gs)

The effects of the foliar application of *Lucky Plant*[®] on multistress plant physiological responses were also examined by the performance of two indicators of the gas exchange process, i.e., stomatal density and stomatal conductance (Table 4.2). Numerous studies documented that abiotic stress alters stomatal density, index, size, and shape and that stomatal density is one of the crucial features of the stomata serving as a reliable indicator of abiotic

stress tolerance in various plant species (Hasanuzzaman *et al.*, 2023; Vinarao *et al.*, 2024; Kaya, 2025), due to their capability to regulate photosynthetic activities including CO₂ and loss of water through transpiration.

The stomatal density of curly lettuce leaves in response to both multistress treatments was significantly affected ($p < 0.05$). The stomatal density of leaves was significantly reduced by mild and moderate treatments by 15.36% and 15.20%, respectively, compared with the control treatment (Table 4.2). These results are corroborated by Vinarao *et al.* (2024) who found that some pea genotypes had reduced stomatal density on both adaxial and abaxial leaf surfaces under water deficit stress. The decrease in the stomatal density under abiotic stress may reduce water loss by transpiration, therefore conserving water within the plant and demonstrating enhanced plant tolerance to stress (Hasanuzzaman *et al.*, 2023).

The interaction between the multistress and foliar application of *Lucky Plant*[®] biostimulant on stomatal density was significantly affected ($p < 0.05$), as shown in Figure 4.9 A-D. The highest number of stomata was 40.33, found in plants treated with a 1.0 g L⁻¹ dose under control conditions (Table 4.2). As shown in Figures 4.7 C and 4.9 C, there was a larger opening of the stomata on leaves that received a 1.0 g L⁻¹ dose of *Lucky Plant*[®] biostimulant. The high number of stomata openings facilitates the influx of CO₂ into the plants for photosynthesis (Kaya, 2025). Interestingly, *Lucky Plant*[®] remarkably increased the number of opening stomata by 56.52% and 57.00% in severely stressed plants at a dose of 1.0 g L⁻¹ and 1.5 g L⁻¹, respectively, compared to the distilled water (Table 4.2). This phenomenon can be explained as the elevated balance between the necessary uptake of CO₂ and the water preservation strategies of plants (Hasanuzzaman *et al.*, 2023). Thus, *Lucky Plant*[®] eventually improves plants' ability to withstand severe stress.

No significant difference was observed under mild and moderate conditions for the stomatal density after the foliar application of *Lucky Plant*[®] at any dose relative to distilled water. Since Jamal Omid *et al.* (2018) reported that trichomes serve as a barrier against water deficit stress in lemon balm (*Melissa officinalis* L.), Figure 4.8 illustrates how the mild and moderate stress levels displayed trichomes, presenting a difference in their resilience to stress as shown by the increase in length of leaf surface trichomes (Sedibe *et al.*, 2013; Khetsha *et al.*, 2022).

Stomatal conductance plays a major role under stress conditions as a crucial parameter for expressing plant-water relations (Naveen *et al.*, 2021). Stomatal conductance was monitored 35, 36, and 37 days after transplant (DAT) before the harvest date (Table 4.2) to show the amount of water vapour and CO₂ exchange between the leaves and the growing environment. The interaction between multistress and *Lucky Plant*[®] biostimulant significantly affected ($p<0.05$) the stomatal conductance based on the three days of measurement. Foliar application of *Lucky Plant*[®] at a dose of 1.5 g L⁻¹ produced positive effects under control treatment by increasing the stomatal conductance compared with the control plants in 35, 36, and 37 DAT. Several studies also reported that exogenously applied phytohormone-based biostimulants elevated the stomatal conductance of sweet basil, maize seedlings, and dry beans under normal growing conditions (Consentino *et al.*, 2023; Tandathu *et al.*, 2024; Mota *et al.*, 2024). Remarkably, Naveen *et al.* (2021) reported that foliar application of BRs at high doses significantly increased the stomatal conductance in mustard (*Brassica juncea* L.) under stressed conditions. Moreover, the stomatal conductance declined by mild, moderate, and severe effects and was not reversed, irrespective of the foliar application of *Lucky Plant*[®] with varying doses compared to control conditions.

4.3.3 The response of biochemical parameters to multistress and biostimulant

Leaf mineral composition

The leaf mineral content of curly lettuce was determined at harvest. Table 4.3 displays the effect of the studied treatments on leaf macro and micro mineral composition (%). The N, P, Mg, K, Ca, Na, Zn, and Mn concentrations were significantly affected ($p<0.05$) by the multistress conditions. The mild, moderate, and severe multistress treatments exhibited a significant decrease in N, P, Mg, K, Ca, Zn, and Mn concentration compared with control conditions in. While Na became highly concentrated or increased under moderate, severe, and mild conditions, respectively, compared to the control. The K and Cu concentrations of curly lettuce leaves significantly decreased when plants were treated with *Lucky Plant*[®] compared to distilled water.

The N content plays an essential role in determining the plant quality. Figure 4.10 shows that the application of *Lucky Plant*[®] biostimulant at a dose of 1.5 g L⁻¹ significantly increased N content under mild conditions, compared with distilled water. These results confirm the findings presented by Sabatino *et al.* (2023) that the foliar application of a biostimulant

containing phytohormones can recover and promote nitrogen use efficiency by approximately 7.7% after it is reduced under stress. Likewise, Giordano *et al.* (2022) depicted that the application of Auxym[®] biostimulants improved the total N content in green lettuce leaves.

The microminerals Fe, Cu and Zn showed no significant differences among treatments. Foliar application of *Lucky Plant*[®] affected Z content under control, mild, moderate, and severe conditions, but no significant differences were observed among the varying doses and distilled water. Therefore, there were no significant differences between the multistress and *Lucky Plant*[®] biostimulant on the concentration of Z (Figure 4.11).

For an overview of the data, Figure 4.12 reports a biplot from the PCA performed to find the most important variations among analysed leaf mineral concentrations of curly lettuce in response to multistress conditions and varying doses of *Lucky Plant*[®] biostimulants. On the biplot PCA, the first principal component (F1) produced the most variance of approximately 70.85%, and the second principal component (F2) accounted for just 13.40%. Overall, variables from the two biplots presented a total variance of 84.25%. PCA revealed that a group of strongly and positively correlated variables included Mn, Mg, P, K, and Ca. These leaf mineral parameters on F1 were associated with improving concentrations at a dose of 0.5 g L⁻¹ on plants grown under control treatments but with little difference relative to distilled water (control). The results of this study are in agreement not only with previous findings on *Amaranthus hybridus* and green lettuce leaves grown under optimal conditions (Ngoroyemoto *et al.*, 2020; Giordano *et al.*, 2022) but also with the findings on chicory plants under water deficit stress (Sabatino *et al.*, 2023) which presented a significant increase in Mg, P, K, and Ca concentrations and enhanced their intracellular bioavailability following the foliar application of Kelpak[®], Auxym[®] and Ecklonia maxima with traces of phytohormones.

Additionally, Zn correlates with N located on the positive side of F1 and is strongly promoted by the foliar-applied *Lucky Plant*[®] at 1.0 g L⁻¹ and 1.5 g L⁻¹ doses in plants grown under control and mild conditions. Contrarily, Na and Cu contents were situated opposite the positive side, exhibiting a negative, better described by the F2 biplot. Furthermore, Na increased with increased multistress treatments in plants treated only with distilled water. In summary, these PCA results are consistent with the ANOVA results obtained using Tukey's LSD at $p < 0.05$. The practical mechanism of a biostimulant containing a phytohormone is to increase the capacity of nutrients to sustain the intercellular ion balance required for the biosynthesis of compounds related to osmosis and contribute to sufficient plant water content

for metabolism (El-Hameda *et al.*, 2024). Conclusively, the use of *Lucky Plant*[®] demonstrated a good contribution to the uptake of nutrients and may reduce the application amount of essential synthetic fertilisers for lettuce cultivation.

Phenolic content

It is well known that abiotic factors, such as wounding and moisture stress, can enhance secondary metabolites (including phenolics) crucial in protecting plant cells against stresses in diverse species (Khetsha *et al.*, 2024). In this study, for a comprehensive analysis of phenolic accumulation, a multivariate analysis was performed considering the multistress conditions (control, mild, moderate, and severe) and *Lucky Plant*[®] (distilled water, 0.5 g L⁻¹, 1.0 g L⁻¹, and 1.5 g L⁻¹) as two main factors on leaf samples obtained at harvest. Table 4.4 shows 43 phenolic compounds detected by the LC-MS untargeted metabolomics method in curly lettuce samples. The PCA was performed to eliminate insignificant differences and summarise the variation in the data by pinpointing important correlations between the combined multi-abiotic stresses and varying doses of *Lucky Plant*[®] biostimulant in relation to changes in phenolic compound accumulation. The two components covered a total variability of 75.8%, with PC1 accounting for 52.4% of the total variation and PC2 providing 23.4% (Figure 4.13). The score plot shows a separation between samples treated with *Lucky Plant*[®] under control conditions and those that only received distilled water under mild, moderate, and severe conditions. Irrespective of the *Lucky Plant*[®] dose, the control (R2_Con_Bio or R1_Con_Bio or R4_Con_Bio) samples tended to be differentiated on the positive side of the PC1 axis. These results suggest that the secondary defence mechanisms were not triggered in plants grown under control conditions. Therefore, the compounds were maintained by the foliar application of the *Lucky Plant*[®] biostimulant. These results partially align with a recent study by Velasco-Clares *et al.* (2025), who also observed that abiotic stress increased the levels of total phenols more than control plants. Nevertheless, plants that received Cytolan[®] Stress biostimulant through priming and buffering had the highest increment of total phenols, while those treated by detoxifying exhibited similar levels to control plants. Contrarily, samples of plants grown under multistress conditions and not treated with the biostimulant (R1_Mil_Bio, R1_Mod_Bio, and R1_Sev_Bio) were dispersed more on the negative side of the PC1 plot and possessed a heterogeneity in comparison to other samples treated with varying doses of *Lucky Plant*[®] but grown under the same conditions. The stimulation effect of *Lucky Plant*[®] biostimulant observed in the current study is in line with a previous study by Consentino *et al.* (2023), who also

pointed out that biostimulants with traces of phytohormones increased phenolic content in sweet basil plants compared to the control plants.

In addition, the heat map analysis represented the insightful selective compounds and identified two major groups clustered into five distinct clusters attributed to different multistress conditions and doses of *Lucky Plant*[®] (Figure 4.15). Samples of the same group are clustered closely, denoting the strong reliability of the identified compounds. For example, on the first bottom cluster, samples of R2_Mod_Bio and R2_Sev_Bio positively correlated with R3_Mil_Bio, R3_Mod_Bio, R3_Sev_Bio, and R4_Sev_Bio on stachyose and verbascose and three other unknown compounds and showed inconsistency with control conditions treated with distilled water, 0.5 g L⁻¹ and 1.5 g L⁻¹. Additionally, severe treatment with 1.5 g L⁻¹ showed similarities with control treatment, suggesting the beneficial action of the biostimulant at this dose. The second cluster revealed that all multistress treatments treated with distilled water and control, irrespective of the biostimulant dose, showed low contents of chicoric acid, quercetin-malonyl-glucoside, and quercetin-O-acetyl-glucoside in comparison with other treatments in this cluster.

Remarkably, samples from severe conditions treated with 1.5 g L⁻¹ dose accumulated high amounts of quercetin-malonyl-glucoside. In the latter group, the second cluster demonstrated a clear pattern attributed to samples of control conditions because of high gentisic acid 5-O-glucoside, kaempferol, caffeoylmalic acid, trifolin, quercetin 3-glucosyl-(1->2)-galactoside, and citroside A, regardless of the biostimulant dose. Contrarily, samples of mild, moderate, and severe stress conditions resulted in low levels of similar compounds corresponding to the foliar application of *Lucky Plant*[®] at varying doses. Francisco *et al.* (2023) reported a significant correlation between kaempferol, and the fresh weight of brassica genotypes generated by mechanical wounding stress, which could perhaps have contributed to the reduction of curly lettuce fresh weight under multi-abiotic stress conditions in the current study. On the contrary, Giordano *et al.* (2022) reported a high concentration of kaempferol in lettuce grown under optimal growth conditions and supplied with a phytohormone-based biostimulant. The *Lucky Plant*[®] biostimulant significantly resulted in the discrepancy between chlorogenic and caftaric acids. Compared to control plants, a phytohormone-based biostimulant improved the contents of total chlorogenic acid and caffeic acid phenols in lettuce leaves (Giordano *et al.*, 2022). It can be seen on the last cluster that samples of severe conditions treated with 1.5 g L⁻¹ dose show high concentrations of quercetin 3-O-glucuronide

followed by quercetin 3-galactoside. Such results agree with those of other authors, such as Giordano *et al.* (2022), who also applied biostimulants containing phytohormones to lettuce which promoted the total flavonoid concentrations such as quercetin-3-*O*-glucuronide and quercetin-3-*O*-glucoside. A study by Francisco *et al.* (2023) further highlighted that the flavonoids derived from quercetin presented the most plant biomass variability under abiotic stress. The biostimulant composed of phytohormones enhanced the phenolic contents in lettuce under abiotic stress (Di Mola *et al.*, 2019).

Apart from unsupervised methods, the supervised PLS-DA classification model was applied to further differentiate the interactive effects of multistress and *Lucky Plant*[®] biostimulants and select significant variables in the curly lettuce phenolics (Figure 4.14). On the PLS-DA score plot, the first two PCs disclosed a total variance similar to that of the PCA score plot. In agreement with the PCA, observations on the PLS-DA illustrated that the application of *Lucky Plant*[®] resulted in the accumulation of distinct phenolic compounds under mild, moderate, and severe conditions, indicating a significant alteration in the phenolic compounds of curly lettuce under multistress due to the foliar application of *Lucky Plant*[®]. Additionally, these samples were distinctly separated from mild, moderate, and severe treatments applied with distilled water, demonstrating a considerable level of generated oxidative stress in control plants.

Moreover, a supervised OPLS-DA model was used to illustrate only biomarkers of phenolic compounds responsible for variation among varying doses of *Lucky Plant*[®] and multistress conditions. The OPLS-DA score plot highlighted two main discriminators, as shown in Figure 4.16. On the OPLS-DA, the T score (Component 1) defines 49% of the variation, and the orthogonal T score (Component 2) accounts for 19.4%. Based on the criterion of the variable importance in projection (VIP) score, phenolic compounds demonstrated the differential distribution on the OPLS-DA model. Phenolic compounds are considered statistically significant with a VIP score >1. As evidenced by the VIP score plot (Figure 4.17), 15 phenolic compounds were significantly upregulated and/or downregulated in response to identified variables displayed on OPLS-DA, including kaempferol, 3-*O*-*p*-Coumaroylquinic acid, citroside A, verbascose, stachyose, gentisic acid 5-*O*-glucoside, and other unknown compounds. For example, the foliar application of 1.5 g L⁻¹ induced a higher amount of unknown phenolic compounds with *m/z*: 922.29 under severe stress (R4_Sev). A dose of 1.5 g L⁻¹ under moderate (R4_Mod) stress conditions is associated with the highest accumulation of

unknown phenolic compounds with m/z : 504.09 and 3-O-p-Coumaroylquinic acid. Likewise, in lettuce, the foliar application of a phytohormone-based biostimulant enhanced the coumaroyl quinic acid isomer content (Giordano *et al.*, 2022). The 3-O-p-Coumaroylquinic acid was associated with mechanical wounding stress (Francisco *et al.*, 2023). Under mild conditions, a dose of 1.0 g L^{-1} (R3_Mil) is characterised by a reduction of gentisic acid 5-O-glucoside and 0.5 g L^{-1} (R2_Mil) with high verbascose concentration.

Figure 4.18 shows a mass spectrum generated to explore the peak intensity of the detected phenolic compounds. A compound observed at a high-intensity base peak is ganoderic acid H at $571.28 \text{ } m/z$ (Table 4.4), an important triterpenoid acid involved in plant wound healing. In addition, the mass peak from the m/z 315.0486 likely characterises the traces of gentisic acid 5-O-glucoside (m/z 315.07233). Some detected ions presented in the mass spectrum were identified as unknown. The total chromatograms of phenolic compound data of curly lettuce extract that were not treated with foliar application of *Lucky Plant*[®] biostimulant under multi-biotic stress conditions are shown in Figure 4.19. Furthermore, the chromatograms display distinct differences from the control, while the mild, moderate, and severe profiles show near-similar attributes regarding detected compounds' intensities (Table 4.4). Therefore, it can be concluded from these chromatograms of control treatments and the findings mentioned above on foliar application of *Lucky Plant*[®] that the oxidative stress generated as a secondary effect of the multi-abiotic stress treatments was reduced by the hormonal composition present in the biostimulant, likely improving the antioxidant defence mechanisms of curly lettuce.

4.4 CONCLUSION

The study highlights the role of a phytohormone-based biostimulant, particularly *Lucky Plant*[®], as a promising tool in mitigating the negative effects of coinciding multi-abiotic stress factors. Multistress significantly reduced yield and inhibited curly lettuce plants' morphophysiological and biochemical parameters, with the most reduction under moderate and severe conditions. The foliar application of *Lucky Plant*[®] biostimulant had the highest effect on the curly lettuce's analysed parameters at 1.0 g L^{-1} and 1.5 g L^{-1} doses. The response of plants to *Lucky Plant*[®] biostimulant depended on the application dose and intensity of multistress conditions. This study, therefore, concludes that the foliar application of *Lucky Plant*[®] biostimulant on lettuce is highly recommended for improving growth and yield under optimal conditions. It can also be used under mild, moderate, and severe conditions to mitigate

the negative effects on morphophysiology and biochemical parameters of stressed plants and promote yield. Future studies should investigate the beneficial effects of *Lucky Plant*[®] biostimulant from the beginning of the stress imposition period throughout the vegetative season. Moreover, further research studies may be important for better understanding the recovery response mechanisms in the open-field production of lettuce.

REFERENCES

- Al-Khayri JM, Rashmi R, Toppo V, Chole PB, Banadka A, Sudheer WN, Nagella P, Shehata WF, Al-Mssallem MQ, Alessa FM, Almaghasla MI. 2023. Plant secondary metabolites: The weapons for biotic stress management. *Metabolites* 13: 716.
- AL-Quraan NA, Al-Ajlouni ZI, Qawasma NF. 2021. Physiological and biochemical characterization of the GABA shunt pathway in pea (*Pisum sativum* L.) seedlings under drought stress. *Horticulturae* 7: 125.
- Aslani Z, Hassani A, Mandoulakani BA, Barin M, Maleki R. 2023. Effect of drought stress and inoculation treatments on nutrient uptake, essential oil and expression of genes related to monoterpenes in sage (*Salvia officinalis*). *Scientia Horticulturae* 309: 111610.
- Baharudin NF, Osman NI. 2023. Plant development, stress responses and secondary metabolism under ethylene regulation. *Plant Stress* 7: 100146.
- Consentino BB, Vultaggio L, Sabatino L, Ntatsi G, Rouphael Y, Bondi C, De Pasquale C, Guarino V, Iacuzzi N, Capodici G, Mauro RP. 2023. Combined effects of biostimulants, N level and drought stress on yield, quality and physiology of greenhouse-grown basil. *Plant Stress* 10: 100268.
- Di Mola I, Cozzolino E, Ottaiano L, Giordano M, Rouphael Y, Colla G, Mori M. 2019. Effect of vegetal-and seaweed extract-based biostimulants on agronomical and leaf quality traits of plastic tunnel-grown baby lettuce under four regimes of nitrogen fertilization. *Agronomy* 9: 571.
- El-Hameda A, Hemida K, Abbas H, Kamel R, Rady MM. 2024. Enhancing water limitation tolerance of tomato plants grown under water-limited irrigation regime by maize grain embryos extract enriched with some bio-stimulant (MEEst). *Labyrinth: Fayoum Journal of Science and Interdisciplinary Studies* 2: 22-34.
- Francisco M, Doghri M, Rodríguez VM. 2023. Time of day of leaf wounding determines plant biomass and affects the interplay between growth and defence in Brassica crops. *Plant Biology* 25: 785-792.
- Gerhards R, Ouidoh FN, Adjogboto A, Avohou VAP, Dossounon BLS, Adisso AKD, Heyn A, Messelhäuser M, Santel HJ, Oebel H. 2021. Crop response to leaf and seed applications of the biostimulant ComCat® under stress conditions. *Agronomy* 11: 1161.

- Giordano M, El-Nakhel C, Carillo P, Colla G, Graziani G, Di Mola I, Mori M, Kyriacou MC, Roupheal Y, Soteriou GA, Sabatino L. 2022. Plant-derived biostimulants differentially modulate primary and secondary metabolites and improve the yield potential of red and green lettuce cultivars. *Agronomy* 12: 1361.
- Guan W, Edwards MG, Gatehouse JA, Gatehouse AM. 2022. Responses of wheat (*triticum aestivum*) to grain aphid (*sitobion avenae*) infestation and mechanical wounding using a cDNA subtractive library approach. *Agricultural Sciences* 13: 715-740.
- Hai NN, Chuong NN, Tu NHC, Kisiala A, Hoang XLT, Thao NP. 2020. Role and regulation of cytokinins in plant response to drought stress. *Plants* 9: 422.
- Hasanuzzaman M, Zhou M, Shabala S. 2023. How does stomatal density and residual transpiration contribute to osmotic stress tolerance?. *Plants* 12: 494.
- Hossain A, Pamanick B, Venugopalan VK, Ibrahimova U, Rahman MA, Siyal AL, Maitra S, Chatterjee S, Aftab T. 2022. Emerging roles of plant growth regulators for plants adaptation to abiotic stress–induced oxidative stress. In *Emerging Plant Growth Regulators in Agriculture* 1-72.
- Isa MM, Kasim KF, Muttalib MFA, Jaafar MN. 2021. Rapid and non-destructive leaf chlorophyll estimation of Fig (*Ficus carica* L.) cv. Iraqi grown on different root zone spatial limitation and controlled porosity level under greenhouse condition. In *IOP Conference Series: Earth and Environmental Science* 765: 012076.
- Jahan MS, Hasan MM, Rahman MA. 2024. Hormones and biostimulants in plants: physiological and molecular insights on plant stress responses. *Frontiers in Plant Science* 15: 1413659.
- Jamal Omid F, Mohajjel Shoja H, Sariri R. 2018. Effect of water-deficit stress on secondary metabolites of *Melissa officinalis* L.: role of exogenous salicylic acid. *Caspian Journal of Environmental Sciences* 16: 121-134.
- Jogawat A, Yadav B, Lakra N, Singh AK, Narayan OP. 2021. Crosstalk between phytohormones and secondary metabolites in the drought stress tolerance of crop plants: a review. *Physiologia Plantarum* 172: 1106-1132.
- Jogawat A, Yadav B, Lakra N, Singh AK, Narayan OP. 2021. Crosstalk between phytohormones and secondary metabolites in the drought stress tolerance of crop plants: A review. *Physiologia Plantarum* 172: 1106-1132.

- Kaya G. 2025. A plant-derived biostimulant Aminolom Enzimatico® application stimulates chlorophyll content, electrolyte leakage, stomata density and root yield of radishes under salinity stress. *PeerJ* 13: e18804.
- Khetsha Z. 2024. Using cytokinin to enhance essential oil biosynthesis of two rose geranium (*pelargonium graveolens* L.) varieties: reunion and Madagascar-type. *Scientific Papers. Series B. Horticulture* 68: 786-805.
- Khetsha ZP, Sedibe MM, Pretorius RJ, Van Der Watt E. 2023. Biostimulants improve the leaf micro-morphology and essential oil biosynthesis of simulated hail-damaged *Pelargonium graveolens* (L'Hér.). *Acta Horticulturae* 1372: 283–294.
- Kumar S, Bhushan B, Wakchaure GC, Meena KK, Kumar, M, Meena NL, Rane, J. 2020. Plant phenolics under water-deficit conditions: biosynthesis, accumulation, and physiological roles in water stress alleviation. In *Plant Phenolics in Sustainable Agriculture* 1: 451-465.
- Lu J, Shao G, Gao Y, Zhang K, Wei Q, Cheng J. 2021. Effects of water deficit combined with soil texture, soil bulk density and tomato variety on tomato fruit quality: A meta-analysis. *Agricultural Water Management* 243: 106427.
- Melo GB, da Silva AG, da Costa AC, Alves da Silva A, Rosa M, Bessa, LA, Rodrigues CR, Castoldi G, Vitorino LC. 2024. Foliar application of biostimulant mitigates water stress effects on soybean. *Agronomy* 14: 414.
- Mota MM, Van der Watt E, Khetsha ZP. 2024. Foliar application of brassinosteroids improves the yield and morpho-physiological characteristics of *Arachis hypogaea* L., *Glycine max* (L.), and *Phaseolus vulgaris* L. *Applied Ecology and Environmental Research* 22: 355–371.
- Naveen N, Kumari N, Avtar R, Jattan M, Ahlawat S, Rani B, Malik K, Sharma A, Singh M. 2021. Evaluation of effect of brassinolide in Brassica juncea leaves under drought stress in field conditions. *Horticulturae* 7: 514.
- Ngoroyemoto N, Kulkarni MG, Stirk WA, Gupta S, Finnie JF, van Staden J. 2020. Interactions between microorganisms and a seaweed-derived biostimulant on the growth and biochemical composition of *Amaranthus hybridus* L. *Natural Product Communications* 15: 1934578X20934228.

- Roy A. 2024. Plant growth regulating phytohormones: The potent targets to alleviate abiotic stress in crop plants—a research update and the way forward. *Journal of Agricultural Science* 16: 10.
- Sabatino L, Consentino BB, Rouphael Y, Baldassano S, De Pasquale C, Ntatsi G. 2023. Ecklonia maxima-derivate seaweed extract supply as mitigation strategy to alleviate drought stress in chicory plants. *Scientia Horticulturae* 312: 111856.
- Sabina S, Jithesh MN. 2021. Mechanical wounding of leaf midrib and lamina elicits differential biochemical response and mitigates salinity induced damage in tomato. *Journal of Applied Horticulture* 23: 3-10.
- Sahu S, Asati BS, Singh J, Kumar N. 2024. Performance of tomato variety Kashi Aman under different growing containers. *International Journal of Advanced Biochemistry Research* 8: 519–522.
- Sedibe MM, Allemann J. 2013. The response of rose geranium (*Pelargonium graveolens* L.) to nitrate and ammonium application. *Acta Horticulturae* 1007: 311–322.
- Sehgal A, Reddy KR, Walne CH, Barickman TC, Brazel S, Chastain D, Gao W. 2022. Individual and interactive effects of multiple abiotic stress treatments on early-season growth and development of two brassica species. *Agriculture* 12: 453.
- Tandathu T, Kotzé E, Van Der Watt E, Khetsha ZP. 2024. Effect of biostimulants and glyphosate on morphophysiological parameters of *Zea mays* (L.) seedlings under controlled conditions. *Agronomy* 14: 2396.
- Turner NC. 2019. Imposing and maintaining soil water deficits in drought studies in pots. *Plant and Soil* 439: 45-55.
- Velasco-Clares D, Navarro-León E, Izquierdo-Ramos MJ, Blasco B, Ruiz JM. 2025. Enhancing drought tolerance in lettuce: the efficacy of the seaweed-derived biostimulant Cytolan® stress applied at different growth stages. *Horticulturae* 11: 157.
- Velasco-Clares D, Navarro-León E, Atero-Calvo S, Ruiz JM, Blasco B. 2024. Is the application of bioactive anti-stress substances with a seaweed-derived biostimulant effective under adequate growth conditions?. *Physiologia Plantarum* 176: e14193.
- Vinarao GB, Ghimire K, Harris DK. 2024. Pilot evaluation of field pea accessions under water deficit conditions. *International Journal of Plant Biology* 15: 1162-1175.

- Wielkopolan B, Frąckowiak P, Wieczorek P, Obrępańska-Stęplowska A. 2022. The Impact of *Oulema melanopus*—Associated bacteria on the wheat defense response to the feeding of their insect hosts. *Cells* 11: 2342.
- Yordanova M, Filipov N. 2023. Effect of the seaweed biostimulant Kelpak® on the growth of cucumber. *Scientific Papers. Series B. Horticulture* 67: 708-713.



Table 4.1: Effect of multistress and foliar application of phytohormone-based biostimulant (*Eco-Stim*[®]) treatments on plant parameters at harvest of curly lettuce.

Treatment	Plant height (plant ⁻¹)	Number of leaves (plant ⁻¹)	Chlorophyll %	Leaf area (cm ²)	Fresh mass (g)	Dry mass (g)
Multistress						
Control	20.68 ± 1.23 ^a	20.98 ± 0.75 ^a	7.51 ± 0.75 ^a	932.90 ± 142.89 ^a	818.50 ± 131.72 ^a	37.94 ± 4.70 ^a
Mild	13.09 ± 0.56 ^b	19.13 ± 0.74 ^b	6.40 ± 0.71 ^b	372.12 ± 40.38 ^b	253.00 ± 21.65 ^b	19.58 ± 0.69 ^b
Moderate	10.05 ± 0.67 ^c	15.44 ± 0.54 ^c	5.24 ± 0.49 ^c	217.26 ± 48.22 ^c	144.33 ± 27.41 ^c	10.50 ± 1.85 ^c
Severe	9.78 ± 0.61 ^c	14.61 ± 0.72 ^d	5.08 ± 0.47 ^c	225.84 ± 50.44 ^c	127.08 ± 18.64 ^c	9.20 ± 1.21 ^c
F-value	453.28	369.67	73.28	197.90	821.50	851.14
P-value	*0.0001	*0.0001	*0.0001	*0.0001	*0.0001	*0.0001
LSD	0.68	0.45	0.37	69.42	31.43	1.32
Biostimulant						
Distilled water	13.40 ± 4.42 ^a	17.33 ± 2.84 ^b	6.20 ± 1.08 ^a	456.15 ± 329.84 ^a	315.00 ± 302.56 ^b	20.36 ± 13.54 ^a
0.5 g L ⁻¹	13.29 ± 4.59 ^a	17.95 ± 3.04 ^a	5.96 ± 1.00 ^{ab}	407.37 ± 314.30 ^a	327.08 ± 285.19 ^{ab}	17.94 ± 11.27 ^b
1.0 g L ⁻¹	13.41 ± 4.68 ^a	17.41 ± 2.79 ^b	6.23 ± 0.79 ^a	447.98 ± 286.23 ^a	256.54 ± 194.99 ^c	18.48 ± 10.34 ^b
1.5 g L ⁻¹	13.51 ± 4.95 ^a	17.47 ± 2.52 ^b	5.68 ± 1.65 ^b	436.63 ± 331.82 ^a	355.50 ± 340.92 ^a	20.50 ± 14.05 ^a
F-value	4.14	3.19	3.90	0.79	14.01	8.533
P-value	0.93	*0.0378	*0.0186	0.51	*0.0001	*0.0003
LSD	0.68	0.45	0.37	69.42	31.36	1.32
Multi × Biosti						
F-value	0.54	3.55	5.07	0.47	6.95	5.13
P-value	0.83	*0.0042	*0.0004	0.88	*0.0001	*0.0003
LSD	1.37	0.90	0.72	138.84	63.2	2.66

Multi × Biosti = Multistress × Biostimulant. Data are means and (±standard errors). Means followed by the same letter within the column for each section are not significant $p > 0.05$. Mean with different letters indicates statistically significant differences at $p < 0.05$. Bold mark and asterisk * indicate $p < 0.05$ (Tukey's LSD).

Table 4.2: Effect of multistress and foliar application of phytohormone-based biostimulant (*Lucky Plant*[®]) treatments on the stomatal density and stomatal conductance of curly lettuce.

Treatment	Stomatal density	Stomatal conductance (mmol m ⁻² s ⁻¹)		
		35 DAT	36 DAT	37 DAT
Multistress				
Control	24.66 ± 10.19 ^a	686.18 ± 118.94 ^a	688.30 ± 60.78 ^a	676.79 ± 91.83 ^a
Mild	20.87 ± 3.87 ^b	426.42 ± 86.29 ^b	424.06 ± 59.87 ^b	388.43 ± 71.27 ^b
Moderate	20.91 ± 3.91 ^b	285.21 ± 74.40 ^c	274.14 ± 46.98 ^c	275.07 ± 74.75 ^c
Severe	22.75 ± 8.82 ^{ab}	225.80 ± 28.27 ^d	200.01 ± 30.82 ^d	206.56 ± 39.50 ^d
F-value	11.36	288.19	391.69	235.08
P-value	*0.0001	*0.0001	*0.0001	*0.0001
LSD	1.98	34.72	307.03	39.07
Biostimulant				
Distilled water	21.00 ± 5.13 ^b	453.78 ± 188.81 ^a	412.13 ± 186.47 ^a	437.58 ± 187.04 ^a
0.5 g L ⁻¹	17.66 ± 2.27 ^c	403.21 ± 210.92 ^b	392.10 ± 182.97 ^a	394.76 ± 178.88 ^b
1.0 g L ⁻¹	27.25 ± 9.72 ^a	318.64 ± 132.55 ^c	358.20 ± 204.71 ^b	329.90 ± 174.51 ^c
1.5 g L ⁻¹	22.63 ± 6.29 ^b	416.31 ± 233.35 ^b	396.55 ± 215.46 ^a	384.62 ± 240.00 ^b
F-value	33.80	20.94	5.08	10.72
P-value	*0.0001	*0.0001	*0.006	*0.0001
LSD	1.97	34.72	30.70	39.07
Multi × Biosti				
Control × DiWater	22.00 ± 1.00 ^{cd}	704.91 ± 34.58 ^b	675.65 ± 47.02 ^{ab}	701.62 ± 51.07 ^{ab}
Control × 0.5 g L ⁻¹	14.00 ± 2.00 ^{fg}	698.96 ± 113.16 ^b	638.51 ± 75.18 ^b	669.97 ± 65.70 ^b
Control × 1.0 g L ⁻¹	40.33 ± 2.08 ^a	510.05 ± 61.77 ^c	716.56 ± 24.69 ^a	580.50 ± 182.24 ^c
Control × 1.5 g L ⁻¹	22.33 ± 2.08 ^{cd}	776.06 ± 104.57 ^a	731.92 ± 51.89 ^a	755.08 ± 20.54 ^a
Mild × DiWater	24.33 ± 0.04 ^c	529.89 ± 21.85 ^c	459.86 ± 11.47 ^c	481.23 ± 13.86 ^d
Mild × 0.5 g L ⁻¹	17.16 ± 0.53 ^{ef}	422.58 ± 52.87 ^d	461.57 ± 23.07 ^c	383.58 ± 21.75 ^e
Mild × 1.0 g L ⁻¹	17.66 ± 0.57 ^{ef}	394.42 ± 58.68 ^{de}	417.39 ± 56.35 ^{cd}	338.41 ± 43.05 ^{efg}
Mild × 1.5 g L ⁻¹	20.50 ± 2.12 ^{de}	357.51 ± 84.77 ^{de}	357.41 ± 71.49 ^{de}	350.52 ± 80.99 ^{ef}
Moderate × DiWater	24.43 ± 1.52 ^c	330.36 ± 72.95 ^{ef}	306.47 ± 9.17 ^{ef}	334.00 ± 58.01 ^{efg}
Moderate × 0.5 g L ⁻¹	23.00 ± 1.00 ^{cd}	260.94 ± 71.74 ^{fg}	250.95 ± 59.22 ^{gf}	285.41 ± 40.29 ^{fgh}
Moderate × 1.0 g L ⁻¹	20.33 ± 4.16 ^{de}	221.15 ± 73.79 ^g	238.23 ± 44.28 ^{gh}	216.61 ± 66.06 ^{hi}
Moderate × 1.5 g L ⁻¹	16.00 ± 1.73 ^{fg}	328.41 ± 40.48 ^{ef}	300.90 ± 34.72 ^{ef}	263.26 ± 103.15 ^{gh}
Severe × DiWater	13.33 ± 1.52 ^g	249.96 ± 25.40 ^g	206.55 ± 33.19 ^{gh}	233.46 ± 15.52 ^{hi}
Severe × 0.5 g L ⁻¹	16.00 ± 1.00 ^{fg}	237.21 ± 13.30 ^g	217.38 ± 21.60 ^{gh}	239.07 ± 42.50 ^{hi}
Severe × 1.0 g L ⁻¹	30.66 ± 3.78 ^b	212.76 ± 27.19 ^g	180.10 ± 35.49 ^h	184.08 ± 29.87 ⁱ
Severe × 1.5 g L ⁻¹	31.00 ± 3.60 ^b	203.29 ± 27.34 ^g	196.00 ± 35.50 ^{gh}	169.63 ± 12.96 ⁱ
F-value	38.12	5.81	3.63	2.42
P-value	*0.0001	*0.0001	*0.0038	*0.03
LSD	3.81	70.00	61.66	78.14

Multi × Biosti = Multistress × Biostimulant, DiWater = Distilled water, DAT = Days after transplant; ND = No data. Data are means and (±standard errors). Means followed by the same letter within the column for each section are not significant $p > 0.05$. Mean with different letters indicates statistically significant differences at $p < 0.05$. Bold mark and asterisk * indicate $p < 0.05$ (Tukey's LSD).



Table 4.3: Effect of multistress and foliar application of phytohormone-based biostimulant (*Lucky Plant*) treatments on the leaf minerals of curly lettuce.

Treatment	Macro mineral concentration (%)					Micro mineral content (%)				
	N	P	Mg	K	Ca	Na	Fe	Zn	Mn	Cu
Multistress										
Control	4.95 ± 0.14 ^a	2.41 ± 0.20 ^a	0.68 ± 0.01 ^a	5.82 ± 1.44 ^a	1.25 ± 0.09 ^a	0.19 ± 0.06 ^c	0.0139 ± 0.0019 ^a	0.0107 ± 0.0024 ^a	0.0051 ± 0.0005 ^a	0.0006 ± 0.0001 ^a
Mild	2.51 ± 0.18 ^b	2.10 ± 0.23 ^b	0.61 ± 0.01 ^b	3.93 ± 0.53 ^b	0.86 ± 0.09 ^b	0.38 ± 0.07 ^b	0.0109 ± 0.0095 ^a	0.0080 ± 0.0014 ^b	0.0034 ± 0.0005 ^d	0.0006 ± 0.0009 ^a
Moderate	2.27 ± 0.16 ^c	1.87 ± 0.18 ^c	0.59 ± 0.01 ^b	3.64 ± 0.96 ^b	0.85 ± 0.09 ^b	0.53 ± 0.12 ^a	0.0142 ± 0.0005 ^a	0.0073 ± 0.0013 ^b	0.0038 ± 0.0004 ^c	0.0006 ± 0.0001 ^a
Severe	2.10 ± 0.09 ^d	1.84 ± 0.24 ^c	0.56 ± 0.01 ^b	3.34 ± 1.01 ^b	0.71 ± 0.06 ^c	0.47 ± 0.12 ^a	0.0131 ± 0.0032 ^a	0.0069 ± 0.0015 ^b	0.0044 ± 0.0059 ^b	0.0005 ± 0.0001 ^a
F-value	1256.87	23.34	60.65	23.57	42.33	32.45	0.10	20.25	33.58	0.51
P-value	*0.0001	*0.0001	*0.0001	*0.0001	*0.0001	*0.001	0.95	*0.0001	*0.0001	0.61
LSD	0.11	0.15	0.02	0.6659	0.1132	0.0754	0.004	0.0011	0.0003	0.0001
Biostimulant dose										
DiWater	2.96 ± 1.27 ^a	2.06 ± 0.29 ^{ab}	0.60 ± 0.04 ^b	4.64 ± 1.73 ^a	1.00 ± 0.19 ^a	0.40 ± 0.16 ^{ab}	0.0108 ± 0.0012 ^a	0.0085 ± 0.0012 ^{ab}	0.0043 ± 0.0007 ^a	0.0006 ± 0.0001 ^a
0.5 g L ⁻¹	2.99 ± 1.23 ^a	2.16 ± 0.30 ^a	0.63 ± 0.06 ^a	4.09 ± 1.29 ^{ab}	0.85 ± 0.18 ^b	0.38 ± 0.15 ^{ab}	0.0129 ± 0.0020 ^a	0.0078 ± 0.0019 ^b	0.0043 ± 0.0008 ^a	0.0006 ± 0.0001 ^{ab}
1.0 g L ⁻¹	2.95 ± 1.20 ^a	2.07 ± 0.20 ^{ab}	0.61 ± 0.05 ^{ab}	4.43 ± 1.04 ^a	0.93 ± 0.27 ^{ab}	0.35 ± 0.15 ^b	0.0138 ± 0.0037 ^a	0.0090 ± 0.0030 ^a	0.0040 ± 0.0007 ^a	0.0005 ± 0.0009 ^b
1.5 g L ⁻¹	3.01 ± 1.26 ^a	1.94 ± 0.41 ^b	0.61 ± 0.05 ^{ab}	3.56 ± 1.39 ^b	0.89 ± 0.28 ^{ab}	0.44 ± 0.18 ^a	0.0143 ± 0.0012 ^a	0.0085 ± 0.0012 ^b	0.0040 ± 0.0009 ^a	0.0005 ± 0.0009 ^b
F-value	0.59	2.81	2.20	4.17	3.41	1.95	1.30	3.18	2.20	3.43
P-value	0.62	0.05	0.16	*0.01	0.06	0.14	0.29	*0.03	0.11	*0.03
LSD	0.11	0.15	0.22	0.66	2.26	0.07	0.004	0.001	0.0003	0.0001
Multi × Biosti										
F-value	2.35	1.68	1.05	0.91	0.50	1.62	0.50	2.79	1.41	0.71
P-value	*0.0406	0.13	0.42	0.53	0.86	0.15	0.86	*0.0175	0.2306	0.6964
LSD	0.22	0.31	0.10	133.18	0.49	0.15	0.009	0.0022	0.0008	0.0002

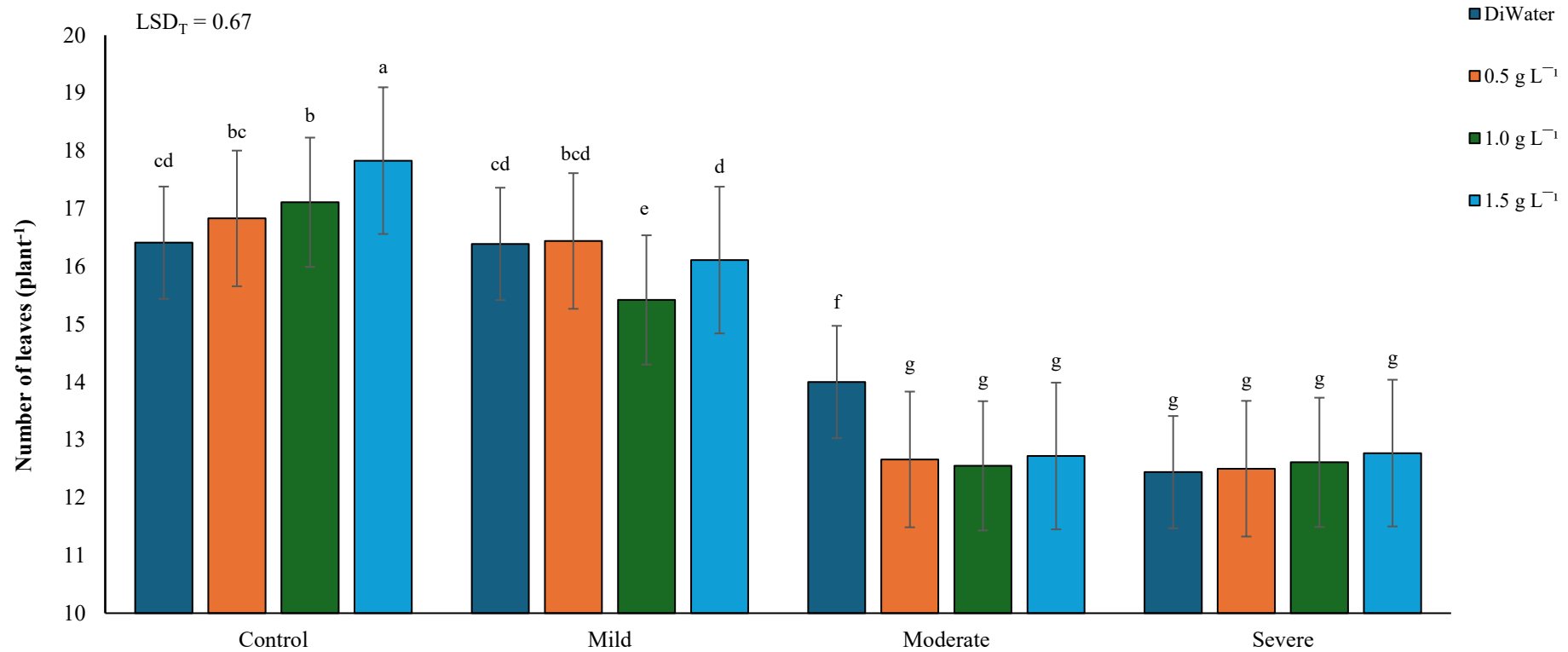
Multi × Biosti = Multistress × Biostimulant, DiWater = Distilled water. Data are means and (±standard errors). Means followed by the same letter within the column for each section are not significant $p > 0.05$. Mean with different letters indicates statistically significant differences at $p < 0.05$. Bold mark and asterisk * indicate $p < 0.05$ (Tukey's LSD).

Table 4.4: Phenolic compounds detected from curly lettuce var. Crispa.

Alig. ID	Phenolic Compounds	Chemical formula	Retention Tim(min)	Mass (M/z)
1827	Stachyose	C ₂₄ H ₄₂ O ₂₁	2.47	665.21436
1878	Stachyose	C ₂₄ H ₄₂ O ₂₁	2.47	711.22058
1439	-	-	2.94	581.18475
343	-	-	2.96	290.0882
2051	Verbascose	C ₃₀ H ₅₂ O ₂₆	3.34	827.2677
2095	Verbascose	C ₃₀ H ₅₂ O ₂₆	3.35	873.27344
2398	UNPD182954	C ₄₈ H ₈₂ O ₄₁	4.09	1313.42786
2405	-	-	4.09	1333.43408
2410	-	-	4.09	1359.43359
2276	Ajugose	C ₃₆ H ₆₂ O ₃₁	4.38	1035.32739
2057	-	-	4.64	841.26532
2186	-	-	4.84	922.29181
2218	-	-	4.84	945.29413
1196	-	-	4.87	495.18497
2323	-	-	4.90	1107.34729
282	-	C ₁₀ H ₂₁ NO ₄ S ₂	4.99	282.08417
1363	-	C ₂₃ H ₃₄ O ₁₆	5.02	565.17603
831	-	-	5.44	426.12723
458	Gentisic acid 5-O-glucoside	C ₁₃ H ₁₆ O ₉	6.66	315.07233
445	Caftaric acid	C ₁₃ H ₁₂ O ₉	7.50	311.04105
457	Gentisic acid 5-O-glucoside	C ₁₃ H ₁₆ O ₉	7.64	315.07193
589	3CQA Chlorogenic acid	C ₁₆ H ₁₈ O ₉	8.09	353.08813
599	-	-	10.19	357.06497
590	Chlorogenic acid	C ₁₆ H ₁₈ O ₉	10.50	353.08817
129	-	-	11.10	210.07637
1721	Quercetin 3-glucosyl-(1->2)-galactoside	C ₂₇ H ₃₀ O ₁₇	11.80	625.14209
365	Caffeoylmalic acid	C ₁₃ H ₁₂ O ₈	11.99	295.04617
1890	-	-	12.17	725.12042
544	3-O-p-Coumaroylquinic acid	C ₁₆ H ₁₈ O ₈	12.48	337.09296
1876	Quercetin-O-(malonyl-glucoside) O-hexoside	C ₃₀ H ₃₂ O ₂₀	12.57	711.14185
860	Citroside A	C ₁₉ H ₃₀ O ₈	12.90	431.19247
1111	Chicoric acid	C ₂₂ H ₁₈ O ₁₂	14.52	473.07343
1209	-	-	14.98	504.09009
1127	Quercetin 3-O-glucuronide	C ₂₁ H ₁₈ O ₁₃	16.00	477.06772
1052	Quercetin 3-galactoside	C ₂₁ H ₂₀ O ₁₂	16.30	463.08893
1032	Kaempferol 3-glucuronide	C ₂₁ H ₁₈ O ₁₂	16.53	461.07346
942	Trifolin	C ₂₁ H ₂₀ O ₁₁	16.73	447.09409
1310	Quercetin -malonyl-glucoside)	C ₂₄ H ₂₂ O ₁₅	17.08	549.08899
1216	Quercetin-O-acetyl-galactoside)	C ₂₃ H ₂₂ O ₁₃	17.60	505.0994
1212	Quercetin-O-acetyl-glucoside)	C ₂₃ H ₂₂ O ₁₃	18.40	505.09821
1247	-	-	18.55	515.12115
303	Kaempferol	C ₁₅ H ₁₀ O ₆	21.97	285.04004
545	Ganoderic acid H	C ₃₂ H ₄₄ O ₉	24.67	571.28925

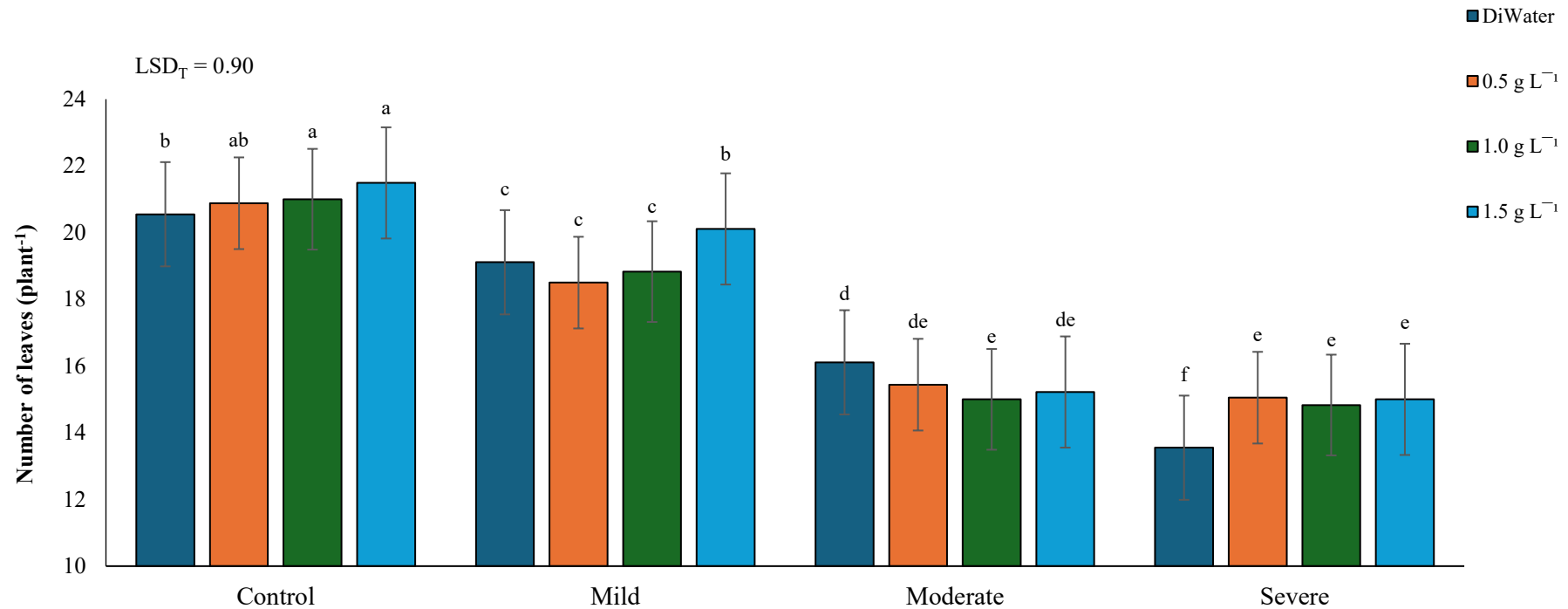


Figure 4.1: Demonstration of the experimental layout of the study.



Multistress levels x Biostimulant Application

Figure 4.2: Number of leaves on the fourth week for curly lettuce treated with varying doses of *Lucky Plant*[®] (0.5, 1.0, and 1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe). The values represent the means of three replications. The lowercase letters above the columns denote statistically significant differences between treatment means at p<0.05 (Tukey's LSD).



Multistress levels x Biostimulant Application

Figure 4.3: Number of leaves at harvest for the curly lettuce treated with varying doses of *Lucky Plant*[®] (0.5, 1.0, and 1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe). The values represent the means of three replications. The lowercase letters above the columns denote statistically significant differences between treatment means at $p < 0.05$ (Tukey's LSD).

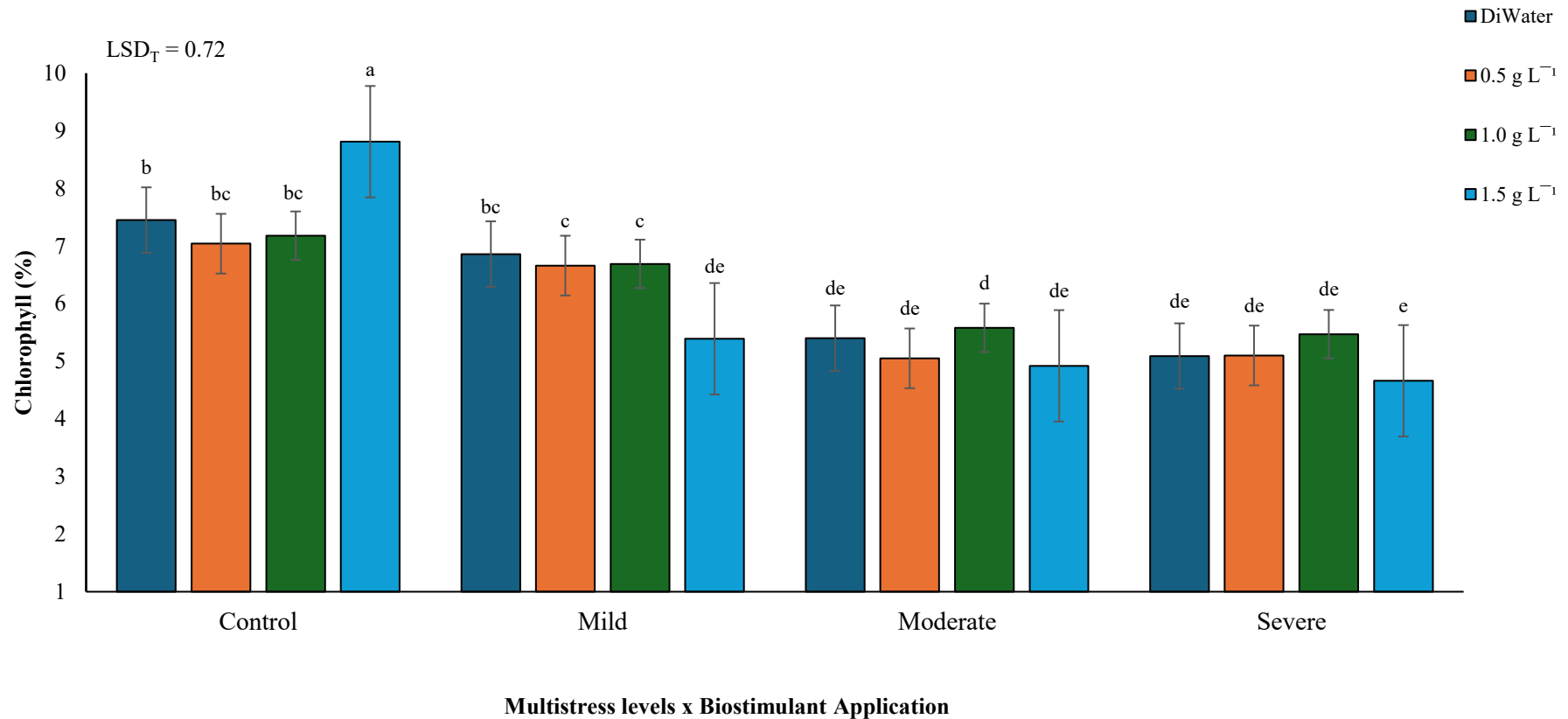
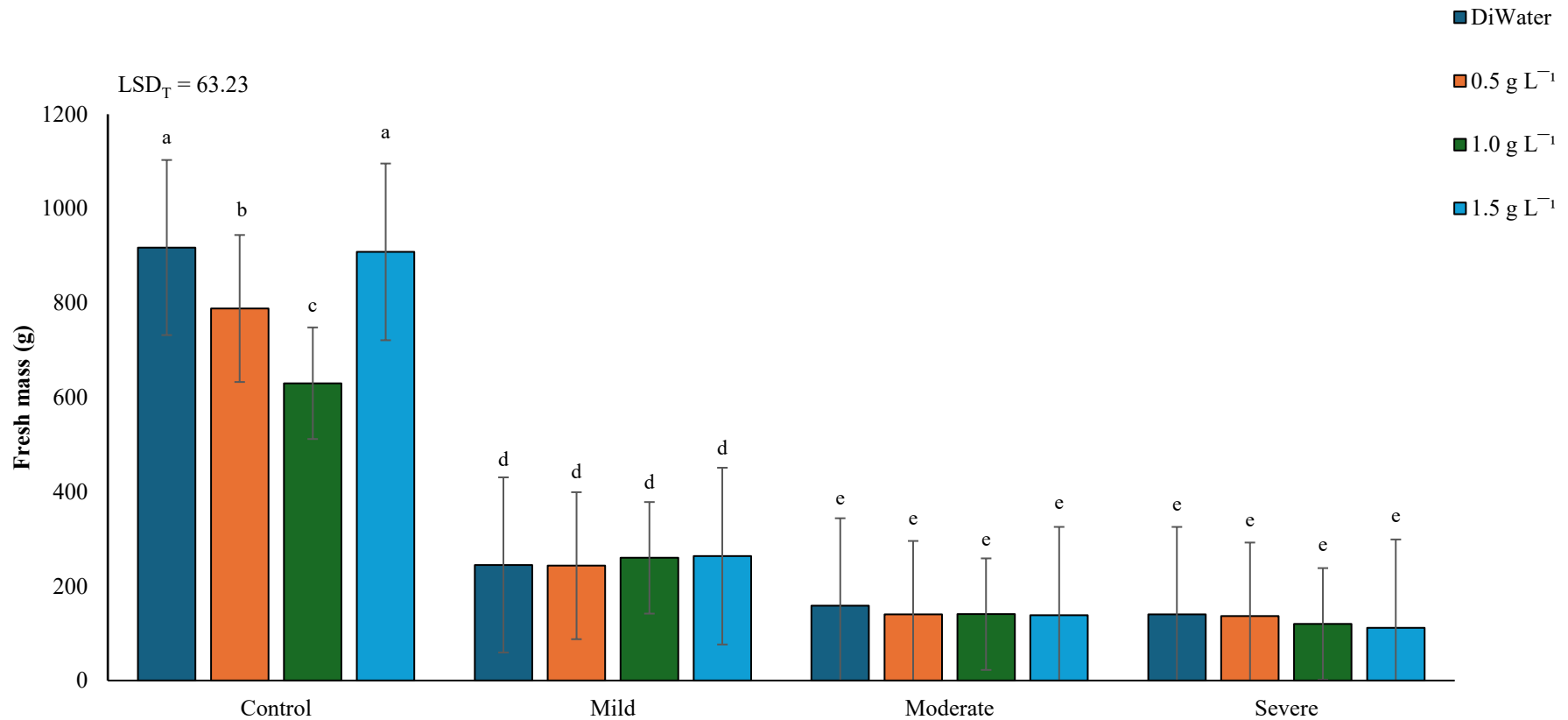


Figure 4.4: Chlorophyll content at harvest for the curly lettuce treated with varying doses of *Lucky Plant*® (0.5, 1.0, and 1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe). The values represent the means of three replications. The lowercase letters above the columns denote statistically significant differences between treatment means at p<0.05 (Tukey's LSD).



Multistress levels x Biostimulant Application

Figure 4.5: Fresh mass at harvest for the curly lettuce treated with varying doses of *Lucky Plant*[®] (0.5, 1.0, and 1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe). The values represent the means of three replications. The lowercase letters above the columns denote statistically significant differences between treatment means at $p < 0.05$ (Tukey's LSD).

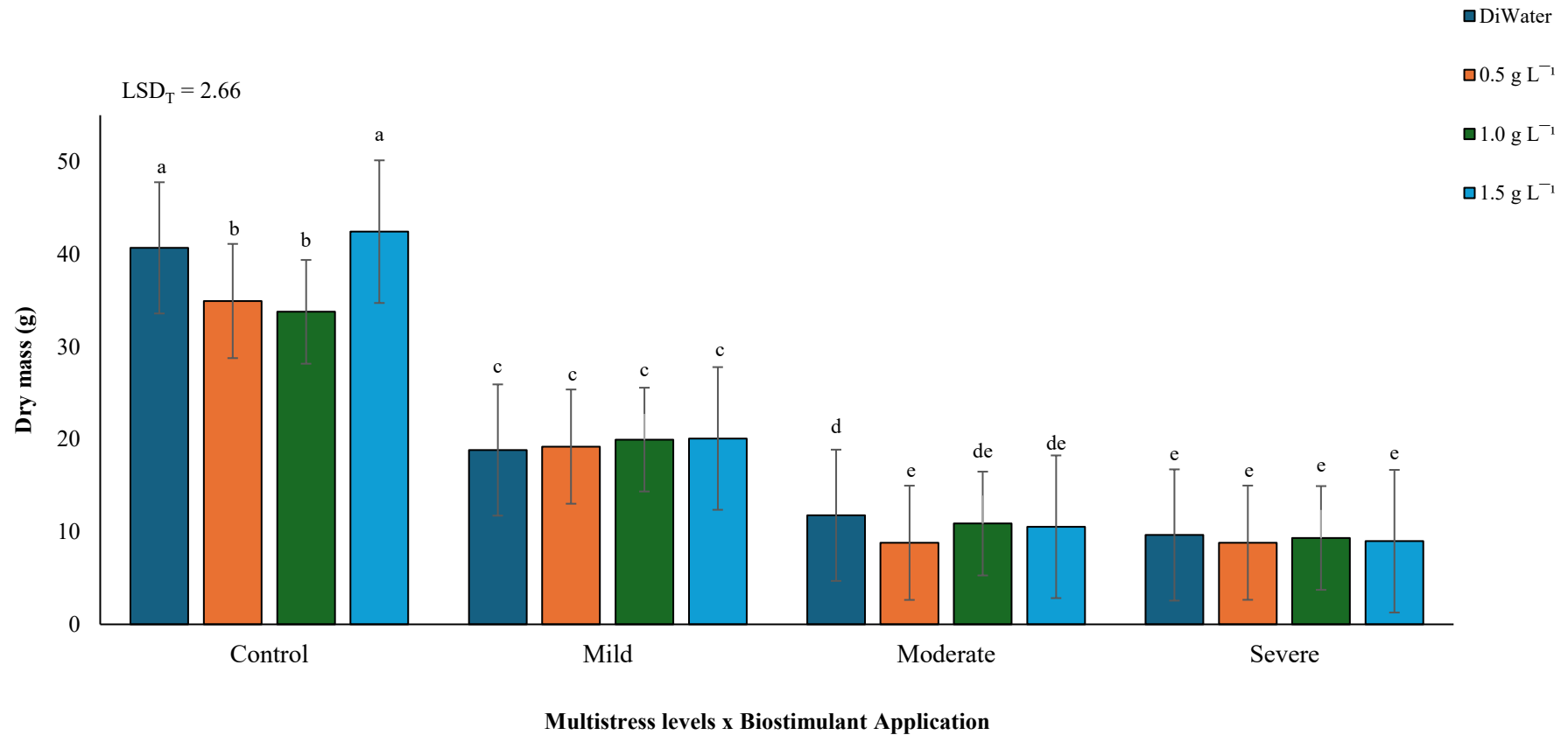


Figure 4.6: Dry mass at harvest for the curly lettuce treated with varying doses of *Lucky Plant*[®] (0.5, 1.0, and 1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe). The values represent the means of three replications. The lowercase letters above the columns denote statistically significant differences between treatment means at $p < 0.05$ (Tukey's LSD).

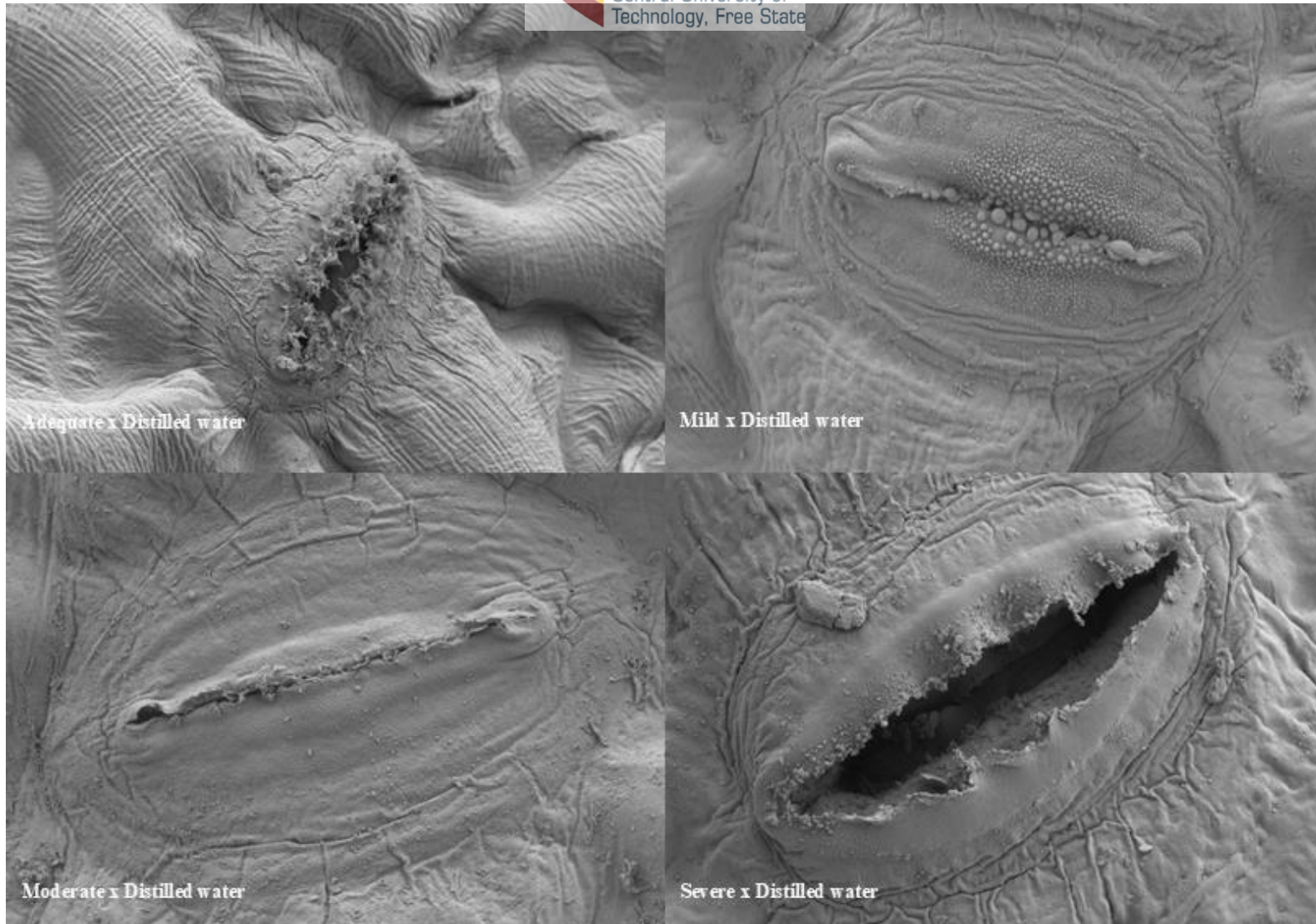


Figure 4.7A: SEM micrographs (10 μm) of the adaxial leaf surface demonstrating the stomatal features across varying doses of distilled water (as control) foliar application under multistress (control, mild, moderate, and severe).

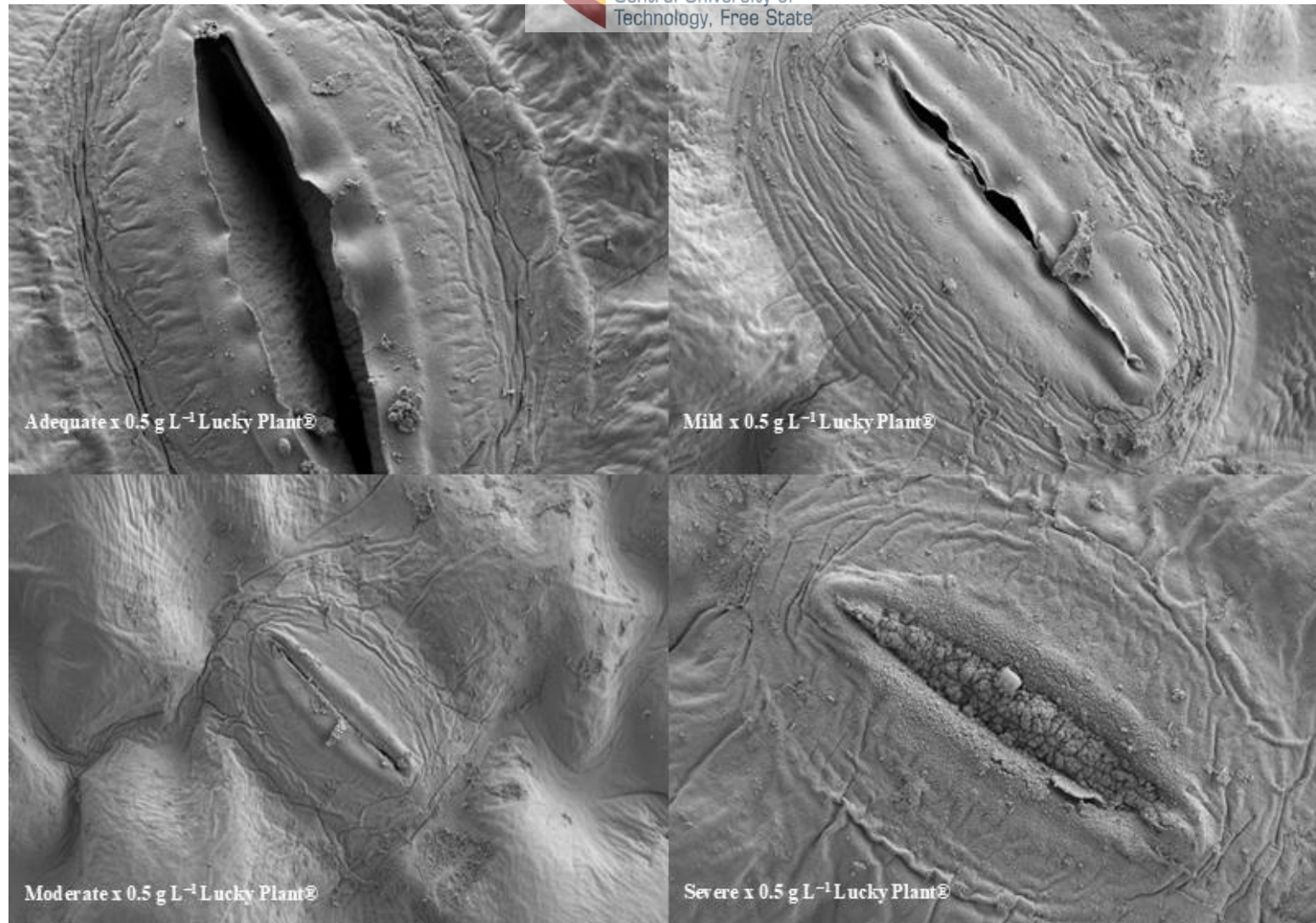


Figure 4.7B: SEM micrographs (10 μ m) of the adaxial leaf surface demonstrating the stomatal features across varying doses of *Lucky Plant*® (0.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe).

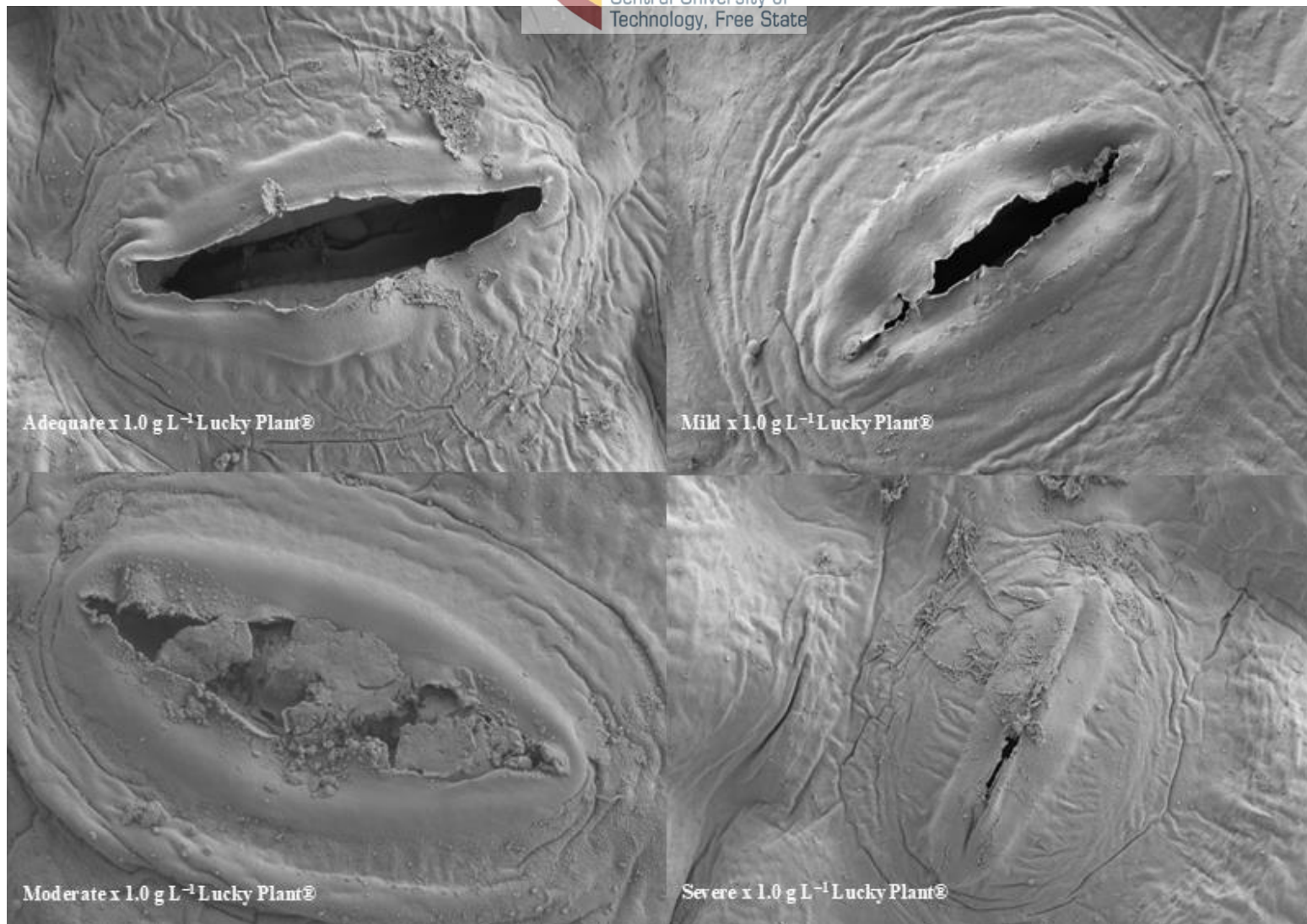


Figure 4.7C: SEM micrographs (10 μm) of the adaxial leaf surface demonstrating the stomatal features across varying doses of *Lucky Plant*® (1.0 g L^{-1}) foliar application under multistress (control, mild, moderate, and severe).

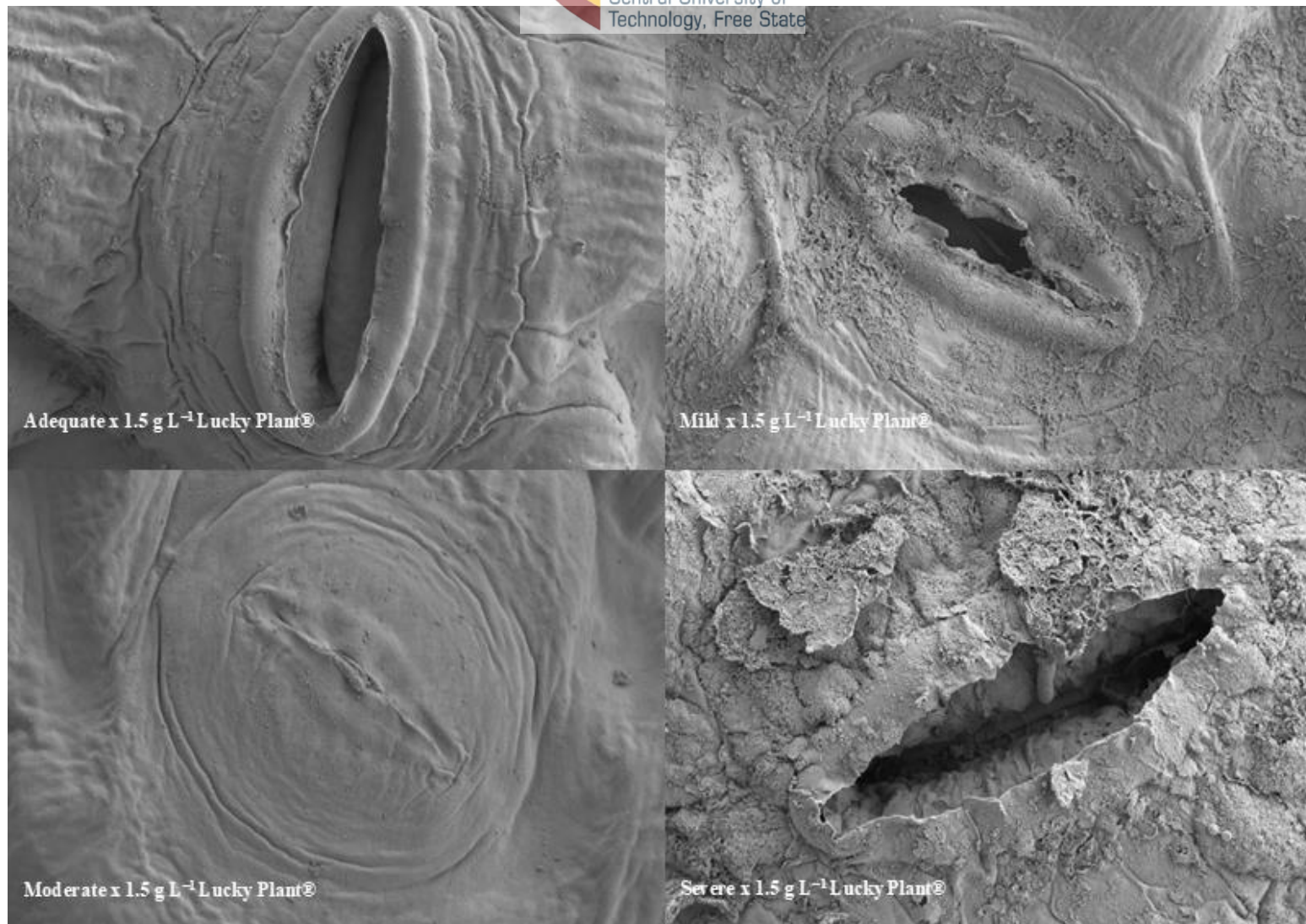


Figure 4.7D: SEM micrographs (10 μm) of the adaxial leaf surface demonstrating the stomatal features across varying doses of *Lucky Plant*® (1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe).

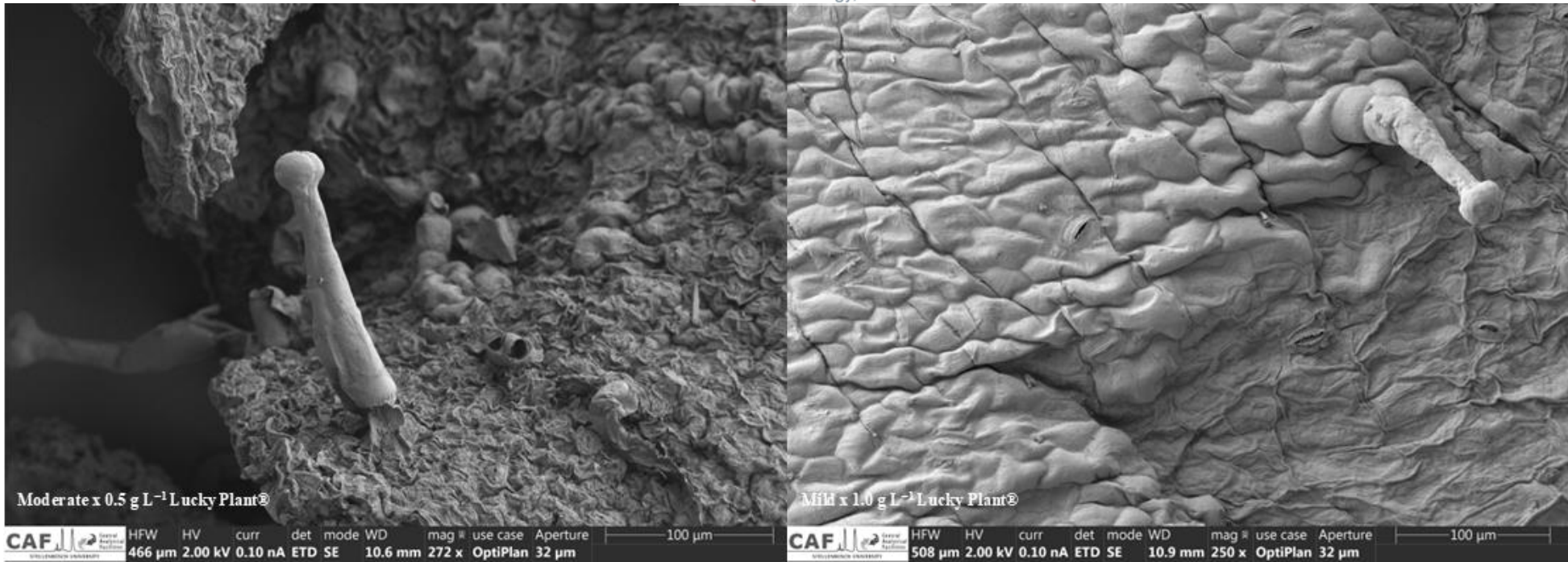


Figure 4.8: Schematic representation (100 µm) of the adaxial leaf surface demonstrating a trichome length capitate/asciiform (Sedibe *et al.*, 2013; Khetsha *et al.*, 2023) where plants were grown under moderate and mild multistress and treated with 0.5 and 1.0 g L⁻¹, respectively.

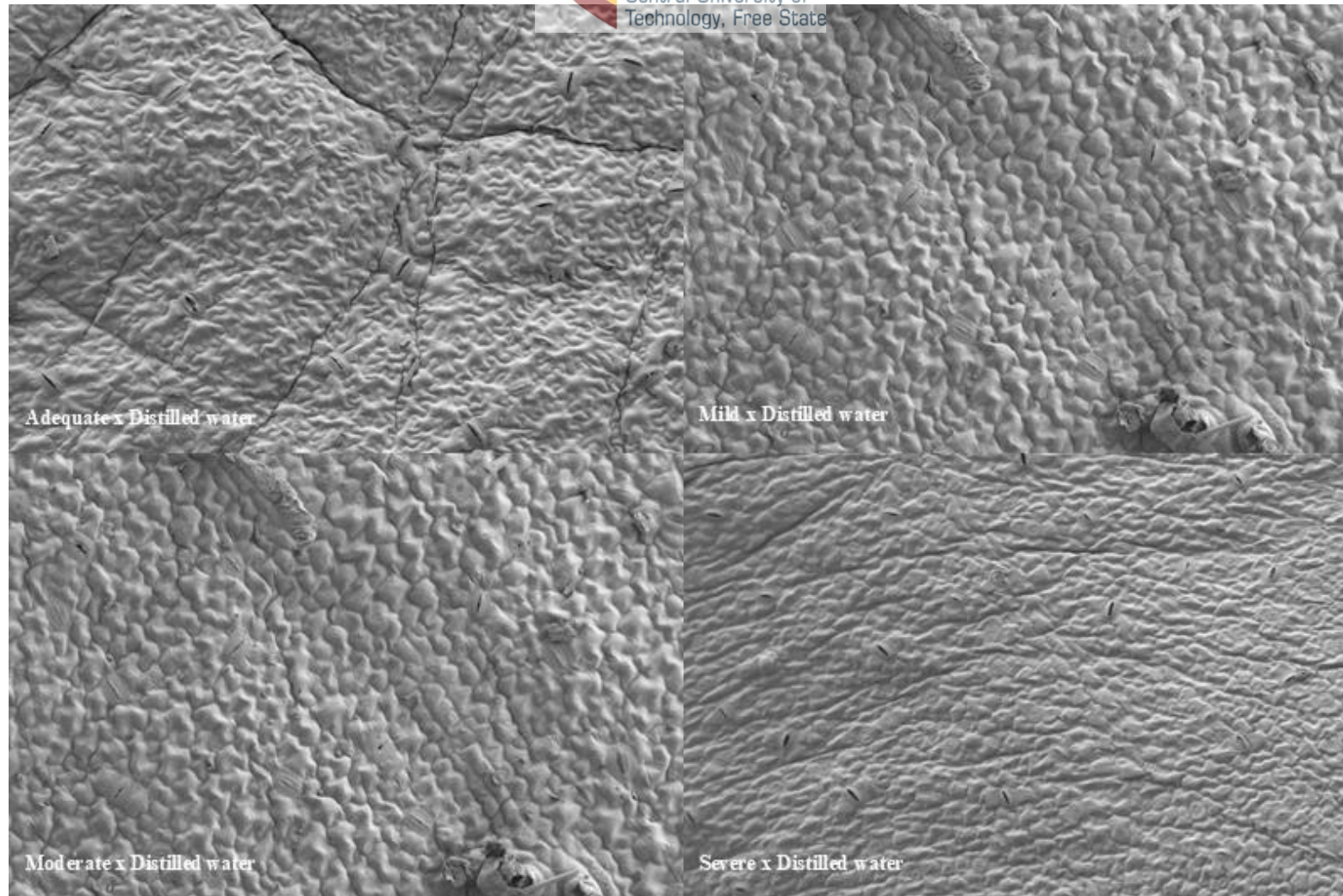


Figure 4.9A: Schematic representation (10 μm) of the adaxial leaf surface demonstrating the stomatal density across varying doses of distilled water (as control) foliar application under multistress (control, mild, moderate, and severe).

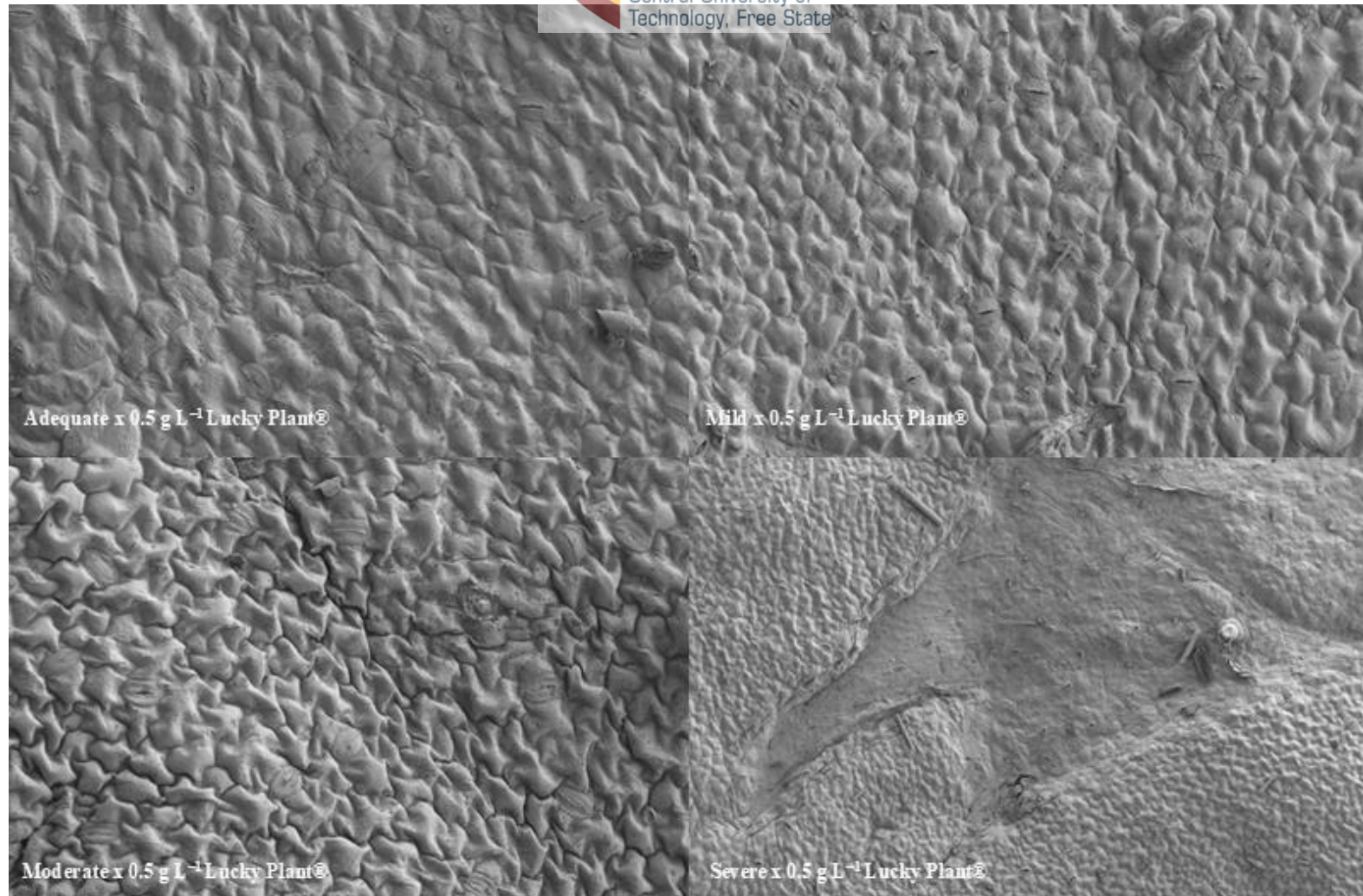


Figure 4.9B: Schematic representation (50 μm) of the adaxial leaf surface demonstrating the stomatal density across varying doses of *Lucky Plant*® (0.5 g L^{-1}) foliar application under multistress (control, mild, moderate, and severe).

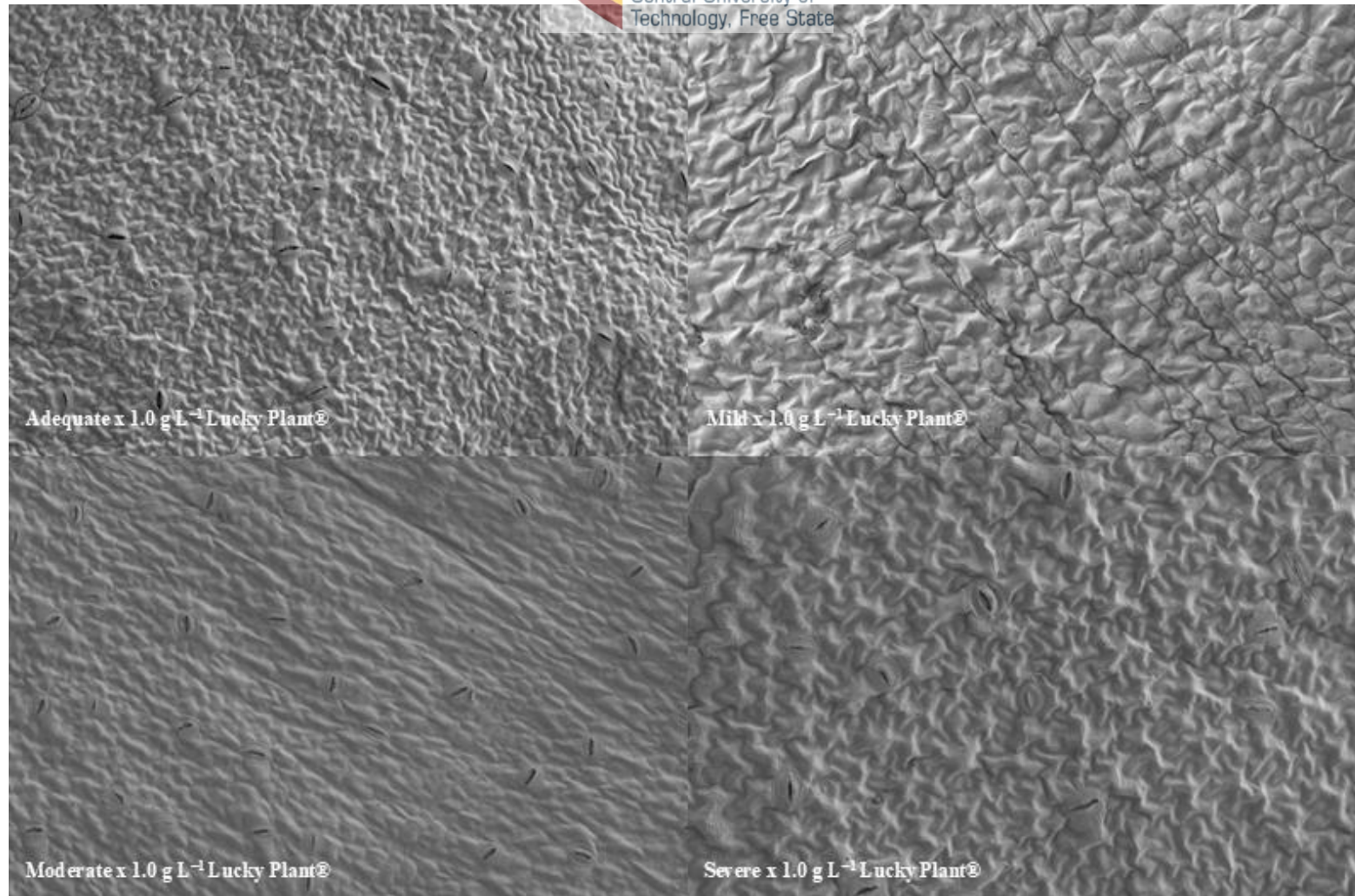


Figure 4.9C: Schematic representation (50 μm) of the adaxial leaf surface demonstrating the stomatal density across varying doses of *Lucky Plant*[®] (1.0 g L^{-1}) foliar application under multistress (control, mild, moderate, and severe).

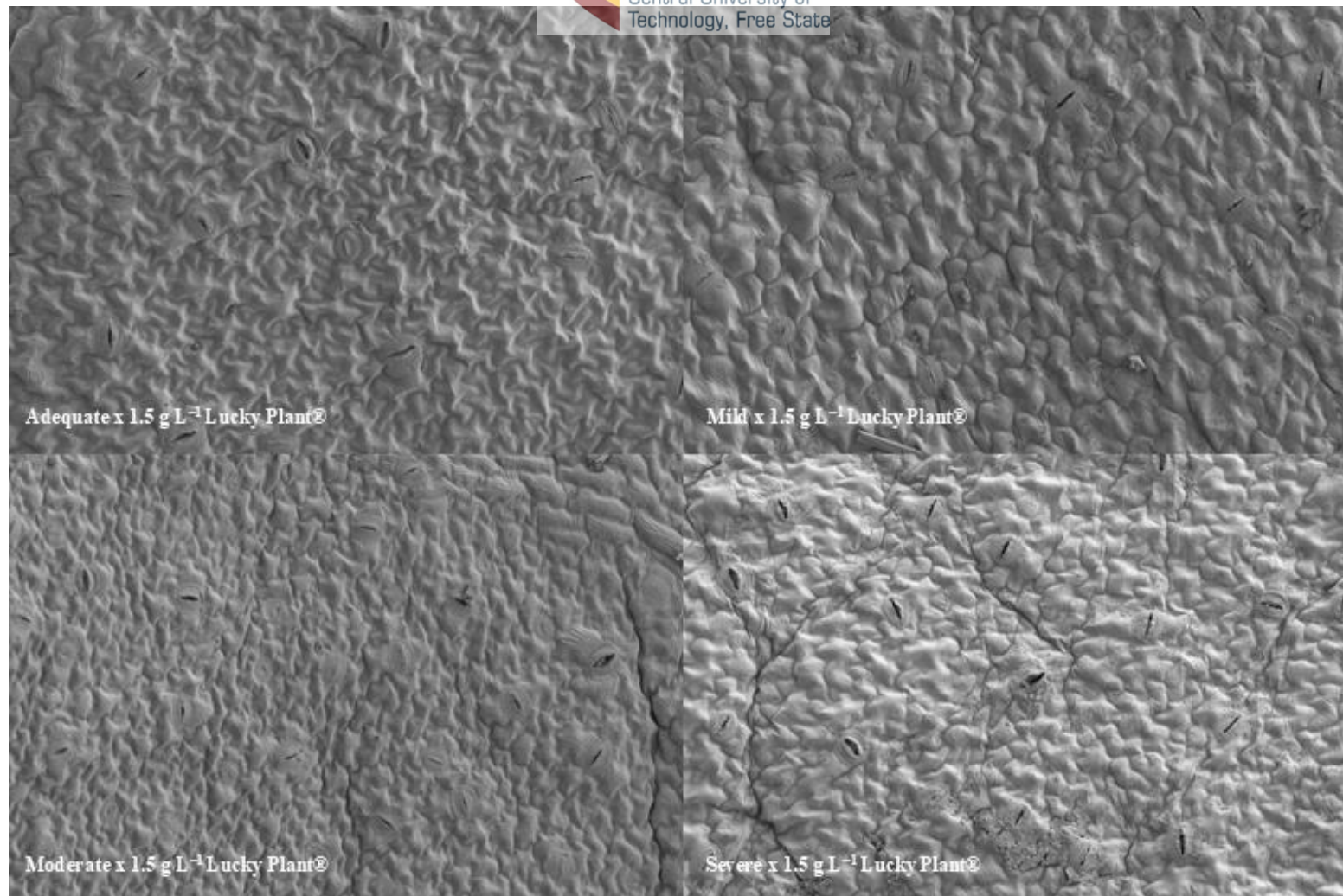
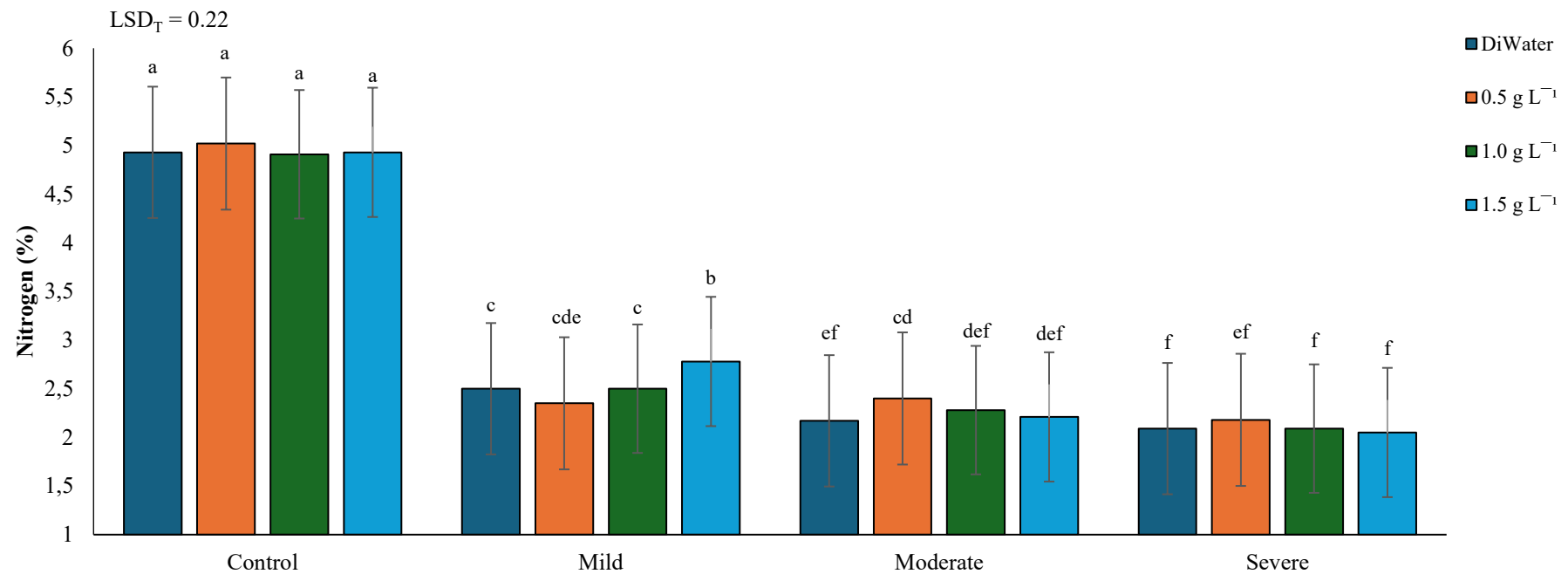
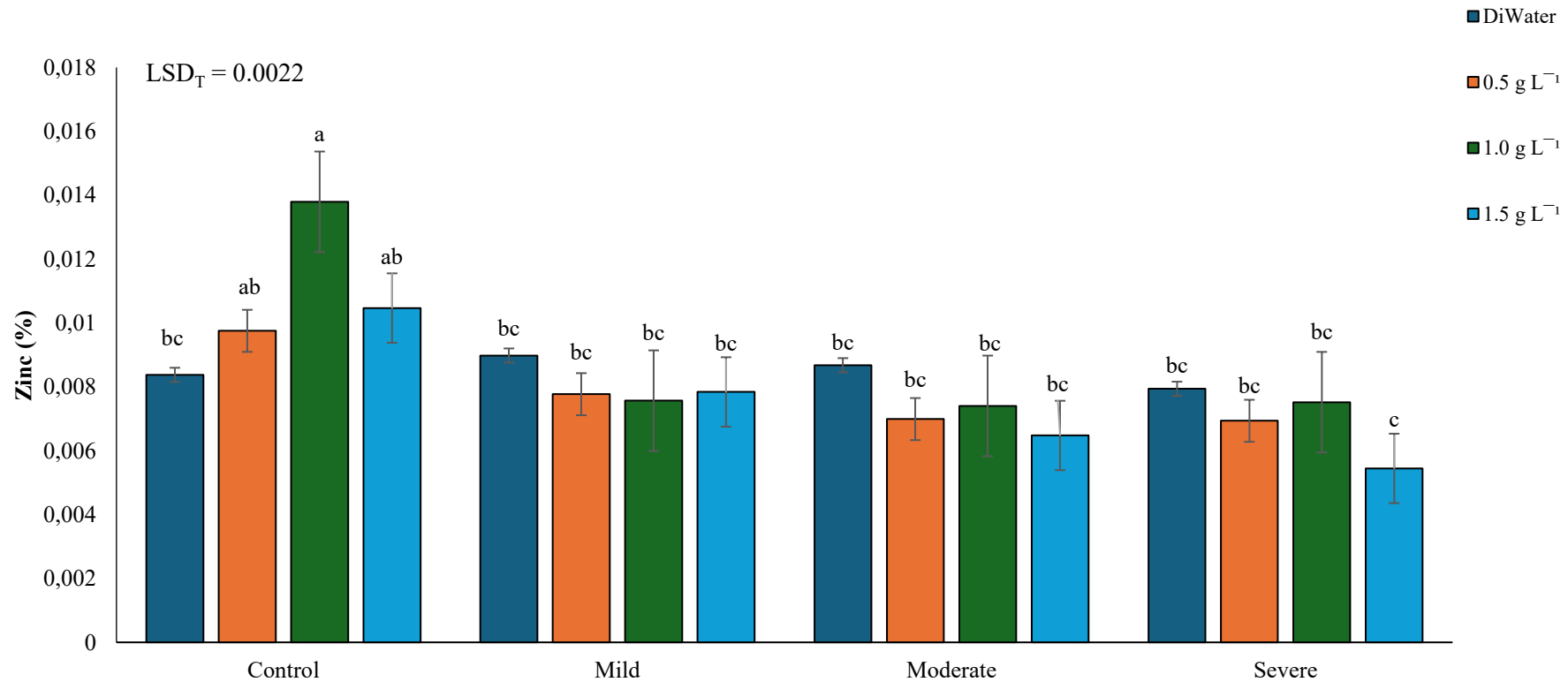


Figure 4.9D: Schematic representation (50 μm) of the adaxial leaf surface demonstrating the stomatal density across varying doses of *Lucky Plant*® (1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe).



Multistress levels x Biostimulant Application

Figure 4.10: Leaf nitrogen content at harvest for the curly lettuce treated with varying doses of *Lucky Plant*[®] (0.5, 1.0, and 1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe). The values represent the means of three replications. The lowercase letters above the columns denote statistically significant differences between treatment means at $p < 0.05$ (Tukey's LSD).



Multistress levels x Biostimulant Application

Figure 4.11: Leaf zinc content at harvest for the curly lettuce treated with varying doses of *Lucky Plant*[®] (0.5, 1.0, and 1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe). The values represent the means of three replications. The lowercase letters above the columns denote statistically significant differences between treatment means at p<0.05 (Tukey's LSD).

Biplot (axes F1 and F2: 84.25 %)

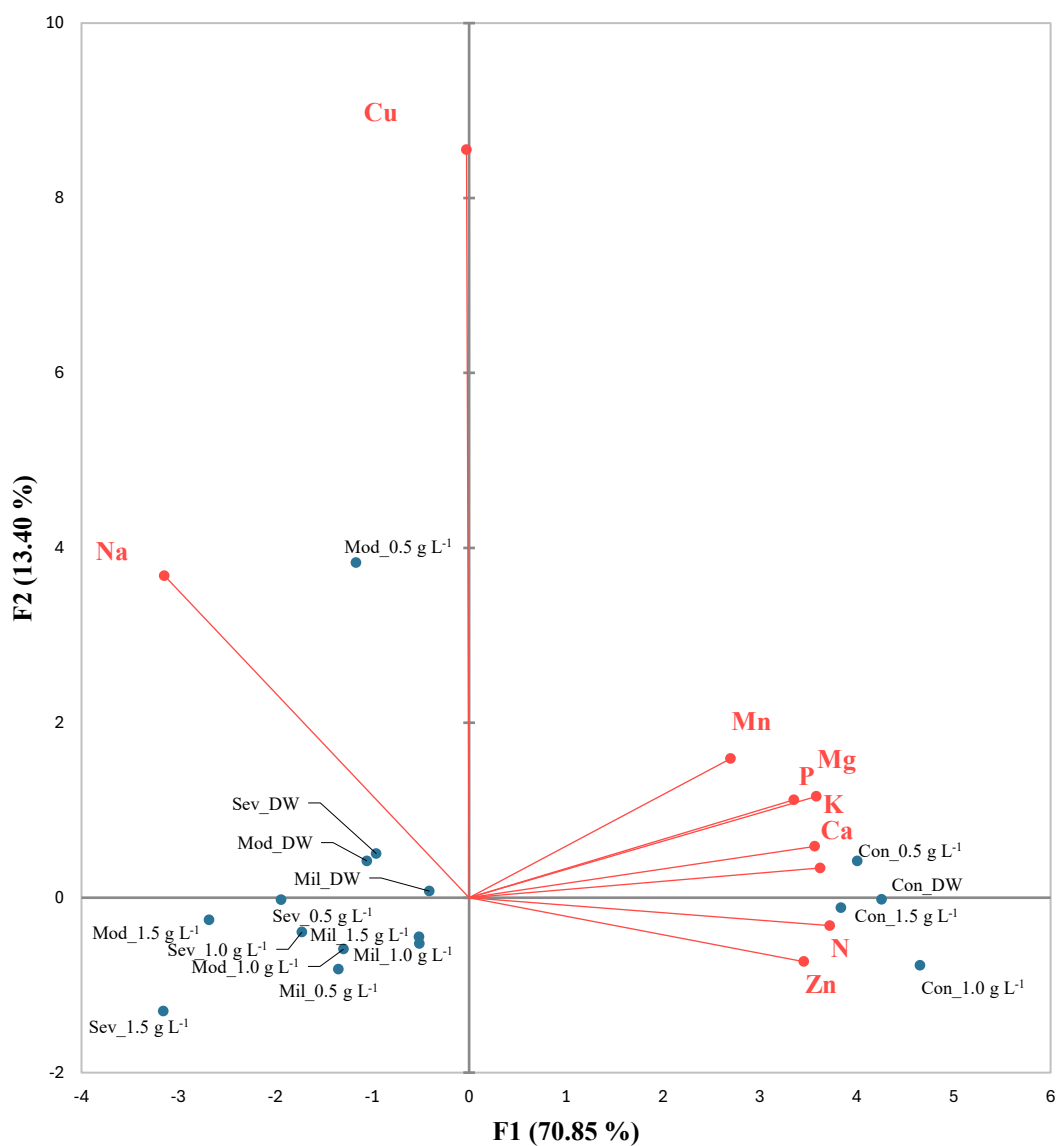


Figure 4.12: PCA of analysed mineral results of curly lettuce treated with varying doses of *Lucky Plant*[®] (0.5, 1.0, and 1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions. **Abbreviations:** Con_DW = Control × Distilled water; Con_0.5 g L⁻¹ = Control × 0.5 g L⁻¹; Con_1.0 g L⁻¹ = Control × 1.0 g L⁻¹; Con_1.5 g L⁻¹ = Control × 1.5 g L⁻¹; Mil_DW = Mild × Distilled water; Mil_0.5 g L⁻¹ = Mild × 0.5 g L⁻¹; Mil_1.0 g L⁻¹ = Mild × 1.0 g L⁻¹; Mil_1.5 g L⁻¹ = Mild × 1.5 g L⁻¹; Mod_DW = Moderate × Distilled water; Mod_0.5 g L⁻¹ = Moderate × 0.5 g L⁻¹; Mod_1.0 g L⁻¹ = Moderate × 1.0 g L⁻¹; Mod_1.5 g L⁻¹ = Moderate × 1.5 g L⁻¹; Sev_DW = Severe × Distilled water; Sev_0.5 g L⁻¹ = Severe × 0.5 g L⁻¹; Sev_1.0 g L⁻¹ = Severe × 1.0 g L⁻¹; Sev_1.5 g L⁻¹ = Severe × 1.5 g L⁻¹.

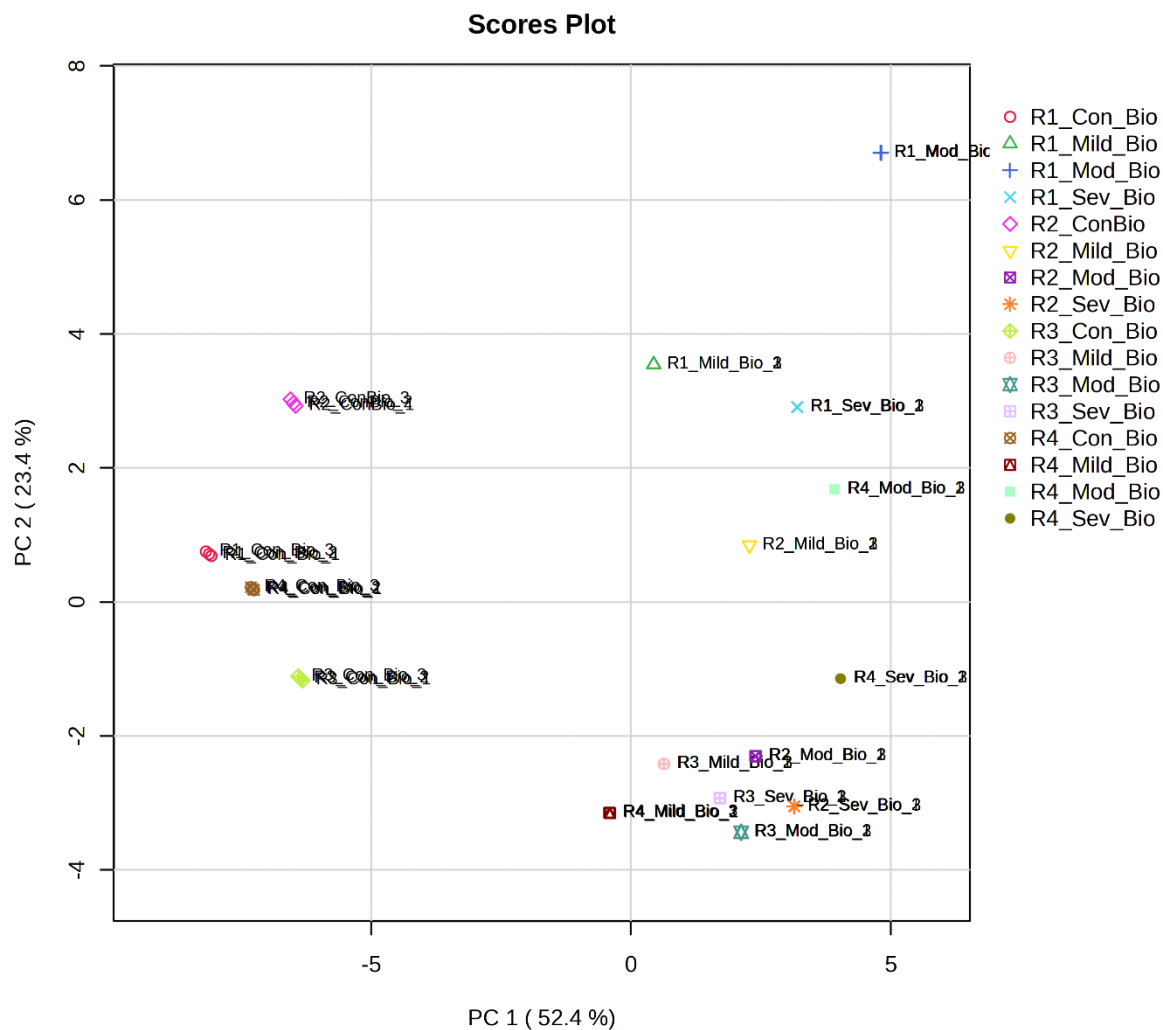


Figure 4.13: Principal Component Analysis (PCA) showing scores plot of phenolics attained by UPLC-MS analysis in curly lettuce treated with varying doses of *Lucky Plant*[®] (0.5, 1.0, and 1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions. **Abbreviations:** R1_Con_Bio = Control (100% × 5 L × Intact plants) and Distilled water; R1_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and Distilled water; R1_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and Distilled water; R1_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and Distilled water; R2_Con_Bio = Control (100% × 5 L × Intact plants) and 0.5 g L⁻¹ *Lucky Plant*[®]; R2_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and 0.5 g L⁻¹ *Lucky Plant*[®]; R2_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and 0.5 g L⁻¹ *Lucky Plant*[®]; R2_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and 0.5 g L⁻¹ *Lucky Plant*[®]; R3_Con_Bio = Control (100% × 5 L × Intact plants) and 1.0 g L⁻¹ *Lucky Plant*[®]; R3_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and 1.0 g L⁻¹ *Lucky Plant*[®]; R3_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and 1.0 g L⁻¹ *Lucky Plant*[®]; R3_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and 1.0 g L⁻¹ *Lucky Plant*[®]; R4_Con_Bio = Control (100% × 5 L × Intact plants) and 1.5 g L⁻¹ *Lucky Plant*[®]; R4_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and 1.5 g L⁻¹ *Lucky Plant*[®]; R4_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and 1.5 g L⁻¹ *Lucky Plant*[®]; R4_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and 1.5 g L⁻¹ *Lucky Plant*[®].

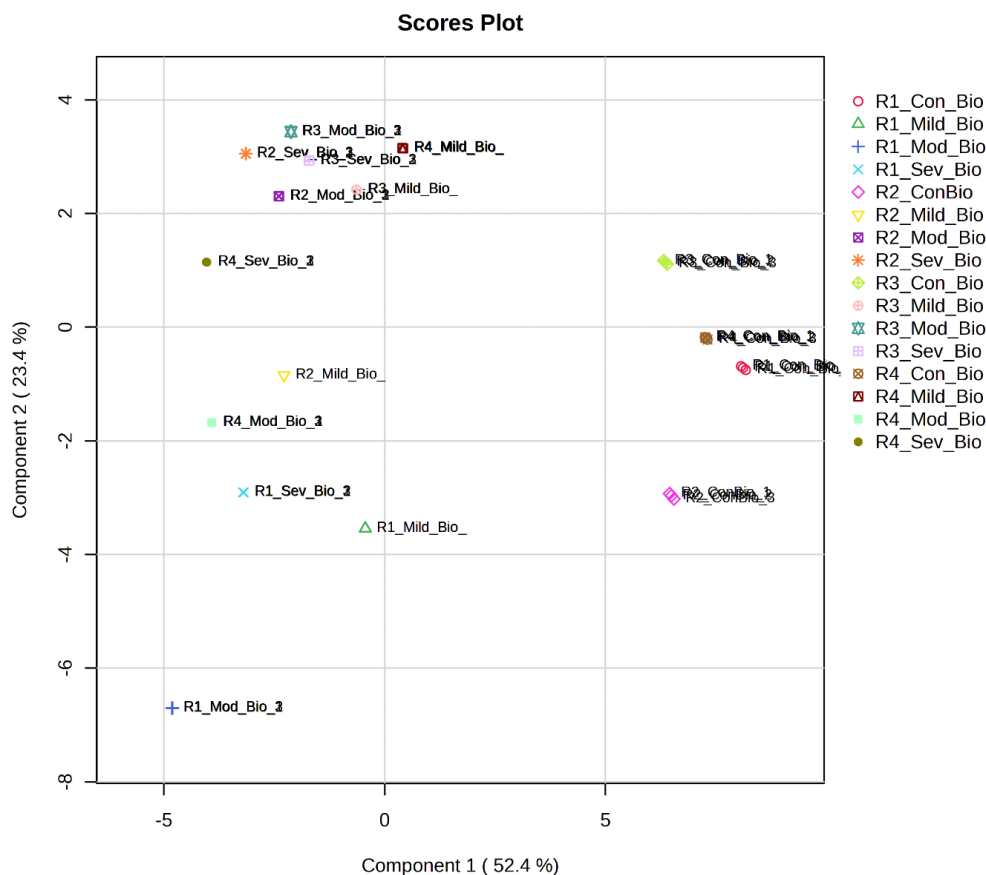


Figure 4.14: Partial least square discrimination analysis (OPLS-DA) score plot showing the phenolic compounds groups found in curly lettuce treated with varying doses of *Lucky Plant*[®] (0.5, 1.0, and 1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions. **Abbreviations:** R1_Con_Bio = Control (100% × 5 L × Intact plants) and Distilled water; R1_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and Distilled water; R1_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and Distilled water; R1_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and Distilled water; R2_Con_Bio = Control (100% × 5 L × Intact plants) and 0.5 g L⁻¹ *Lucky Plant*[®]; R2_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and 0.5 g L⁻¹ *Lucky Plant*[®]; R2_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and 0.5 g L⁻¹ *Lucky Plant*[®]; R2_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and 0.5 g L⁻¹ *Lucky Plant*[®]; R3_Con_Bio = Control (100% × 5 L × Intact plants) and 1.0 g L⁻¹ *Lucky Plant*[®]; R3_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and 1.0 g L⁻¹ *Lucky Plant*[®]; R3_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and 1.0 g L⁻¹ *Lucky Plant*[®]; R3_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and 1.0 g L⁻¹ *Lucky Plant*[®]; R4_Con_Bio = Control (100% × 5 L × Intact plants) and 1.5 g L⁻¹ *Lucky Plant*[®]; R4_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and 1.5 g L⁻¹ *Lucky Plant*[®]; R4_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and 1.5 g L⁻¹ *Lucky Plant*[®]; R4_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and 1.5 g L⁻¹ *Lucky Plant*[®].

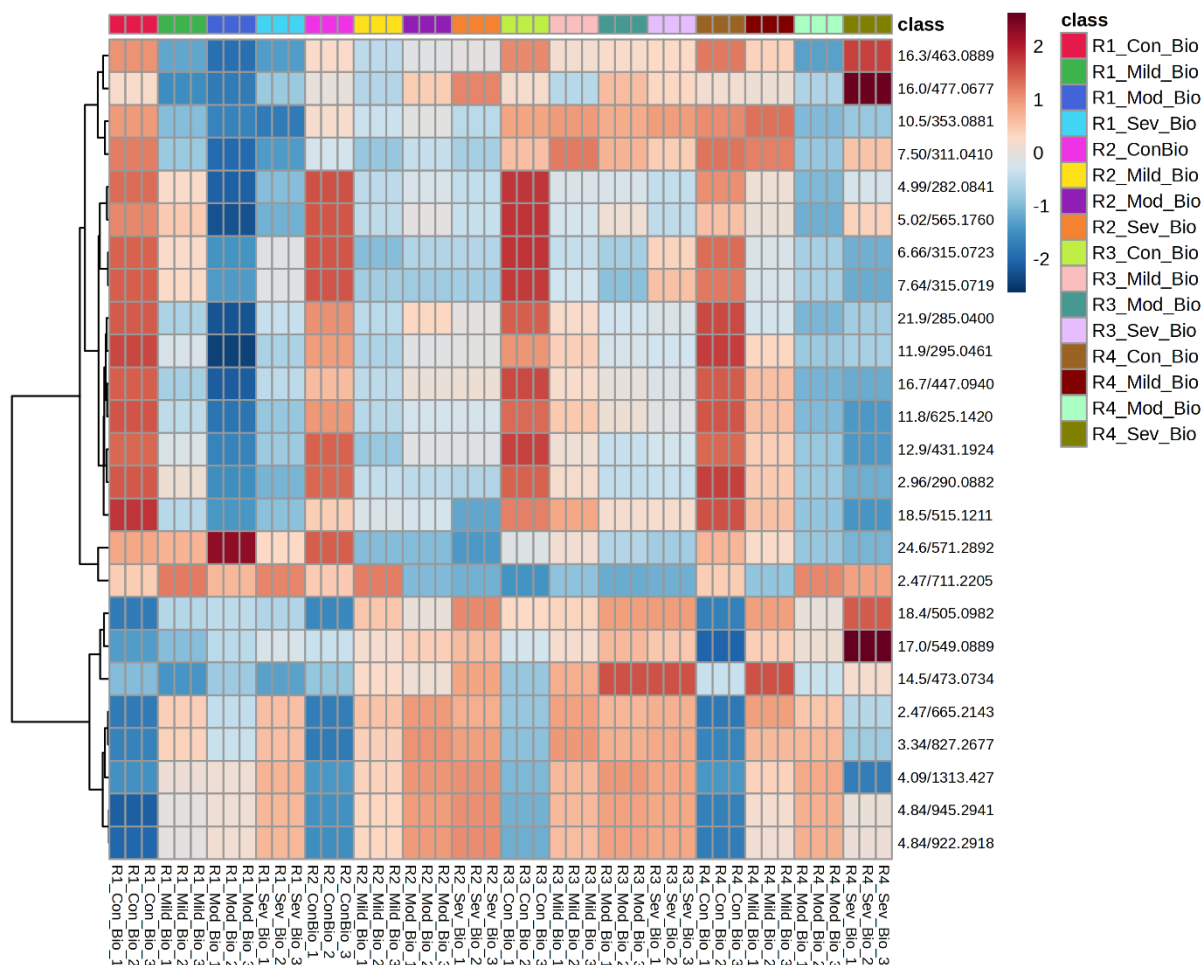


Figure 4.15: Heat map of unsupervised hierarchical clustering analysis (HCA) showing the phenolic compounds found in curly lettuce treated with varying doses of *Lucky Plant*[®] (0.5, 1.0, and 1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions. The coloured parts correspond to the contents of various phenolic compounds (for high red and low blue shades). Rows illustrate phenolic compounds, while columns illustrate treatments. **Abbreviations:** R1_Con_Bio = Control (100% × 5 L × Intact plants) and Distilled water; R1_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and Distilled water; R1_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and Distilled water; R1_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and Distilled water; R2_Con_Bio = Control (100% × 5 L × Intact plants) and 0.5 g L⁻¹ *Lucky Plant*[®]; R2_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and 0.5 g L⁻¹ *Lucky Plant*[®]; R2_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and 0.5 g L⁻¹ *Lucky Plant*[®]; R2_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and 0.5 g L⁻¹ *Lucky Plant*[®]; R3_Con_Bio = Control (100% × 5 L × Intact plants) and 1.0 g L⁻¹ *Lucky Plant*[®]; R3_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and 1.0 g L⁻¹ *Lucky Plant*[®]; R3_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and 1.0 g L⁻¹ *Lucky Plant*[®]; R3_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and 1.0 g L⁻¹ *Lucky Plant*[®]; R4_Con_Bio = Control (100% × 5 L × Intact plants) and 1.5 g L⁻¹ *Lucky Plant*[®]; R4_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and 1.5 g L⁻¹ *Lucky Plant*[®]; R4_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and 1.5 g L⁻¹ *Lucky Plant*[®]; R4_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and 1.5 g L⁻¹ *Lucky Plant*[®].

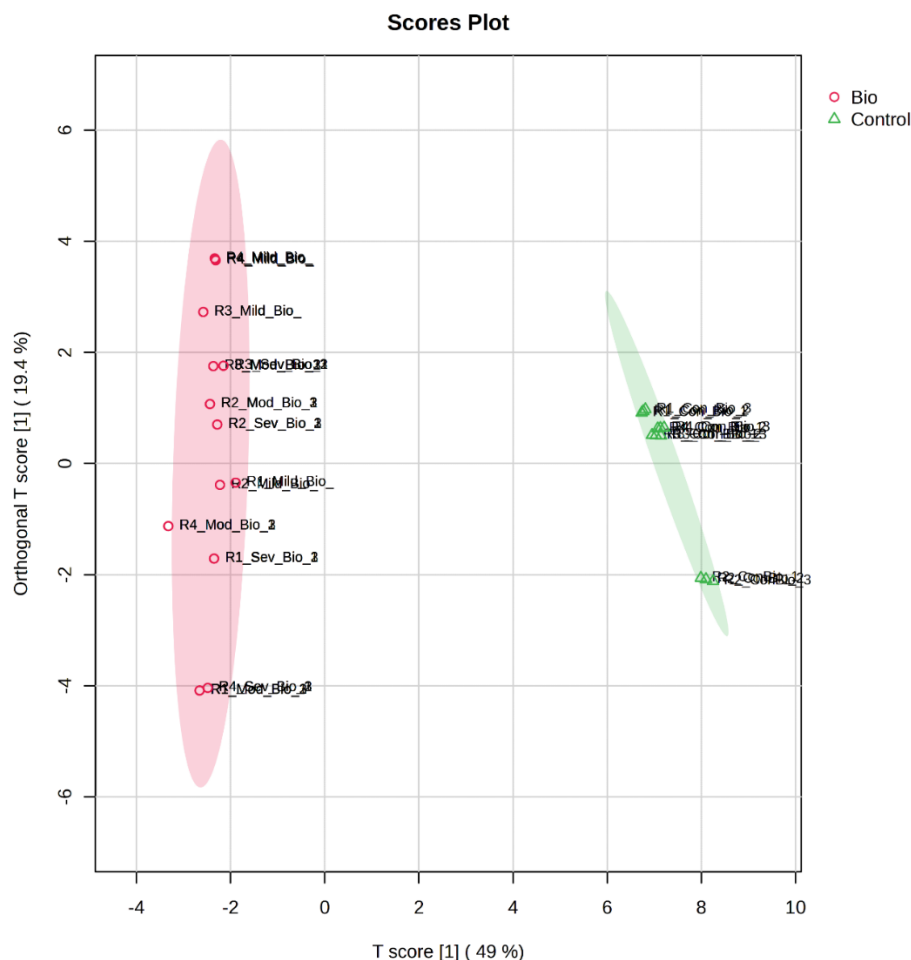


Figure 4.16: Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) score plot supervised modelling displaying the phenolic compounds groupings found in curly lettuce treated with varying doses of *Lucky Plant*[®] (0.5, 1.0, and 1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions. The legends: red circles and green triangles indicate data groupings. **Abbreviations:** R1_Con_Bio = Control (100% × 5 L × Intact plants) and Distilled water; R1_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and Distilled water; R1_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and Distilled water; R1_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and Distilled water; R2_Con_Bio = Control (100% × 5 L × Intact plants) and 0.5 g L⁻¹ *Lucky Plant*[®]; R2_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and 0.5 g L⁻¹ *Lucky Plant*[®]; R2_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and 0.5 g L⁻¹ *Lucky Plant*[®]; R2_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and 0.5 g L⁻¹ *Lucky Plant*[®]; R3_Con_Bio = Control (100% × 5 L × Intact plants) and 1.0 g L⁻¹ *Lucky Plant*[®]; R3_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and 1.0 g L⁻¹ *Lucky Plant*[®]; R3_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and 1.0 g L⁻¹ *Lucky Plant*[®]; R3_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and 1.0 g L⁻¹ *Lucky Plant*[®]; R4_Con_Bio = Control (100% × 5 L × Intact plants) and 1.5 g L⁻¹ *Lucky Plant*[®]; R4_Mild_Bio = Mild (60% × 1.25 L × Leaf wounded) and 1.5 g L⁻¹ *Lucky Plant*[®]; R4_Mod_Bio = Moderate (40% × 3.5 L × Leaf wounded) and 1.5 g L⁻¹ *Lucky Plant*[®]; R4_Sev_Bio = Severe (40% × 1.25 L × Leaf wounded) and 1.5 g L⁻¹ *Lucky Plant*[®].

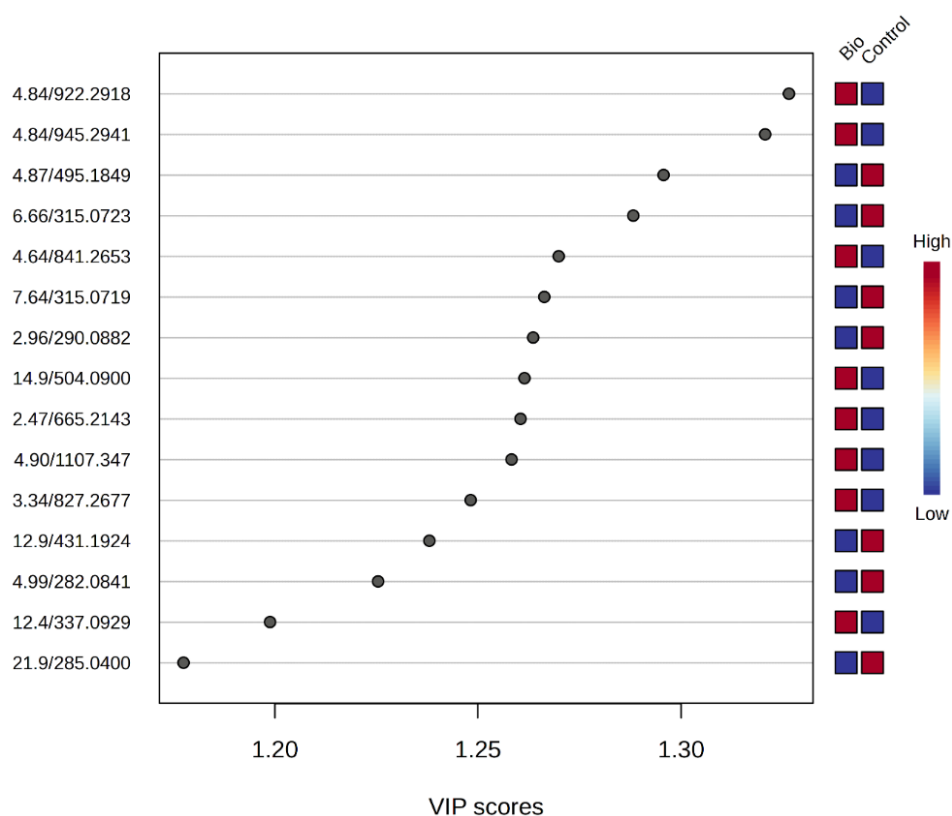


Figure 4.17: Variables Importance in Projection (VIP) scores in OPLS-DA assigned to phenolic compounds data found in curly lettuce treated with varying doses of *Lucky Plant*[®] (0.5, 1.0, and 1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions. Mini-coloured blocks on the right represent the relative content of each of the phenolic compounds. High levels are in red and low levels are in blue.

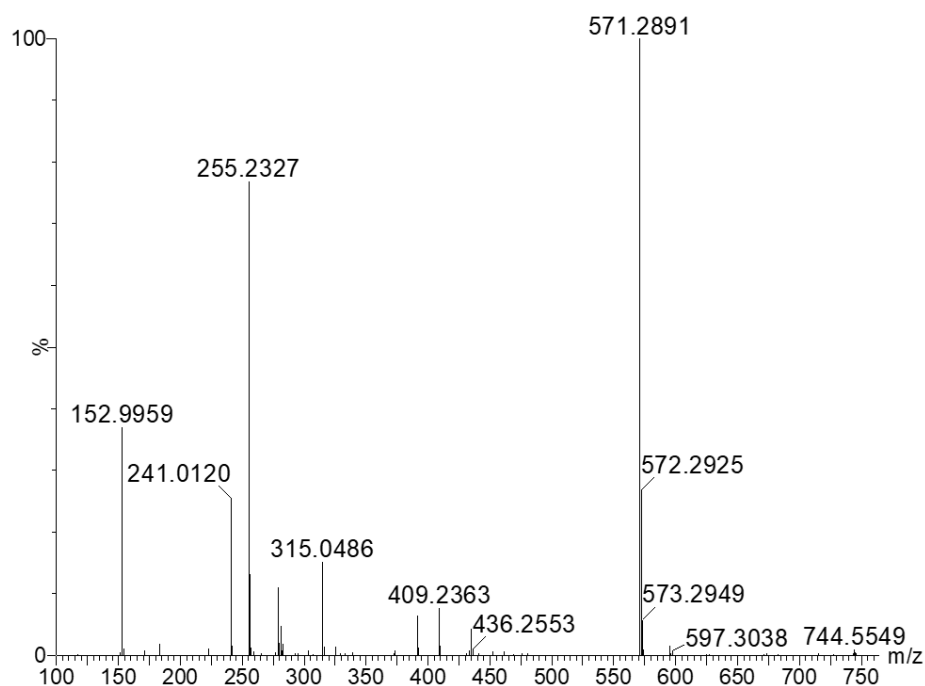


Figure 4.18: Mass spectrum of phenolic compounds data of curly lettuce treated with varying doses of *Lucky Plant*[®] (distilled water, 0.5, 1.0, and 1.5 g L⁻¹) foliar application under multistress (control, mild, moderate, and severe) conditions.

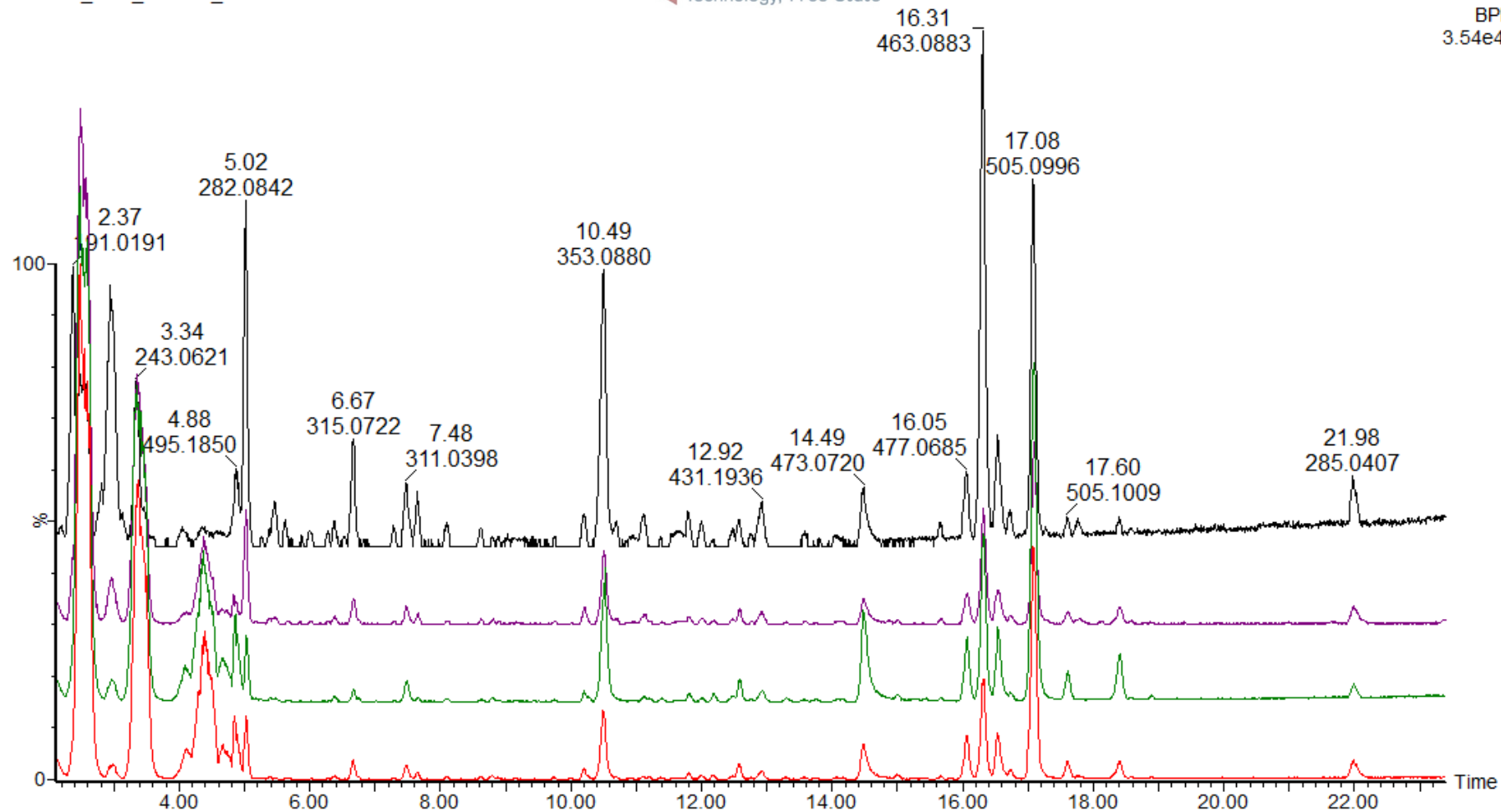


Figure 4.19: Total ions chromatogram of phenolic compounds data of curly lettuce not treated with *Lucky Plant*[®] biostimulant (distilled water) foliar application under multistress (control, mild, moderate, and severe) conditions. Black = R1_Con_Bio1 (control and distilled water); Purple = R1_Mild_Bio1 (mild and distilled water); Red = R2_Mod_Bio1 (moderate and distilled water); and Green = R1_Sev_Bio1 (severe and distilled water), R stands for replicate number.

CHAPTER 5

SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1 SUMMARY

Lettuce is an economically crucial vegetable with a high nutritional value that benefits human consumption. Like other horticultural crops, lettuce production is frequently exposed to individual and combined environmental stresses due to its sessile nature. Multi-abiotic stressors can substantially impact food security and disrupt the economic stability of agricultural crop producers. Multiple abiotic stress factors have been reported to reduce horticultural crop yields and quality by 50–70% worldwide. Plant tolerance to abiotic stresses depends on timing, duration, frequency, intensity, affected tissues, and phenological phases. The urgent need for crop management to find strategies to help horticultural crops cope with the adverse effects caused by multi-abiotic stress is vital for sustainable agriculture and improving horticultural crop production. Biostimulants are recognised as agronomic tools due to their beneficial effects on plants, including improving plant performance and tolerance to harsh environmental stresses (Mandal *et al.*, 2023). In the current study, plants conveyed different strategies for adapting to the adverse effects of abiotic stress, which resulted in poor plant performance. It was evident that when two or more abiotic stress factors occur, it leads to a severe decline in plant height, number of leaves, leaf area, chlorophyll content, stomatal conductance, nutrient acquisition, leaf fresh mass, dry mass, and high accumulation of phenolic content in leaves. The lack of scientifically validated studies to support the use of phytohormone-based biostimulants as an approach to enhance plant development, mitigate damage, and recover from abiotic stress. Phytohormones regulate the protective responses of plants against abiotic and biotic stress by either direct or crosstalk. Therefore, the current study was established to evaluate the recovery response of curly lettuce to multi-abiotic stress effects using phytohormone-based biostimulants as an alternative mitigation strategy. Indeed, the foliar application of a phytohormone-based biostimulant acts as a plant stress repair mechanism and counteracts abiotic stress. The *Lucky Plant*[®] biostimulant increased the number of leaves, chlorophyll content, stomatal conductance, and stomatal density under multi-abiotic stress conditions. Nonetheless, it promoted the uptake of essential nutrients and intensified the accumulation of phenolics in severely stressed plants.

5.2 SYNTHESIS

This chapter presents the synthesis of findings to briefly report on the two specific objectives of this study, which were comprehensively discussed in Chapters 3 and 4. The effects of multi-abiotic stress (water deficit, wounding, and plant bag size) on curly lettuce (*Lactuca sativa* L. var. Crispa) yield and morphophysiological and biochemical parameters was the first

objective. This was followed by the effects of foliar application of phytohormone-based biostimulant in varying doses on yield and the recovery response of curly lettuce (*Lactuca sativa* L. var. Crispa) grown under extreme multi-abiotic stress.

5.2.1 Effects of multi-abiotic stress on curly lettuce yield and morphophysiological and biochemical parameters

The current study obtained the best morphology results at 100% irrigation water regime and 5 L plant bag and intact plant as control treatments with the highest plant height, number of leaves, leaf area, fresh mass, and dry mass. Physiology showed differences with the high chlorophyll content at 40% water deficit regime on intact plants. Meanwhile, stomatal conductance was higher in the control treatments. The combination with 60% or 40% water regimes and 1.25 L plant bags showed strong negative impacts on morphophysiological parameters. In contrast, these same treatments showed increased Zn and Na content. In addition, the PCA biplot Na showed an increment under low water supply in small plant bags (3.5 L and 1.25 L). Despite the mechanical wounding treatments, the leaf mineral balance of curly lettuce was achieved at 100% and 80% water deficit and 5 L plant bag.

The water deficit regimes at 60% and 40% stress intensity emerged as a key factor observed in multivariate analysis, which showed a high accumulation of phenolic content in leaves for osmotic adjustment, as shown by the increase in chicoric acid, chlorogenic acid 5CQA, caftaric acid, chlorogenic acid 3CQA and 4"-O-Acetylmyricitrin phenolic compounds known to be biomarkers of stress-responsive compounds. This study revealed plants' complex responses to multi-abiotic stress as the performance of most parameters was reduced. Furthermore, it was found that adequate performance can be maintained using 40% less water in 5 L plant bags during the lettuce plant cycle, allowing a substantial amount of water to be saved in agriculture.

5.2.2 Foliar application of phytohormone-based biostimulant in varying doses on yield and the recovery response of curly lettuce (*Lactuca sativa* L. var. Crispa) grown under extreme multi-abiotic stress

It has been demonstrated that the application of phytohormone-based biostimulants can alleviate the detrimental effects of individual abiotic stress, but there is limited information on extreme multi-abiotic stress. The foliar application of *Lucky Plant*[®] significantly influenced the morphophysiological and biochemical aspects of curly lettuce. The curly lettuce showed a good response to foliar application of *Lucky Plant*[®] at a dose of 1.0 g L⁻¹ and 1.5 g L⁻¹, with higher

values registered under control. The beneficial effect of *Lucky Plant*[®] biostimulant was evident in severe conditions that reported higher values in the increase in the number of leaves. Regarding other morphological parameters like plant height, leaf area, fresh mass, and dry mass, no significant differences were reported following the foliar application of *Lucky Plant*[®]. Interesting results were obtained in physiological parameters, including stomatal conductance, chlorophyll, and stomatal density.

According to the analysis of variance, the foliar application of *Lucky Plant*[®] at a dose of 0.5 g L⁻¹, 1.0 g L⁻¹, and 1.5 g L⁻¹ enhanced the N content significantly under mild, moderate, and severe stress conditions. Furthermore, the PCA biplot revealed that *Lucky Plant*[®] significantly facilitated nutrient uptake and assimilation to leaves, as exhibited by increased P, K, Mg, Ca, Zn, and Mn contents under control compared to distilled water. In the multivariate analysis, significant separations were observed in phenolic content between control (R1_Con_Bio) and mild (R1_Mil_Bio), moderate (R1_Mod_Bio), and severe (R1_Sev_Bio) treatment levels received distilled water only as control. The further increase in extreme multistress levels reduced the accumulation gentisic acid 5-O-glucoside, kaempferol, caffeoylmalic acid, trifolin, quercetin 3-glucosyl-(1->2)-galactoside, and citroside A. Thus, the maximum contents were obtained at control and minimum levels in mild, moderate, and severe stress conditions irrespective of the foliar *Lucky Plant*[®] application at varying doses. A high content of quercetin-malonyl-glucoside, quercetin 3-O-glucuronide, and quercetin 3-galactoside was linked with 1.5 g L⁻¹ dose of *Lucky Plant*[®] under severe conditions. The VIP score plot showed that a dose of 1.0 g L⁻¹ increased 3-O-p-Coumaroylquinic acid under moderate stress conditions.

This study highlights the potential of phytohormone-based biostimulants to mitigate the effects of abiotic stresses (water deficit, plant bag size, and mechanical wounding) by acting on primary or secondary metabolism to improve plant capability to face adverse multi-abiotic conditions. The findings showed that the applied dose and level of multistress influenced the analysed parameters in the recovery response of curly lettuce to *Lucky Plant*[®] biostimulants. These are interesting results, particularly for emerging growers, to improve crop quality and achieve the desired yield under extreme multi-stress conditions.

5.3 IMPLICATIONS

The existing scientific research extensively focused on single abiotic stressors in different horticulture species. Hence, there is currently enough knowledge on the impacts of individual

abiotic stresses. The current study results show that coinciding multi-abiotic stressors are more detrimental to the yield, morphophysiological, and biochemical parameters of lettuce plants. The beneficial effects of the foliar application of the *Lucky Plant*[®] biostimulant were evident in the recovery and enhancement of morphophysiological and biochemical parameters of curly lettuce grown under extreme multi-abiotic stress conditions. However, not all morphological parameters were significantly recovered under moderate and severe multi-abiotic stress. In practice, these results suggest that integrating phytohormone-based biostimulants such as *Lucky Plant*[®] can be crucial in mitigating the detrimental effects of extreme multi-abiotic stress, improving the yield and inducing distinct tolerance in sustainable horticultural crop production. In the future, applying *Lucky Plant*[®] biostimulant at an initial stage of stress occurrence may be necessary for early stimulation and the long-term recovery of stressed plant mechanisms.

5.4 RECOMMENDATIONS

Based on the findings of the current study, the following key recommendations are proposed to offer alternative management practices to the challenges posed by multi-abiotic stresses in lettuce and related plant species:

- Lettuce cultivation can achieve maximum productivity using a 5 L plant bag, irrigating with 40% (120 mL) in root media bulk density of approximately 0.18 g cm⁻³ under a greenhouse environment. It is recommended that this be adopted to conserve the available water resources with limited irrigation while achieving a satisfactory yield.
- The foliar application of *Lucky Plant*[®] at a dose of 1.0 g L⁻¹ and 1.5 g L⁻¹ can increase lettuce's growth, quality, and yield under stress and optimal growing conditions.
- The study encourages investigating different application methods, like applying directly to root media via fertigation and drenching to facilitate quick uptake of the biostimulant.
- Further research in the open field will be necessary to assess the efficiency of the foliar application of *Lucky Plant*[®] in the recovery of plants and mitigation of the negative effects of multi-abiotic stress.

5.5 CONCLUSION

The study comprehensively assessed the adverse effects of water deficit stress, mechanical wounding, and plant bag sizes as simultaneous multi-abiotic stress on the yield,

morphophysiological and biochemical parameters of lettuce plants, and their recovery response to the *Lucky Plant*[®] phytohormone-based biostimulant. The study concludes that the curly lettuce subjected to multiple stress combinations showed impaired growth, inhibited physiology, unbalanced mineral nutrient contents, intensified secondary metabolites, and reduced yield. The research further demonstrated that an appropriate selection of plant bag size plays a critical role in minimising water losses and optimising the growth, yield, and quality of lettuce. Thus, 5 L plant bags emerged as the best-performing option for lettuce cultivation in this context. Foliar application of *Lucky Plant*[®] biostimulant is an effective mitigation strategy for enhancing the number of leaves, stomatal conductance, stomatal density, chlorophyll, leaf nutrient composition, and phenolic compounds counteracting multi-abiotic stress. However, *Lucky Plant*[®] at low and high doses did not significantly recover nearly all variables of yield and morphology, except the number of leaves. However, *Lucky Plant*[®] generated a slight increment in nearly all variables of yield and morphology compared to control. Notably, the findings present crucial information for sustainable agriculture, prioritising solutions for multi-abiotic stress conditions to protect food security.

Table 5.1: Reporting the null hypothesis for the study: “Morphophysiological and biochemical recovery response of lettuce (*Lactuca sativa* L. var. Crispa) to multistress and the application of a phytohormone-based biostimulant”.

Objectives	Null hypothesis	Implication
Effects of multi-abiotic stress (water deficit, wounding, and plant bag size) on curly lettuce (<i>Lactuca sativa</i> L. cv. Crispa) on yield and morphophysiological and biochemical parameters.	Rejected	The test was significant at most levels.
Effects of phytohormone-based biostimulant concentrations on yield and the recovery of lettuce (<i>Lactuca sativa</i> L.) grown under extreme multi-abiotic stress.	Rejected	The test was significant at most levels.

APPENDIX

Published part of the article extracted from Chapter 1



Phytohormone-Based Biostimulants as an Alternative Mitigating Strategy for Horticultural Plants Grown Under Adverse Multi-Stress Conditions: Common South African Stress Factors

Zenzile Khetsha^{1*}, Elmarie Van Der Watt², Maxson Masowa^{1,3}, Lesetja Legodi¹, Sanelisiwe Satshi¹,
Lethabo Sadiki¹ and Kenoni Moyo¹

¹Department of Agriculture, Faculty of Health and Environmental Sciences, Central University of
Technology, Free State, Bloemfontein, South Africa; ²Soil, Crop and Climate Sciences, Faculty of
Natural and Agricultural Sciences, University of the Free State, Bloemfontein, South Africa; ³Agricultural
Research Council-Vegetable, Industrial and Medicinal Plants, Pretoria, South Africa

*Corresponding author: zkhetsha@cut.ac.za

Abstract

Worldwide, it has been recorded extensively that plants are subjected to severe abiotic and biotic stressors. The scientific research community has widely reported that multi-abiotic stressors cause horticultural crop losses, accounting for at least 50 to 70% of the crop yield and quality losses. Therefore, this review focused on the detrimental effects caused by abiotic stress factors occurring in single-, combined- and multi-cell stresses on horticultural plants worldwide, along with the best production systems practices for mitigation during and post-single and combined abiotic or multi-stress damages. A conclusion and recommendation could be reached using the pool of research material, which constituted research articles, reviews, book chapters, thesis, research short communications and industrial short communications from at least twenty-five years ago. Findings showed that some of the leading abiotic stresses are single- and combined abiotic stressors like water deficit, salinity, soil pH, phosphate deficiency, wounding, soil density and pot size. Established commercial and smallholder farmers are globally adapting to plant growth regulators and biostimulants as part of their production systems. However, as much as the effectiveness of biostimulants containing humic acids, algal extracts, plant growth-promoting microorganisms and phytohormones has been reported to promote plant development under multi-stress, only a few studies are focusing on organic phytohormone-based biostimulants on horticultural crops grown under adverse multi stress factoring. In conclusion, the review recommends alternative solutions for emerging South African farmers and growers who cannot afford agricultural insurance options and energy alternatives on the common single- and combined abiotic- or multi-stress-factors.

Keywords: abiotic stress; moisture stress; plant growth regulators; salinity stress; wounding stress

Cite this as: Khetsha, Z., Van Der Watt, E., Masowa, M., Legodi, L., Satshi, S., Sadiki, L., & Moyo, K. (2024). Phytohormone-Based Biostimulants as an Alternative Mitigating Strategy for Horticultural Plants Grown Under Adverse Multi-Stress Conditions: Common South African Stress Factors. *Caraka Tani: Journal of Sustainable Agriculture*, 39(1), 167-193. doi: <http://dx.doi.org/10.20961/carakatani.v39i1.80530>

INTRODUCTION

Environmental factors such as salinity, drought, cold, moisture stress, and a variety of biotic stresses, including herbivory, are estimated to lower plant growth and crop yield by up to 50%

worldwide (Khetsha, 2020; Habib et al., 2021; Turan et al., 2021; Ramzan and Younis, 2022). According to Franzoni (2020), only 3.5% of the worldwide geographical area is unaffected by

* Received for publication November 19, 2023
Accepted after corrections February 29, 2024

Recovery response mechanisms of horticultural plants to multi-stress: plant-based biostimulants as a potential alternative strategy for lettuce (*Lactuca sativa* L.) in South Africa

S. Satshi¹, M.M. Masowa^{1,2}, E. Van Der Watt³ and Z.P. Khetsha^{1,a}

¹Central University of Technology, Free State, Bloemfontein, South Africa; ²Agricultural Research Council - Vegetable, Industrial and Medicinal Plants, Pretoria, South Africa; ³University of the Free State, Bloemfontein, South Africa.

Abstract

Plants are subjected to severe abiotic and biotic stresses worldwide. Global warming and climate change are increasing water scarcity and water-saving strategies, especially in coastal agricultural lands and arid and semi-arid regions, including South Africa. A wide range of horticultural crops encounter single or combined environmental stresses such as water deficit, substrate bulk density, potting size, and wounding stress throughout the production cycle, resulting in significant yield losses. Under these abiotic stress conditions plant physiology and biochemical regulations are governed by a complex network of interactions in hormonal pathways crosstalk between the different phytohormones. Plant-based biostimulants have been widely reported to sustainably improve nutrient use efficiency, seed germination rate, crop performance, yield, plant nutritional quality, fresh produce shelf life, and tolerance against multiple environmental stresses. Therefore, this short review explores the effects of these adverse multi-stress conditions on horticultural plants, as well as the recovery responses to the use of plant-based biostimulants as an alternative strategy. Lastly, this review also provides scientific morphophysiological responses of lettuce (*Lactuca sativa* L.) to the use of plant-based biostimulants as a potential strategy.

Keywords: abiotic stress, horticultural crops, pot size, water deficit, wounding, bulk density

INTRODUCTION

Horticultural crops are exposed to abiotic and biotic environmental stresses worldwide. Abiotic stresses are caused by inanimate factors such as drought, heat, cold, heavy metals, salinity, and mechanical damage. In contrast, biotic stress is caused by animated creatures such as herbivores, bacteria, diseases, and weeds (Clément et al., 2023). Environmental stresses affect plants at all development stages, from seed germination to growth and reproduction, causing severe crop yield losses and threatening food security globally. Abiotic and biotic stresses constrain agricultural production, with an estimation to lower crop yield globally by up to 70% annually (Ali et al., 2020).

Hence, achieving Sustainable Development Goal Two of a world with zero hunger by 2030, set by the United Nations in 2015 (Persaud and Dagher, 2021), remains challenging. As predicted by the Food and Agricultural Organization of the United Nations (FAO) (2023), hunger affected 691 million to 783 million people in 2022 around the world. On the other hand, the world is under pressure to urgently increase agricultural productivity to meet the food demands of the rising population. The United Nations (2022) estimated the current world population to be 8.1 billion and is expected to reach 9.7 billion by 2050. Commercial and emerging farmers encounter abiotic and biotic stresses such as water deficit, mechanical damage, insect herbivory, root media bulk density, and pot size occurring alone or simultaneously combined, altering the morphophysiological and biochemical attributes of

^aE-mail: zkhetsa@cut.ac.za

