

The Beneficial effects of *Trichoderma harzianum* T-22 in barley (*Hordeum vulgare* L.) roots under salt stress

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By

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Thesis abstract

Soil salinity is an important problem that impacts agriculture globally. A sustainable approach for improving productivity is to adopt beneficial microorganisms to enhance the supply of soil nutrients to plants in stressful environments. Our work is showing that the fungus *Trichoderma harzianum* T-22 enhances barley growth and nutrient uptake in saline conditions. The fungus symbiotically lives inside the roots and triggers beneficial biochemical and metabolic changes. This project has broad implications for applying beneficial plant-microbe interactions to improve agricultural productivity.

Chapter 1 covers the history and performance of endophytic fungi applied to crops as an alternative or supplement to the use of plant genetics or soil management to alleviate salt stress in crops. We focus primarily on root-associated microorganisms. The root-soil zone is the first point of defence for the plant against salt moving into the plant with the transpiration stream. It has dynamic biogeochemical processes driven by diverse metabolites released by the plant root and associated soil microorganisms. Fungal endophytes associated with some crops not only protect against plant pathogens and pests but also impart strong tolerance against several abiotic stresses in crops, including salinity. This is achieved via inducing systemic resistance, increasing the levels of metabolites such as pathogen protectants and osmolytes, activating antioxidant systems to prevent damage caused by ROS, and modulating plant growth phytohormone levels. Colonization by endophytic fungi improves nutrient uptake and maintains ionic homeostasis by modulating ion accumulation, thereby restricting the transport of Na^+ to leaves and ensuring a low cytosolic $\text{Na}^+:\text{K}^+$ ratio in plants. This literature review has been submitted after external review to the journal *Plant and Soil* for publication. Following there is an addendum (**Section 1.6**) covering investigations and application of the endophyte-plant interactions using in metabolomics and lipidomics. The addendum aims to provide an overview of the necessity to investigate plant-fungal interactions and its influence on plant metabolism. Lastly, the chapter gives a brief description of the main experimental, technical and instrumental methods employed in this project.

Chapter 2 determines the effect of the salt tolerant beneficial endophyte, *Trichoderma harzianum* strain T-22, on the growth and development of barley under optimal and saline conditions. Two barley genotypes were used in the study, cv. Vlamingh as a salt tolerant cultivar and cv. Gairdner as a salt sensitive cultivar. Barley was chosen as it is not only an agriculturally and industrially important crop but is also the most salt tolerant cereal crop. Two experimental setups were used for this study. In the first experiment, agar was used as the growth medium for plants. This was performed to observe and determine the effect

of fungus on the growth of plants under controlled conditions. In the subsequent experiment, sandy loam soil was used as the matrix to grow plants and to emulate agricultural scenarios. Light microscopy was used to confirm the association of the fungus within roots. The results confirm the positive effect of this fungus under controlled and saline conditions in both experiments. Various parameters were measured to confirm the effects of salt and fungus on both genotypes under controlled and saline conditions. This study suggests that inoculation of salt sensitive plants with *T. harzianum* T-22 may ameliorate the effects of salinity and improve plant growth.

Chapter 3 describes the role of *Trichoderma harzianum* T-22 in alleviating NaCl-induced stress in two barley genotypes (cv. Vlamingh and cv. Gairdner) by mapping metabolites and lipids using GC-MS for polar metabolites and LC-MS for lipids. This was performed to provide insights into the biochemical changes in barley roots treated with fungus during the early stages of interaction. *T. harzianum* increased the root length of both genotypes under controlled and saline conditions. The fungus reduced sugars in both genotypes and caused no changes in organic acids under saline conditions. Amino acids decreased only in cv. Gairdner in fungal-inoculated roots under saline conditions. Triacylglycerols (TAGs) were the substantially increased lipids in inoculated roots of both genotypes under saline conditions. This study shows that the fungus imparts adaptation or tolerance mechanism to cv. Vlamingh and in cv. Gairdner by remodelling lipids mainly from glycerolipids after salt stress.

Chapter 4 examined the role of *Trichoderma* strain T-22 on two barley genotypes (cv. Vlamingh and cv. Gairdner) grown in saline soil as soil is an important substrate for *Trichoderma*. Biomass results described in Chapter 2 of this thesis clearly show the positive effect of this fungus on both genotypes under control and saline conditions as measured using several physiological parameters. Here, the aim of this part of study was to determine the metabolites and lipids modified in roots grown in saline soil following endophyte inoculation that are involved in conferring positive effects on barley plants as mentioned in **Chapter 2**. We employed gas chromatography and liquid chromatography both coupled to mass spectrometry to analyse metabolites and lipids in inoculated and uninoculated roots of both genotypes under control and saline conditions.

Chapter 5 explains the application of mass spectrometry imaging (MSI), liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QToF-MS), inductively coupled plasma mass spectrometry (ICP-MS), and X-ray fluorescence (XRF) to determine the spatial distribution of metabolites, lipids and a range of elements, such as K⁺ and Na⁺, in seeds of two barley genotypes with contrasting germination phenology (Australian

barley varieties Mundah and Keel). This chapter was published in Front Plant Sci (Gupta et al. 2019). Following this, an addendum (**Section 5.10**) is added to this chapter where the effect of *T. harzianum* was measured on germination of four barley genotypes used in this project. The results showed that the fungus improves germination efficiency of the sensitive genotypes, suggesting its role in reducing the adverse effects of salt stress on germination and growth of the plant.

The final **Chapter 6** describes the application and importance of mapping the biochemical changes involving metabolites and lipids, imparted due to the inoculation of the fungus in roots of barley seedlings and provides a view on future perspectives of research of plant-fungal interactions for plant growth promotion which can be useful for sustaining agricultural productivity.

Declaration

This is to certify that

- (i) The thesis comprises only my original work, except where indicated in the Preface,
- (ii) due to acknowledgement has been made in the text to all other materials used,
- (iii) the thesis is less than 100 000 words in length, exclusive of tables, maps, bibliographies and appendices

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Preface

The candidate has contributed to more than 50% of the content of this thesis and is the primary author of all publications derived from this thesis.

The growth of the plants including the salt treatment, harvesting, extraction and analysis were carried out by the candidate. Dr Thusitha Rupasinghe contributed to the interpretation of LC-MS based lipidomics analysis. The elemental profiling of seminal roots by ICP-MS and analysis of data was performed by Dr Damien Callahan. Dr Allison Van de Meene provided help in the use of light microscopy and physiological analysis of roots.

Project supervisors Prof Ute Roessner, Assoc. Prof Penelope Smith and Dr. Siria Natera were involved in the design of all experiments, and supervision of the candidate.

All authors discussed the results and their implications and provided feedback on the manuscripts at all stages.

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friend, my soul-mate, my partner as the strongest support system and always be by my side. Nimesh is the only person who can appreciate my quirkiess, my jitters and sense of humour. There are no words to convey how much I love him. He has been a true inspiration, a great supporter and has unconditionally loved me during my good and bad times. He has been non-judgmental of me and instrumental in instilling confidence. These past several years have been a tough journey, both academically and personally. I truly thank Nimesh for sticking by my side, even when I was irritable and miserable at times. I am sure that what we both have learned a lot about life and reinforced our assurance and fortitude to each other thus thriving a colourful life we can cherish forever

Publications

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Presented in Chapter 1

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Presented in Chapter 5

Abbreviations

Abbreviation	Full name
°C	Celsius degrees
(Put, diamine)	Putrescine
(Spd, triamine)	spermidine
(Spm, tetramine)	Spermine
μ-XRF	Micro-Xray fluorescence
0h	Zero hour
AA	Ascorbic Acid
ABA	Abscisic Acid
ACar	Acyl Carnitines
ACN	Anthracnose
ADGGA	Acyl Diacylglycerol Glucuronide
AMDIS	Automated Mass Spectral Deconvolution and Identification System
AMF	Arbuscular Mycorrhizal Fungi
ANOVA	Analysis of Variance
APX	Ascorbate Peroxidases
AsA	Ascorbate
ASH-GSH	Ascorbate-Glutathione
ATCC	American Type Culture Collection
AUC	Area Under the Curve
BFTFA	Bis-(trimethylsilyl)-Trifluoroacetamide
BHT	butylated hydroxytoluene
BPA	Bioplatforms Australia
BRs	Brassinosteroids
C	Control
CAT	Catalase
CE	Ceramides
Cer-AP	Ceramide alpha-hydroxy fatty acid-phytospingosine,
Cer-NDS	Ceramide non-hydroxyfatty acid-dihydrosphingosine
Cer-NS	Ceramide non-hydroxyfatty acid-sphingosine
CF	Control + Fungus
CFU	Colony-forming unit
CKs	Cytokinins
CPT	cholinephosphotransferase
CPT	CDP-choline:DAG cholinephosphotransferase
cv	Cultivar
DAG	Diacylglycerol

DEG	Differentially expressed genes
DGAT	Acyl-CoA:DAG acyltransferase
DGDG	Digalactosyldiacylglycerol
DHAR	Dehydroascorbate Reductase
DNA	Deoxyribonucleic Acid
EC	Electrical Conductivity
ECC	Extracted Compound Chromatogram
EI	Electron Impact
ENC	Endophytic Colonized
ERM	Ericoid Mycorrhizal
ESI-QToF-MS	Electrospray ionization (ESI) quadrupole time-of-flight (QTOF) mass spectrometry (MS)
ESP	Exchangeable Sodium Percentage
ETHY	Ethylene
FA	Fatty Acid
FAS	Fatty acid synthesis
FDR	False Discovery Rate
G3P	Glycerol-3-Phosphate
G3P	Glycerol-3-phosphate
GA	Gibberellins
GC	Gas chromatography
GC	Gairdner Control
GCF	Gairdner Control + Fungus
GLs	Glycerolipids
GPAT	Acyl acyl-CoA:G3P acyltransferase
GPs	Glycerophospholipids
GR	Glutathione Reductases
GS	Gairdner Salt
GSF	Gairdner Salt + Fungus
GSH	Glutathione
HCA	Hierarchical Cluster Analysis
HexCer-AP	Hexosylceramide alpha-hydroxy fatty acid- phytospingosine
HKT1	High Affinity Potassium Transporter 1
IAA	Indole Acetic Acid
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
ILCNC	International Lipid Classification and Nomenclature Committee
IST	Induced Systemic Tolerance
KEGG	Kyoto Encyclopedia of Genes and Genomes
kV	Kilovolts

LC	Liquid Chromatography
LC-QToF-MS	Liquid chromatography quadrupole time-of-flight mass spectrometry
LPA	Lyso-Phosphatidic Acids
LPAT	Acyl-CoA:LPA acyltransferase
LPAT	Acyl-CoA:LPA acyltransferase
LPC	Lysophosphatidylcholines
m/z	Mass to charge ratio
MAG	Monoacylglycerol
MALDI	Matrix-Assisted Laser Desorption Ionization
mbar	Millibar
MDA	Malondialdehyde
MDHAR	Monodehydroascorbate reductase
MGDG	Monogalactosyldiacylglycerol
mL	Milliliter
mM	Millimolar
MS	Mass Spectrometry
MSI	Mass Spectrometry Imaging
NCRIS	National Collaborative Research Infrastructure Scheme
NENC	Non-Endophytic Colonized
NIST	National Institute of Standards and Technology
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
OCT	Optimal Cutting Temperature
P5CS	Pyroline-5-Carboxylate Synthase
PA	Polyamines
PA	Phosphatidic Acid
PAP	PA phosphatase
PBQC	Pooled Blank Quality Control
PC	Phosphatidylcholine
PCA	Principle Component Analysis
PDA	Potato Dextrose Agar
PDCT	PC:DAG cholinephosphotransferase
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PGPM	Plant Growth Promoting Microorganisms
PI	Phosphatidylinositol
PI-Cer	Ceramide Phosphoinositol
PK	Polyketides
PM	Plasma Membrane
POD	Peroxidase
ppb	Parts per billion
ppm	Parts per million

PS	Phosphatidylserine
PSD	Phosphatidylserine decarboxylase
PSS	Phosphatidylserine synthase
Rh	Rhodium
RLD	Root Length Density
RMS	Root Mean Square
RNA	Ribonucleic acid
RNA-Seq	RNA Sequencing
ROC	Receiver operating characteristic
ROS	Reactive Oxygen Species
RT	Retention Times
RT-qPCR	Quantitative reverse transcription PCR
S	Salt
S/N	Single to Noise
SAR	Sodium Adsorption Ratio
SCEM	Super Cryo Embedding Medium
SF	Salt + Fungus
SL	Sphingolipids
SOD	Superoxide Dismutase
SQDG	Sulfoquinovosyldiacylglycerol
SRL	Seminal root length
ST	Sterol lipids
T-22	<i>Trichoderma harzianum</i>
TAGs	Triacylglycerols
Ta-N13	Trichoderma isolate
TCL	Target Component Library
TIC	Total Ion Count
TOF	Time-of-Flight
TRL	Total Root Length
TXD	<i>Trichoderma lixii</i> ID11D
UDP	Uridine 5' -diphosphate
US	United States
v/v	Volume/volume
VC	Vlamingh Control
VCF	Vlamingh Control + Fungus
VIC	Victoria
Vpp	Voltage peak to peak
VS	Vlamingh Salt
VSF	Vlamingh Salt + Fungus
XRF	X-ray Fluorescence

Table of Contents

Thesis abstract	ii
Declaration.....	v
Preface	vi
Acknowledgements	vii
Publications.....	ix
Abbreviations.....	x
Table of Contents	xiv
List of Tables	xix
List of Figures	xxi
List of Supplemental Tables.....	xxxv
List of Supplemental Figures.....	xxxviii
1. Literature Review	1
1.1 Soil salinity affects agriculture globally.....	3
1.2 Effects of salt stress on above-ground and below-ground organs of plants.....	4
1.3 The root-soil interface/ The rhizosphere.....	5
1.4 Alleviating salt stress by association with microorganisms.....	6
1.4.1 Beneficial effects due to plant root interactions with endophytic fungi.....	6
1.5 Mechanisms of salt tolerance in ENC plants.....	9
1.5.1 Increase in total biomass.....	9
1.5.2 Alteration in root architecture.....	9
1.5.3 Osmoregulation.....	10
1.5.3.1...Proline.....	11
1.5.3.2...Sugars.....	14
1.5.3.3...Organic acids.....	15
1.5.3.4...Polyamines.....	15
1.5.4 Nutrient acquisition and ionic homeostasis.....	16
1.5.4.1...Phosphorus.....	18
1.5.4.2...Nitrogen.....	18
1.5.4.3...Na ⁺ :K ⁺ ratio.....	19
1.5.5 Oxidative stress.....	20
1.5.6 Photosynthesis.....	24
1.5.7 Hormonal regulation.....	27
1.5.8 Perspectives and future directions.....	27
1.5.9 Acknowledgements.....	29
1.6 Addendum.....	30
1.6.1 Metabolomics and lipidomics as tools to determine the biochemistry of root-microbe interactions.....	30
1.6.2 Significance of this Thesis.....	32
1.6.3 Aims of this Thesis.....	33
2. The role of <i>Trichoderma harzianum</i> T-22 in plant growth and development under controlled and soil environment.....	36
2.1 Introduction.....	37
2.2 Materials and Methods.....	39
2.2.1 Seeds used in this study.....	39

2.2.2	Fungal isolate and growth conditions used in this study.....	39
2.2.3	Seed preparation.....	39
2.2.4	Fungal inoculant preparation.....	39
2.2.5	Determining fungal association with roots using brightfield microscopy.....	40
2.2.5.1	Fungal root interactions on agar medium.....	40
2.2.5.1.1.	Agar medium total root length analysis.....	42
2.2.5.1.2.	Fungal root interactions in soil medium.....	43
2.2.5.1.3.	Soil medium shoot and root length, weight and chlorophyll content determination.....	44
2.3	Results.....	45
2.3.1	Light microscopy.....	45
2.3.2	Root architecture.....	45
2.3.2.1	Effect of fungus and salt on physiological parameters in barley grown on agar.....	45
2.3.2.2	Effect of fungus and salt stress on seminal root length of cv. Vlamingh and cv. Gairdner.....	49
2.3.3	Root and shoot dry weight.....	51
2.3.3.1	Root to shoot ratio.....	51
2.3.4	Chlorophyll content.....	55
2.4	Discussion.....	56
2.5	Conclusion.....	58
3.	Investigation of the biochemical changes in two barley genotypes inoculated with a beneficial fungus <i>Trichoderma harzianum</i> T-22 under salt stress.....	59
3.1	Introduction.....	61
3.2	Materials and Method.....	65
3.2.1	Plant Materials and growth conditions.....	65
3.2.2	Fungal isolates and growth conditions.....	65
3.2.3	Material and chemicals.....	65
3.2.4	Plant growth conditions and inoculation with <i>T. harzianum</i>	65
3.2.5	Staining and visualization of roots using Light Microscopy.....	66
3.2.6	GCMS untargeted analysis for polar metabolites.....	67
3.2.7	LCMS untargeted analysis for lipid analysis.....	68
3.2.8	Data preprocessing and statistical analysis.....	68
3.3	Results.....	70
3.3.1	Fungal association within roots confirmed using microscopy.....	70
3.3.2	Plant growth and root development.....	70
3.3.3	The effect of inoculation with <i>T. harzianum</i> and salt stress to polar metabolites in roots.....	70
3.3.4	Global untargeted lipid profiling of barley roots.....	77
3.3.5	Significant differences in the lipid profiles under saline conditions.....	81
3.3.6	Targeted MS/MS lipid analysis.....	82
3.3.7	Salt stress induced changes in GPs and GLs in cv. Vlamingh and cv. Gairdner.....	88
3.3.7.1	Salt stress strongly affects GP lipids in cv. Vlamingh.....	88
3.3.7.2	Salt stress strongly affects GL levels in both genotypes.....	89
3.3.8	Changes in Glycerophospholipids (GP) and Glycerolipids (GL) in fungal inoculated roots of cv. Vlamingh and cv. Gairdner under control conditions.....	94
3.3.8.1	<i>T. harzianum</i> affected more PCs in cv. Vlamingh and more PEs in cv. Gairdner.....	94
3.3.8.2	Increased glycerolipids in inoculated roots of cv. Gairdner under control conditions.....	94
3.3.9	Effect of salt stress and fungal inoculation on GP and GL profiles in cv. Vlamingh and cv. Gairdner.....	95
3.3.9.1	<i>T. harzianum</i> changed PCs and PEs under saline conditions.....	95
3.3.9.2	Triglycerols - the class of lipids most affected by <i>T. harzianum</i> under saline conditions.....	95

3.4	Discussion.....	96
3.4.1	Total Root length is a good indicative root trait to measure plant growth.....	97
3.4.2	Fungus changes osmolytes in roots as a protective adaptation to salt stress.....	97
3.4.3	Alterations in the lipid profiles in response to salt stress.....	100
3.4.3.1...	Changes in membrane phospholipids in response to salt stress.....	100
3.4.3.2...	Changes in membrane phospholipids in inoculated roots of both genotypes under control and saline conditions.....	101
3.4.3.3...	Alterations in membrane glycolipids in response to salt stress.....	102
3.4.3.4...	Alterations in membrane glycolipids in inoculated roots of both genotypes under control and saline conditions.....	103
3.4.3.5...	Changes in membrane neutral lipids after salt stress.....	104
3.4.3.6...	Changes in membrane neutral lipids in inoculated roots of both genotypes under control and saline conditions.....	105
3.5	Conclusion.....	106
4.	Biochemical changes in two barley genotypes inoculated with a beneficial fungus <i>Trichoderma harzianum</i> Rifai T-22 grown in saline soil.....	108
4.1	Introduction.....	110
4.2	Materials and Methods.....	112
4.2.1	Plant Material and growth conditions.....	112
4.2.2	Fungal isolates and growth conditions.....	112
4.2.3	Material and chemicals.....	112
4.2.4	Plant growth conditions and inoculation with <i>T. harzianum</i>	112
4.2.5	Shoot and root length, weight and chlorophyll content determination.....	113
4.2.6	GCMS untargeted analysis for polar metabolites.....	113
4.2.7	LCMS untargeted analysis for lipid analysis.....	114
4.2.8	Data preprocessing and statistical analysis.....	114
4.3	Results.....	115
4.3.1	Effect of fungus and salt stress on seminal root length of cv. Vlamingh and cv. Gairdner.....	115
4.3.2	Root and shoot dry weight.....	116
4.3.3	Root to shoot ratio.....	117
4.3.4	Chlorophyll content.....	117
4.3.5	The effect of inoculation with <i>T. harzianum</i> and salt stress on polar metabolites in roots.....	117
4.3.6	Untargeted lipid profiling of barley roots.....	127
4.3.7	Targeted MS/MS lipid analysis.....	130
4.3.7.1...	Salt stress induced changes in cv. Vlamingh and cv. Gairdner.....	135
4.3.7.2...	The effect of fungus in roots of cv. Vlamingh and cv. Gairdner under control conditions.....	140
4.3.7.3...	Changes in lipids in roots of cv. Vlamingh and cv. Gairdner due to the inoculation of fungus under saline conditions.....	140
4.3.7.3.1	<i>T. harzianum</i> changed glycerophospholipids and glycerolipids under saline conditions.....	140
4.4	Discussion.....	142
4.4.1	Changes in osmolytes in roots of cv. Vlamingh and cv. Gairdner under salt stress.....	144
4.4.2	Changes in polar metabolites in inoculated roots of cv. Vlamingh and cv. Gairdner under non-saline conditions.....	145
4.4.3	Changes in polar metabolites in inoculated roots of cv. Vlamingh and cv. Gairdner after salt stress.....	145
4.4.4	Changes in the lipid profiles in response to salt stress.....	147

4.4.5	Salt stress resulted in higher changes in membrane phospholipids in cv. Vlammingh as compared to cv. Gairdner.....	148
4.4.6	Changes in membrane phospholipids in inoculated roots of both genotypes under control and saline conditions.....	149
4.4.7	Changes in membrane neutral lipids after salt stress.....	150
4.4.8	Changes in membrane neutral lipids in inoculated roots of both genotypes under control and saline conditions.....	151
4.5	Conclusion.....	152
5.	Spatio-Temporal Metabolite and Elemental Profiling of Salt Stressed Barley Seeds During Initial Stages of Germination by MALDI-MSI and μ -XRF Spectrometry.....	154
5.1	Introduction.....	157
5.2	Material and methods.....	159
5.2.1.	Plant material.....	159
5.2.2.	Seed germination and sample collection.....	159
5.2.3.	Chemicals.....	160
5.2.4.	MALDI-MSI: Sample preparation and imaging.....	160
5.2.5.	MALDI-MSI: Analysis, data processing, detection and annotations.....	161
5.2.6.	ICP-MS: Sample preparation and analysis.....	162
5.2.7.	μ -X-ray fluorescence analysis.....	163
5.2.8.	LC-QToF-MS: Sample preparation and analysis for flavonoid detection.....	163
5.2.9.	LC-QToF-MS:Data processing and analysis.....	164
5.3	Results.....	164
5.3.1.	Overview of experimental workflow.....	164
5.3.2.	Germination assay: Germination and seedling growth under salt stress.....	164
5.3.3.	MALDI-MSI of lipids in barley seeds during germination.....	167
5.3.4.	Lipids detected immediately after imbibition and after 8 hours of salt stress.....	171
5.3.5.	Spatial segmentation of the MALDI-MSI data using an unsupervised clustering approach using SCiLS Lab.....	172
5.3.6.	Confirmation of tentative flavonoid using MS/MS.....	175
5.3.6.1...	MALDI-MS/MS.....	175
5.3.6.2...	LC-MS/MS.....	176
5.3.7.	Level of tentative flavonoid increases in response to salinity stress in four out of seven barley genotypes.....	177
5.3.8.	Elemental concentrations in seeds.....	177
5.4	Discussion.....	181
5.5	Conclusions.....	186
5.6	Acknowledgements.....	186
5.7	Author Contributions Statements.....	186
5.8	Conflict of Interest Statement.....	186
5.9	Contribution to the Field Statement.....	187
5.10	Addendum.....	188
5.10.1.	Introduction.....	188
5.10.2.	Materials and methods.....	189
5.10.2.1...	Plant Material.....	189
5.10.2.2...	Seed germination and Sample Collection.....	189
5.10.2.3...	Fungal inoculant preparation.....	190
5.10.3.	Results.....	190
5.10.4.	Discussion.....	192

5.10.5 Conclusion.....	193
6. General Discussion.....	194
6.1 Introduction.....	195
6.2 <i>Trichoderma harzianum</i> T-22 – a bioagent for seed priming and growth of cereal crops under salt stress.....	197
6.3 Osmolytes are greatly affected due to inoculation of the fungus after salt stress.....	198
6.4 Lipid modifications in inoculated barley roots in response to salt stress.....	200
6.5 Conclusion and Future perspectives.....	201
7. Appendices.....	205
8. References.....	216

List of Tables

Table 1.1	Examples of studies on effects of salt stress and endophytic fungi on osmotic regulation in plants	13
Table 1.2	Examples of studies on the effects of salinity and endophytic fungi on nutrient concentration and ionic ratios in plants	17
Table 1.3	Examples of studies on the effects of salinity and endophytic fungi on lipid peroxidation in plants. Abbreviations in the table stand for: CAT- catalase, GR- glutathione reductase, SOD- superoxide dismutase, APX- ascorbate peroxidase, ABA- abscisic acid, MDA- malondialdehyde, SA- salicylic acid, POD- peroxidase	23
Table 1.4	Examples of studies on the effects of salinity and endophytic fungi on photosynthesis in plants	26
Table 2.1	Percentage increase in total root lengths, as compared in three treatments in two barley genotypes, cv. Vlamingh and cv. Gairdner (n=3 per treatment group)	48
Table 2.2	Percentage increase in seminal root length, as compared in three treatments in two barley genotypes, cv. Vlamingh and cv. Gairdner.	50
Table 2.3	Physiological parameters measured in cv. Vlamingh and cv. Gairdner under four treatment conditions. Root and shoot fresh weight were measured immediately after harvest and dry weights of both were measured after drying in oven at 75 oC for 48 h. Chlorophyll content was measured using SPAD meter. n = 6 for all treatments and results are presented as mean $\pm$ standard error for all except root:shoot ratio. Asterisks denote significance for $P < 0.05$ (one asterisk) and $P < 0.01$ (two asterisks) as determined by Student's t-test	54
Table 3.1	Summary of the number of identified lipid species from roots of two barley genotypes (cv. Vlamingh and cv. Gairdner) from four treatment groups control C, control inoculated with fungus CF, salt (200 mM NaCl) S and salt inoculated with fungus SF. Analysis was performed using LC-TripleTOF-MS (positive ionization mode)	85
Table 3.2	Number of Glycerophospholipids as defined by Student's t-test ($P < 0.05$, FDR) with a 2-fold change or greater, found for two barley genotypes in three treatments	87

Table 3.3	Number of Glycerolipids as defined by Student's t-test ($P < 0.05$, FDR) with a 2-fold change or greater, found for two barley genotypes in three treatments	87
Table 4.1	Summary of the number of identified lipid species from roots of two barley genotypes (cv. Vlammingh and cv. Gairdner) from four treatment groups- control C, control inoculated with fungus CF, salt (200 mM NaCl) S and salt inoculated with fungus SF. Analysis was performed using an LC-TripleTOF-MS (positive ionization mode).	133
Table 4.2	Number of significantly altered lipid species ($P < 0.05$) with ≥ 1.5 fold-changes in relative abundance between the three treatment group comparisons for two barley genotypes	135
Table 5.1	Percentage of individual lipid classes in two treatments of Mundah and Keel. The values are total percentages of annotated lipids at all timepoints	170
Table 5.2	Theoretical and observed m/z for the tentative flavonoid, Gancaonin F from MALDI-MS/MS and LC-MS/MS	176
Table 5.3	Theoretical and observed m/z for the tentative flavonoid, Gancaonin F from MALDI-MS/MS and LC-MS/MS	180
Table 5.4	Germination assay of four barley genotypes at four time points under different salt (NaCl) concentrations. Twenty seeds were germinated for each time point and treatment. The number shown is the number of seeds that germinated (Color gradients represent the decline in the number of seeds germinated- black is twenty seeds germinated, darker grey represents nineteen seeds germinated, dark grey represents eighteen seeds germinated, grey represents seventeen seeds germinated and light grey represents sixteen seeds germinated)	191

List of Figures

- | | | |
|------------|---|----|
| Figure 1.1 | <p>Potential beneficial effects of root colonisation of plants by endophytic, symbiotic fungi in saline soil conditions, summarised from the literature. Salinity results in reduced root biomass due to salt-induced inhibition of cell division and affect the total biomass yield (1) (left). Plant colonized with endophytic fungi improves biomass accumulation by modifying root architecture and increased nutrient absorption (1a) (right). Salt accumulation creates competition for nutrient uptake and transport. This results in imbalance of the ionic composition of plant, affecting plant's physiological traits (2) (left). Endophytic fungi improve expression of genes and upregulate several cation transporters, resulting in improved nutrient uptake and maintenance of ionic homeostasis (2a) (right). Increase of salt in soil lowers soil water potential resulting in cellular dehydration (3) (left). Endophytic fungi negate this effect by mediating accumulation of osmolytes consequently improving plant's water status (3a) (right). Increasing salinity causes oxidative stress due to imbalance in reactive oxygen species generation and quenching activities of antioxidants (4) (left). Endophytic fungi improve the antioxidant systems of plants reducing oxidative stress under salt stress (4a) (right). Salt stress hinders photosynthesis by reduced uptake of magnesium and decreasing chlorophyll concentration which eventually reduces carbon dioxide supply to RuBisCo (5) (left). Endophytes has a positive effect on photosynthesis under salt stress (5a) (right). See text for relevant references and further details.</p> | 7 |
| Figure 1.2 | <p>Salinity stress induced osmotic stress tolerance mechanisms in plants. Increase in salt in soil lowers the soil water potential of plant cells. This reduces water uptake by plants and consequently causes cellular dehydration (1) (left). To combat this issue, plants accumulate osmolytes, such as proline, sugars, polyamines in higher concentration. Osmolytes accumulation results in lowering of cellular water potential and maintains a favourable gradient for water uptake from soil to roots. Endophytic fungi alleviate osmotic stress by influencing the expression of specific genes, P5CS, pyrroline-5-carboxylate synthase (1a) (right), involved in the biosynthesis of osmolyte proline, activation of starch degrading enzyme, glucan-water dikinase (1b) (right) and forming tripartite symbiosis with roots and rhizobia (1c) (right) to elevate the accumulation of sugars and by increasing the biosynthesis of polyamines such as spermidine and spermine (1D) (right). See text for relevant references and further details.</p> | 11 |

Figure 1.3	Oxidative stress tolerance mechanisms in salt stressed plants. Increase in salinity causes oxidative stress in plants due to redox imbalance between ROS (reactive oxygen species) and antioxidants. This results in molecular and cellular damage and membrane peroxidation eventually disturbing the normal functioning of the cell. Several enzymatic antioxidants pathways are induced to combat salt stress that include catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR). Ascorbate (AsA), glutathione (GSH), carotenoids are non-enzymatic antioxidants that are produced to counteract the adverse consequences of salt stress. In endophytic colonized (ENC) plants, the tolerance mechanism is reinforced by activating an efficient antioxidant system that abates the oxidative damage caused due to salt stress.	21
Figure 2.1	Flow chart of experimental setup for plant growth conditions and inoculation with fungus.	41
Figure 2.2	Experimental steps for first experiment of the study. A, 48 hours germinated seedlings of barley genotype cv. Vlammingh; B, Micro hydroponics system used for interaction between fungus and roots; C, 20 × 20 cm petri plates with agar media use	42
Figure 2.3	Images showing growth of plants for second experiment of the study. Images are captured at, A, 3 days; B, 6 days; C, 9 days; and D, 18 days from sowing	44
Figure 2.4	Light microscopy images of barley genotype cv. Gairdner over seven days. A1-G1, control barley roots; A2-G2, roots inoculated with <i>T. harzianum</i> T-22 (1×10^8 CFU g ⁻¹ of seedling). Orange color arrows in D2 represent fungal spores; yellow arrows in E2, F2, and G2 represent fungal hyphae. Scale = 0.1 mm. n = 3	46
Figure 2.5	WinRHIZO root scans of barley roots for cv. Vlammingh and cv. Gairdner genotypes. A- Vlammingh C, B- Vlammingh CF, C- Vlammingh S, D-Vlammingh SF; E- Gairdner C, F- Gairdner CF, G- Gairdner S, H- Gairdner SF	47

Figure 2.6	Total root length of barley roots of two genotypes, (A) cv. Vlamingh and (B) cv. Gairdner in four treatments for plants grown on agar plates. X axis represent treatments and Y axis represent lengths in cm. n = 3. Three color bars above graphs represent different comparisons: black indicates comparisons between salt and control, light brown indicates comparisons between control + fungus and control and cyan green indicates comparisons between salt + fungus and salt. Asterisks denote significance for $P < 0.05$ (one asterisk) and $P < 0.01$ (two asterisks) as determined by Student's t-test.	48
Figure 2.7	Seminal root length of barley roots of two genotypes, cv. Vlamingh and cv. Gairdner under four treatment conditions for seedlings grown in soil. X axis represent treatments and Y axis represent lengths in cm. n = 6 for both genotypes. Three colour bars above graphs represent different comparisons: black indicates comparisons between salt and control, light brown indicates comparisons between control + fungus and control and cyan green indicates comparisons between salt + fungus and salt. Asterisks denote significance for $P < 0.05$ (one asterisk) and $P < 0.01$ (two asterisks) as determined by Student's t-test	50
Figure 2.8	Averages and std errors of showing fresh and dry weight of roots of barley genotypes cv. Vlamingh (A) and cv. Gairdner (B) under four treatment conditions. X axis represent the four treatment conditions and Y axis represent weights in grams. n = 6 for both genotypes. Asterisks denote significance for $P < 0.05$ (one asterisk) and $P < 0.01$ (two asterisks) as determined by Student's t-test. Blue color indicates significant reduction and orange color indicates significant increase	53
Figure 2.9	Leaf chlorophyll content of two barley genotypes cv. Vlamingh and cv. Gairdner measured using SPAD meter. X axis represent the four treatments and Y axis represent chlorophyll index. Three colour bars above graphs represent different comparisons: black indicates comparisons between salt and control, light brown indicates comparisons between control + fungus and control and cyan green indicates comparisons between salt + fungus and salt. Asterisks denote significance for $P < 0.01$ (two asterisks) as determined by Student's t-test.	55

Figure 3.1	Principal Component Analysis (PCA) and heatmaps of four treatments in cv. Vlamingh and cv. Gairdner. a- PCA of Control- C, Control + fungus- CF, Salt- S and Salt + fungus- SF in cv. Vlamingh; b- PCA of Control- C, Control + fungus- CF, Salt- S and Salt + fungus- SF in cv. Gairdner; c and d- heatmap obtained from hierarchical cluster analysis (HCA) of all identified metabolites in cv. Vlamingh and cv. Gairdner, respectively. The legend on the bottom indicates the sample groups and legends on the right are the identified metabolites.	72
Figure 3.2	Logarithmic ratios of representative sugars, organic acids and amino acids in roots of barley cv. Vlamingh (V) and cv. Gairdner (G) of salt grown compared to control grown (VS/VC and GS/GC); control with fungus compared to control grown (VCF/VC and GCF/GC); and salt with fungus compared to salt grown (VSF/VS and GSF/GS). Values that are significantly higher ($P < 0.05$, FDR) are indicated by *. A threshold of ± 2 -fold change is indicated by a dashed line.	74
Figure 3.3	Metabolome and pathway view of alanine, aspartate and glutamate metabolism for metabolites with 2-fold change or greater in roots of barley cv. Vlamingh and cv. Gairdner under four treatments a- Metabolome view showing all matched pathways arranged by p values on Y-axis and pathway impact values on X-axis. The node colour is based on its p value and the node radius is determined on their pathways impact values. b- Pathway view showing current metabolic pathway achieved by clicking the corresponding node on the metabolome view as given in 3a. Blue colour indicates default node and matched nodes are given in red colour. The compound names can be obtained via mouse over tool-tip.	76
Figure 3.4	Pathway coverage graph for metabolites with 2-fold change or greater in roots of barley cv. Vlamingh and cv. Gairdner under four treatments. Top ten biochemical pathways that were affected are shown on X-axis and percentage of coverage is given on Y-axis	77

- Figure 3.5 Heatmap showing the relative abundances of detected lipid species in samples from four treatments in two barley genotypes. Heatmap represents unsupervised hierarchical clustering of lipid species (rows). The row displays identified lipid species and the column represents the samples. Relative lower abundance of lipids in samples was displayed in blue, whilst relative higher abundance of lipids was displayed in red. Clustering was performed using Euclidean distance matrices on in the R package heatmap. VC- control treated cv Vlamingsh; VCF- control and fungus treated cv Vlamingsh, VSF- salt and fungus treated cv Vlamingsh; GC- control treated cv Gairdner; GCF- control and fungus treated cv Gairdner, GSF- salt and fungus treated cv Gairdner. n = 6 for all treatments. salt- 200mM NaCl. 79
- Figure 3.6 Principle component analysis (PCA) of the mass features (m/z_RT) extracted from lipid profiles of dissected roots of two barley genotypes grown under control and saline conditions with and without fungus. PC 1 versus PC 2 displays the separation between control and salt treatment for cv. Vlamingsh (A) and cv. Gairdner (B). VC- control treated cv. Vlamingsh; VCF- control and fungus treated cv. Vlamingsh, VSF- salt and fungus treated cv. Vlamingsh; GC- control treated cv. Gairdner; GCF- control and fungus treated cv. Gairdner, GSF- salt and fungus treated cv. Gairdner. n = 6 for all treatments. salt= 200 mM NaCl 80
- Figure 3.7 Venn diagram summary of the number of significantly altered features identified by one-way ANOVA and post-hoc analysis of the lipid profiles from roots of two barley genotypes under salt stress and with and without fungal inoculation. The number represent significantly altered features regardless of their directionality (upregulation and downregulation). Numbers appearing in overlapped sections are common between treatments. Four analysis performed are Vlamingsh salt (VS) compared to Vlamingsh control (VC), Vlamingsh salt and fungus (VSF) compared to Vlamingsh salt (VS), Gairdner salt (GS) compared to Gairdner control (GC) and Gairdner salt and fungus (GSF) compared to Gairdner salt (GS). 82

Figure 3.8	Principle component analysis (PCA) of the identified lipids from dissected roots of two barley genotypes grown under control and saline conditions with and without fungus. PC 1 versus PC 2 displays the separation between control and salt treatment for cv. Vlamingh (A) and cv. Gairdner (B). VC- control treated cv. Vlamingh; VCF- control and fungus treated cv. Vlamingh, VSF- salt and fungus treated cv. Vlamingh; GC- control treated cv. Gairdner; GCF- control and fungus treated cv. Gairdner, GSF- salt and fungus treated cv. Gairdner. n = 6 for all treatments. salt =200 mM NaCl.	84
Figure 3.9	Total percentage of identified lipid species in each lipid class in roots of two barley genotypes grown under control and saline conditions with and without fungus. Major classes identified were the phospholipids- phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), triacylglycerol (TAG), diacylglycerol (DAG), monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), acyl diacylglycerol glucuronide (ADGGA) and ceramides. A total of 271 lipids were identified and number for each class is given with the corresponding percentage	86
Figure 3.10	Logarithmic ratios of representative phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) content in roots of barley cv. Vlamingh and cv.Gairdner of salt grown (200 mM NaCl) compared to control grown; control with fungus grown compared to control grown; salt and fungus grown compared to salt grown. Values that are significantly higher ($P < 0.05$, FDR) are indicated by *. A threshold of ± 4 -fold change is indicated by a dashed line.	90
Figure 3.11	Logarithmic ratios of representative phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (LPE) content in roots of barley cv. Vlamingh and cv. Gairdner of salt grown (200 mM NaCl) compared to control grown; control with fungus grown compared to control grown; salt and fungus grown compared to salt grown. Values that are significantly higher ($P < 0.05$, FDR) are indicated by *. A threshold of ± 4 -fold change is indicated by a dashed line.	91

Figure 3.12	Logarithmic ratios of representative acyl diacylglyceryl glucuronide (ADGGA), monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), monoacylglycerol (MAG) and diacylglycerol (DAG) content in roots of barley cv. Vlammingh and cv. Gairdner of salt grown (200 mM NaCl) compared to control grown; control with fungus grown compared to control grown; salt and fungus grown compared to salt grown. Values that are significantly higher ($P < 0.05$, FDR) are indicated by *. A threshold of ± 4 -fold change is indicated by a dashed line.	92
Figure 3.13	Logarithmic ratios of Triacylglycerol (TAG) content in roots of barley cv. Vlammingh and cv. Gairdner of salt grown (200 mM NaCl) compared to control grown; control with fungus grown compared to control grown; salt and fungus grown compared to salt grown. Values that are significantly higher ($P < 0.05$, FDR) are indicated by *. A threshold of ± 4 -fold change is indicated by a dashed line.	93
Figure 3.14	PC-derived TAG synthesis. Brown arrows indicate reactions involved in de novo DAG1 synthesis. Red arrows indicate reactions involved in PC-derived DAG2 synthesis. Enzymatic reactions are embedded in green boxes: A- modification of FA esterified to PC, B- reversible CPT or phospholipase C or phospholipase D/PAP DAG production. CPT- CDP-choline:DAG cholinephosphotransferase, DGAT- acyl-CoA:DAG acyltransferase, FAS- fatty acid synthesis, LPAT- acyl-CoA:LPA acyltransferase, GPAT- acyl acyl-CoA:G3P acyltransferase, PDCT- PC:DAG cholinephosphotransferase, PAP- PA phosphatase. Substrates are DAG- diacylglycerol, G3P- glycerol-3-phosphate, LPA- lyso-phosphatidic acids, PA- phosphatidic acid. Figure adapted from Bates (2012)	101
Figure 3.15	De novo TAG synthesis using the Kennedy pathway. Brown arrows indicate reactions involved in de novo DAG1 synthesis. Enzymatic reactions are embedded in green boxes: A- FA elongation, DGAT- acyl-CoA:DAG acyltransferase, FAS- fatty acid synthesis, LPAT- acyl-CoA:LPA acyltransferase, GPAT- acyl acyl-CoA:G3P acyltransferase, PAP- PA phosphatase. Substrates are DAG- diacylglycerol, G3P- glycerol-3-phosphate, LPA- lyso-phosphatidic acids, PA- phosphatidic acid. Figures adapted from Bates (2012)	105

Figure 4.1	Principal Component Analysis (PCA) score plot for two barley genotypes - cv. Vlamingh and cv. Gairdner from four treatments - control, control + fungus, salt, and salt+ fungus - and quality control samples. Abbreviations: G- cv. Gairdner, V- cv. Vlamingh, C- control treated roots, S- Salt treated roots (200 mM NaCl), CF- control + fungus, SF- salt + fungus, PBQC- pooled biological quality control samples	118
Figure 4.2	Principal Component Analysis (PCA) score plot and Heatmaps for cv. Vlamingh (a and c) and cv. Gairdner (b and d) under four treatments- C- control treated roots, S- Salt treated roots (200 mM NaCl), CF- control + fungus, SF- salt + fungus. The fungal inoculated samples (VCF and VSF) separate from the uninoculated samples (VC and VS) in cv. Vlamingh on component 1 (PC1). The salt treatment contributed to distinct separation on PC2 in cv. Vlamingh. In cv. Gairdner, PC1 clearly separates treatment GS from the other three treatments (GCF, GSF and GC). The heatmaps represent unsupervised hierarchical clustering of lipid species (rows). The row displays identified lipid species and the column represents the samples. Relative lower abundance of metabolites is displayed in blue, whilst relative higher abundance of metabolites are displayed in red. Clustering was performed using Euclidean distance matrices on in the R package heatmap.	120
Figure 4.3	Logarithmic ratios of representative sugars, organic acids and amino acids in roots of barley cv. Vlamingh (V) of salt grown compared to control grown (VS/VC); control with fungus compared to control grown (VCF/VC); and salt with fungus compared to salt grown (VSF/VS). Values that are significantly different ($P < 0.05$, FDR) are indicated by *. A threshold of ± 4 -fold change is indicated by a dashed line.	122
Figure 4.4	Logarithmic ratios of representative sugars, organic acids and amino acids in roots of barley cv. Gairdner (G) of salt grown compared to control grown (GS/GC); control with fungus compared to control grown (GCF/GC); and salt with fungus compared to salt grown (GSF/GS). Values that are significantly higher ($P < 0.05$, FDR) are indicated by *. A threshold of ± 4 -fold change is indicated by a dashed line.	123

- Figure 4.5 Overview of pathway analysis, corresponding pathway view and significantly changed compound - L-glutamate in cv. Vlamingh treatment control + fungus (CF) compared to control (C). (a): metabolome view- pathway impact is given on X-axis and log (p value) on Y-axis. The pathway impact is calculated as the sum of the importance measures of the matched metabolites normalized by the sum of the importance measures of all metabolites in each pathway. The node colour is based on its p value and the node radius is determined based on their pathway impact values. Red colour indicates the most significant pathway and light-yellow colour indicates least significant pathway. (b): Corresponding pathway view for node colour given in yellow. Alanine, aspartate and glutamate metabolism is highlighted by pointing and clicking on hyperlinked nodes (yellow colour above in 'a'). (c): Single metabolite node. L-glutamate is highlighted as an example to compare its concentration between CF and C in cv. Vlamingh. 125
- Figure 4.6 Metabolome view for all identified metabolites of barley cv. Vlamingh (V) and cv. Gairdner (G) of salt grown compared to control grown (VS/VC-a and GS/GC-d); control with fungus compared to control grown (VCF/VC-b and GCF/GC-e); and salt with fungus compared to salt grown (VSF/VS-c and GSF/GS-f). Metabolome view shows all matched pathways arranged by p values on Y-axis and pathway impact values on X-axis. The node colour is based on their p value and the node radius is determined on their pathways impact values. 126
- Figure 4.7 Principle Component Analysis (PCA) and heatmap of the mass features (m/z_RT) extracted from lipid profiles from roots of two barley genotypes (cv. Vlamingh and cv. Gairdner) grown under control and saline conditions, with and without fungus. 11a- PCA showing scores plot for detected m/z_RT for Control- C, Control + fungus- CF, Salt- S and Salt + fungus- SF in cv. Vlamingh and cv. Gairdner; 11b- heatmap obtained from hierarchical cluster analysis (HCA) of all detected m/z_RT features in cv. Vlamingh and cv. Gairdner. The labels on the bottom indicate the sample groups. 128

Figure 4.8	Venn diagram summary of the number of significantly altered features identified by one-way ANOVA and post-hoc analysis of the lipid profiles from roots of two barley genotypes under salt stress and with and without fungal inoculation. The number represent significantly altered features regardless of their directionality (upregulation and downregulation). Numbers appearing in overlapped sections are common between treatments. Four analysis performed are cv. Vlamingh salt (VS) compared to cv. Vlamingh control (VC), cv. Vlamingh salt and fungus (VSF) compared to cv. Vlamingh salt (VS), cv. Gairdner salt (GS) compared to cv. Gairdner control (GC) and cv. Gairdner salt and fungus (GSF) compared to cv. Gairdner salt (GS).	129
Figure 4.9	Principle component analysis (PCA) of the identified lipids from roots of two barley genotypes grown under control and saline conditions, with and without fungus. VC- control treated cv. Vlamingh; VCF- control and fungus treated cv. Vlamingh, VSF- salt and fungus treated cv. Vlamingh; GC- control treated cv. Gairdner; GCF- control and fungus treated cv. Gairdner, GSF- salt and fungus treated cv. Gairdner. PC 1 versus PC 2 shows separation between VSF from other groups. n = 6 for all treatments. PC2 failed to show any separation between treatments or genotypes	131
Figure 4.10	Principle component analysis (PCA) of the identified lipids from roots of two barley genotypes grown under control and saline conditions, with and without fungus. VC- control treated cv. Vlamingh; VS- salt treated cv. Vlamingh; VCF- control and fungus treated cv. Vlamingh; GC- control treated cv. Gairdner; GS- salt treated cv. Gairdner; GCF- control and fungus treated cv. Gairdner, GSF- salt and fungus treated cv. Gairdner. PC 1 versus PC 2 shows the separation between SF and other groups in cv. Vlamingh (a). PCA failed to show any clear separation in cv. Gairdner (b). n = 6 for all treatments.	132

- Figure 4.11 Volcano plots showing the three treatment group comparisons from roots of two barley genotypes, cv. Vlamingh and cv. Gairdner grown under control and saline conditions, with and without fungus. VC- control treated cv. Vlamingh; VS- salt treated cv. Vlamingh; VCF- control and fungus treated cv. Vlamingh, VSF- salt and fungus treated cv. Vlamingh; GC- control treated cv. Gairdner; GS- salt treated cv. Gairdner; GCF- control and fungus treated cv. Gairdner, GSF- salt and fungus treated cv. Gairdner. The horizontal axis plots the fold change between the two groups (on a log scale), while the vertical axis represents the $P < 0.05$ for a t-test of differences between samples (on a negative log scale). The black dots represent non-significant lipids whereas pink dots represent significantly changed lipids. 134
- Figure 4.12 Logarithmic ratios of representative phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) content in roots of barley cv. Vlamingh and cv. Gairdner of salt grown (200 mM NaCl) compared to control grown; control with fungus grown compared to control grown; salt and fungus grown compared to salt grown. Values that are significantly different ($P < 0.05$) are indicated by *. A threshold of ± 2 -fold change is indicated by a black dashed line and a threshold of ± 4 -fold change is indicated by a red dashed line. $n = 6$ for all treatments 137
- Figure 4.13 Logarithmic ratios of representative phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS) content in roots of barley cv. Vlamingh and cv. Gairdner of salt grown (200 mM NaCl) compared to control grown; control with fungus grown compared to control grown; salt and fungus grown compared to salt grown. Values that are significantly different ($P < 0.05$) are indicated by *. A threshold of ± 2 -fold change is indicated by a black dashed line and a threshold of ± 4 -fold change is indicated by a red dashed line. $n = 6$ for all treatments 138

Figure 4.14	Logarithmic ratios of representative diacylglycerol (DAG) and triacylglycerol (TAG) content in roots of barley cv. Vlammingh and cv. Gairdner of salt grown (200 mM NaCl) compared to control grown; control with fungus grown compared to control grown; salt and fungus grown compared to salt grown. Values that are significantly different ($P < 0.05$) are indicated by *. A threshold of ± 2 -fold change is indicated by a black dashed line and a threshold of ± 4 -fold change is indicated by a red dashed line. $n = 6$ for all treatments	139
Figure 5.1	Overall workflow for MALDI-MSI, ICP-MS and LC-MS analysis of barley seeds. Figure modified from (Sarabia et al, 2018).	166
Figure 5.2	Average mass spectrum between m/z 780-890 acquired from barley seed cv. Mundah after 24 hours of germination. Each peak illustrates a specific lipid species (or isotope) that can be selected to display the distribution of the lipid across the section. The images obtained at $50 \times 50 \mu\text{m}$ raster size display the different spatial distribution of the lipids across different timepoints, different treatments and between the two genotypes. To ensure the reproducibility of detection of the lipids, three independent seed sections were analysed for each mass spectrometry imaging experiment. Distribution of the m/z 782.5466 PC(36:4) signal in sections of control (a) and salt treated (b) seeds at 24 hours are shown in the inset panel. Data was normalized using Root Mean Square.	168
Figure 5.3	Different lipid species displaying different spatial distribution. A: scanned image of Mundah seed at 48 hours in control conditions; B: scanned image of Mundah seed at 48 hours in salt conditions; A-I and B-I: m/z 496.338 LPC(16:0) showing spatial distribution only in endosperm of both seeds with different intensities in control and salt conditions. A-II and B-II: m/z 757.574 PA(40:2) showing spatial distribution in entire seed with different intensities in control and salt treated seed; A-III and B-III: m/z 820.530 PC(36:4) showing spatial distribution in the aleurone layer and embryo of control and salt treated seeds. Intensity scale for both images was set to 0-50%. Data was normalized using Root Mean Square.	169

- Figure 5.4 A flavonoid (m/z 365.102) with AUC close to 0.8 at 16, 24 and 48 hours in Mundah embryos and at 72 hours in Keel embryos. A, B and C: scanned images of Mundah seeds at 16, 24 and 48 hours of salt treatment; D: scanned image of Keel seed at 72 hours of salt treatment; A', B' and C': Embryo region showing high intensity of the flavonoid in Mundah salt treated seeds at 16, 24 and 48 hours; D': Embryo region showing high intensity of the flavonoid in Keel salt treated seed at 72 hours. Intensity scale was set to 0-10% for all seeds. Data was normalized using Root Mean Square. 174
- Figure 5.5 Combined MS and MS/MS spectra of m/z 365.1 $[M+H]^+$ derived from on tissue MALDI-MS/MS and LC-MS/MS respectively. A mass spectrum between 365.090-365.115 is shown in the figure. Two peaks, m/z 365.10243 (shown in bold) and m/z 365.10582 are clearly shown corresponding to a tentative flavonoid and dihexose respectively. Inset Figure 5A shows the mass spectrum analysed using LC-MSMS for a tentative flavonoid with retention time of 6.719 mins giving seven fragments. Three fragments (marked with asterisks) match with fragments obtained from MALDI-MS/MS given in Figure S6. Inset Figure 5B displays the mass spectrum for a dihexose sugar analysed using LC-MSMS with retention time of 4.49 mins and displaying two fragments- m/z 203.052 and 185.041. 175
- Figure 5.6 Change in the sodium concentrations identified by ICP-MS in Mundah and Keel seeds after 24 hours and 48 hours in control and salt treated conditions. Concentration of sodium mg g⁻¹/dry weight (n = 4). Scales over the bar graph indicates SDs. Asterisks on the top of each bar highlight the significance levels between two treatments at individual timepoints (*, P < 0.001). 178
- Figure 5.7 A protocol for seed priming with *Trichoderma harzianum* T-22. a – *Trichoderma harzianum* T-22 grown for seven days to obtain conidiophores, b – Priming of barley seeds with 1 x 10⁸ CFU ml⁻¹ conidia in 2 mL Eppendorf, c – Seeds germinating on petri plates with control and saline (250 mM NaCl) agar. 190

Figure 6.1 Summary for agar study and soil study from metabolomics results 202
given in **Chapter 3** and **Chapter 4** in salt and salt + fungus treatments
in cv. Vlamingh and cv. Gairdner. Arrows pointing up indicates
increase in the metabolites whereas arrows pointing downwards
indicate decrease in metabolites.

List of Supplemental Tables

- Table S1. Identified metabolites from roots of two barley genotypes (cv. Vlammingh and cv. Gairdner) using gas chromatography- mass spectrometry. X-fold changes for treatment groups CF and S are compared against treatment C in both genotypes. X-fold changes for treatment SF is compared against treatment S in both genotypes. The identified metabolites are categorised in five categories- amino acids, organic acids, sugars, others and unknowns
- Table S2. Table S2. Average normalized responses of identified metabolites in roots of two barley genotypes- cv. Vlammingh and cv. Gairdner. Normalization was performed using fresh frozen weights of all samples and internal standard
- Table S3. Table S3. Detected m/z_RT mass features using MSDIAL from roots of two barley genotypes (cv. Vlammingh and cv. Gairdner). Four treatment groups were analysed- control, control + fungus, salt and salt+ fungus
- Table S4. Table S4. Annotated lipids returned with matches from MSDIAL library in roots of two barley genotypes Four treatments were analysed for both genotypes- control, control + fungus, salt and salt + fungus
- Table S5. Table S5. Identified lipids with MS2 Spectra in roots of two barley genotypes- Vlammingh and Gairdner. Four treatments were analysed for both genotypes- control, control + fungus, salt and salt + fungus GC- Gairdner control treated roots, GCF- Gairdner control+fungus roots, GS- Gairdner salt treated uninoculated roots, GSF- Gairdner inoculated and treated with salt roots, VC-Vlammingh control treated roots, VCF- Vlammingh control+fungus roots, VS- Vlammingh salt treated uninoculated roots, VSF- Vlammingh inoculated and treated with salt roots
- Table S6. Table S6. Fold changes of identified PCs- phosphatidylcholines and LPCs- lysophosphatidylcholines from roots harvested in agar study from four treatments- C-Control, S- Salt, CF- Control+Fungus, SF- Salt+Fungus, Three comparisons were performed: S/C- salt vs control; CF/C- control+fungus/control; SF/S- salt+fungus/salt. V-Vlammingh, G-Gairdner
- Table S7. Table S7. Fold changes of identified PEs- phosphatidylethanolamines LPEs- lysophosphatidylethanolamines from roots harvested in agar study from four treatments- C-Control, S- Salt, CF- Control+Fungus, SF- Salt+Fungus, Three comparisons were performed: S/C- salt vs control; CF/C- control+fungus/control; SF/S- salt+fungus/salt. V-Vlammingh, G-Gairdner

- Table S8. Table S8. Fold changes of identified DAG- diacylglycerol, TAG- triacylglycerol, MAG- monoacylglycerol, MGDG- monogalactosyl diacylglycerol, DGDG- digalactosyl diacylglycerol, ADGGA- Acyl diacylglyceryl glucuronide, from roots harvested in agar study from four treatments- C-Control, S- Salt, CF- Control+Fungus, SF- Salt+Fungus, Three comparisons were performed: S/C- salt vs control; CF/C- control+fungus/control; SF/S- salt+fungus/salt. V-Vlamingh, G-Gairdner
- Table S9. Table S9. Fold changes of identified DAG- diacylglycerol, TAG- triacylglycerol, MAG- monoacylglycerol, MGDG- monogalactosyl diacylglycerol, DGDG- digalactosyl diacylglycerol, ADGGA- Acyl diacylglyceryl glucuronide, from roots harvested in agar study from four treatments- C-Control, S- Salt, CF- Control+Fungus, SF- Salt+Fungus, Three comparisons were performed: S/C- salt vs control; CF/C- control+fungus/control; SF/S- salt+fungus/salt. V-Vlamingh, G-Gairdner
- Table S10. Supplementray Table S10. List of identified and unknwon metabolites from GC-MS analyses in roots of cv. Vlamingh and cv. Gairdner from four treatments- control, control + fungus (*Trichoderma harzianum*), salt (200 mM NaCl) and salt + fungus
- Table S11. Supplementary Table S11. Identified metabolites from roots of two barley genotypes (cv. Vlamingh and cv. Gairdner) using gas chromatography- mass spectrometry. X-fold changes for treatment groups CF and S are compared against treatment C in both genotypes. X-fold chnages for treatment SF is compared against treatment S in both genotypes. The identified metabolites are categorised in five categories- amino acids, organic acids, sugars, others and unknowns
- Table S12. Supplementary Table S12. m/z_RT features detected using LC-ESI-QqTOF-MS analyses in positive ionization mode in roots of two barley genotypes (cv. Vlamingh and cv. Gairdner) under four treatments. GC- control roots in cv. Gairdner, GCF- inoculated roots of cv. Gairdner undert control conditions, GS- salt treated roots of cv. Gairdner, GSF- inoculated roots of cv. Gairdner under saline conditions, VC- control roots in cv. Vlamingh, VCF- inoculated roots of cv. Vlamingh undert control conditions, VS- salt treated roots of cv. Vlamingh, VSF- inoculated roots of cv. Vlamingh under saline conditions
- Table S13. Supplementary Table S13. Identified, annotated and unknown lipid species detected using LC-ESI-QqTOF-MS analyses in positive ionization mode in roots of two barley genotypes (cv. Vlamingh and cv. Gairdner) under four treatments. GC- control roots in cv. Gairdner, GCF- inoculated roots of cv. Gairdner undert control conditions, GS- salt treated roots of cv. Gairdner, GSF- inoculated roots of cv. Gairdner under saline conditions, VC- control roots in cv. Vlamingh, VCF- inoculated roots of cv. Vlamingh undert control conditions, VS- salt treated roots of cv. Vlamingh, VSF- inoculated roots of cv. Vlamingh under saline conditions; m/z- mass to charge ratio, RT- retention time, Category: Identified- lipid molecular species

with MS2 spectra, Annotated- lipid molecular species identified without MS2 spectra, Unknowns- mass features with no match

Table S14. Supplementary Table S14. Fold changes for all identified lipid species detected using LC-ESI-QqTOF-MS analyses in positive ionization mode in roots of two barley genotypes (cv. Vlamingham and cv. Gairdner) under four treatments. GC- control roots in cv. Gairdner, GCF- inoculated roots of cv. Gairdner under control conditions, GS- salt treated roots of cv. Gairdner, GSF- inoculated roots of cv. Gairdner under saline conditions, VC- control roots in cv. Vlamingham, VCF- inoculated roots of cv. Vlamingham under control conditions, VS- salt treated roots of cv. Vlamingham, VSF- inoculated roots of cv. Vlamingham under saline conditions

For details on Supplementary Tables, refer to the hyperlink provided in Chapter 7

List of Supplemental Figures

A schematic of the experimental workflow is provided in Figure 1. MALDI-MSI analysis was performed for seeds grown under control and saline conditions and harvested at six different time points, namely 0, 8, 16, 24, 48, and 72 hours post imbibition(Supplementary Figure 7-1). Sublimation of sectioned tissue with DHB matrix was used for MALDI-MSI positive mode ionisation to image the spatial distribution of a wide range of lipids (m/z 150–2800) in the seed sections. ICP-MS (for total elemental analysis) and μ -XRF spectrometry (to obtain the spatial information of targeted elements) was also performed for three time points (0, 24, and 48 hours post imbibition).....	164
Supplementary Figure 7-1 Timepoints for barley genotypes germinated for the collection of samples. Arrows indicate timepoints for collection of samples. I: 0 hours, II: 8 hours, III: 16 hours, IV: 24 hours, V: 48 hours, VI: 72 hours post aeration. S: sterilization of seeds, A: overnight aeration, GC: growth cabinet (dark, constant 17°C), RP: radicle protrusion, C: coleoptile emergence, Germination period: starts from emergence of radicle (visible germination) until final sample collection (72hours).	206
Supplementary Figure 7-2 Tissue distribution of m/z 796.52 PC(34:2) observed in three independent replicates at 16 hours in Mundah control and salt treated seeds. A, B, C: scanned image of Mundah control seed replicates 1,2 and 3; A-I, B-I, C-I: single colour filter for m/z 796.52 [M+K] ⁺ showing the intensity scale of 0-100%. A-II, B-II, C-II: colour gradient filter to visualize the distribution of m/z 796.52 [M+K] ⁺ with intensity scale of 0-100%. D, E, F: scanned image of Mundah salt treated seed replicates 1, 2 and 3; D-I, E-I, F-I: single colour filter for m/z 796.52 [M+K] ⁺ showing the intensity scale of 100%. D-II, E-II,F-II: colour gradient filter tovisualize the distribution of m/z 796.52 [M+K] ⁺ with intensity scale of 100%.	207
Supplementary Figure 7-3 Distribution of total lipid classes at all time points in cv. Mundah and cv. Keel germinating seed under control and salt-treated conditions. The X-axis shows the six time points for control and 5 time points for salt treated samples. The Y-axis indicates the number of lipids putatively identified at each time point. The putative lipid identities shown in this graph were established using an accurate mass precursor ion search (<5 ppm) of the LIPID MAPS database. Note: These identities are not collapsed into single ID and hence there is repetition of masses at different time points.	208
Supplementary Figure 7-4 ROC plot for m/z 615.494 [M+Na] ⁺ DAG(34:2) from Mundah endosperm. AUC was 0.087 showing high intensity of this ion in control seed of Mundah at 72 hours. A: Mundah control 72 hours seed scanned image, B: Mundah salt treated 72 hours seed scanned image, A': Mundah control 72 hours showing DAG(34:2) in the endosperm region, B': Mundah endosperm region for salt treated seed at 72 hours. C: ROC plot for m/z 615.494 showing true positive rate higher than 0.8. D: Intensity box plot chart for two selected endospermic regions. Red lines indicate sub regions created in SCiLS Lab. Here, red bordered regions are the endosperms. Intensity scale was set to 0-100%.	209
Supplementary Figure 7-5 The intensity box plots and ROC plots for a putative flavonoid (m/z 365.102) observed in Mundah at 16, 24 and 48 hours. A, B, C: The intensity box plots at 16, 24 and 48 hours. The box part contains a rectangle divided by a horizontal line, that represents a median intensity. Lower and upper bounds of the box represent the total number of spectra with intensities below these lines in one quarter and three quarters respectively. The cloud part of the plot shows scattering of spectra of a given region by intensity of a given m/z interval. Blue dots represent the spectra in which intensities of a given m/z interval are between the lower and upper quantiles whereas red dots represent the spectra whose intensities are outliers. A', B', C': ROC plots at 16, 24 and 48 hours showing the discrimination capabilities of given m/z for two (control and salt) regions with AUC values of 0.867, 0.952 and 0.907 respectively.	210
Supplementary Figure 7-6 The intensity box plots and ROC plots (AUC value of 0.953) for a putative flavonoid (m/z 364.0947) observed in Keel at 72 hours.	211
Supplementary Figure 7-7 MS/MS fragments of precursor ion with m/z 365.102 [M+H] ⁺ . Nine product ions were detected with m/z 347.0920, m/z 337.1032, m/z 335.0918, m/z 333.0760, m/z 323.0553, m/z 309.0397, m/z 307.0603, m/z 297.0395 and m/z 281.0445. Asterisks represent fragments matched with LC-MS/MS analysis shown in figure 5.	211
Supplementary Figure 7-8 Line graph of normalised log responses of m/z 365.102 at 6 different timepoints (8 hours, 16 hours, 24 hours, 48 hours and 72 hours) in seven genotypes under control and saline conditions measured using LC-QToF-MS. X-axis indicates timepoints and Y-axis indicate normalized log response.....	212

Supplementary Figure 7-9 The elemental maps of S, P, K and Cl in germinating barley seeds of Mundah under control and saline conditions. Each image indicates the relative distribution of the specific element captured at 10 ms/pixel.	213
Supplementary Figure 7-10 The elemental maps of S, P, K and Cl in germinating barley seeds of Keel under control and saline conditions. Each image indicates the relative distribution of the specific element captured at 10 ms/pixel.	214

Chapter 1-
Literature Review

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Alleviation of Salinity Stress in Plants by Endophytic Plant-Fungal Symbiosis: Current Knowledge, Perspectives and Future Directions

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Abstract

Salinization of soil with sodium chloride ions inhibits plant functions, causing reduction of yield of crops. Salt tolerant microorganisms have been studied to enhance crop growth under salinity. This review describes the performance of endophytic fungi applied to crops as a supplement to plant genetics or soil management to alleviate salt stress in crops. This is achieved via inducing systemic resistance, increasing the levels of beneficial metabolites, activating antioxidant systems to scavenge ROS, and modulating plant growth phytohormones. Colonization by endophytic fungi improves nutrient uptake and maintains ionic homeostasis by modulating ion accumulation, thereby restricting the transport of Na⁺ to leaves and ensuring a low cytosolic Na⁺:K⁺ ratio in plants. Participating endophytic fungi enhance transcripts of genes encoding the high Affinity Potassium Transporter 1 (HKT1) and the inward-rectifying K⁺ channels KAT1 and KAT2, which play key roles in regulating Na⁺ and K⁺ homeostasis. Endophytic-induced interplay of strigolactones play regulatory roles in salt tolerance by interacting with phytohormones. Future research requires further attention on the biochemical, molecular and genetic mechanisms crucial for salt stress resistance requires further attention for future research. Furthermore, to design strategies for sustained plant health with endophytic fungi, a new wave of exploration of plant-endophyte responses to combinations of stresses is mandatory.

Keywords: endophytic fungi, biochemical changes, ionic homeostasis, osmoregulation, hormones, salinity, roots, soil, inoculants, microorganisms

1.1 Soil salinity affects agriculture globally

The beginning of the 21st century has been marked by global scarcity of water resources, increased environmental pollution and salinization of soil and fresh water. Two major threats for agricultural sustainability are increased human population and reduction in arable land available for crop cultivation (Shahbaz and Ashraf 2013). Several environmental stresses such as high winds, extreme temperatures, drought, salinity and flood have impacted on the production and cultivation of agricultural crops. Among these, soil salinity is one of the most significant environmental stresses resulting in major reductions in cultivatable land area, and decreased crop productivity and quality. It is estimated that 50% of all arable land will be impacted by salinity by 2050 (Shrivastava and Kumar 2015) and that globally, soil salinity results in more than US\$12 billion in annual losses due to reduced crop productivity (Jägermeyr and Frieler 2018). Salinity is recognized as the main threat to environmental resources in several countries, affecting almost 1 billion ha worldwide, which represents about 7% of the earth's continental area (Shrivastava and Kumar 2015). Consequently, it is important to understand the crop responses to this major soil and plant stress to minimize economic loss and improve food security.

Soil is defined as being saline when the electrical conductivity (EC) of the saturation extract (EC_e) in the root zone exceeds 4 dSm⁻¹ at 25°C and has an exchangeable sodium of 15% (w/v). Salinization also includes excessive accumulation of ions such as calcium (Ca²⁺), magnesium (Mg²⁺), sodium (Na⁺), sulphates (SO₄²⁻), and chlorides (Cl⁻) in the soil, inhibiting plant growth and cellular functions. The most abundant ion in most salt-affected soils is Na⁺ and hence the exchange phase is dominated by Na⁺. A secondary process often associated with saline soils is alkalinisation, creating a condition known as sodicity. This results in the degradation of soil physical properties and porosity, leading to reduced water and air flow and increased soil hardness and crusting.

Apart from affecting soil physical properties, high soil salinity directly and adversely affects plants- both native vegetation and introduced crops, severely affecting seed germination, root growth, and the physiological functions of crops (Oster and Jayawardane 1998). It has been estimated that worldwide 20% of total cultivated and 33% of irrigated agricultural land is affected by high salinity. This is mainly due to the toxicity of the salt ions directly on the plant cells but also through general osmotic effects of the soil around the roots of the plant. High osmotic potentials at the soil-root interface reduce the ability of the plant to absorb water from the soil (Machado and Serralheiro 2017).

Native plants have evolved mechanisms to tolerate low rainfall and high salinity over hundreds of thousands of years (Steffen et al. 2009). However, in the past 200 years, human activities have intensely disrupted the natural hydrological balance in many regions of the globe. This has resulted in significant consequences for the distribution of salt in all landscapes leading to severe degradation of both natural and agricultural environments. It is predicted that the total area of land affected by salinity will increase drastically over the next few

decades if effective solutions are not implemented. These solutions would involve significant changes to our present systems of management including research and development of strategies to improve salt tolerance in crops and improve mechanisms to mitigate its consequences (Rengasamy 2002; Rengasamy 2006).

1.2 Effects of salt stress on above-ground and below-ground organs of plants

Plants have two major systems, the above-ground organs (shoots) and below-ground organs (roots). Each system has morphological, physiological and anatomical differences that affect plant performance differently (Gregory 2007). However, while these two systems grow and function as a separate site for the uptake of nutrients and other resources, they are coupled, and their functions need to form an integrated system. The above-ground system is highly dependent on the development of below-ground organs and without a sufficiently developed root system, the above-ground system cannot fully mature (de Willigen and van Noordwijk 1987).

Salinity limits vegetative and reproductive development by inducing physiological dysfunctions, and this has profound implications on different harvested organs such as leaf, stem, root, shoot, fruit or grain. The complex phenomenon of tolerance and response to salt stress involves dynamic changes in growth, physiology, metabolic pathways and gene expressions (Atkinson and Urwin 2012; Munns and Tester 2008). Strategies used to mitigate against salt stress include proline accumulation within cells (Matysik et al. 2002), modulation of hormones and accumulation of glycine betaine and polyols (Gupta and Huang 2014). They also involve generation of nitric oxide (NO) and compounds to combat formation of reactive oxygen species (ROS). NO directly or indirectly triggers expression of several redox-regulated genes. NO also reacts with lipid radicals thus preventing lipid oxidation, exerting a protective effect by scavenging superoxide radicals and formation of peroxynitrite that can be neutralised by other cellular processes. NO also helps in the activation of many antioxidant enzymes including catalase (CAT), ascorbate or thiol-dependent peroxidases (APX), glutathione reductases (GR) and superoxide dismutase (SOD).

The effect of salinity on leaf growth, biomass production and grain yield on several crops are well documented (Hasanuzzaman et al. 2013; Munns et al. 2011; Munns and Tester 2008; Sun et al. 2014). The extent to which plants are damaged by salinity depends on several factors including species, genotype, plant growth phase, ionic strength, duration of salinity exposure, composition of salinizing solution and, most importantly, which plant organ is exposed (Robin et al. 2016).

Munns (2005) hypothesized that salinity damage in plants occurs in two temporal phases. The first phase of growth reduction occurs rapidly after exposure and is due to an osmotic effect, while the second phase, which is a slower process, is due to the accumulation of salt ions, mainly in older leaves. Early symptoms of the second phase of growth reduction include damage to old leaves and a reduced photosynthetic capacity (Munns et al. 2006). At the plant

organ level, shoots have been demonstrated to be more sensitive to salinity than roots (Munns and Tester 2008). However, roots are exposed to salinity stress before leaves and can respond rapidly through changes in elongation (Rahnama et al. 2011) and function (Shelden et al. 2016). The roots are crucial for a myriad of physiological processes including water and nutrient uptake, preventing toxic substances from reaching photosynthetic tissue, signal exchange with shoots, anchoring of plants, and providing mechanical support to the above-ground organs.

1.3 The root-soil interface/ The rhizosphere

Roots and their growing substrate are intrinsically connected, and they mutually influence each other at all stages of plant life (Gregory 2006). The interface between roots and the soil is a complex and often ill-defined zone. Compounds are released from roots into the surrounding soil matrix resulting in changes to its chemical and physical properties. The narrow zone of soil that surrounds and is influenced by plant roots is known as the rhizosphere. The term rhizosphere was first defined over a century ago by Hiltner (1904) and recently, redefined by Pinton et al. (2007) as the most dynamic interface on earth that includes soil influenced by the root, along with the root tissues. The rhizosphere is home to a vast number of microorganisms (Morgan et al. 2005; Pinton et al. 2007), and consists of three distinct zones: a) the endorhizosphere, which includes part of the cortex and endodermis in which microbes occupy the apoplastic space; b) the rhizoplane, which is the medial zone immediately next to the root consisting of the root surface and mucilages; and c) the ectorhizosphere, which extends from the rhizoplane out into the bulk soil (Lynch 1990).

The root system architecture is greatly influenced by soil conditions (Rich and Watt 2013), including nutrient gradients and concentrations of nitrate and phosphorus (Ho et al. 2005; Paterson et al. 2006). Roots also affect the surrounding nutrient composition by the release of organic compounds that play a vital role in mineralizing nutrients. The compounds released from the roots into the surrounding soil are generally part of rhizodeposits (Jones et al. 2009), which include a range of substances from sloughed-off root cells and tissues, mucilages, volatiles, and soluble lysates and exudates from damaged and intact cells (Curl and Truelove 1986; Dakora and Phillips 2002; Watt 2009). Abiotic factors influence the root system (Bekkara et al. 1998; Brimecombe et al. 2000; Groleau-Renaud et al. 1998; Watt and Evans 1999) with roots responding by secreting a different combination of compounds to protect against negative effects and encourage positive microbial interactions (Badri and Vivanco 2009). These secreted compounds usually induce an interactive metabolic cross-talk involving diverse biosynthetic networks and pathways.

Root exudates include both secretions (including mucilage) that are actively released from the root and diffusates which are passively released because of osmotic differences between soil solution and the root cells (McNear Jr 2013). Inorganic root exudates include ions, water, ubiquitous H^+ and electrons. Although the concentration of inorganic compounds make up far less of the root exudate composition compared to organic compounds but their role is still

significant (Khorassani 2008; Uren 2000). Organic compounds can be classified into high molecular weight compounds, such as complex molecules including polysaccharides secreted by root cap cells and epidermal cells at the apical zone, and low molecular weight compounds that include arabinose, fructose, glucose, amino acids, organic acids, plant hormones and phenolic compounds (Bertin et al. 2003). Due to the richness of inorganic and organic compounds in rhizodeposits, the rhizosphere is home to specialised microbes that are able to utilise these compounds as an energy source.

Several recent and comprehensive reviews have been written covering the diversity and activity of microorganisms at within roots and in the rhizosphere, as well as the functions and effects of microorganisms in nutrient turnover and supply to the plant (Garcia et al. 2016; Jacoby et al. 2017; Smith and Smith 2011; Udvardi and Poole 2013). In the following section, the use of microorganisms as one of the key approaches used to alleviate abiotic stresses, with the focus on using fungi as a major beneficial microbe will be discussed.

1.4 Alleviating salt stress by association with microorganisms

Diverse metabolic and genetic strategies used by plant-associated microbes can reduce the impact of salt stress and other abiotic stresses arising from extreme environmental conditions (Gopalakrishnan et al. 2015; Singh 2014). Induced Systemic Tolerance (IST) is the term used to describe microbe-mediated induction of abiotic stress responses (Meena et al. 2017). In these beneficial situations, rhizosphere microorganisms not only perceive and respond to signal molecules secreted by plant roots, they also release diverse signalling molecules that influence plants, resulting in increased biotic and abiotic stress resistance or tolerance, as well as root development and plant growth (Zhang et al. 2017a). Microbial interactions with plants induce several local and systemic responses that improve the metabolic capacity of plants to respond to salt stress (Nguyen et al. 2016). This microorganisms-based plant biotechnology has proven to be more efficient in many cases than plant breeding and genetic modification approaches (Smith 2014).

1.4.1 Beneficial effects due to plant root interactions with endophytic fungi

In recent years the ability of mycorrhizal fungi to induce tolerance against salt stress in crops has been documented (Gangwar and Singh 2018) (**Figure 1.1**). In a mycorrhizal association, the fungus colonizes the host plant's root tissues, either intracellularly as in arbuscular mycorrhizal fungi (AMF), or forms extracellular exchange mechanisms outside of the root cells, as in ectomycorrhizal fungi. Thus, mycorrhiza fungi can be categorised as endo- inside plant tissue, or ecto- associated with the external rhizosphere or not penetrating root cells. For the purpose of clarity, this review will only focus on endomycorrhizal (termed as endophytic for this review) fungi.

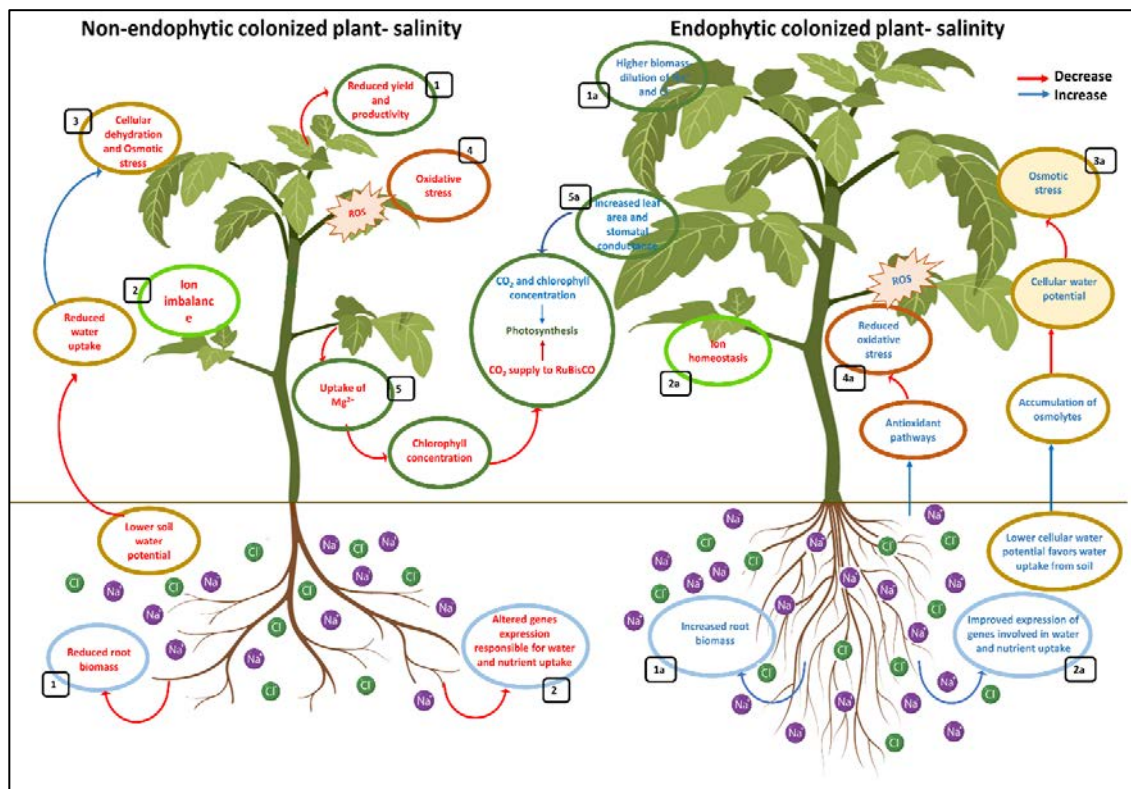


Figure 1.1. Potential beneficial effects of root colonisation of plants by endophytic, symbiotic fungi in saline soil conditions, summarised from the literature. Salinity results in reduced root biomass due to salt-induced inhibition of cell division and affect the total biomass yield (1) (left). Plant colonized with endophytic fungi improves biomass accumulation by modifying root architecture and increased nutrient absorption (1a) (right). Salt accumulation creates competition for nutrient uptake and transport. This results in imbalance of the ionic composition of plant, affecting plant's physiological traits (2) (left). Endophytic fungi improve expression of genes and upregulate several cation transporters, resulting in improved nutrient uptake and maintenance of ionic homeostasis (2a) (right). Increase of salt in soil lowers soil water potential resulting in cellular dehydration (3) (left). Endophytic fungi negate this effect by mediating accumulation of osmolytes consequently improving plant's water status (3a) (right). Increasing salinity causes oxidative stress due to imbalance in reactive oxygen species generation and quenching activities of antioxidants (4) (left). Endophytic fungi improve the antioxidant systems of plants reducing oxidative stress under salt stress (4a) (right). Salt stress hinders photosynthesis by reducing uptake of magnesium and decreasing chlorophyll concentration which eventually reduces carbon dioxide supply to RuBisCo (5) (left). Endophytes have a positive effect on photosynthesis under salt stress (5a) (right). See text for relevant references and further details.

Penetration and colonisation of plant roots appears to be essential for some endophytic fungal strains that are reported to promote plant growth and provide protection against pathogens. For example, some species belonging to the genus *Trichoderma* can colonize local sites (Metcalf and Wilson 2001) on roots, mediated by hydrophobins- (Viterbo et al. 2004) and expansin-like proteins (Brotman et al. 2008) present in the outermost cell wall layer that coats the fungal cell surface. Other rhizosphere-competent *Trichoderma* spp. colonize entire root surfaces for long periods of time (Harman 2000; Thrane et al. 1997) or penetrate the epidermis and the cortex (Yedidia et al. 1999). Once hyphae penetrate roots, a series of fungal

bioactive compounds can be produced inducing plant biochemical mechanisms (Harman 2006). The callose-enriched wall appositions in the root cell limit the growth of the *Trichoderma* spp. to a small area (epidermis and cortex), preventing the entry of *Trichoderma* spp. into the vascular stele (Hermosa et al. 2012; Yedidia et al. 1999). Arbuscular mycorrhiza fungi (AMF) are another group of endophytic fungi. Their hyphae penetrate plant cells, producing structures that are either balloon-like (vesicles) or dichotomously branching invaginations (arbuscules) as a means of nutrient exchange. The fungal hyphae do not in fact penetrate the protoplast (i.e. the interior of the cell), but invaginate the cell membrane. Dark septate endophytic (DSE) fungi are also root endophytes, characterized by intense dark pigmentation and the formation of septate and melanized hyphae and occasionally microsclerotia (Knapp et al. 2015; Yuan et al. 2016). They can be found in plant cortical cells inter- and intracellularly and are present in several environments (Li et al. 2019; Santos et al. 2017). In contrast to the vast information on AMF, information on the role of DSE fungi in the ecosystem is limited.

Colonization of several crops with endophytic fungi has been reported to induce systemic resistance to pathogens, mitigate stress by increasing the levels of protective metabolites and osmoprotectants, activate antioxidant systems to prevent damage caused by ROS, decreasing salt induced root respiration and modulate the phytohormone profile to minimize salt effects on growth of plants (Ghaffari et al. 2016; Jogawat et al. 2013; Li et al. 2017; Nia et al. 2012; Rewald et al. 2015; Zhang et al. 2019a). These effects are in coordinated to improve plant growth and resilience to salinity stress. These ameliorative effects can be evaluated in terms of improved plant growth exhibited by endophyte colonized (ENC) plants in comparison to non-endophytic (NENC) colonized plants.

Salinity triggers a decrease in stomatal conductance, thus decreasing the CO₂:O₂ ratio and increasing photorespiration (Kangasjärvi et al. 2012). This causes an increase in stomatal resistance to transpiration and an increase in the rate of tissue respiration. Under these conditions, photosynthetic capacity is limited, and the plant uses its own photo-assimilates, resulting in decreased growth. Rewald et al. (2015) showed that in NENC *Ulmus glabra* seedlings there was a significant increase in fine root respiration under salt stress as compared to their ENC counterparts. This suggested that colonization by endophytic fungi can prevent a major increase of root respiration under moderate NaCl stress, enabling trees to deploy more assimilated C for growth and, theoretically, improve defence mechanisms against other stress factors occurring in urban environments.

Endophytic fungi are effective against several root diseases (Azcón-Aguilar and Barea 1997; Borowicz 2001) and impart stress tolerance to plants (Duc et al. 2018; Evelin et al. 2019; Yasmeen et al. 2019), but can also enhance susceptibility to biotrophic leaf pathogens (Gernns et al. 2001; Waller et al. 2005). These endophytes have been frequently reported to not only protect against plant pathogens and pests but also impart strong tolerance against several abiotic stresses in crops (Gangwar and Singh 2018).

In the past decade, significant progress has been made to understand several mechanisms of salt tolerance imparted by endophytic fungi. In the following sections, current understanding of biochemical and physiological changes that occur in salt stressed plants inoculated with endophytic fungi will be covered. This will include advances made recently toward better understanding of the mechanisms that contribute to salt stress alleviation in ENC plants. Finally, gaps in our understanding of the mechanisms will be identified and research challenges to be met in future studies will be discussed.

1.5 Mechanisms of salt tolerance in ENC plants

1.5.1 Increase in total biomass

Total biomass is usually evaluated as an indicator of the plant's ability to tolerate salinity. Several studies have highlighted that endophytic fungi impart salinity tolerance in host plants by virtue of higher biomass as compared to NENC plants. Endophytic fungus colonization has been demonstrated to increase biomass in *Zea mays* L. (Rho et al. 2018), soybean (Hamayun et al. 2017), *Vochysia divergens* Pohl (Farias et al. 2019), *Solanum lycopersicum* (Azad and Kaminskyj 2016), *Brassica juncea* (Ahmad et al. 2015), *Oryza sativa* L. (Saddique et al. 2018) and, *Triticum aestivum* L. (Zhang et al. 2019b).

The total biomass can also be assessed by measuring plant relative growth rate (plant weight increment per plant weight unit). This includes measurement of the net assimilation rate (NAR) (the increase in plant weight per leaf area unit), the leaf area ratio (LAR) and root relative growth rate (RGR_{plant}). Balliu et al. (2015) investigated the effects of commercially available AMF inoculant (*Glomus* sp. mixture) on growth and nutrient acquisition in tomato (*Solanum lycopersicum* L.) plants grown in media with different levels of salinity. Salinity stress immediately and significantly reduced the LAR, NAR and RGR_{plant} in NENC as compared to ENC plants. Similarly, Sallaku et al. (2019) showed that AMF alleviates the salinity stress in cucumber plants by extending their root length and root surface area and even more through enhancing their photosynthetic rate (NAR) as compared to NENC plants.

1.5.2 Alteration in root architecture

Root branching and root system architecture play a significant role in determining the composition of exudates (Badri and Vivanco 2009). Changes in the root system architecture for regulating salt acquisition and translocation are crucial for enhancing plant resistance to salt stress (Jung and McCouch 2013). Barley plants experienced a decline in primary root growth under saline conditions due to salt-induced inhibition of cell division and elongation of root epidermal cells, while simultaneously stimulating lateral root development (Rahnama et al. 2011). Endophytic fungi can modulate the plant's ability to modify root architecture (Salope-Sondi et al. 2015; Vahabi et al. 2016). Yun et al. (2018) observed that the length and volume of roots were greater in ENC than in NENC maize plants under saline conditions and similar observations have been reported in *Hordeum vulgare* (Waller et al. 2005) and *Oryza sativa* L. (Kord et al. 2019). Improved root systems enable the plant to utilize water and

minerals from non-saline areas until exploitation of areas affected by salt cannot be avoided (Jogawat et al. 2013). Though few studies have shown the ability of endophytic fungi to alter root architecture under saline conditions for beneficial purposes, much remains to be investigated on endophytic fungi influenced root architecture for better water and nutrient uptake in saline conditions.

1.5.3 Osmoregulation

Upon exposure to saline environments, plants undergo a reduction in water absorbing capacity from the soil, disrupting cell water relations and inhibiting cell expansion. In order to negate these effects, plants employ osmoregulation as a mechanism to tolerate salt stress (Munns and Tester 2008). This is achieved by accumulation of osmolytes in the form of proline, glycine betaine, sugars, organic acids, polyamines and amino acids contributing to osmotic adjustment (Hasegawa et al. 2000). These osmolytes, often termed as compatible solutes, are organic compounds of low molecular weight that are water soluble and non-toxic at high concentrations (Chen and Murata 2011).

Under salt stress, ENC plants have been shown to possess higher osmotic potential than NENC plants (Contreras-Cornejo et al. 2014) due to accumulation of osmolytes (Ahmad et al. 2015; Song et al. 2015) (Fig. 2). Osmolytes are also involved in quenching reactive oxygen species (ROS), maintaining membrane integrity, and stabilizing enzymes. Osmolytes are also described as osmoprotectants (Azad and Kaminskyj 2016; Li et al. 2017). Endophytic symbiosis can influence the concentration and profile of polyamines and organic acids in plants (Chen et al. 2019; Zhao et al. 2014). Polyamines help retain ion homeostasis in plant cells by enhancing the uptake of nutrients and water (Pang et al. 2007). Organic acids may increase the availability of nitrogen, phosphorus and potassium (N, P and K) in soil (Samolski et al. 2012). The role of specific osmolytes in improving salt tolerance in ENC plants are discussed below.

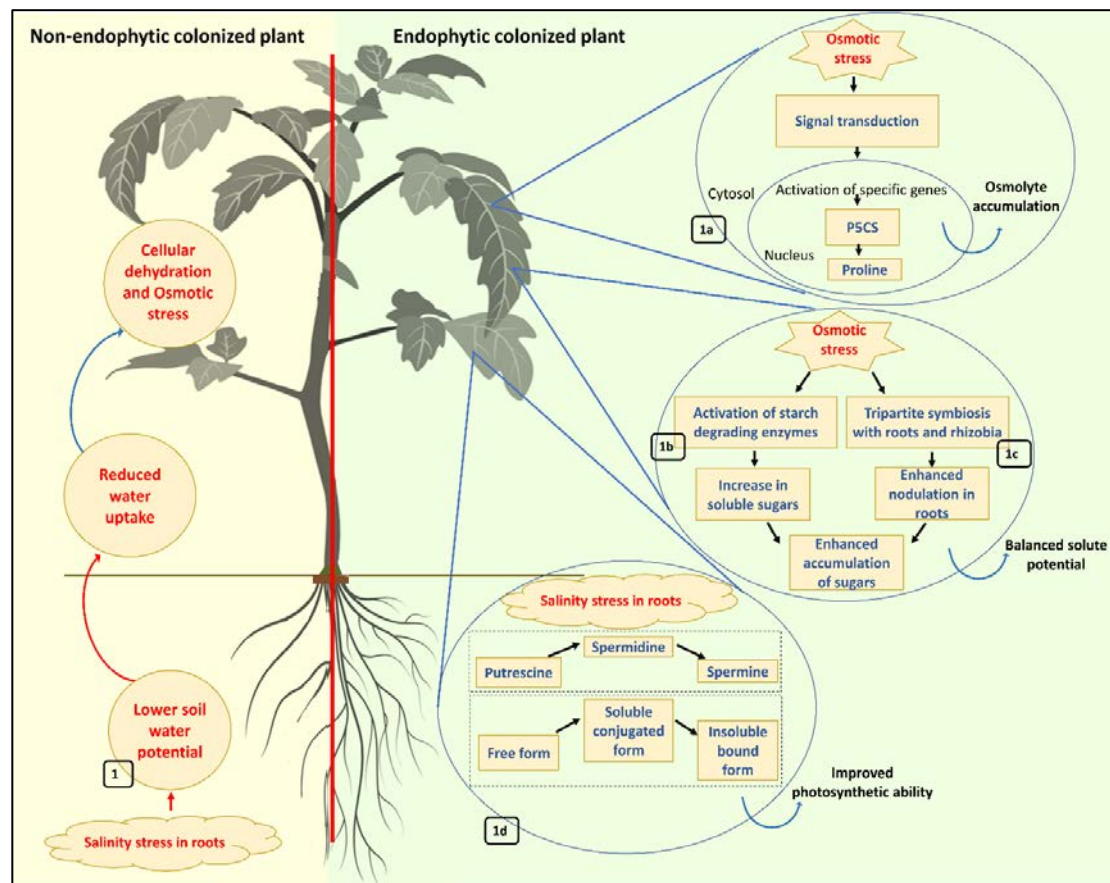


Figure 1.2. Salinity stress induced osmotic stress tolerance mechanisms in plants. Increase in salt in soil lowers the soil water potential of plant cells. This reduces water uptake by plants and consequently causes cellular dehydration (1) (left). To combat this issue, plants accumulate osmolytes, such as proline, sugars and polyamines in higher concentration. Osmolyte accumulation results in lowering of cellular water potential and maintains a favourable gradient for water uptake from soil to roots. Endophytic fungi alleviate osmotic stress by influencing the expression of specific genes, P5CS, pyrroline-5-carboxylate synthase (1a) (right), involved in the biosynthesis of the osmolyte proline, activation of starch degrading enzyme, glucan-water dikinase (1b) (right) and forming tripartite symbiosis with roots and rhizobia (1c) (right) to elevate the accumulation of sugars and by increasing the biosynthesis of polyamines such as spermidine and spermine (1D) (right). See text for relevant references and further details.

1.5.3.1 Proline

Proline is one of the most common osmoprotectants that accumulates in plants during salt stress, thereby ameliorating the negative effects of salinity. Proline has been observed to protect cell walls under osmotic stress, protect protein integrity and to increase enzymatic activity by acting as a molecular chaperone. Proline also has a role in scavenging ROS and shows singlet oxygen quenching ability (Kaur and Asthir 2015). Despite these benefits, there are conflicting reports on the role of endophytic fungi in proline accumulation in salt stressed plants. Several studies reported increases in proline contents in ENC plants compared to NENC plants, while others have reported lower proline contents in ENC plants (Table 1). Higher proline content in ENC plants has been attributed to – (i) favouring a decline in ionic influx

inside cellular masses thus helping plants to maintain their osmotic balance; (ii) increasing the expression of the gene encoding Pyrroline-5-carboxylate synthase (P5CS) enzyme which is involved in proline biosynthesis; and (iii) increasing activity of the P5CS enzyme (Rawat et al. 2016). Besides its role as an osmolyte proline can act as a stress marker. In ENC tomato plants, proline accumulation was reduced when the toxic effects of salinity were reduced following colonization of an endophytic fungus, *Piriformospora indica* (Abdelaziz et al. 2019).

Table 1.1 Examples of studies on effects of salt stress and endophytic fungi on osmotic regulation in plants

Study No.	Salt level (mM NaCl)	Plant	Fungus	Parameters assessed	Effects of		References
					Salinity	Endophytic fungi on salt stressed plants	
1	0, 100	<i>Zea mays</i>	<i>Yarrowia lipolytica</i>	Shoot proline content, total flavonoid, total phenolics, phytohormone analysis	Increased proline, total flavonoid, total phenolics, phytohormones	Controlled the production of proline	Jan et al. (2019)
2	0, 100, 200, 300	<i>Hordeum vulgare</i>	<i>Epichloë bromicola</i>	Free, soluble conjugated and insoluble bound forms of polyamine (proline), putrescine, spermidine and spermine content	Increased proline	Proline, spermidine, total spermine - increased under higher salt stress conditions. Putrescine, free form of spermine - significantly decreased at higher salt treatments.	Chen et al. (2019)
3	0, 50	<i>Solanum lycopersicum</i>	<i>Piriformospora indica</i>	Shoot proline content	Highly increased	Significantly reduced	Abdelaziz et al. (2019)
4	40, 100, 175, 250	<i>Medicago truncatula</i>	<i>Piriformospora indica</i>	Shoot proline content	Continually enhanced in line with the increased salt concentration	Significantly increased compared to un-colonized plants	Li et al. (2017a)
5	0, 100, 200	<i>Brassica juncea</i>	<i>Trichoderma harzianum</i>	Oil and proline content, pigments, enzymatic assay	Increased with maximum accumulation of 59.12% at 200mM NaCl	Further increase to 70.37%	Ahmad et al. (2015)
6	0, 150	<i>Triticum aestivum</i>	<i>Trichoderma longibrachiatum</i>	Water content in leaves and roots, chlorophyll content, shoot proline content	Increased	Highest increase in plants pretreated with fungus under 150 mM NaCl stress	Zhang et al. (2016)
7	0, 70, 150, 240	<i>Oryza sativa</i>	Five isolates of <i>Trichoderma sp.</i>	Leaf water content, chlorophyll content, proline content, membrane stability, lipid peroxidation and expression of stress related genes	All parameters assessed increased	Further increased	Rawat et al. (2016)
8	0, 100, 200, 300, 400, 500	<i>Triticum aestivum</i>	<i>Piriformospora indica</i>	Total biomass, photosynthetic pigments, compatible solutes	All parameters assessed increased	Further increased	Zarea et al. (2012)

1.5.3.2 Sugars

In salt stressed plants, the accumulation of total soluble sugars, such as glucose, sucrose, dextrins and maltose, serves as an osmoprotection as they can stabilize the cell membrane and protoplast. These sugars also protect water soluble enzymes from high intracellular concentrations of inorganic ions (Liang et al. 2018). The synthesis of soluble sugars from starch and sucrose in plants is upregulated by the activities of sucrose anabolizing enzymes such as α - and β -amylase, which convert starch into dextrins and maltose, respectively (Preiss 2018). Sucrose phosphate synthase and sucrose synthase catalyse the synthesis of sucrose, while β -fructofuranosidase catalyses the breakdown of sucrose to glucose and fructose (Peng et al. 2016). In plants grown under saline conditions, sucrose undergoes decomposition in order to meet the requirements for glucose (Munns and Tester 2008).

There have been reports that show the role of endophytic fungi in enhancing accumulation of soluble sugars in salt stressed plants (Qi and Zhao 2013; Uma Shaanker 2014; Zhang et al. 2019b). These sugars act as chemoattractant signals to soil rhizobia (el Zahar Haichar et al. 2014). These chemoattractants can direct movement to microorganisms in response to chemical gradients- a behaviour known as chemotaxis. This chemotactic response of microorganisms to root exudates play key role in initiating communication between plant roots and microbes. Yang et al. (2015) reported that the colonization by *Phomopsis liquidambari* could stimulate sugar secretion from the rhizodeposition of sloughed off cells and root debris of rice, thereby providing carbon to the endophytic fungi. Another study of *P. liquidambari* on peanut showed increased soluble sugar contents in leaves. This was due to the ability of the fungus to form tripartite symbiotic associations with peanut roots and rhizobia. This tripartite association significantly enhanced peanut nodulation (Zhang et al. 2017b). Here, sucrose derived from photosynthesis was transported to bacterial inoculated root nodules and was hydrolysed by sucrose synthase into UDP-glucose and fructose. This was due to the allocation of more carbon by the endophyte toward peanut and rhizobia symbionts by increased soluble sugar content, leading to more active nodule carbon metabolism in ENC plants.

Furthermore, Sherameti et al. (2005) also suggested that one of the major starch-degrading enzymes, glucan-water dikinase, activated by the fungus in colonized roots, is responsible for the increase in soluble sugars in ENC plants. Similar results were obtained by Ghabooli (2014) with *Piriformospora indica* increasing the level of soluble sugars, including glucose, fructose, and sucrose, in inoculated plants under salt stress conditions. Recently, Zhang et al. (2019a) demonstrated that *T. harzianum* improved salt tolerance of cucumber seedlings by enhancing accumulation of sugars. This results in adjustment of the osmotic potential for cellular water retention and turgor maintenance, thereby minimizing the adverse effects of salt stress by balancing the solute potential (Bai et al. 2019).

1.5.3.3 Organic acids

Other important osmolytes in plants are organic acids such as citric acid and malic acid. They are found in plant vacuoles and the regulation of their metabolism plays a crucial role in providing tolerance to salt stress (Guo et al. 2010). Fungal endophytes have been reported to induce the release of organic compounds by the roots (Yang et al. 2015; Zhang et al. 2014), thus influencing the concentrations and profile of organic acids in plants. One of the major plant nutritional disorders associated with increased salinity in soil is iron (Fe) deficiency. Endophytes can enhance Fe acquisition by their host through their ability to secrete organic acids which chelate and solubilise iron in the soil (Chen et al. 1998; Khan et al. 2006). A study by Zhao et al. (2014) demonstrated that the release of organic acids from endophytes, resulted in ferric solubilization to form organic ferric salts that can be assimilated directly by plants under saline conditions. It has also been shown that ENC plants have better nutrient uptake capacity and distribution within plant tissues due to modulation of the root architecture and nutrient availability in the soil. These benefits are imparted by increases in organic acids produced by ENC plants (Samolski et al. 2012; Zhao et al. 2014). Limited research has been done on understanding the mechanisms underlying the changes in organic acids in ENC plants, thus this topic calls for further investigation.

1.5.3.4 Polyamines

Polyamines (PA) are low molecular weight nitrogenous aliphatic molecules that participate in physiological processes such as activation of antioxidant systems, cell growth and development, and in cellular osmoregulation in plants under salt stress (Singh et al. 2018). PA also regulate ion channels, either by direct binding or via PA-induced signalling molecules (ROS and NO). PAs also regulate the activity of ion channels indirectly by membrane depolarization. The hyperpolarization-activated Ca^{2+} influx and the NO-induced release of intracellular Ca^{2+} result in a higher cytoplasmic Ca^{2+} concentration, which is a major component in general stress responses such as stomatal movements (Wani 2018; Williams 1997). They are either present in free, soluble conjugated (covalently conjugated with small molecules such as phenolic acids) or insoluble (bound with macromolecules such as proteins, DNA and RNA) forms. These compatible solutes accumulate under salt stress and include putrescine (Put, diamine), spermidine (Spd, triamine) and spermine (Spm, tetramine) (Minocha et al. 2014; Todorova et al. 2013).

Differences in PA (Put, Spd, Spm) responses under salt-stress have been reported in several species (Singh et al. 2018) and it remains unclear which polyamine plays the major role in imparting salt tolerance. Chen et al. (2019) demonstrated that the putrescine content was significantly reduced in ENC plants compared to NENC plants in high stress conditions whereas spermidine and spermine content showed the opposite pattern. It was suggested that salinity stress tolerance induced by endophytic fungus *Epichloë bromicola* correlated with enhanced conversion of putrescine to spermidine and spermine. The fungus also

converted the free forms and soluble conjugated forms of polyamines to insoluble bound forms of polyamines.

Modulation of the polyamine pool to help tolerate salt stress by arbuscular mycorrhizal fungi (AMF) is well explored (Evelin et al. 2009). However, research on polyamine metabolism during the interactions between endophytic fungi and plants under salt stress is underrepresented and many questions remain unanswered. For example, most plant polyamine research relates to changes in free polyamines, and where polyamine conjugates have been measured, substantial changes have been detected. The precise role of polyamines, free or conjugated, in ENC plants remains unclear. Further investigations, focusing on understanding endophyte-facilitated modulation of polyamines, including the intracellular localization of free polyamines and conjugates associated with salt tolerance in plants, is needed. Already some of the key genes involved in the biosynthetic pathways have been cloned making it possible to manipulate polyamine metabolism using molecular genetic approaches (Malmberg et al. 1998). Hence, genetic manipulation of polyamine levels in ENC plants may allow valuable insights into the role of these compounds especially in studies of plant tolerance to salt stress.

1.5.4 Nutrient acquisition and ionic homeostasis

High salt (Na^+ and Cl^-) in the soil disturbs nutrient availability by imposing competition during uptake, translocation or distribution within the plant. This may suppress nutrient associated activities resulting in undesired ratios of $\text{Na}^+:\text{K}^+$, $\text{Na}^+:\text{Ca}^{2+}$, and $\text{Ca}^{2+}:\text{Mg}^{2+}$ (Munns et al. 2011). This in turn results in imbalance among ionic composition of the plant subsequently affecting plants physiological traits (Hasegawa et al. 2000; Munns et al. 2006). However, endophytic symbiosis has been shown to improve assimilation of nutrients and assist in maintenance of ionic homeostasis in host plants grown in saline conditions (**Table 1.2**).

Although the effects of AM fungi on plant nutrient acquisition are commonly discussed based on the differences of nutrient concentration in plant tissues, the relative uptake rate of nutrient elements (RUR) has recently been suggested as a better tool to distinguish the differences among treatments over a short period, as the nutrient concentration could be largely influenced by the dilution effect of fast growth in young plants. Balliu et al. (2015) found that RUR values of ENC tomato plants grown in both non-saline and moderate saline conditions were higher than in non-inoculated seedlings. Similarly, another study showed the enhancement effect of AMF inoculation on the nutrient uptake capacity of cucumber seedlings after salt stress (Sallaku et al. 2019).

Table 1.2 Examples of studies on the effects of salinity and endophytic fungi on nutrient concentration and ionic ratios in plants

Study. No.	Salt level (mM NaCl)	Plant	Fungus	Parameters assessed	Effects of		References
					Salinity	Endophytic fungi on salt stressed plants	
1	0, 140	<i>Cucumis sativus</i>	<i>Phoma glomerata</i> LWL2 and <i>Penicillium</i> sp. LWL3	Na ⁺ , K ⁺ , Ca ²⁺ , Mg content	Significant increases in Na ⁺ and decreases in K ⁺ , Mg ²⁺ and Ca ²⁺ levels	Significantly higher levels of K ⁺ , Mg ²⁺ and Ca ²⁺ ions, particularly for plants associated with <i>Penicillium</i> sp. and <i>P. glomerata</i> and uptake of Na ⁺ inhibited	(Waqas et al. 2012)
2	0, 100, 200	<i>Zea mays</i>	<i>Piriformospora indica</i>	Na ⁺ , K ⁺ content	Increased Na ⁺ in roots and shoots, K ⁺ in shoots and decreased K ⁺ in roots	Significantly decreased levels of Na ⁺ and K ⁺ in roots and increase in shoots	Yun et al. (2018)
3	0, 75, 100	<i>Arabidopsis thaliana</i>	<i>Piriformospora indica</i>	Transcript levels of several genes known to encode proteins involved in Na ⁺ and K ⁺ homeostasis and the abiotic stress marker gene Relative to Dessication A (<i>RD29a</i>)	Increased expression of the stress marker gene, <i>RD29a</i> , expression level of <i>AtHKT1</i> and K ⁺ content in roots and shoots. Decreased Na ⁺ content in roots and shoots	Decreased expression of the stress marker gene, <i>RD29a</i> , further decrease in Na ⁺ content. Further increased expression level of <i>AtHKT1</i> and K ⁺ content	Abdelaziz et al. (2017)
4	0, 100	<i>Arabidopsis thaliana</i>	<i>Trichoderma virens</i> and <i>trichoderma atroviride</i>	Na ⁺ content	Decreased Na ⁺ content in roots	Further decreased Na ⁺ content in roots	Contreras-Cornejo et al. (2014)
5	0, 150, 300, 450, 600	<i>Hordeum vulgare</i>	<i>Epichloe</i>	Na ⁺ , C, P, N, K ⁺ content, C:N, C:P, Na ⁺ :K ⁺ , N:P ratios	Increased Na ⁺ , N, P and K ⁺ contents, ionic ratios, no significant effect on C content	Further increased N, P and K ⁺ contents, N:P ratios. Decreased C:N, C:P, Na ⁺ :K ⁺ ratios	Song et al. (2015)
6	0, 100, 300	<i>Hordeum vulgare</i>	<i>Piriformospora indica</i>	Na ⁺ , K ⁺ , Ca ²⁺ , ionic ratios	Increased Na ⁺ , Ca ²⁺ , Decreased K ⁺ :Na ⁺ , Ca ²⁺ :Na ⁺ ratios	Increased K ⁺ , K ⁺ :Na ⁺ , Ca ²⁺ :Na ⁺ . Decreased Na ⁺ content	Ghabooli (2014)
7	0, 50, 100, 150	<i>Lolium arundinaceum</i>	<i>Neotyphodium coenophialum</i>	Na ⁺ , K ⁺ , Ca ²⁺ , and Mg ²⁺ content in leaves, roots and sheath	At lower salt concentration- in leaves, decreased K ⁺ , similar Na ⁺ , Ca ²⁺ unaffected; in sheath, decreased K ⁺ , similar Na ⁺ and Ca ²⁺ . At higher salt concentration- in leaves, decreased Na ⁺ , similar K ⁺ , Mg ²⁺ unaffected	At lower salt concentration- in leaves, increased K ⁺ , similar Na ⁺ , Ca ²⁺ unaffected; in sheath, increased K ⁺ , similar Na ⁺ and Ca ²⁺ . At higher salt concentration- in leaves, increased Na ⁺ , similar K ⁺ , Mg ²⁺ increased at all concentrations	Yin et al. (2014)

1.5.4.1 Phosphorus

Phosphorus (P) and nitrogen (N) are two of the most important and essential elements for plant growth with crucial roles in cell function and metabolism (Uchida 2000). Increased salt in soil occludes P to plants due to its precipitation with other cations (de Aguilar et al. 1979), thereby creating soil-induced P deficiency in plants. This affects the normal growth of the plant and causes older leaves to die prematurely (Niu et al. 2012). Increased P acquisition in ENC plants under saline conditions is attributed to (i) increased availability of phosphates in soil due to the conversion of insoluble phosphates into soluble forms through the process of acidification, chelation and exchange reactions; (ii) ability of endophytic fungi to absorb P at lower thresholds owing to the expression of a high affinity Pi transporter, PiPT, and (iii) ability of endophytic fungi to interact with diverse rhizobacteria which have inorganic phosphate-solubilizing capabilities by virtue of production of a variety of organic acids and acid phosphatases (Johri et al. 2015; Meena et al. 2010; Ngwene et al. 2016; Singh et al. 2009; Srividya et al. 2009; Swetha and Padmavathi 2016). This effective P uptake in ENC plants aids in transporting absorbed phosphorus to leaves, prompting plant growth; increasing absorption of nutrients and biomass accumulation (Wu et al. 2019), consequently alleviating the adverse effects of salinity.

1.5.4.2 Nitrogen

Nitrogen plays a crucial role in cell function and metabolism (Chokshi et al. 2017). Plants absorb N as nitrate (NO_3^-), ammonium (NH_4^+) ions, and also as organic compounds such as amino acids and peptides (Rentsch et al. 2007; Tegeder and Rentsch 2010) but absorption is compromised by salinity due to N immobilisation. Nitrate reductase (NR, E.C.1.6.6.1) catalyses reduction of NO_3^- to NO_2^- and its activity is nitrate-inducible. The NR activity is the limiting step in the conversion of NO_3^- to amino acids (Campbell 1999). Nitrate reductase activity in leaves is largely dependent on nitrate flux from roots (Ferrario-Méry et al. 1998) and is severely affected by osmotic stress induced by NaCl (Baki et al. 2000). A number of reports have shown that endophytic fungal colonization assists in N uptake under stress conditions (Khan et al. 2011a; Sherameti et al. 2005; Song et al. 2015). Recruitment of N in endophytic interactions differs from mycorrhizal interactions in which the fungus preferentially recruits ammonium rather than nitrate from the soil (Boukcim and Plassard 2003; Gage 2004). Song et al. (2015) showed that in ENC plants, N content increased in both the shoots and roots with increasing salt concentrations. The fungus was suggested to be involved in the cell's antioxidant and ROS-scavenging enzymes where N is an essential component (Cabot et al. 2014; Khan et al. 2014). Another study by Sherameti et al. (2005) showed a significant increase in growth of ENC plants that was proposed to be associated with a stimulation of the NADH-dependent NR, the key enzyme of nitrate assimilation in plants.

1.5.4.3 Na⁺:K⁺ ratio

Increased levels of Na⁺ in cells impairs important biochemical mechanisms required for plant growth and survival. Sodium accumulation alters cellular Na⁺:K⁺ ratios thereby reducing the availability of K⁺ that is required for activity of various enzymes and for the regulation of osmotic pressure and stomatal closure. Increased Na⁺ also competes with K⁺, disrupting cellular metabolism in roots and leaf tissues (Abdelaziz et al. 2017). This eventually increases the Na⁺:K⁺ ratios in the cytosol, and subsequently disrupts enzyme activity, protein synthesis, turgor maintenance, photosynthesis and stomatal movement (Evelin et al. 2019).

High Na⁺:K⁺ ratios in plants indicate higher levels of stress. Hence, plants must maintain low levels of Na⁺ to be able to resist the deleterious effects of salinity. ENC plants consistently have lower Na⁺:K⁺ ratios than NENC plants under saline conditions. Reza Sabzalilian and Mirlohi (2010) demonstrated that the toxic effect of Na⁺ was mitigated in grasses inoculated with endophytic fungi by increasing K⁺ concentration and thus maintaining the Na⁺:K⁺ ratio in plants. Similar results were found by Song et al. (2015) and Alikhani et al. (2013) where endophytic fungi modulated ion accumulation in colonized barley plants by decreasing the foliar Na⁺:K⁺ ratio. Restricting the transport of Na⁺ to leaves and ensuring a low cytosolic Na⁺:K⁺ ratio are important ways plants can increase their tolerance to high salt levels (Berthomieu et al. 2003; Cuin et al. 2003). Increase of K⁺ concentration is also related to mechanisms that control turgor pressure (Beckett and Hoddinott 1997). Song et al. (2015) also showed that the lower Na⁺:K⁺ ratios observed in ENC plants decreased the level of toxic ions and osmotic influence on plants under salt stress. Another study on barley plants inoculated with endophytic fungi showed a decreased Na⁺:K⁺ ratio compared to uninoculated plants, indicating that this ratio is a reliable indicator of the severity of salt stress, or for screening plant genotypes for high Na⁺ tolerance (Ghabooli 2014) (Table 2).

Plants control Na⁺ homeostasis through a variety of membrane proteins, antiporters, nonspecific cation channels, Na⁺ and K⁺ transporters, vacuolar ATPases and aquaporins, and the plasma membrane (PM) (Grabov 2007). Recently, Abdelaziz et al. (2017) postulated a molecular basis of establishing a balanced ion homeostasis of Na⁺:K⁺ ratio in ENC plants. Inoculated *Arabidopsis* plants had enhanced transcript levels of the genes encoding the high Affinity Potassium Transporter 1 (HKT1) and the inward-rectifying K⁺ channels KAT1 and KAT2, which play key roles in regulating Na⁺ and K⁺ homeostasis. Subsequently, lower Na⁺:K⁺ ratios were confirmed in the *Arabidopsis* line gl1-HKT:AtHKT1;1 that expresses an additional AtHKT1;1 copy driven by the native promoter. This study demonstrated that endophytic colonization promotes plant growth under saline conditions by modulating the expression level of the major Na⁺ and K⁺ ion channels, which helps in the establishment of a balanced ion homeostasis of Na⁺ and K⁺ under salt stress conditions (Abdelaziz et al. 2017).

1.5.5 Oxidative stress

Salt stress (osmotic and ionic stress) also interferes with proper cellular functions of plants due to enhanced production of ROS, which can lead to oxidative damage in several cellular components such as lipids, proteins and DNA (Gupta and Huang 2014). ROS consist of a group of chemically reactive oxygen molecules such as hydroxyl radical ($\text{OH}^\cdot$), H_2O_2 , $\text{O}_2^\cdot$ and O^{2-} and are produced as a result of interrupted pathways in plant metabolism that cause transfer of high energy electrons to molecular oxygen (Gill and Tuteja 2010). Broad host range endophytic fungi can confer effective tolerance to ROS under abiotic stress conditions such as salinity (Mastouri et al. 2010; Rodriguez et al. 2008). (Redman et al. 2011); Singh et al. (2011) reported that exposure to high salt conditions caused ROS accumulation in tomato, rice, panic grass, and dunegrass without endophytic associations, whereas the ENC plants had decreased ROS accumulation through various pathways (**Figure 1.3**).

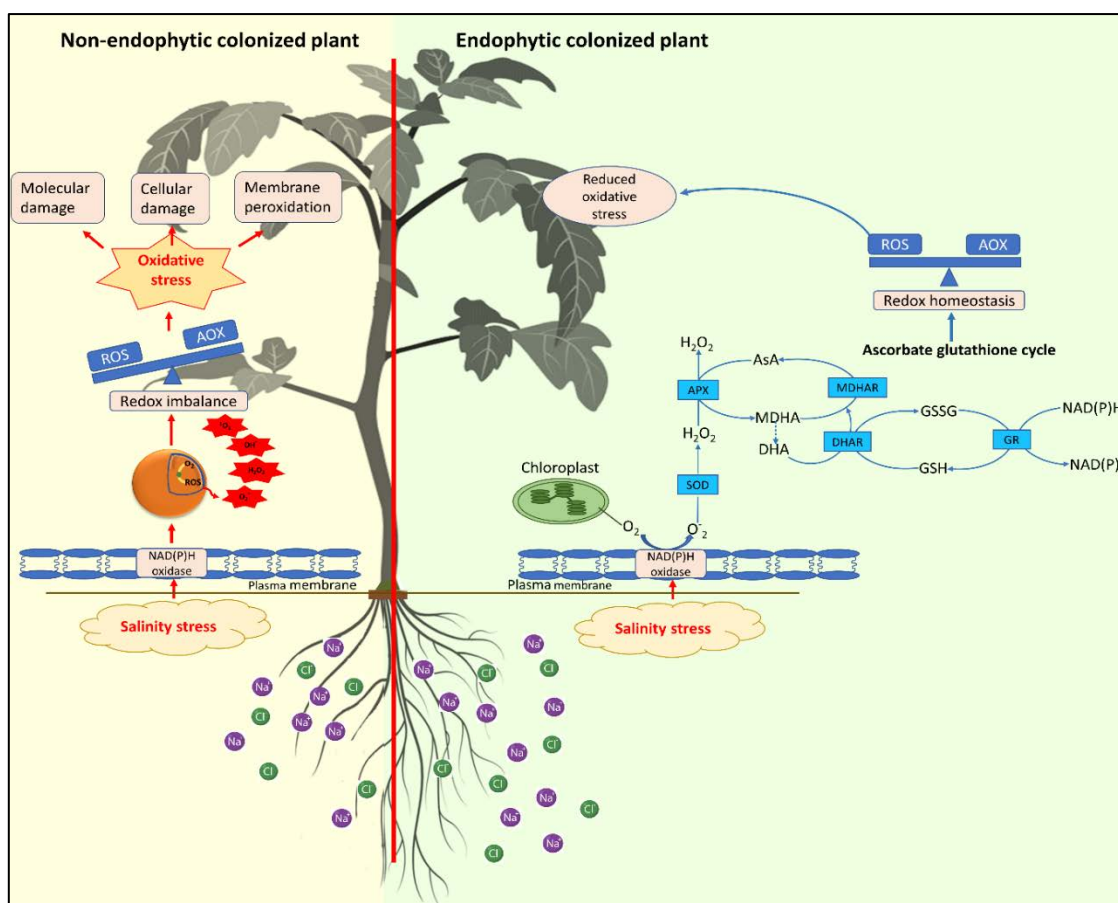


Figure 1.3. Oxidative stress tolerance mechanisms in salt stressed plants. Increase in salinity causes oxidative stress in plants due to redox imbalance between ROS (reactive oxygen species) and antioxidants. This results in molecular and cellular damage and membrane peroxidation eventually disturbing the normal functioning of the cell. Several enzymatic antioxidants pathways are induced to combat salt stress that include catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR). Ascorbate (AsA), glutathione (GSH), carotenoids are non-enzymatic antioxidants that are produced to counteract the adverse consequences of salt stress. In endophytic colonized (ENC) plants, the tolerance mechanism is reinforced by activating an efficient antioxidant system that abates the oxidative damage caused due to salt stress.

Plants have two ways to counteract the adverse consequences of ROS, mainly enzymatic and non-enzymatic antioxidative systems. The enzymatic system includes catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR), dehydroascorbate reductases (DHAR) and monodehydroascorbate reductases (MDHAR). The non-enzymatic antioxidant system consists of ascorbic acid (AsA), glutathione (GSH), carotenoids and osmoprotectants that play roles in quenching toxic by-products of ROS.

Baltruschat et al. (2008) reported increased activity of CAT, APX, GR and DHAR in the root tissues of barley under saline conditions. Increased activity of DHAR was seen in *P. indica* colonized barley leading to detoxification of ROS and an enhanced ratio of ascorbic acid to neutralize oxygen free radicals (Foryer and Noctor 2000). Azad and Kaminskyj (2016) used

H₂O₂ localization as a proxy to assess accumulation of ROS and showed that ENC plants had lower H₂O₂ levels in their leaves following NaCl-stress, confirming the role of endophytes to reduce stress-induced ROS generation.

Also, Zhang et al. (2016) reported that ENC plants had higher SOD, peroxidase (POD) and CAT activity suggesting that the coordination of POD and CAT activity along with SOD activity played a central protective role in the O₂⁻ and H₂O₂ scavenging process in ENC plants (Ahmad et al. 2015). Increased activity was a result of increased expression of the genes encoding the enzymes (Zhang et al. 2016). Under saline conditions, endophytic colonization also increases the concentrations of non-enzymatic antioxidant molecules such as AsA, GSH and carotenoids in plants as shown by several studies (Jan et al. 2019; Jogawat et al. 2013; Prasad et al. 2013; Waller et al. 2005).

Salinity increases the level of lipid peroxidation (Hernández 2019; Yu et al. 2020) which results in higher membrane permeability and loss of ions from the cells (Gupta and Huang 2014). NaCl treatment of ENC plants resulted in higher rates of lipid peroxidation in salt-sensitive plants than in salt-tolerant plants suggesting that the rate of lipid peroxidation can be used as an indicator to measure how effectively ENC plants cope with salt stress (Baltruschat et al. 2008). Another study showed that ENC plants contained higher ascorbate concentrations in roots compared with NENC plants, while the ratio of ascorbate to dehydroascorbate was not significantly altered and CAT, APX, GR, DHAR and MDHAR activities were increased. These changes were consistent with the decrease of leaf lipid peroxidation observed in these experiments (Waller et al. 2005). Similar results were shown by Mastouri et al. (2010) and Zhang et al. (2001) where ENC plants had significantly reduced accumulation of lipid peroxides than control plants under salt stress. Malondialdehyde (MDA), a product of lipid peroxidation, is generally regarded as an indicator of free radical damage to cell membranes caused by oxidative stress. Zhang et al. (2016) reported that salt stressed ENC plants had a 15% reduction in MDA compared to salt stressed NENC plants. **Table 1.3** lists some of the studies reporting changes in lipid compositions due to endophytic symbiosis in salt stressed plants.

Table 1.3 Examples of studies on the effects of salinity and endophytic fungi on lipid peroxidation in plants. Abbreviations in the table stand for: CAT-catalase, GR- glutathione reductase, SOD- superoxide dismutase, APX- ascorbate peroxidase, ABA- abscisic acid, MDA- malondialdehyde, SA-salicylic acid, POD- peroxidase

Study. No.	Salt level (mM NaCl)	Plant (Family)	Fungus	Parameters	Effects of		References
					Salinity	Endophytic fungi on salt stressed plants	
1	0, 100, 200, 300	<i>Oryza sativa</i>	<i>Piriformospora indica</i>	Total soluble proteins, lipid peroxidation (measured thiobarbituric acid reactive substances in shoots and roots), free proline content, and enzyme antioxidants (catalase (CAT: EC1.11.1.6), glutathione reductase (GR: EC1.6.4.2), superoxide dismutase (SOD:EC 1.15.1.1), ascorbate peroxidase (APX:EC 1.11.1.11)) activity	Increased lipid peroxidation, SOD, APX, CAT, GR; Decreased total soluble proteins, free proline content	Increased total soluble proteins, proline, further increase in SOD, APX, CAT, GR; Decrease in lipid peroxidation	Bagheri et al. (2013)
2	0, 100	<i>Glycine max</i>	<i>Fusarium verticillioides</i>	Lipid peroxidation, antioxidant enzyme analysis, gibberellins, ABA, salicylic acid	Increased ABA, SA, lipid peroxidation; Decreased CAT, SOD, POD, SA, ABA	Increased CAT, SOD, POD; Decreased lipid peroxidation, ABA, SA significantly	Radhakrishnan et al. (2013)
3	0, 100	<i>Glycine max</i>	<i>Fusarium verticillioides</i> and <i>Humicola sp.</i>	Protein content, catalase activity, ABA content, SA content, lipid peroxidation (measured in terms of MDA content)	Increased ABA, SA, lipid peroxidation; Decreased CAT, SOD, POD, SA, ABA, lipid peroxidation	Significant three-fold reduction in MDA level, ABA, SA; Increased CAT, SOD, POD	Radhakrishnan et al. (2015)
4	0, 100, 175, 250	<i>Medicago truncatula</i>	<i>Piriformospora indica</i>	Proline, MDA, Sodium ion, antioxidant enzymes	Increased MDA, Na^+ in shoots, slight increase in proline content; Decreased POD, SOD, CAT	Highest increased in proline content with increase in POD, SOD, CAT; decreased MDA, Na^+ in shoots	Li et al. (2017a)
5	0, 200	<i>Cucumis sativus</i>	<i>Trichoderma harzianum</i>	Antioxidant enzymes, K^+ content, K^+/Na^+ ratio, Na^+ content, ethylene levels, MDA levels as a measure of lipid peroxidation	Increased MDA levels, Na^+ content, ethylene levels; Decreased antioxidant enzymes, K^+ content, K^+/Na^+ ratio	Improved activities of antioxidant enzymes, increased K^+ content, K^+/Na^+ ratio; decreased Na^+ content, ethylene levels, MDA levels	Zhang et al. (2019)

1.5.6 Photosynthesis

Salt stress hinders photosynthesis resulting in an enormous loss in crop productivity (Sudhir and Murthy 2004). Salt stress has been shown to degrade D1 and D2 proteins of the photosystem II reaction centre. These proteins play crucial roles in protein phosphorylation coupled with the flow of electrons (Jansen et al. 1996). Salt stress also results in decreased photosynthetic pigments by reducing the activity of enzymes that synthesize them. Osmotic shock resulting from salt stress leads to reduced leaf area and decrease in stomatal and mesophyll conductance (Chaves et al. 2009). This limits CO₂ availability and assimilation which consequently affects RuBisCO (Seemann and Critchley 1985). Decreasing CO₂ assimilation also increases the risk of the accumulation of electrons in thylakoid membranes and predisposes the photosynthetic apparatus to increased energy dissipation. Thus, to dissipate this energy, photosystem II loses excess electrons causing injury to photosynthetic tissues and affecting the net photosynthetic rate (Redondo-Gómez et al. 2010).

Plants can protect the photosystems from light induced inhibition and damage in several ways such as minimizing harvesting of light and dispersion of excess energy by non-photochemical quenching (NPQ) (Lima Neto et al. 2015). An increase in NPQ can limit quantum yield (Baker 2008) but ENC plants are reported to have lower NPQ, therefore symbiosis enhances photosynthetic efficiency by proficient conversion of harvested light into chemical energy and minimizing NPQ (Pehlivan et al. 2017). Endophytic fungi are also known to reinforce these mechanisms and reduce the negative effects of salinity on plant photosynthetic capacity (Jogawat et al. 2013; Molina-Montenegro et al. 2018). **Table 1.4** lists some of the studies in the last decade on effect of salinity and endophytes on photosynthesis in plants. Endophytic symbiosis combats the negative effects of salt stress on photosynthesis in several ways. ENC plants have shown improved water status resulting in larger leaf area and higher stomatal conductance and eventually better assimilation of carbon dioxide (Zarea et al. 2012).

Magnesium (Mg) is one of the essential macronutrients for plant growth and is involved in numerous physiological and biochemical processes such as photosynthesis, enzyme activation and synthesis of nucleic acids and proteins (Chen et al. 2018). It is the central atom of the tetrapyrrole ring of chlorophyll *a* and *b* molecules, which are the major pigments for photosynthetic light absorption (Mayland 1990). Salt reduces uptake of Mg²⁺ thus also reducing the concentration of chlorophyll in leaves (Sudhir and Murthy 2004). ENC plants maintain higher chlorophyll concentration by improving the uptake of Mg²⁺ (Jogawat et al. 2013; Yin et al. 2014) and this leads to maintenance of plastid integrity and enhanced photosynthetic efficiency (Johnson et al. 2014).

Another way in which endophytes induce defence systems in plants under saline conditions is by upregulating the ascorbate-glutathione (ASH-GSH) cycle; for example Kumar et al. (2012) described that during salt stress, the endophytic fungus *P. indica* maintains a high antioxidative environment by defence system priming, especially the ascorbate–glutathione (ASH–GSH) cycle leading to maintenance of plastid integrity and therefore enhanced photosynthetic efficiency in colonised plants during abiotic stress (Johnson et al. 2014). ENC plants also confer the benefit of maintaining the integrity of photosystem II by repairing salt-induced degradation of D1/D2/Cytb 559 complex by the accumulation of glycine betaine in ENC plants (Rivero et al. 2014). Glycine betaine is also known to stabilise PSII pigment-protein complexes and protect the activities of RuBisCO and rubisco activase enzymes responsible for fixing CO₂ in AM fungi (Talaat and Shawky 2014).

Table 1.4 Examples of studies on the effects of salinity and endophytic fungi on photosynthesis in plants

Study. No.	Salt level (mM NaCl)	Plant	Fungus	Parameters	Effects of		References
					Salinity	Endophytic fungi on salt stressed plants	
1	0, 200, 300	<i>Oryza sativa</i>	<i>Piriformospora indica</i>	Photosynthetic pigment content [chlorophyll (Chl) a, Chl b]	Decreased	Increased	Jogawat et al. (2013)
2	0, 50, 150	<i>Lactuca sativa</i> (lettuce var. Romaine) and <i>Solanum lycopersicum</i> (tomato var. MoneyMaker)	<i>Colobanthus quitensis</i> (AFE001) and <i>Deschampsia antarctica</i> (AFE002)	The net photosynthesis rate (A), and transpiration rate (EC), water use efficiency (WUE) for photosynthesis as the ratio between photosynthetic rate and transpiration (A/EC)	Decreased Amax, WUE	Significantly increased Amax, WUE	Molina-Montenegro et al. (2018)
3	0, 100, 200, 300, 400, 500	<i>Triticum aestivum</i>	<i>Piriformospora indica</i> and <i>Azospirillum</i> spp.	Photosynthetic pigments (Chl a, b, ab)	Decreased	Significantly increased with inoculation of both organisms	Zarea et al. (2012)
4	0, 300, 500	<i>Solanum lycopersicum</i>	<i>Fusarium culmorum</i>	Photosystem II (PSII) efficiency	Decreased	Increased	Azad and Kaminskyj (2016)

1.5.7 Hormonal regulation

Induction of phytohormones is also one of the strategies plants use to mitigate abiotic stresses that ultimately enhance plant growth and productivity in stressful environments (Ryu and Cho 2015). Phytohormones, often regarded as plant growth regulators, are compounds that are derived from plant biosynthetic pathways acting either locally or via transport to other sites within the plant to mediate growth, development and nutrient allocation (Peleg and Blumwald 2011). These include abscisic acid (ABA), gibberellins (GA), ethylene (ETHY), cytokinins (CKs), brassinosteroids (BRs) and auxins, particularly indole acetic acid (IAA). To initiate suitable plant responses to environmental stimuli, there is interplay between these hormones to modulate biochemical and physiological processes (Saeed et al. 2017).

It is known that some strains of endophytic fungi can produce plant hormones, especially gibberellins (GAs), to help the plant to tolerate or avoid abiotic stress (Contreras-Cornejo et al. 2009; Khan et al. 2011b; Waller et al. 2005). Hamayun et al. (2010) reported that inoculation with the endophytic fungi *Phoma herbarum* showed increased plant biomass and elevated production of active GAs including GA₁, GA₃, GA₄, and GA₇ in salt-stressed soybean. Similar results were shown by Waqas et al. (2012), where salt-stressed cucumber plants inoculated with *Penicillium* sp. had larger shoot growth and plant biomass that was attributed to the secretion of bioactive GAs. A study on salt-stressed cucumber plants inoculated with *Trichoderma asperellum* Q1 alleviated the suppression effects of salt stress by altering the phytohormone levels (IAA, GA and ABA) and the phosphate solubilization ability (Lei and Zhang 2015). Three bioactive GAs, i.e. GA₄, GA₉ and GA₁₂ were more abundant in ENC plants grown under salt stress compared to NENC plants (Khan et al. 2011c), and this mitigated the adverse effects of salinity and improved growth.

Endophytic symbiosis under saline conditions has a positive influence on the endogenous concentration of auxins (Contreras-Cornejo et al. 2009). Contreras-Cornejo et al. (2014) evaluated the expression of the auxin-responsive marker gene *DR5:uidA* which was upregulated in ENC plants compared to their counterparts under saline conditions speculating that, by providing auxins, *Trichoderma* spp. could restore auxin homeostasis and, consequently growth and development could be normalized when grown under salt stress.

1.5.8 Perspectives and future directions

Evolution has led to complex interactions between a wide diversity of microorganisms and plants; many of them resulting in the establishment of a symbiotic relationship between them (Hassani et al. 2018). These interactions beneficially impact plant survival, biodiversity, fitness and ecosystem function (Bai et al. 2018; Rosier et al. 2016; Sasse et al. 2018). Growing evidence indicates that endophytic associations can also be important for plant fitness, development of the immune system, tolerance to abiotic stresses, nutrient acquisition and disease suppression

(Hiruma et al. 2016; Khan et al. 2015; Khare et al. 2018; Soliman et al. 2015; Terhonen et al. 2016; Zuccaro et al. 2014). This review highlights some of the numerous mechanisms by which endophytic symbiosis promotes salt tolerance in plants. However, there are several challenges and issues that future research should address for comprehensive understanding of these mechanisms. It is well established how osmotic adjustment in plants under salt stress via enhanced accumulation of osmolytes is achieved using endophytic symbiosis. However, the biochemical, molecular and genetic mechanisms are largely unexplored. Therefore, there is a need to understand these phenomena by investigating genes encoding enzymes used for the synthesis of molecules that are crucial for salt stress resistance. Therefore, dedicated research into unravelling the molecular basis of osmolyte accumulation in ENC plants will broaden our understanding of the mechanisms involved.

In recent years, new compounds, such as polyamines, and strigolactones have been implicated in improving plant tolerance to salt stress (Fahad et al. 2015). Strigolactones (SL) play regulatory roles to combat abiotic stress, including salinity, and in order to be fully effective, they need to modulate and interact with other phytohormones, especially auxin and ABA. SLs are also involved in several aspects of plant development; suppression of secondary branches in shoots, regulation of leaf senescence, stimulation of internode length and induction of endophytic symbiosis (de Saint Germain et al. 2013; Lopez-Raez et al. 2017; Yamada et al. 2014). This group of sesquiterpene lactones is responsible for hyphal branching and successful colonisation within roots by producing 5-deoxy-strigol, followed by the formation of a pre-penetration apparatus (Genre et al. 2005). Recently, SL secreted by roots of *Arabidopsis thaliana* was found to act as a signal molecule for colonization of endophytic *Mucor* sp. (Rozpadek et al. 2018). Studies on auxin and ABA involvement with endophytes under salt stress has been explored, but further research is required to investigate the role of strigolactones secreted by ENC plants in ameliorating salt stress.

The root is the primary location in plants that senses salt stress. The PM constitutes the interface between a cell and its surroundings and plays an important role in cell wall biosynthesis, ion transport, endocytosis, sensing of environmental stimuli, and cellular signal transduction (Mansour et al. 2015). PM lipids and proteins in salt tolerant plants are protected from oxidative attack through enhanced antioxidant systems, a mechanism that minimizes lipid and protein oxidation while retaining PM integrity (Mansour 2013). Though lipid peroxidation has been elucidated in ENC plants under salt stress, lipid metabolism in the PM in root tissues is yet to be investigated. Hence future research that evaluates how endophytic symbiosis influences these changes under saline conditions is warranted.

Limited studies are available to understand the role of endophytic fungi in modifying the photosynthetic capacity of plants to alleviate the negative effects of salinity as described in previous sections. Salt stress has been shown to degrade proteins of the PSII reaction centre. These proteins play fundamental roles in phosphorylation of proteins (Jansen et al. 1996). Studies in the past have focused on understanding how AMF symbiosis acts to maintain the integrity of PSII showing the upregulation of the genes encoding these proteins under salt stress (Chen et al. 2017). However, research on maintenance of these proteins by endophytic fungi under salt stress is a field to explore.

Metabolomics is increasingly being utilized for generating deep insights into abiotic stress responses. Several studies have focused on exploring and discovering compounds that stimulate ENC plant growth by alleviating stress using various technologies (Chetia et al. 2019; Kusari and Spiteller 2012; Mazlan et al. 2019; Tawfike et al. 2018). However, molecular signalling mechanisms employed by endophytic fungi under saline conditions are yet to be explored. The high-throughput mass spectrometric profiling of cellular metabolites of plant-associated endophytes under the influence of salt stress could help to reveal the level of interference by the stressor in overall cellular homeostasis. Thus, future 'omics-based research is required to generate comprehensive information on specific plant-endophytic fungi-salt stress systems to resolve facts behind precise mechanisms of stress tolerance in crop plants.

Although this review covers mechanisms and strategies employed by plants under salt stress, in nature plants often face multiple biotic and abiotic stresses instead of a single stress. These combinations of stresses exert more complex effects on plant fitness which eventually results in potential differences from the responses elicited under single stresses. (Bai et al. 2018) demonstrated that tomato developed integrated responses via genetic components and cross-talk of signalling pathways under combined salinity and pathogen stresses. This shows that plants must have evolved to mitigate a combination of stresses. Addressing specific questions related to multiple stresses such as how beneficial microorganisms and pathogens or combined abiotic stresses interact would facilitate the design of strategies for sustained plant health under diverse environmental stresses.

In conclusion, directing future research on endophytic symbiosis under salinity in order to understand the above-mentioned challenges will help improve our knowledge and understanding of the mechanisms of endophyte facilitated salinity tolerance in host plants.

1.5.9 Acknowledgements

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1.6 Addendum

1.6.1 Metabolomics and lipidomics as tools to determine the biochemistry of root-microbe interactions

In recent years, the applications of metabolomics to analyse the metabolite complement (metabolome) of the rhizosphere is emerging, as it is an unbiased way to understand complex physiological, pathological, symbiotic and other relationships among the inhabitants of the rhizosphere (Bhalla et al. 2005). Metabolite composition in the rhizosphere is mainly governed by the nature of root exudates, secretions from microbes and other soil organisms. On the other hand, the nature of these root exudates also directly or indirectly affects microbial growth in the rhizosphere (Reuben et al. 2008). Plant-microbe interaction in the rhizosphere also covers many aspects of plant growth and chemicals involved in nutrient and carbon cycling. A substantial number of these chemicals are present in the form of plant primary and secondary metabolites. While some metabolites enhance growth, others are involved in antimicrobial activities. These effects are not confined to the microbes but also extend to the plants owing this rhizosphere. Therefore, gaining knowledge about the metabolites involved in plant-microbe interactions will help us better understand this ecological niche (Reuben et al. 2008).

Metabolomics and lipidomics utilise state-of-the-art analytical technologies such as chromatography, mass spectrometry and nuclear magnetic resonance (NMR). This field is not only confined to metabolite and lipid profiling; it also encompasses a much broader approach, including identification of compounds, their quantitation, comparisons between genotypes and treatments, data analysis and the development of metabolic models, in a given tissue at a given time. The metabolome of an organism includes the entire range of metabolites and lipids whose profiling is in practice unattainable with a single method of extraction or analytical technique. Thus, various analytical methods and techniques have been used for comprehensive metabolic profiling (Bhalla et al. 2005). Some of the most common techniques are discussed below.

Gas chromatography (GC) is a high resolving chromatographic separation technique used mainly for separation of volatile and semi-volatile compounds. GC coupled with mass spectrometers (GC-MS), such as quadrupole MS, tandem MS, orbitraps, time-of-flight (TOF) and QTOF-MS offers high chromatographic efficiency and molecular selectivity of compounds of interest. GC-MS has enabled various studies to investigate, for example, organic compounds and sugars from root exudates in *Helianthus annuus* (Bowsher et al. 2015), and fatty acids of wheat, lupin and maize in root mucilage (Read et al. 2003). GC-MS has also been used to study symbiotic nitrogen fixation in legume roots (Desbrosses et al. 2005). Another study by Menotta et al. (2004) used GC-MS to identify molecules involved in signalling during presymbiotic interaction between the host plant, *Tilia Americana*, and ectomycorrhizaa, *Tuber borchii*. Bilal et al., 2018 (Bilal et al. 2018) used GC-MS to analyze the phytohormones, IAA and GA in culture filtrate of two endophytic fungi,

Aspergillus fumigatus TS1 and *Fusarium proliferatum* BRL1. Recently, GC-MS was used by Guo et al. (2019b) to investigate volatile organic compounds (VOCs) of 47 different fungal species from different functional guilds. The study showed that fungal VOCs were expressed in phylum- and eco function-based patterns.

Another technique used widely to identify numerous metabolites that are sequestered in plants compartments, are labile or degraded at high temperature and are difficult to derivatize, is liquid chromatography mass spectrometry (LC-MS) (Karlovsky 2008). Tugizimana et al. (2019); Tugizimana et al. (2018a) performed an untargeted LC-MS metabolomic strategy to elucidate metabolome changes in the anthracnose-causing *C. sublineolum*, a member of one of the most widespread and economically detrimental genera of plant pathogenic fungi. The results showed a metabolic variation trajectory using chemometric modelling. This comprised three distinct stages that metabolically described the adaptation of the fungus to diminished nutrients. Tugizimana et al. (2018a) also investigated the metabolic alterations of three sorghum cultivars interacting with *C. sublineolum*. This study revealed key characteristics of the biochemical mechanism underlying *C. sublineolum*–sorghum interactions and provided insights with potential applications in crop breeding. Another study by Vinale et al. (2017) used LC-QTOF-MS/MS to investigate the metabolome of two beneficial fungi grown together. The results indicated that the presence of the fungal counterpart modified the metabolome of another by modulating the biosynthesis of secondary metabolites.

Metabolomics allows detection of thousands of metabolites from a homogenate of a complex community in a single measurement using mass spectrometry. However, the use of a homogenate destroys the spatial organisation of host-microbe associations. Mass Spectrometry Imaging (MSI) is a technique that allows the visualization of the spatial metabolome of samples in situ. Using this technique, a two- or three-dimensional image is created by taking measurements across an individual pixel basis across a tissue section (Heyman and Dubery 2016; Rao et al. 2016; Wheatcraft et al. 2014). Recent technical advancements in matrix-assisted laser desorption ionization MSI (MALDI-MSI) now allows imaging of metabolites with a spatial resolution in the lower micrometer range (Feenstra et al. 2017; Gilmore et al. 2019).

The use of MALDI-MSI technology to study plant-microbe interactions has been demonstrated in previous reports. For example, hesperidin, a flavonone produced by citrus plants associated with *Xylella fastidiosa* infections, was imaged using MALDI-MSI on petiole and leaf transverse sections of infected and uninfected plants. This suggested a potential role of hesperidin as a phytoanticipin involved in plant-microbe interactions. Phytoanticipins are low molecular weight defense compounds that are produced by plants from pre-existing constituents, after infection by microbes (VanEtten et al. 1994). In another work, organic acids, amino acids, sugars and lipids

were identified and mapped using MALDI-MSI in root nodules formed by symbiotic associations between *Medicago truncatula* and *Sinorhizobium meliloti* (Ye et al. 2013). Recently, Barbosa et al. (2018) identified and determined the spatial distribution of peptides, proteins, and secondary metabolites present in galls of tomato roots (*Solanum lycopersicum*) induced by root-knot nematode *Meloidogyne incognita*. The study characterised glycerophospholipids suggesting their involvement in infection and maintenance of nematode feeding cells as well as root development.

1.6.2 Significance of this Thesis

High salinity in soils is one of the major causes of decline in cereal crop yield and significantly affects agricultural sustainability worldwide. In the past few decades, the use of salt tolerant microbial inoculants that enhance crop growth in saline soils has become an alternative strategy to improve salt tolerance of crops (de Zelicourt et al. 2013; Nadeem et al. 2014; Souza et al. 2015).

Barley (*Hordeum vulgare* L), a member of the Poaceae grass family, is an ancient cereal grain that was domesticated around 10,000 years ago (Nevo 2013). This crop was originally used as human food but developed into a feed, malting and brewing grain due to the rise to prominence of wheat and rice (Baik and Ullrich 2008). Today, approximately 75% of barley's global production is used for animal feed, 20% is malted for alcoholic and non-alcoholic beverages and 5% is used as an ingredient in a myriad of food products (Blake et al. 2010).

Barley is one of the most important cereal crop after rice (*Oryza sativa*), wheat (*Triticum aestivum*) and maize (*Zea mays*) (Ferreira et al. 2016). It not only grows in gradually more adverse environments such as saline soils but is also highly adaptable to other stressful conditions such as cold, drought and alkaline soils. Due to this, barley is more suited to cultivation in higher altitudes and latitudes and almost any environment in the world than other cereal crops (Baik and Ullrich 2008). Even though barley is commonly regarded as a salt-sensitive (glycophyte crop), it exhibits more salt tolerance than other cereal crops consequently making it a great model to study salt tolerance traits in cereal crops (Saisho and Takeda 2011). Barley also exhibits a typical cereal germination process, allowing the results for this crop to be applied to other cereal crops (Gorzolka et al. 2016).

The ability of the endophyte *Trichoderma harzianum* to promote plant growth has been extensively studied. Its role in conferring salt tolerance on its host plant is also well documented. However, the effect of colonisation on the metabolome and lipidome of plants grown in saline soil, and the spatial distribution of the metabolites in roots has not been explored. Therefore, this study will firstly aim to develop approaches for successful association of the endophyte *T. harzianum* strain T-22 with barley roots to be confirmed using brightfield microscopy. Secondly,

analysis of the metabolome and lipidome of roots interacting with *T. harzianum* T-22 will be done by using metabolomic techniques such as GC-MS and LC-MS with the aim to investigate metabolites and lipids that may potentially play a role in conferring growth promotion in barley under saline conditions. MALDI-MSI will also be employed to study the spatial distribution of metabolites involved in the plant-endophyte interactions during the response to salinity in barley roots.

Lastly, early stages of germination in barley seeds will be studied using four high-end analytical techniques — MALDI-MSI, liquid chromatography-tandem mass spectrometry (LC-QToF-MS/MS), inductively coupled plasma mass spectrometry (ICP-MS), and micro X-ray fluorescence (μ -XRF), to give new insights into the effects of salinity on metabolites including lipids, as well as elements including Na^+ and K^+ during germination. This combined approach provides unique insights into the spatially resolved coordination of metabolic processes that are sequestered among the germinating embryo axis, the lipid rich scutellum, the nutritive endosperm, the digestive enzyme secreting aleurone and the outer pericarp cell layers during the early stages of seed germination, a critical stage of successful plant growth.

1.6.3 Aims of this Thesis

The main objective of this thesis is to investigate the metabolites and lipids involved in growth promotion and responses to salinity stress in barley roots interacting with the beneficial fungi *T. harzianum* T-22 using high-end metabolomics techniques of GC-MS, LC-MS and MALDI-MSI.

Chapter 2 describes the effect of the salt tolerant beneficial endophyte, *T. harzianum* strain T-22, on the growth and development of barley under optimal and saline condition. Two barley genotypes were used in the study, cv. Vlammingh as a salt tolerant cultivar and cv. Gairdner as a salt sensitive cultivar (Gupta et al. 2019). Barley was chosen as it is not only an agriculturally and industrially important crop but is also the most salt tolerant cereal crop (Hill et al. 2016). Two experimental setups were used for this study. In the first experiment, agar was used as the growth medium for plants. This was performed to observe and determine the effect of fungus on the growth of plants under controlled conditions. In the subsequent experiment, sandy loam soil was used as the matrix to grow plants and to emulate agricultural land scenarios. The results confirm the positive effect of this fungus under controlled and saline conditions in both experiments. Various parameters were measured at the end of each study, to confirm the effects of salt and fungus on both genotypes under controlled and saline conditions. Roots were also immediately frozen in liquid nitrogen and stored in a $-80\text{ }^{\circ}\text{C}$ freezer until extraction for metabolomic analyses as described in the following Chapters. This study suggests that inoculation of salt sensitive plants with *T. harzianum* T-22 may ameliorate the effects of salinity and improve plant growth.

Chapter 3 investigates the changes in polar metabolites and lipids in roots of both genotypes mentioned in Chapter 2. For this, a combination of two high-end analytical techniques- gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) was used to give new insights into the metabolism of roots. It investigates the biochemical changes in barley roots treated with fungus during the early stages of interaction. Hierarchical cluster analysis, unsupervised principal component analysis and pairwise comparisons allowed identification of metabolites and lipid species in barley roots. This combined approach provides unique insights into the metabolome and lipidome in barley roots influenced by the inoculation of endophytic fungi identifying metabolic processes that are induced during this interaction.

Chapter 4 uses the same approach mentioned in **Chapter 3**. However, since soil is an important substrate for *Trichoderma*, investigation of barley roots was performed which were harvested from saline soil. The aim of this Chapter was to determine the metabolites and lipids modified in roots grown in saline soil following endophyte inoculation that are involved in conferring positive effects on barley plants as mentioned in **Chapter 2**. This study provides evidence of the molecular adaptations enacted by metabolites and lipid species in barley roots from genotypes with differing levels of salinity tolerance under these conditions. cv. Vlamingh displayed significantly high increase of polar metabolites and lipids within roots inoculated with fungus under saline conditions and an opposite pattern was observed in cv. Gairdner. This points to these findings as potential targets for engineering of salt tolerant crops, especially TAGs with highly unsaturated fatty acids.

Chapter 5 covers analysis using mass spectrometry imaging (MSI), liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QToF-MS), inductively coupled plasma mass spectrometry (ICP-MS), and X-ray fluorescence (XRF) to determine the spatial distribution of metabolites, lipids and a range of elements, such as K^+ and Na^+ , in seeds of two barley genotypes with contrasting germination phenology (Australian barley varieties Mundah and Keel). The detection and tentative identification of more than 200 lipid species belonging to seven major lipid classes (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, prenol lipids, sterol lipids and polyketides) that differed in their spatial distribution based on genotype (Mundah or Keel), time post-imbibition (0 to 72 hours), or treatment (control or salt) was performed. This study integrates data obtained from three mass spectrometry platforms together with μ -XRF to yield information on the localization of lipids, metabolites and elements improving our understanding of the germination process under salt stress at a molecular level. Lastly, the effect of inoculation of *T. harzianum* T-22 on seeds of four barley genotypes used in **Chapter 2** and **Chapter 5** was investigated.

The final **Chapter 6** describes the application and importance of mapping the biochemical changes involving metabolites and lipids, imparted due to the inoculation of the fungus in roots of barley seedlings and provides a view on future perspectives of plant-fungal interactions for plant growth promotion which can be useful for sustaining agricultural productivity.

Chapter 2

**The role of *Trichoderma harzianum* T-22 in plant growth and development
under controlled and soil environment**

2.1 Introduction

Agriculture is one of the most important practices developed by humans for their survival on earth. However, increasing global population places mounting pressure on global food security that greatly relies on agricultural crops and their productivity to sustain the population. Despite huge advancements in technology and un-precedent use of chemical fertilizers, recent surveys show that around 870 million people are hunger-stricken due to various resource scarcities. Therefore, there is a need to increase agricultural production by at least 50% to meet the demands of human population growth that is expected to reach 10 billion by 2050 (FAO 2017).

Soil salinity is one of the major global issues impacting agricultural productivity and sustainability. This problem is projected to increase in future due to climate change with increased sea level and impact on coastal areas resulting in agricultural land inundation and increased temperature that will eventually increase evaporation resulting in further salinization (Shahid et al. 2018). Therefore, there is a need to achieve agricultural sustainability by utilizing and implementing techniques of farming which would increase production of crops while also conserving and protecting the environment and its natural sources.

Sustainable agriculture relies on the interaction between plants and useful microbes resulting in a symbiotic relationship where plants benefit in a number of ways such as enhanced growth and better yield (Ishaq 2017; Kumar and Verma 2019; Kumari et al. 2019; Shelake et al. 2019). Microbes (mainly bacteria and fungi) that thrive in the rhizosphere (the narrow zone of soil that surrounds, and is influenced by, plant roots) play an important role in agriculture by protecting plants against several biotic (Gangwar and Singh 2018; Johnson et al. 2014) and abiotic stresses (Harman and Uphoff 2019; Khan et al. 2015; Kumar et al. 2019; Kumar et al. 2012; Mastouri et al. 2010) while, simultaneously, reducing reliance on agro-chemicals. Hence, these microbes, also known as plant growth promoting microorganisms (PGPM) are important tools for sustainable and environmentally friendly solutions to agricultural problems.

The use of endophytic fungi as a means of improving plant quality and growth, is extensively reported in the scientific literature (Carroll 1992; Schulz et al. 1999). They are also involved in nutrient supply, environmental acclimatization, biotic and abiotic stress protection, growth promotion and enhancing the community biodiversity of host plants (Berg 2009; Gond et al. 2010; Pandey et al. 2011). Examples of PGPM include several species of Ascomycota fungi belonging to the genus *Trichoderma*. *Trichoderma spp.* have been known as biocontrol agents for decades (Mastouri et al. 2010). *Trichoderma spp.* are known to draw nutrients from plants by facultative symbiosis and in return boost fertility and immunity by coordinated genetic responses that alter the transcriptome, proteome and metabolome in host plants (Akter et al. 2019). In recent years, *Trichoderma spp.* have been studied in plants under abiotic stress and have been

reported to improve the survival of plants in hostile environments by increasing plant tolerance (Gangwar and Singh 2018; Harman et al. 2004b; Khomari et al. 2018; Mastouri et al. 2010; Yasmeen and Siddiqui 2018).

The root system is the main interface with the soil and the soil microbiome in vascular plants. It is also the first organ that encounters soil born biotic and abiotic stress. Root architecture is largely under genetic control (Bray and Topp 2018) but can also be significantly influenced by environmental factors. Therefore, understanding changes in the root morphology in response to abiotic stress, particularly salinity, may be helpful in describing the role of roots not only in ion uptake but also for control of water loss and nutrient uptake through aerial parts of the plant. Among all the possible root traits that can be measured on root samples extracted from plant studies, the specific root length and the root length density are of particular interest. However, one must know the total root length (TRL) in the studied plants as a prerequisite for calculating specific root length and the root length density (Delory et al. 2017). Several studies have used this trait to measure the effect of salt stress in various cereal crops such as rice (Kakar et al. 2019), wheat (Rahnama et al. 2019) and barley (Sarabia et al. 2019). Furthermore, biomass production is an important trait to breed productive plant species that can be grown in various types of biotic and abiotic stress conditions to optimize production practices (Edwards et al. 2014; Freschet et al. 2015).

In this chapter, the effect of the salt tolerant beneficial endophyte, *Trichoderma harzianum* strain T-22, on the growth and development of barley under optimal and saline conditions, was investigated. Two barley genotypes were used in the study, cv. Vlamingh as a salt tolerant cultivar and cv. Gairdner as a salt sensitive cultivar (Gupta et al. 2019). Barley was chosen as it is not only an agriculturally and industrially important crop but is also the most salt tolerant cereal crop (Hill et al. 2016). Two experimental setups were used for this study. In the first experiment, agar was used as the growth medium for plants. This was performed to observe and determine the effect of fungus on the growth of plants under controlled conditions. In the subsequent experiment, sandy loam soil was used as the matrix to grow plants and to emulate agricultural land scenarios. Light microscopy was used to confirm the association of fungus within roots. The results confirm the positive effect of this fungus under controlled and saline conditions in both experiments. The salt concentration chosen for this study was 200mM NaCl. A small experiment was conducted before selecting this concentration where fungal hyphae was grown on saline media (data not shown). The data showed that the fungus was successful in growing upto 200mM NaCl and thus this concentration was chosen for all further studies. Various parameters were measured to confirm the effects of salt and fungus on both genotypes under controlled and saline conditions. This study suggests that inoculation of salt sensitive plants with *T. harzianum* T-22 may ameliorate the effects of salinity and improve plant growth.

2.2 Materials and Methods

2.2.1 Seeds used in this study

Two barley genotypes (malting variety) were used in the study, cv. Vlamingh as a salt tolerant cultivar and cv. Gairdner as a salt sensitive cultivar, based on their known differences in germination phenology and salinity tolerance (Gupta et al. 2019). These cultivars come from different breeding programs (Sarabia 2019). Cultivar Vlamingh seed had a 95% germination rate after four days treatment with 200 mM NaCl whereas for cv. Gairdner, the germination rate was 75% (Gupta et al., 2019). In a glasshouse study (data not shown) comparing control and saline conditions (200 mM NaCl) cv. Vlamingh had higher biomass production than other tested cultivars and cv. Gairdner the lowest. All seeds were sourced from the University of Adelaide, Australia.

2.2.2 Fungal isolate and growth conditions used in this study

Freeze dried pellets of *Trichoderma harzianum* strain T-22 (ATCC 20847) were sourced from the American Type Culture Collection (ATCC, Washington, DC). The pellet was resuspended in sterile distilled water and kept at room temperature (25 °C) undisturbed overnight. After initial incubation, the suspension was mixed well and inoculated on Potato Dextrose Agar (PDA) (Merck, Australia) prepared as per manufacturer's instructions. The plates were incubated at 25 °C in the dark for 5 d followed by light for 3 d. Conidia were observed after eight days of incubation. The plates were stored at room temperature prior to experimental setup.

2.2.3 Seed preparation

To initiate fungal association with barley roots, seeds from both genotypes were sterilized by immersion in 70% ethanol for 1 min and rinsed 4-5 times in sterile 18.2 Ω deionized water followed by immersion in 1.0% (w/v) sodium hypochlorite for 10 min and rinsed thoroughly in sterile 18.2 Ω deionized water 6–7 times. Seeds were then imbibed overnight (~16 h) in sterile 18.2 Ω deionized water with continuous aeration. Sterilized seeds were transferred to agar plates containing Whatman paper soaked in 5 mL 18.2 Ω deionized water. Plates were then sealed with parafilm, wrapped in aluminium foil and kept at 45° angle in a growth chamber maintained at 17 °C constant temperature with no light. Plates were unwrapped after 48 hours and transferred to a growth chamber maintained at 17 °C for 16 h light and 10 °C for 8 h dark cycles for five days.

2.2.4 Fungal inoculant preparation

Simultaneously to seed preparation in Section 2.2.3, a fresh conidial suspension was prepared by flooding a PDA plate, with observable conidia, with sterile 18.2 Ω deionized water. The subsequent fungal suspension was collected and passed through a miracloth (Sigma-Aldrich, Australia) filter to separate conidia from hyphae. The suspension was diluted to yield 1×10^8 colony-forming unit (CFU) ml⁻¹ conidia.

2.2.5 Determining fungal association with roots using brightfield microscopy

Fungal association with barley roots was performed using seeds of cv. Gairdner only. Fifteen 7d old germinated seedlings were inoculated by immersion in a conidial suspension of 10 mL of 1×10^8 CFU g⁻¹ of seedling in a 10 mL conical centrifuge tube (Thermo Fisher Scientific, USA). The fungal association was monitored daily from 24 h post-inoculation using light microscopy. Daily microscopic observations were continued until fungal hyphae were observed associated with roots. A modified method from Vierheilig et al. (1998) was used for staining roots. Briefly, roots were cleared using 10% (v/v) KOH for 3 min and rinsed several times with tap water. Cleared roots were boiled for 3 min in 5% (v/v) Shaeffer black ink-vinegar solution with pure white household vinegar (5% acetic acid). Roots were then destained by rinsing in pure water for 20 min. This allowed observation of hyphae structures within the roots and distinguished colonized roots from non-colonized roots.

For light microscopy, roots were immediately transferred to a 50 mL conical centrifuge tube (Thermo Fisher Scientific, USA) containing 40 mL of 18.2 Ω deionized water until observed under the microscope. A Leica MZ FLIII Microscope was used to observe fungus associated with roots. A Leica DC 300F camera (Leica Microsystems, Australia) was used to capture the images. In order to process the images obtained, Irfan View Software was used (Pomeranz et al. 2013). The lens was adjusted to blank and the filter was set from visible to 100% photon. The configuration was set to brightfield and a blank image was captured using plain white paper to adjust the white balance. All images acquired from the tissues were subsequently processed using Irfan View Software.

2.2.5.1 Fungal root interactions on agar medium

A flow-chart showing the experimental setup for plant growth conditions and inoculation with fungus is given in **Figure 2.1**. For fungal root interactions on agar medium, 36 seeds from each genotype were sterilized as per **Section 2.2.3**. Simultaneously, a fresh conidial suspension was plated on PDA to yield conidia for inoculation as per **Section 2.2.4**.

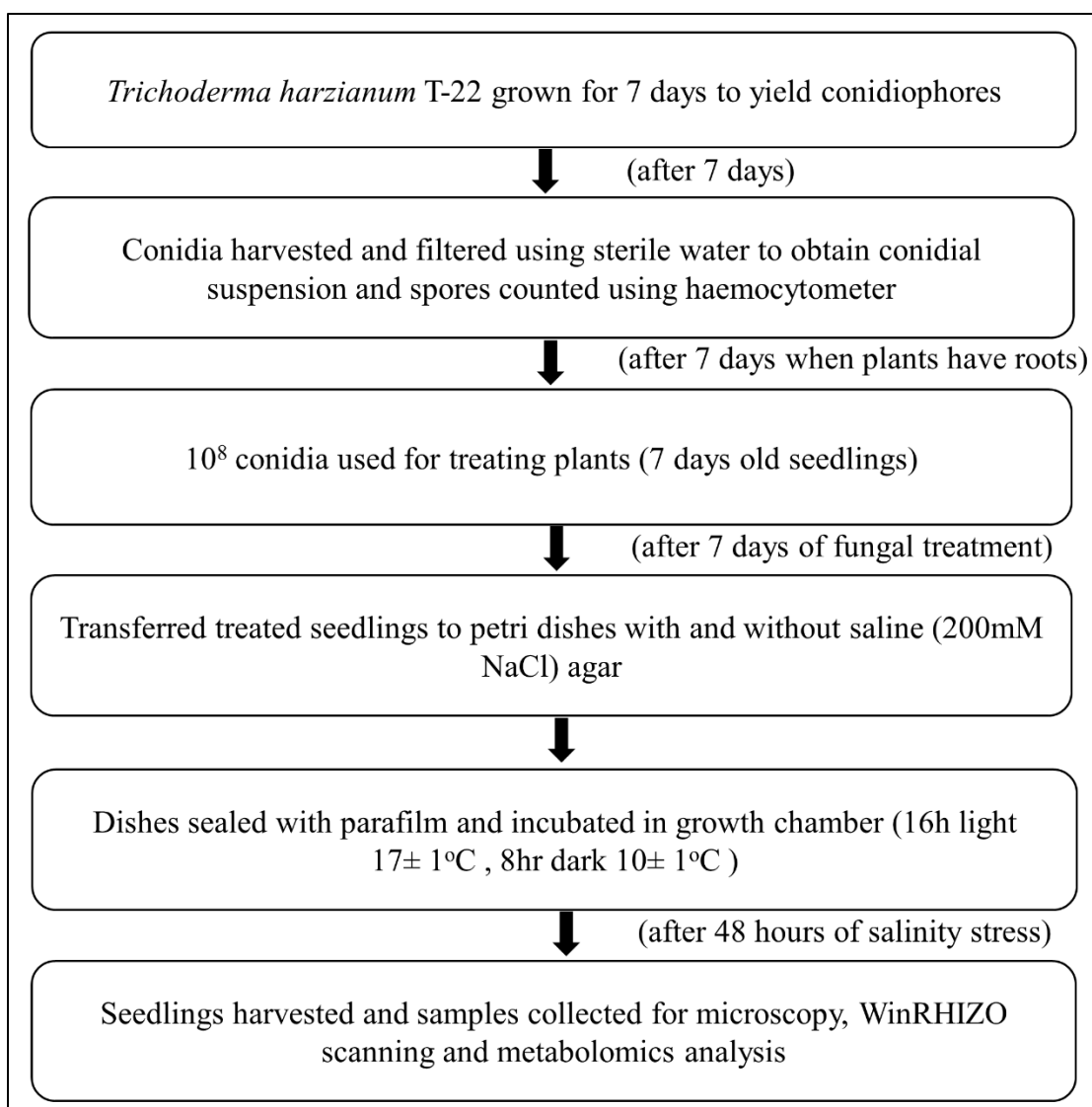


Figure 2.1 Flow chart of experimental setup for plant growth conditions and inoculation with fungus.

After 7 d of germination, 18 seedlings from each genotype were inoculated with conidial suspension of 10 mL of 1×10^8 CFU g⁻¹ of seedling in a 10 mL conical centrifuge tube (Thermo Fisher Scientific, USA) and another 18 seedlings from each genotype were treated with 10 mL of sterile 18.2 Ω deionized water. Seedlings were placed in a growth chamber maintained at 17 °C for 16 h light and 10 °C for 8 h dark cycles for 7 d. Seven days were chosen for interaction between roots and fungus after confirming the presence of fungal hyphae on day seven as established in **Section 2.2.5**. After 7 d, 9 inoculated and 9 uninoculated seedlings from each genotype were transferred to either 1% (w/v) agar plates containing 200 mM NaCl or 1% (w/v) agar plates containing no NaCl (**Figure 2.2**). A total of nine replicates were allocated for each condition and each condition was assigned as follows, 'C' (control), uninoculated seeds with no salt stress; 'CF' (no salt + fungus), fungal inoculated seeds with no salt stress; 'S' (negative control), uninoculated

seeds with salt stress; and 'SF' (salt + fungus), fungal inoculated seeds with salt stress. The agar plates were again transferred to a growth chamber maintained at 17 °C for 16 h light and 10 °C for 8 h dark cycles for 48 h to introduce short term salt stress to growing seedlings.

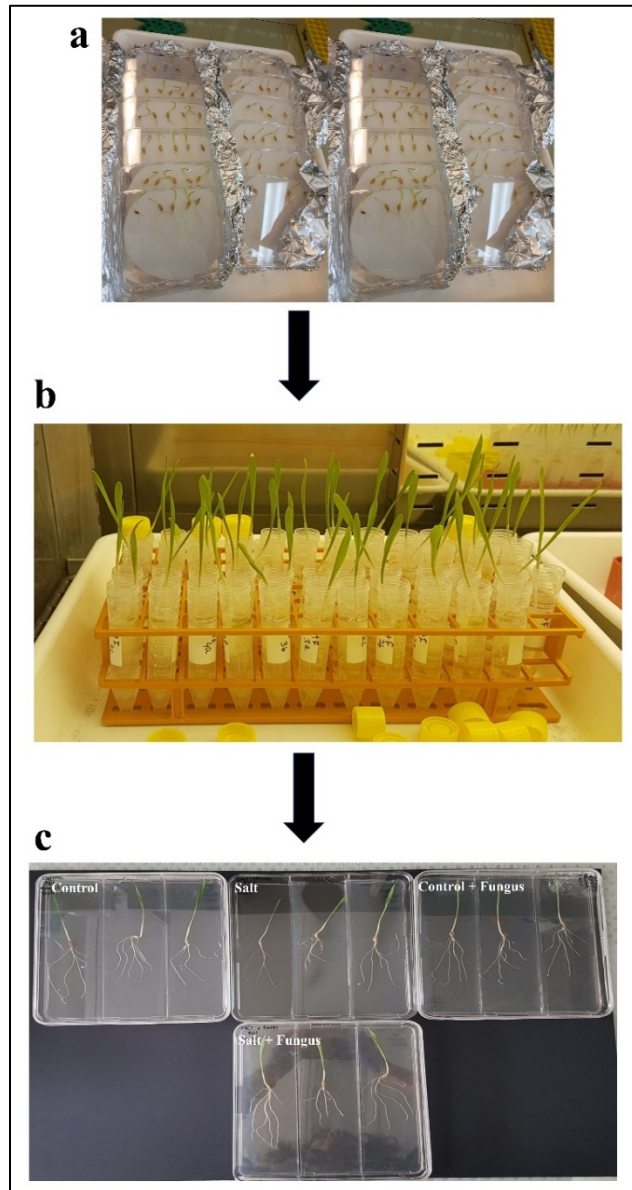


Figure 2.2 Experimental steps for first experiment of the study. A, 48 hours germinated seedlings of barley genotype cv. Vlammingh; B, Micro hydroponics system used for interaction between fungus and roots; C, 20 × 20 cm petri plates with agar media use

2.2.5.1.1. Agar medium total root length analysis

All plants were harvested after 48 hours. Roots from six replicates from each condition were immediately frozen in liquid nitrogen and stored in a -80 °C freezer until extraction for metabolomic analyses (Discussed in **Chapter 3**). The remaining 3 roots from all conditions were

immersed in water and scanned using a flatbed scanner followed by analysis using WinRHIZO software (Regent Instruments, Canada) to determine total root length (TRL). Student's t-test was used for statistical analyses.

2.2.5.1.2. Fungal root interactions in soil medium

For fungal root interactions in soil, a fresh conidial suspension was prepared as per **Section 2.2.4.** and 48 seeds prepared as per **Section 2.2.3.** Sterilized seeds were transferred to cylindrical PVC plastic pipes filled with sandy loam (Fultons, Australia). Soil was pasteurised by steam for 1 h at 85 °C and rapidly cooled by blowing air through the mix. The cylindrical plastic pipes were placed in a growth cabinet maintained at 17 °C constant temperature with no light (**Figure 2.3**) to allow germination of the sown seeds. After 48 h, the conditions of the growth cabinet were adjusted to 17 °C for 16 h light and 10 °C for 8 h dark cycles for the remainder of the experiment. The average water holding capacity of soil in each pot was 300 mL with approximately 20-30 mL water loss per day, whether taken up by plant or evaporated to the environment. Prior to salt treatment, all plants were given an equal amount of water (200 mL) every alternate day.

After 7 d, 24 seedlings from each genotype were inoculated with a conidial suspension of 25 mL of 1×10^8 CFU g⁻¹ of seedling and 24 seedlings from each genotype received an equal amount of sterile 18.2 Ω deionized water. After 7 d post inoculation, salt treated seedlings received 50 mM NaCl and 50 mL of sterile 18.2 Ω deionized water (to prevent salt precipitation) until the NaCl concentration in each pipe reached 200 mM NaCl. A total of twelve replicates were allocated for each condition and each condition was assigned as follows, 'C' (control), uninoculated seeds with no salt stress; 'CF' (no salt + fungus), fungal inoculated seeds with no salt stress; 'S' (negative control), uninoculated seeds with salt stress; and 'SF' (salt + fungus), fungal inoculated seeds with salt stress. Plants were harvested 48 h after the 200 mM NaCl treatment reached its final level.

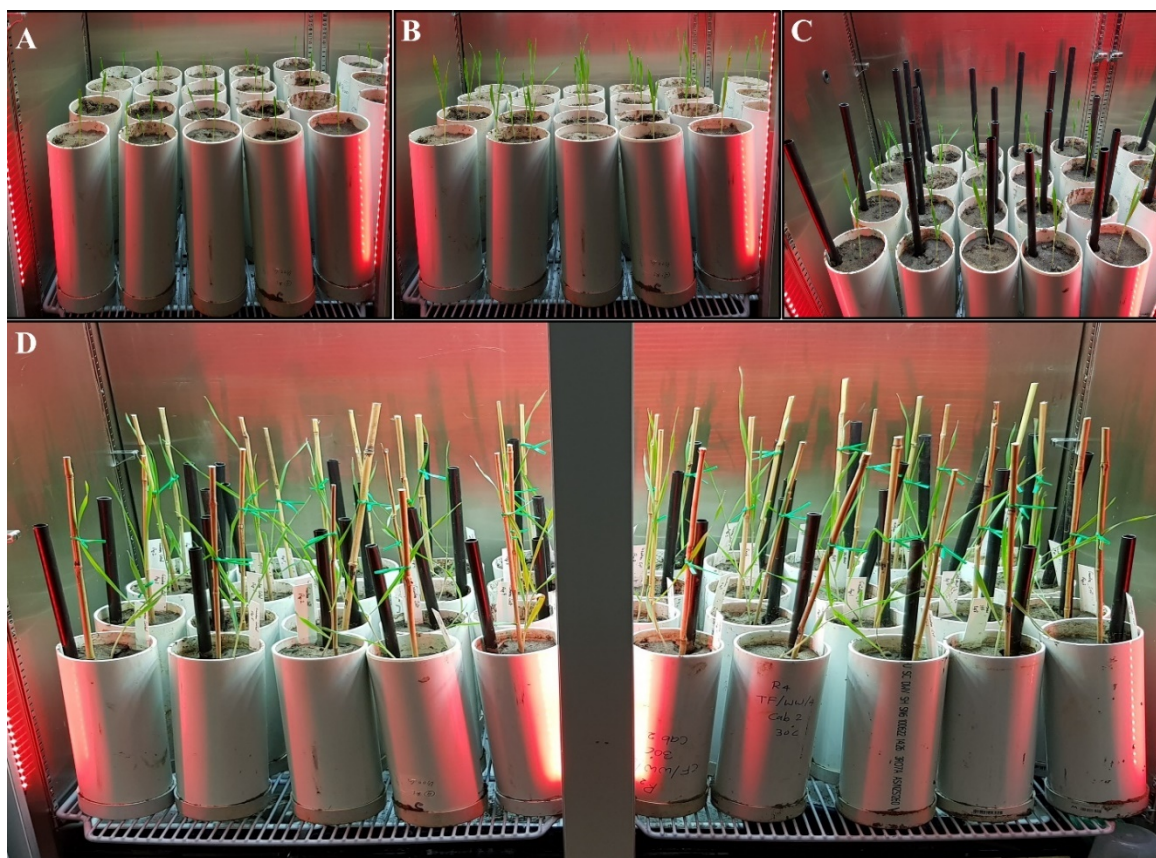


Figure 2.3 Images showing growth of plants for second experiment of the study. Images are captured at, A, 3 days; B, 6 days; C, 9 days; and D, 18 days from sowing

2.2.5.1.3. Soil medium shoot and root length, weight and chlorophyll content determination

The roots from 6 harvested plants from each condition and genotype were immersed in water and scanned using a flatbed scanner followed by analysis using WinRHIZO software (Regent Instruments, Canada) to determine total root length (TRL). Shoot lengths were determined at harvest by measuring above ground shoot length with a ruler. The aerial parts of the plants were separated and dried in an oven (75 °C) for 48 h and weighed using a weighing scale. The chlorophyll content was measured as spad value (SPAD-502Plus, Konica, Europe). The remaining 6 plants were harvested, and roots were immediately frozen in liquid nitrogen and stored in a -80 °C freezer until extraction for metabolomic analyses (Discussed in **Chapter 4**).

2.3 Results

2.3.1 Light microscopy

Light microscopy showed the presence of fungal hyphae in all treated roots confirming the presence of *T. harzianum* T-22 in agar study after harvest at day 7 after inoculation (**Figure 2.4**). It was noticed that all inoculated root replicates at day 5 failed to show fungal hyphae whereas at day 7, all three inoculated root replicates showed the presence of hyphae on the root surface. Therefore, day 7 was chosen as the timepoint for interaction. On the contrary, no hyphae were observed in uninoculated roots from both barley genotypes.

2.3.2 Root architecture

2.3.2.1 Effect of fungus and salt on physiological parameters in barley grown on agar

The root length of all samples was compared using WinRHIZO root-scanning software. **Figure 2.5** shows root scans for both genotypes in all treatments. Average TRL in four treatments in both genotypes (three replicates each) was calculated to measure the increase or decrease in root length. An increase in average TRL in treatment CF as compared to treatment C by 6.04% ($P < 0.05$) and 9.90% ($P < 0.01$) in cv. Vlamingh and cv. Gairdner, respectively, was observed. Average TRL in SF roots compared to S roots increased by 14.90% ($P < 0.05$) and 35.10% ($P < 0.05$) in cv. Vlamingh and cv. Gairdner, respectively. To compare the effect of fungus to control plants, average TRL was compared between SF and C roots with an increase of 7.0% ($P < 0.01$) and 3.0% ($P < 0.01$) in cv. Vlamingh and cv. Gairdner, respectively (**Figure 2.6** and **Table 2.1**).

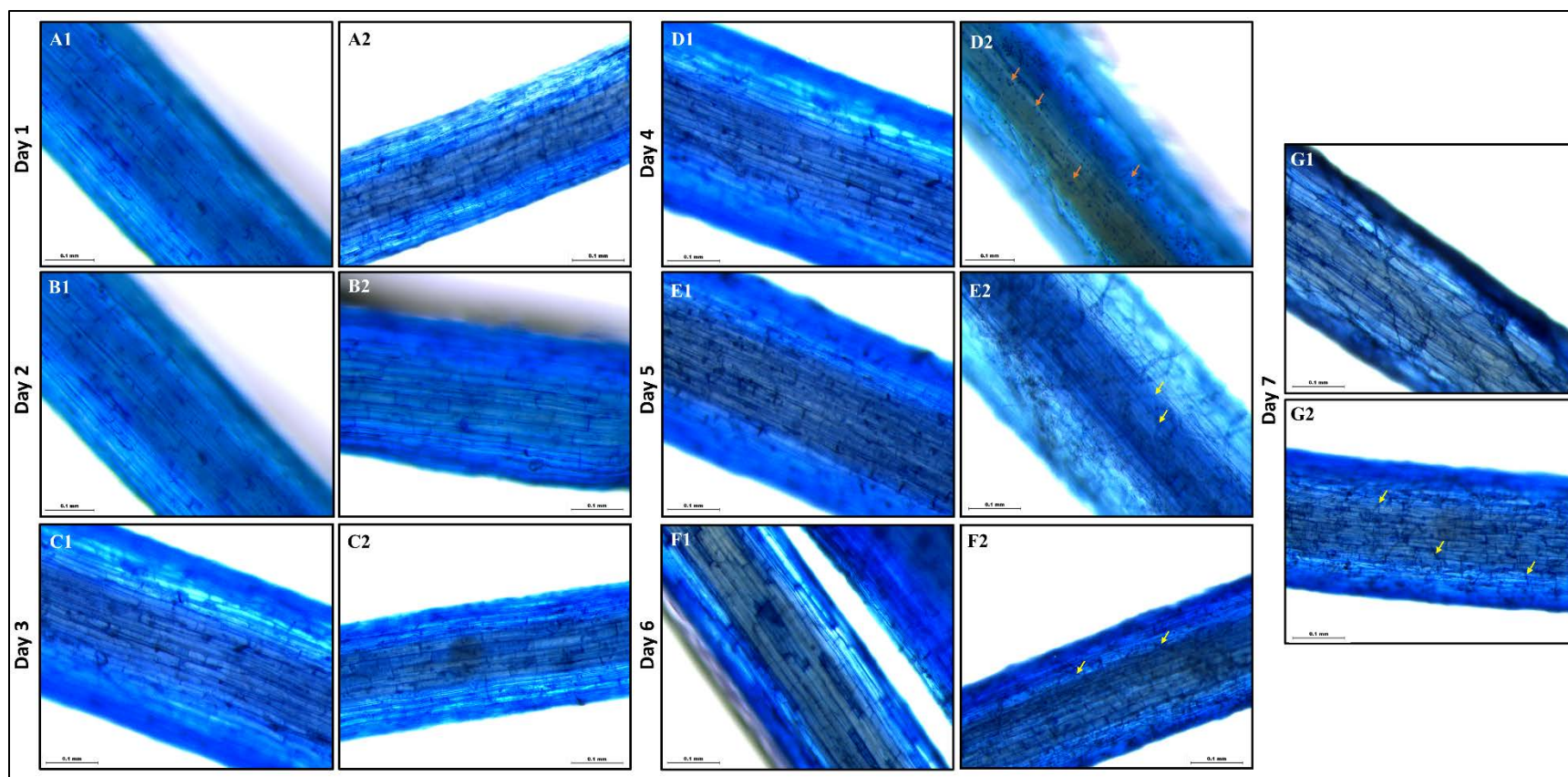


Figure 2.4 . Light microscopy images of barley genotype cv. Gairdner over seven days. A1-G1, control barley roots; A2-G2, roots inoculated with *T. harzianum* T-22 (1×10^8 CFU g⁻¹ of seedling). Orange color arrows in D2 represent fungal spores; yellow arrows in E2, F2, and G2 represent fungal hyphae. Scale = 0.1 mm. n =3

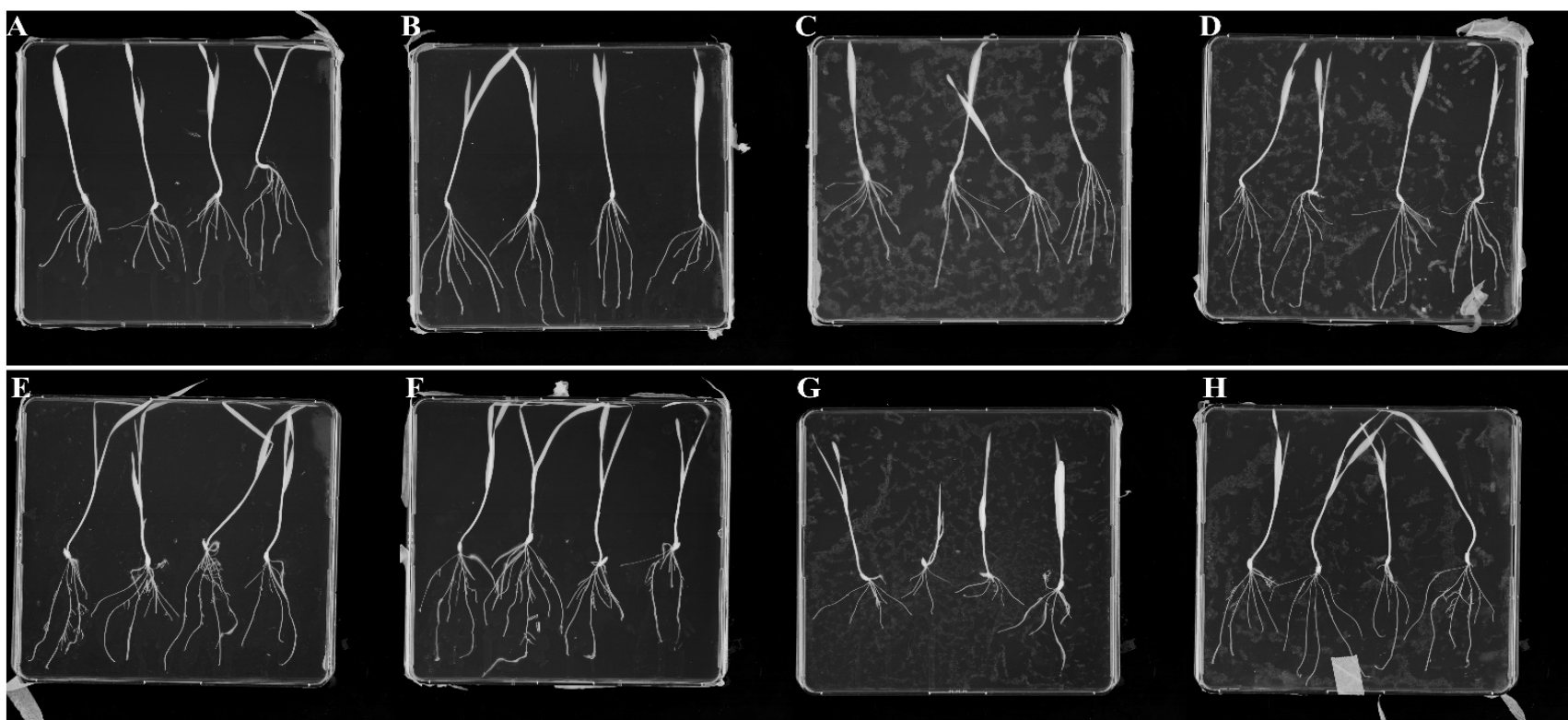


Figure 2.5 WinRHIZO root scans of barley roots for cv. Vlamingh and cv. Gairdner genotypes. A- Vlamingh C, B- Vlamingh CF, C- Vlamingh S, D-Vlamingh SF; E- Gairdner C, F- Gairdner CF, G- Gairdner S, H- Gairdner SF

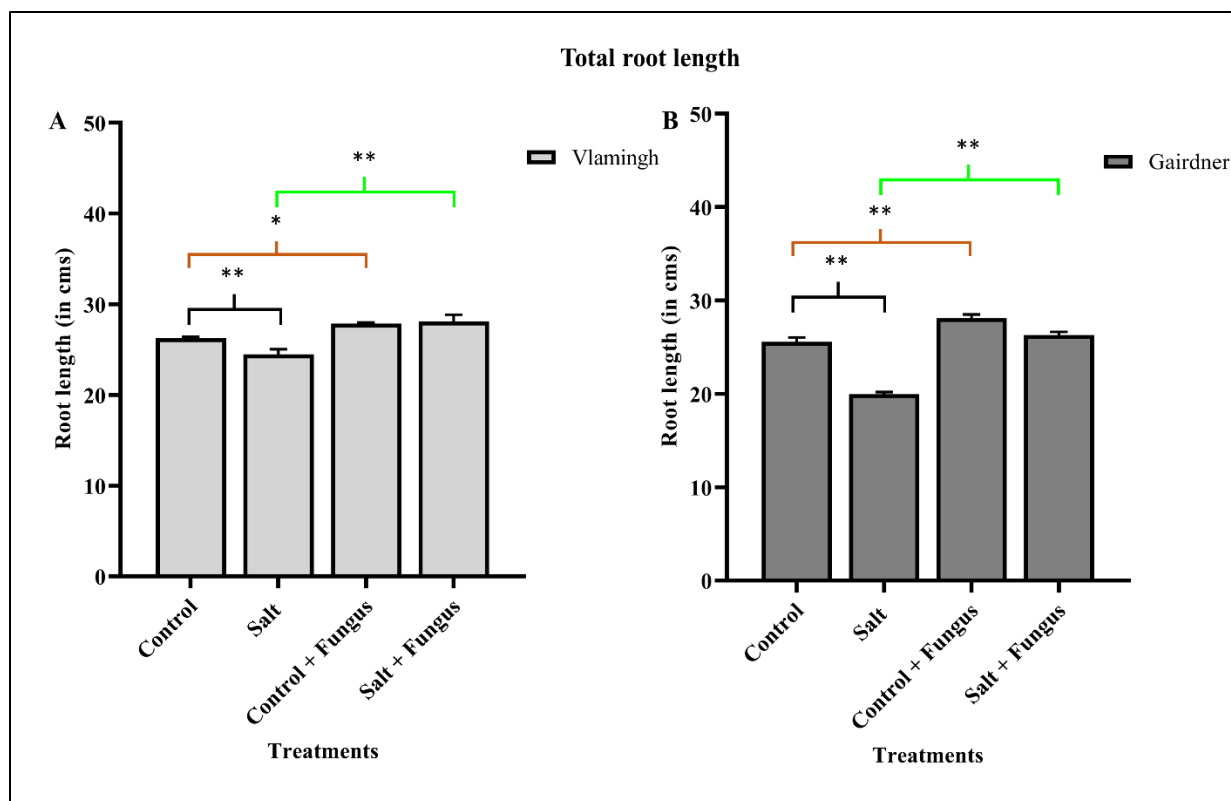


Figure 2.6 Total root length of barley roots of two genotypes, (A) cv. Vlamingh and (B) cv. Gairdner in four treatments for plants grown on agar plates. X axis represent treatments and Y axis represent lengths in cm. $n = 3$. Three color bars above graphs represent different comparisons: black indicates comparisons between salt and control, light brown indicates comparisons between control + fungus and control and cyan green indicates comparisons between salt + fungus and salt. Asterisks denote significance for $P < 0.05$ (one asterisk) and $P < 0.01$ (two asterisks) as determined by Student's t-test. Error bars indicate standard error of the mean.

Table 2.1 Percentage increase in total root lengths, as compared in three treatments in two barley genotypes, cv. Vlamingh and cv. Gairdner ($n=3$ per treatment group).

Treatments	Increase in TRL (in %)	
	Vlamingh	Gairdner
CF vs C	6.04	9.90
SF vs S	14.90	35.11
SF vs C	7.00	3.01

2.3.2.2 Effect of fungus and salt stress on seminal root length of cv. Vlamingh and cv. Gairdner grown in sandy loam soil

Nodal roots could not be distinguished from seminal roots due to soil aggregates in both genotypes. Therefore, average total seminal root length was measured. This method of measurement provided a reasonably fast and accurate alternative for phenotyping of seedlings. Average seminal root length (SRL) was measured for all conditions in both genotypes. When grown in control conditions the sensitive genotype cv. Gairdner had higher SRL than the tolerant genotype cv. Vlamingh. The patterns of growth for salt and fungal treated seedlings grown in soil were similar to those for seedlings grown on agar plates. Although SRL was increased in CF compared to C in both genotypes, the increase was greater in cv. Gairdner (3.50%) than in cv. Vlamingh (0.35%) (**Figure 2.7** and **Table 2.2**).

Salt treatment reduced the SRL for both genotypes in control seedlings (14.0% ($P < 0.01$) for cv. Vlamingh and 23.0% ($P < 0.01$) for cv. Gairdner) but when seedlings were grown with fungus and treated with salt (SF), SRL increased significantly by 20.0% ($P < 0.01$) in cv. Vlamingh and 28.50% ($P < 0.05$) in cv. Gairdner. It was also observed that SRL in seedlings grown with fungus and treated with salt recovered better than control (C) seedlings in cv. Vlamingh (3.21% ($P < 0.05$)). However, the SRL for seedlings grown with fungus and treated with salt in cv. Gairdner was slightly reduced as compared to control seedlings (-0.9%).

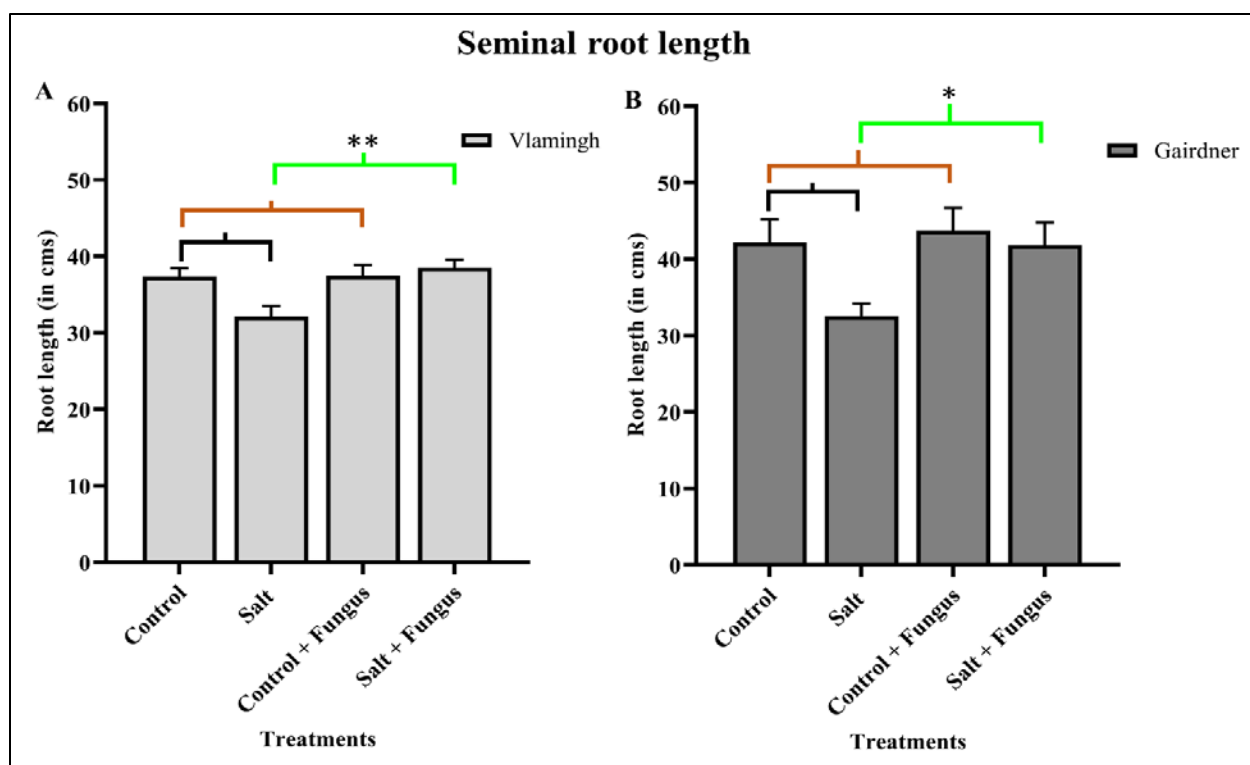


Figure 2.7 Seminal root length of barley roots of two genotypes, cv. Vlamingh and cv. Gairdner under four treatment conditions for seedlings grown in soil. X axis represent treatments and Y axis represent lengths in cm. $n = 6$ for both genotypes. Three colour bars above graphs represent different comparisons: black indicates comparisons between salt and control, light brown indicates comparisons between control + fungus and control and cyan green indicates comparisons between salt + fungus and salt. Asterisks denote significance for $P < 0.05$ (one asterisk) and $P < 0.01$ (two asterisks) as determined by Student's t-test. Error bars indicate standard error of the mean.

Table 2.2 Percentage increase in seminal root length, as compared in three treatments in two barley genotypes, cv. Vlamingh and cv. Gairdner.

Treatments	Increase in SRL (%)	
	Vlamingh	Gairdner
CF vs C	0.35	3.51
SF vs S	19.95	28.48
SF vs C	3.21	-0.95

2.3.3 Root and shoot dry weight

The effects of fungal inoculation on root and shoot dry weight under control and saline conditions were measured (**Figure 2.8** and **Table 2.3**). When grown in control conditions, the tolerant genotype cv. Vlamingh had higher root dry weight than the sensitive genotype cv. Gairdner. In CF compared to C, root dry weight increased in both genotypes. However, these were not significant. Root dry weight in cv. Vlamingh increased from 0.24 g in C to 0.27 g in CF and in cv. Gairdner, it increased from 0.21 g in C to 0.30 g in CF when compared to inoculated seedlings without salt.

Salt stress reduced root dry weight in both genotypes but the effect was greater in cv. Vlamingh (from 0.24 g in C to 0.16 g in S) as compared to cv. Gairdner (from 0.21 g in C to 0.16 g in S) but when seedlings were grown with fungus and treated with salt (SF), root dry weight significantly increased from 0.16 g in S to 0.28 g in SF ($P < 0.01$) in cv. Vlamingh and from 0.16 g in S to 0.22 g in SF ($P < 0.01$) in cv. Gairdner. It was also observed that root dry weight in seedlings grown with fungus and treated with salt recovered better than control (C) seedlings in both genotypes and the effect was higher in cv. Vlamingh.

Shoot dry weight was measured to determine the above ground plant biomass (**Table 2.3**). In treatment CF, shoot dry weight increased in cv. Vlamingh but reduced in cv. Gairdner. But the difference was not significant in either of the genotypes. It was found that shoot dry weight reduced in both genotypes after salt stress. However, it was significant only in cv. Vlamingh but not in cv. Gairdner. But when seedlings were grown with fungus and treated with salt (SF), a significant increase in shoot dry weight in cv. Vlamingh (from 0.31 g in S to 0.51 g in SF) with $P < 0.05$ was found. Though the shoot dry weight increased from 0.38 g in S to 0.50 g in SF in cv. Gairdner, it was not statistically significant possibly due to high variation between samples.

2.3.3.1 Root to shoot ratio

The root to shoot ratio was measured to assess the overall health of the plants in all treatments (**Table 2.3**). When grown in control conditions, root to shoot ratio was higher in the tolerant genotype cv. Vlamingh as compared to the sensitive genotype cv. Gairdner. Under salt stress, the root to shoot ratio of plants decreased in cv. Vlamingh from 0.6 in C to 0.52 in S and in cv. Gairdner, there was a slightest difference between control and salt treated seedlings (from 0.43 in C to 0.42 in S). The effect of fungal inoculation without salt (CF) resulted in decreased root to shoot ratio in cv. Vlamingh (0.53) as compared to uninoculated seedlings under no salt (C) (0.60). However, the root to shoot ratio increased in inoculated seedlings in cv. Gairdner (0.68) when compared to uninoculated seedlings (0.43) under no salt conditions. But when grown with fungus and treated with salt, root to shoot ratio was increased in both genotypes (from 0.52 to 0.55 in cv. Vlamingh and from 0.42 to 0.44 in cv. Gairdner) with the effect better observed in cv. Gairdner

where seedlings showed better root to shoot ratio than seedlings grown under control conditions without fungus.

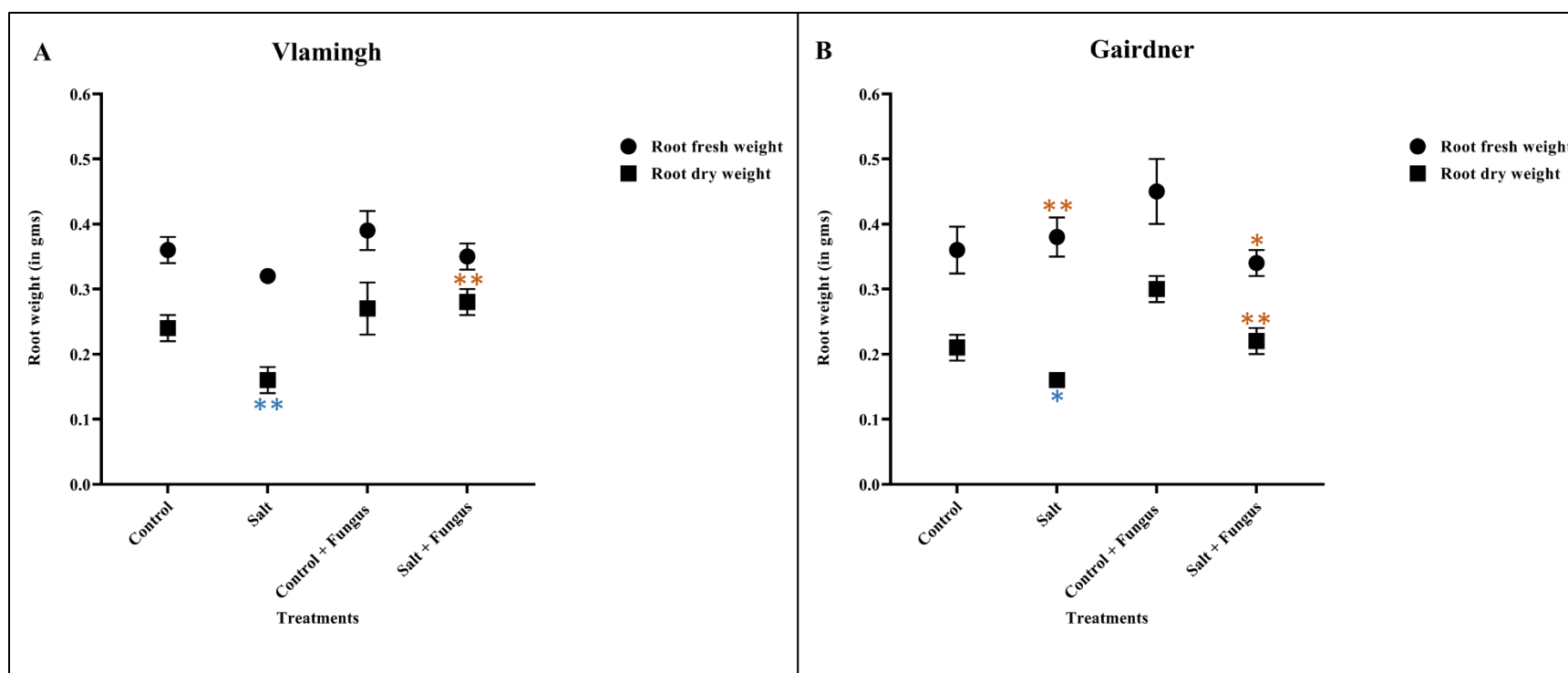


Figure 2.8 Averages and std errors of showing fresh and dry weight of roots of barley genotypes cv. Vlamingh (A) and cv. Gairdner (B) under four treatment conditions. X axis represent the four treatment conditions and Y axis represent weights in grams. n = 6 for both genotypes. Asterisks denote significance for $P < 0.05$ (one asterisk) and $P < 0.01$ (two asterisks) as determined by Student's t-test. Blue color indicates significant reduction and orange color indicates significant increase

Table 2.3 . Physiological parameters measured in cv. Vlamingh and cv. Gairdner under four treatment conditions. Root and shoot fresh weight were measured immediately after harvest and dry weights of both were measured after drying in oven at 75 °C for 48 h. Chlorophyll content was measured using SPAD meter. n = 6 for all treatments and results are presented as mean $\pm$ standard error for all except root:shoot ratio. Asterisks denote significance for $P < 0.05$ (one asterisk) and $P < 0.01$ (two asterisks) as determined by Student's t-test.

	Control		Salt		Control + Fungus		Salt + Fungus	
Measurements (g)	Vlamingh	Gairdner	Vlamingh	Gairdner	Vlamingh	Gairdner	Vlamingh	Gairdner
Root fresh weight	0.36 $\pm$ 0.02	0.36 $\pm$ 0.04	0.32 $\pm$ 0.01	0.38 $\pm$ 0.03**	0.35 $\pm$ 0.3	0.45 $\pm$ 0.05	0.35 $\pm$ 0.3	0.3 $\pm$ 0.02*
Root dry weight	0.24 $\pm$ 0.02	0.21 $\pm$ 0.02	0.16 $\pm$ 0.02**	0.16 $\pm$ 0.01*	0.27 $\pm$ 0.04	0.3 $\pm$ 0.02	0.28 $\pm$ 0.02**	0.22 $\pm$ 0.02**
Shoot fresh weight	0.69 $\pm$ 0.05	0.76 $\pm$ 0.05	0.5 $\pm$ 0.04**	0.66 $\pm$ 0.01	0.82 $\pm$ 0.03	0.74 $\pm$ 0.05	0.74 $\pm$ 0.06*	0.7 $\pm$ 0.09
Shoot dry weight	0.4 $\pm$ 0.05	0.49 $\pm$ 0.02	0.31 $\pm$ 0.04**	0.38 $\pm$ 0.02	0.51 $\pm$ 0.05	0.44 $\pm$ 0.06	0.51 $\pm$ 0.05*	0.5 $\pm$ 0.09
Chlorophyll content	34.32 $\pm$ 0.67	32.42 $\pm$ 0.40	26.14 $\pm$ 0.85**	18.09 $\pm$ 0.57**	36.85 $\pm$ 0.56**	36.62 $\pm$ 0.62**	34.54 $\pm$ 0.36**	35.19 $\pm$ 0.26**
Root: shoot ratio	0.6	0.43	0.52	0.42	0.53	0.68	0.55	0.44

2.3.4 Chlorophyll content

Leaf chlorophyll content was measured to examine the effect of fungal inoculation with *T. harzianum* T-22 on the photosynthetic potential of both genotypes. Under control conditions, the chlorophyll content was higher in the tolerant genotype cv. Vlamingh as compared to the sensitive genotype cv. Gairdner. But when seedlings were grown with the fungus and without salt (CF), the chlorophyll content increased in both genotypes and the effect was higher in cv. Gairdner (**Table 2.3** and **Figure 2.9**).

Salt stress greatly reduced the chlorophyll content in both genotypes, but the difference was greater in cv. Gairdner where the chlorophyll content reduced from 32.42 in C to 18.09 in S as opposed to the effect observed in cv. Vlamingh where the chlorophyll content was 34.32 in C and reduced to 26.14 in S. But when seedlings were grown with the fungus and treated with salt, the chlorophyll content was significantly increased in both genotypes making it equivalent to or better than those for untreated control seedlings (**Figure 2.9**).

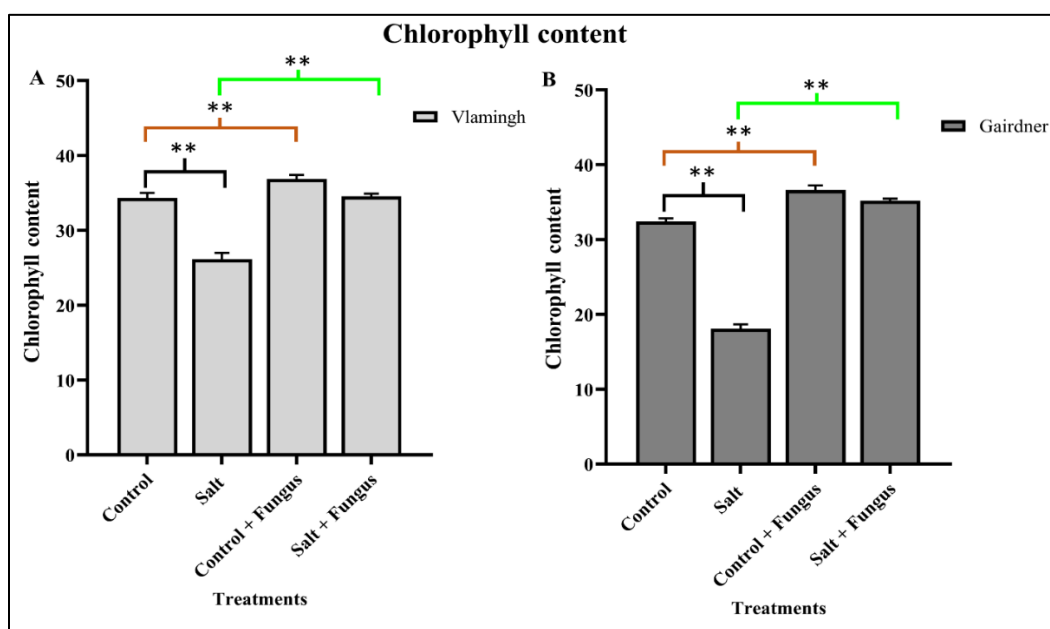


Figure 2.9 Leaf chlorophyll content of two barley genotypes cv. Vlamingh and cv. Gairdner measured using SPAD meter. X axis represent the four treatments and Y axis represent chlorophyll index. Three colour bars above graphs represent different comparisons: black indicates comparisons between salt and control, light brown indicates comparisons between control + fungus and control and cyan green indicates comparisons between salt + fungus and salt. Asterisks denote significance for $P < 0.01$ (two asterisks) as determined by Student's t-test. Error bars indicate standard error of the mean.

2.4 Discussion

Salinity is one of the most significant environmental challenges limiting crop productivity around the world. Salt stress first affects the root system, inducing osmotic stress and causing reduced water availability. In the long term, salt stress induces ion toxicity resulting in nutrient imbalances in the cytosol. In this study, we investigated whether fungal inoculation with the endophytic ascomycete, *T. harzianum* T-22 could protect barley genotypes (Vlamingh and Gairdner) from the effects of salt stress. A matrix of treatments was established where barley was uninoculated or inoculated with fungus and then grown either in control conditions or exposed to salt stress. Colonization by fungus was observed seven days after inoculation and so the salt treatment was introduced at this stage of growth. Plants were grown either on agar plates (where conditions could be controlled more carefully to reduce contamination when metabolites were extracted) or in soil.

To determine treatment effects, TRL was measured to analyze plant development. Salinity reduced root length of both genotypes in this study. According to Acosta-Motos et al. (2017) and Sheldon et al. (2016), this could be due to inhibitory impacts of salts and by restriction of cell division and cell expansion in the root apical meristem. The effect of salinity was more severe in the sensitive genotype cv. Gairdner as compared to tolerant genotype cv. Vlamingh, confirming the greater salt tolerance potential of the latter genotype. The effect of fungal inoculation in control treated roots showed considerable increase in root lengths of both genotypes with a greater effect in cv. Gairdner suggesting growth promoting characteristics of the fungus (Altomare et al. 1999; Björkman et al. 1998; Contreras-Cornejo et al. 2009).

In the agar study, salt treatment of uninoculated seedlings caused a reduction in TRL that was larger for the sensitive cultivar Gairdner. However, seedlings inoculated with *T. harzianum* T-22 had TRL longer than control untreated seedlings and almost the same as CF seedlings suggesting that the fungus was able to mitigate the negative effects of NaCl. These results are similar to those for tomato treated with *T. harzianum* T-22 (Mastouri et al. (2012).

In the subsequent soil trial, various parameters were assessed to analyze the effect of fungal inoculation in both barley genotypes grown in sandy loam soil. Like other cereals, barley plants have a typical fibrous root system consisting of seminal roots and post embryonic nodal roots. Seminal root lengths were greatly reduced in both genotypes under salt stress. However, the reduction was greater in cv. Gairdner as compared to cv. Vlamingh. The results are in alignment with our glasshouse study where cv. Vlamingh grew better than cv. Gairdner under saline conditions. This study further confirms the ability of cv. Vlamingh to adapt physiologically to the early impact of salinity stress and confirms greater salt tolerance potential of cv. Vlamingh as compared to cv. Gairdner.

For plants grown in soil and inoculated with fungus, the protective effects of fungal inoculation were similar to those seen in the agar grown seedlings. Salt treatment of uninoculated seedlings caused a significant reduction in SRL, root and shoot dry weight and chlorophyll content. In the same salt treatment, these parameters for seedlings inoculated with *T. harzianum* T-22 were equivalent to or better than those for untreated control seedlings and in most cases very similar to those for CF seedlings. In this experiment the effects of salt on cv. Gairdner were more marked than for cv. Vlamingh but the result of inoculation with fungus were similar.

Cultivar cv. Gairdner with fungal inoculation showed increase in seminal root length as compared to cv. Vlamingh under controlled and saline conditions. The patterns of growth and effects of the fungus were similar in both agar plates and soil. Though the preference of the fungus to colonize one genotype over the other needs to be further validated, it can be proposed that the use of *T. harzianum* T-22 in salinity affected crops may assist in reducing the effects of salt stress.

Salt stress decreased root to shoot ratio in cv. Vlamingh. There was also a slight reduction in seminal root length in salt stressed seedlings as compared to control seedlings and overall root biomass was also reduced significantly. This suggests that while cell elongation was maintained, the roots were possibly exhibiting reduced radial expansion. Similar results were observed in maize and barley in other studies (Lemcoff et al. 2006; Sharp et al. 1988; Shelden et al. 2013). In contrast, root to shoot ratio in cv. Gairdner under saline conditions was almost similar to control seedlings. This may be attributed to the reduction in above ground while maintaining the below ground biomass (determined by root and shoot fresh and dry weight measurements in salt compared to no salt treatments). This characteristic favors the retention of toxic ions in roots, thereby controlling their translocation to the aerial parts. This response is a typical mechanism of plant resistance under saline conditions (Cassaniti et al. 2009; Cassaniti et al. 2012).

In CF, root to shoot ratio was observed to be higher in cv. Gairdner as compared to cv. Vlamingh. This was mainly due to the increase in root biomass which is supported by the higher seminal root length in cv. Gairdner in fungal treatment compared to cv. Vlamingh. In treatment SF as compared to S, it is worth mentioning that although there was an increase in seminal root length in cv. Gairdner as compared to cv. Vlamingh, root to shoot ratio decreased in the former genotype. It is possible that the fungus promoted the production of nodal and lateral roots under salt stress in the sensitive genotype post inoculation. This could be an adaptive mechanism of the sensitive genotype for better nutrient and water absorption needed for resistance against salt stress. From the above results, it is clear that salt treatment has an effect on root and shoot weight (reducing it) but growth with the fungus restores growth close to or better than normal confirming the positive role of the fungus to help plants cope with the adverse effects of salt stress.

Damage to the chloroplast is an important aspect in the effect of salinization on leaf cells. Salinity reduced the chlorophyll content in both genotypes in this study. Ashraf and Foolad (2005) and Roy and Srivastava (2000) reported the reduction in chlorophyll contents in rice and wheat under saline conditions to be associated with disturbances in membrane stability. Khan et al. (2009) also reported a significant decrease in sensitive genotypes of wheat in comparison to tolerant genotypes, which is aligned with results obtained in this study where the sensitive genotype cv. Gairdner had lower chlorophyll content than the tolerant genotype cv. Vlamingh under salt stress.

In treatment CF, a higher chlorophyll content was found in both genotypes when compared to C suggesting increased photosynthetic capacity in these plants. The photosynthetic capacity and carbon production directly relate to growth parameters. This is also supported by the findings of greater biomass in both genotypes in fungal inoculated seedlings. In treatment SF when compared to S, chlorophyll content was higher in both genotypes. Presumably, fungal association with plants improves the plant's capacity of gas exchange and the efficiency of photochemistry and non-photochemistry of photosystem II. Similar results were observed in soybean plants (Khan et al. 2011) and Bermudagrass (Xie et al. 2017) inoculated with an endophyte under saline conditions.

2.5 Conclusion

This study demonstrated the beneficial effect of *T. harzianum* T-22 under no salt and salt stress conditions in two barley genotypes cv. Vlamingh and cv. Gairdner with contrasting germinating phenology and tolerance to salinity. Various physiological parameters were measured demonstrating different potential roles of the fungus in improving the ability of plants to grow under saline conditions. The patterns of growth and effects of the fungus were similar in both agar plates and soil, confirming that the fungal treatment allowed the seedlings to grow normally even when grown under salt stress. Therefore, this study provides further validation for the effectiveness of *Trichoderma spp.* in mitigating salt stress in cereal crops. In the following chapters, the harvested root tissue (inoculated and uninoculated) from agar study and soil were subjected to metabolomics analyses to determine the changes in metabolites and lipids under control and saline conditions. Since the results on root length were similar for agar study and soil, this suggest that metabolites and lipids extracted from roots grown on agar plates are biologically valid to compare with the results from soil study.

Chapter 3

Investigation of the biochemical changes in two barley genotypes inoculated with a beneficial fungus *Trichoderma harzianum* Rifai T-22 under salt stress

Abstract

Increases in soil salinity reduce growth and yield of agricultural plants by inhibiting plant functions. Salinization of soil is increasing because of the pressure of population on food supply. Use of genetically modified crops and plant breeding techniques are being used to produce plants tolerant to salt stress. However, interaction of fungal endophytes with the crop plants can also improve tolerance and is a less expensive approach. Here, the role of *Trichoderma harzianum* T-22 in alleviating NaCl-induced stress in two barley genotypes (cv. Vlamingh and cv. Gairdner) has been investigated. Metabolomics using GC-MS for polar metabolites and LC-MS for lipids was employed to provide insights into the biochemical changes in barley roots treated with fungus during the early stages of interaction. *T. harzianum* increased the root length of both genotypes under controlled and saline conditions. The fungus reduced sugars in both genotypes and caused no change in organic acids under saline conditions. Amino acids decreased only in cv. Gairdner in fungal-inoculated roots under saline conditions. Triacylglycerols (TAGs) were the most abundant lipids in inoculated roots of both genotypes under saline conditions. This study shows that the fungus imparts adaptation or tolerance mechanism to cv. Vlamingh and in cv. Gairdner by remodelling lipids mainly from glycerolipids class after salt stress

3.1 Introduction

Soil salinity is becoming a significant problem worldwide as it is encountered in all climates (Shahbaz and Ashraf 2013). Soils are rendered saline due to deposition of salt either by natural or anthropogenic processes. It is estimated that 50% of all arable land will become impacted by salinity by 2050. Consequently, it is important to understand the crop response to salinity to minimize economic loss and improve food security. It is estimated that globally, US\$12 to 28 billion are lost annually due to reductions in crop productivity (FAO 2013). These environmental conditions play a major role in limiting plant productivity by reducing both yield and quality of the crop.

The effect of salinity on leaf growth, biomass and grain yield, and oxidative damage on several crops are well documented (Machado and Serralheiro 2017; Oster and Jayawardane 1998; Shahbaz and Ashraf 2013; Shrivastava and Kumar 2015). At the plant organ level, shoots are more sensitive to salinity than roots (Gregory 2008). However, roots are first exposed to salinity stress and respond to it rapidly. Soil salinity is responsible for reducing root growth greatly in salt-stressed plants (Abbas et al. 2015; Negrão et al. 2017; Rahnama et al. 2011). The extent of the damage caused by salinity depends on the plant's developmental stage, duration of the salt stress, the amount of salt in the growth medium, and the crop genotype (Hu et al. 2017; Saqib et al. 2013). Hence, the study of plant growth, especially roots, enables a statistical inference on how well plants can grow under (or adapt to) certain environmental conditions. This statement also holds for very young plants – which is highly beneficial for experimental evaluation. Therefore, the study of plant root growth moved into the focus of biologist in recent years (Mühlich et al. 2008). Among all the possible root traits that can be measured on root samples extracted from plant studies, the specific root length (SRL) and the root length density (RLD) are of particular interest. However, one must know the total root length (TRL) in the studied plants as a prerequisite for calculating SRL and RLD (Delory et al. 2017). Several studies have used this trait to measure the effect of salt stress in various cereal crops such as rice (Kakar et al. 2019), wheat (Rahnama et al. 2019) and barley (Sarabia et al. 2019).

The roots are crucial for a myriad of physiological processes including water and nutrient uptake, anchoring of plants and providing mechanical support to the aboveground organs. Roots also affect surrounding nutrient composition by the release of organic compounds that play a vital role in mineralizing nutrients (de Willigen and van Noordwijk 1987; Hasanuzzaman et al. 2013; Munns et al. 2011; Munns and Tester 2008). The area surrounding the root system, the rhizosphere, not only consists of root tissues but is also home to a vast number of microorganisms that influence plant functions. Salt stress influences the root system (Munns 2005; Munns et al. 2006; Robin et al. 2016; Sun et al. 2014) with roots responding by secreting a different combination of compounds to protect against negative effects and encourage positive microbial

interactions (Munns and Tester 2008). These secreted compounds usually induce an interactive metabolic crosstalk involving diverse biosynthetic networks and pathways. The complex phenomenon of tolerance to cope with salt stress involves dynamic changes in gene expression, metabolic pathways and physiological adaptations (Gregory 2006). Plants employ various mitigation strategies to deal with salt stress including proline accumulation within cells (Hartmann et al. 2008), activation of antioxidant enzymes and production of compounds to combat reactive oxygen species (ROS) formation, modulation of hormones, and accumulation of glycine betaine and polyols (Hiltner 1904). In the past few decades it has become clear that interaction with salt tolerant microbial inoculants can enhance crop growth in saline soils (Lynch 1990; Morgan et al. 2005; Pinton et al. 2007). Microbes that interact with plants have a diverse array of metabolic and genetic strategies that reduce the impact of salt stress and other abiotic stresses arising from extreme environmental conditions (Ho et al. 2005; Lynch and Brown 2001).

Rhizosphere microorganisms not only perceive and respond to signal molecules secreted by plant roots, they also release diverse signalling molecules that act on plants and result in increased biotic and abiotic stress resistance or tolerance, as well as root development and plant growth (Jones et al. 2009). Several reviews have documented the microbial diversity and activity near roots as well as the functions and effects of microorganisms in nutrient turnover and supply to the plant under salt stress (Abdelaziz et al. 2019; Jan et al. 2019; Yun et al. 2018). Significant progress has been made in the past decade to understand the mechanisms of salt tolerance imparted by endophytic fungi. *Trichoderma* species are one group of rhizosphere microbes that impart beneficial effects on plants under saline conditions. Several studies have been reported showing positive role of several strains from *Trichoderma* species under salt stress. Contreras-Cornejo et al. (2014) reported that *T. virens* promoted *Arabidopsis* growth through auxin response pathway to modulate root system architecture and activate auxin regulated gene expression. Another study by (Contreras-Cornejo et al. 2014) showed that plants inoculated with *Trichoderma atroviride* developed a more branched root system as compared to uninoculated *Arabidopsis* seedlings. Further, *T. atroviride* showed enhanced shoot growth and increased chlorophyll content, demonstrating the beneficial effects of fungus under salt stress. Mastouri et al. (2010) also showed that *T. harzianum* strain T22 increased root length in tomato plants when compared to uninoculated plants under salt stress. Another strain from *Trichoderma spp.*, *Trichoderma longibrachiatum* T6, enhanced root length in wheat plants under saline conditions (Zhang et al. 2016).

Colonization of several crops with endophytic fungi has been reported to mitigate stress by increasing the levels of protective metabolites and osmoprotectants, activate antioxidant systems to prevent damage caused by ROS, and modulate the phytohormone profile to minimize salt effects on growth of plants (Curl and Truelove 1986; Dakora and Phillips 2002; McNear Jr

2013; Uren 2000; Watt 2009). There have been reports that show the role of endophytic fungi enhancing accumulation of total soluble sugars (TSSs) in salt stressed plants (Bertin et al. 2003; Clarholm 1985; Khorassani 2008). These sugars act as chemoattractant signals to soil rhizobia (Coleman 1986). Yang et al. (2015) reported that the colonization of *Phomopsis liquidambari* could stimulate sugar secretion from the roots of rice, thereby providing carbon to the endophytic fungi. A study by Zhao et al. (2014a) demonstrated that the release of organic acids from roots colonized by endophytes, resulted in ferric solubilization to form organic ferric salts that can be assimilated directly by plants under saline conditions. Plants colonized by endophytes often have a greater capacity for nutrient uptake and distribution due to modulation of the root architecture and nutrient availability in the soil. These benefits are imparted by an increase in organic acids produced by plants colonized by endophytic fungi (Badri and Vivanco 2009; Vancura and Kunc 2012).

Most of the efforts in characterizing and defining the biochemical changes related to response or defense mechanisms during plant-microbe interactions have been driven by targeted approaches or by measuring only one class of metabolites (Tugizimana et al. 2018b). These methodologies play an important role in elucidating the main elements of interactions that include dormant signal transduction enzymes, chromatin modification and transcription factors (Lambers et al. 2009; Padgham 2009; Prithiviraj et al. 2019). However, there are still gaps in holistically understanding the dynamism and complexity of molecular mechanisms involved in the interactions, mainly due to the complexity of the multi-layered biological information networks involved (Tugizimana et al. 2018b). There are many studies that describe the interactions between the endophytes and host plants and their effects but the metabolic contributions of these endophytes to their host plants is less well documented with few studies available involving the early stages of interaction. The early stage of interaction between plants and microbes is crucial in priming plants to respond to the environment according to the nature of the microbes. Previous studies have suggested that the alteration of plant metabolic responses during this stage correspond to the environment in which these interactions occur (Grayson 2013). Therefore, using metabolomics approach to assess (qualitatively and quantitatively) all measurable metabolites without any preconception or pre-selection is a powerful tool to provide insights into the biochemical mechanisms that underpin various physiological responses.

Given the increasing number of studies dealing with root trait measurements, we estimated the TRL of harvested root samples inoculated with the beneficial endophytic fungi *Trichoderma harzianum* strain T-22 grown under control and saline conditions in this study. Two barley genotypes (cv. Vlamingsh and cv. Gairdner) with contrasting germination phenology and salinity tolerance were used for this study. Further, we determined the changes in polar metabolites and lipids in roots of both genotypes. For this, we used a combination of two high-end analytical

techniques- gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) to give new insights into the metabolism of roots. Barley was chosen as it is not only an agriculturally and industrially important crop but is also the most salt tolerant cereal crop (Shelden et al. 2016). This combined approach provides unique insights into the metabolome and lipidome in barley roots influenced by the inoculation of endophytic fungi identifying metabolic processes that are induced during this interaction.

3.2 Materials and Method

3.2.1 Plant Materials and growth conditions

Two barley genotypes (Vlamingh and Gairdner) were selected based on their known differences in germination phenology and salinity tolerance (Gupta et al. 2019). All seeds were sourced from The University of Adelaide, Australia.

3.2.2 Fungal isolates and growth conditions

Freeze dried form of endophyte *Trichoderma harzianum* strain T-22 (ATCC 20847) was sourced from The American Type Culture Collection (ATCC, Washington, DC). The pellet obtained in the ampoule was suspended with sterile distilled water and kept at room temperature (25°C) undisturbed overnight. On the following day, the suspension was mixed well and inoculated on Potato Dextrose Agar (39 g/L PDA powder in de-ionized water) in solid medium on a petri plate. The plates were incubated at 25°C in dark for 5 days followed by light for 3 days. Conidia was seen growing after 8 days of incubation. The plates were stored at room temperature prior to setting up the symbiosis interaction.

3.2.3 Material and chemicals

Analytical or mass spectrometric grades solvents and reagents were purchased from Merck Millipore (Bayswater, VIC, Australia). 18.2 Ω deionized water was obtained from Synergy UV Millipore System (Millipore, USA). Chemicals such as 2,5-dihydroxy benzoic acid (DHB), elemental red phosphorus and Supra pure® nitric acid (70%) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Embedding and freezing supplies were purchased from Section-Lab Co. Ltd (Tokyo, Japan). Sectioning supplies were purchased from Grate HDS (Ringwood, Australia). Lysing Matrix Tubes with 0.5 g Lysing Matrix D (1.4 mm ceramic spheres) were purchased from MP Biomedicals (Seven Hills, NSW, Australia). PDA was purchased from Sigma-Aldrich.

3.2.4 Plant growth conditions and inoculation with *T. harzianum*

A flow-chart showing the experimental setup for plant growth conditions and inoculation with fungus is given in Chapter 2, Figure 2.1. Twenty-four seeds from each variety were sterilized in 70% ethanol for 1 min. They were then rinsed 4-5 times in sterile 18.2 Ω deionized water before being treated with 1.0% (v/v) bleach for 10 min, followed by thorough washing in sterile 18.2 Ω deionized water 6–7 times. Seeds were then imbibed overnight (~16 h) in sterile 18.2 Ω deionized water with continuous aeration. Simultaneously, fresh conidial suspension was plated on PDA to yield conidia for inoculation after 7 days. On the following day, seeds were transferred to agar plates containing Whatman paper soaked in 5ml 18.2 Ω deionized water. Plates were then sealed with parafilm, wrapped in aluminium foil and kept in a growth chamber maintained at 17°C constant temperature with no light. Plates were unwrapped after 48 hours of germination period

and transferred to a growth chamber maintained at 17°C for 16 hours light and 10°C for 8 hours dark cycles for five days.

After seven days, twelve seedlings from each variety were inoculated with conidial suspension of 10 ml of 1×10^8 CFU g⁻¹ of seedling in a 10 ml tube. Twelve control seedlings from each variety were treated with an equal amount of sterile 18.2 Ω deionized water. Seedlings were kept in growth chamber maintained at 17°C for 16 hours light and 10°C for 8 hours dark cycles for 7 days. This was done to facilitate fungal association with growing roots. After 7 days of treatment, six of twelve inoculated seedlings from each variety were transferred to 1% agar plates containing 200 mM salt (NaCl) concentration and remaining six seedlings were transferred to 1% agar plates containing 0mM (control). Seven days were chosen for symbiosis between roots and fungus after confirming the presence of fungal hyphae on day seven which was visualized using brightfield microscopy. Six uninoculated seedlings from each variety were also transferred to 1% agar plates containing 200 mM salt (NaCl) concentration and remaining six seedlings were transferred to 1% agar plates containing 0mM (control). Therefore, six replicates with four treatment groups were created for each variety (control 0mM abbreviated as 'C', control with inoculum abbreviated as 'CF', salt given as 'S' 200 mM NaCl and salt 200 mM NaCl with inoculum described as 'SF'. The agar plates were again transferred to a growth chamber maintained at 17°C for 16 hours light and 10°C for 8 hours dark cycles for 48 hours to introduce short term salt stress to growing seedlings. Previous studies showed that a significant stress response was induced by the application of salt stress for 48 hours, altering the spatial distribution in tissue (Sarabia 2019). Therefore, this timepoint was chosen for this study.

The seedlings were harvested after 48 hours. Shoots were collected for biomass measurements, roots were immediately frozen in liquid nitrogen, and stored in a -80°C freezer until extraction for metabolomics analyses. Roots were also immediately frozen in Super Cryo Embedding Medium (SCEM, Section Lab) using liquid nitrogen and stored at -80°C into 50 ml Conical Centrifuge Tubes (Thermo Fisher Scientific, USA) until subjected to cryosectioning. Three seedlings from each treatment for each variety were used for WinRhizo scanning to measure root morphological parameters.

3.2.5 Staining and visualization of roots using Light Microscopy

A modified method from Vierheilig et al., 1998 (Vierheilig et al. 1998) was used for staining roots. Here, roots were cleared using 10% KOH for 3 minutes and then rinsed several times with tap water. Cleared roots were boiled for 3 minutes in a 5% Shaeffer black ink-vinegar solution with pure white household vinegar (5% acetic acid). Roots were then destained by rinsing in pure water for 20 minutes. This allowed observation of hyphae structures within the roots and also distinguished colonized roots from non-colonized roots.

For light microscopy, 14 days old roots were harvested from plants inoculated and uninoculated with fungus grown under control conditions to confirm fungal association with roots. Upon harvest, roots were immediately transferred to falcon tubes containing MilliQ water until observed under microscope. A Leica MZ FLIII Microscope was used to observe roots association with fungus. Leica DC 300F camera was used to capture the images. In order to process the images obtained from brightfield microscopy, Irfan View Software was used (Pomeranz et al. 2013). The lens was adjusted to blank and the filter was set from visible to 100% photon. The configuration was set to brightfield and a blank image was captured using a plain white paper to adjust the white balance. Further, all images were acquired from the tissues and processed using Irfan View Software.

3.2.6 GCMS untargeted analysis for polar metabolites

Fifty (50 ± 2) mg of fresh frozen root tissue harvested from both varieties of barley were extracted in 500 μL of 100% MeOH (Scharlau), containing 4% v/v of [$^{13}\text{C}_6$] Sorbitol/[$^{13}\text{C}_5$ ^{15}N] Valine (0.5 mg mL^{-1}). The tissue was homogenized using a cryomill (Bertin Technologies) using a program (6100-3 x 45 x 45) at -10°C . Homogenized tissue was then shaken at 800 rpm ($97.6 \times g$) for 15 min at 30°C on a thermomixer (Eppendorf), centrifuged for 15 min at 13000 rpm ($15860 \times g$). The supernatant was then transferred into a fresh 2 mL tubes and 500 μL MilliQ water was then added to the remaining pellet. The mixture was then vortexed and centrifuged at 13000 rpm ($15860 \times g$). The supernatants were combined and 50 μL of the supernatant for each sample was completely dried down using a speed vacuum dryer (John Morris Scientific Pty. Ltd, Australia). Samples were then transferred to crimp vials and sealed with metal caps for analysis. Pooled Biological Quality Control (PBQC) samples were prepared by pooling 50 μL aliquots from each sample solution.

The dried extracts were derivatized with methoxyamine hydrochloride in pyridine and bis-(trimethylsilyl)-trifluoroacetamide (BFTFA) as described by Dias et al., 2015 (Dias et al. 2015). Briefly, 30 mg mL^{-1} methoxyamine hydrochloride in pyridine was added to all samples and derivatized at 37°C for 120 minutes with mixing at 500 rpm ($61.0 \times g$). The samples were further incubated on a thermomixer (500 rpm/ $61.0 \times g$) maintained at 37°C for 30 minutes by adding 20 μL N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA). Each sample was then allowed to rest for 60 minutes prior to injection. GC-MS system comprising of a Gerstel 2.5.2 Autosampler, a 7890A Agilent gas chromatograph and a 7000 Agilent triple-quadrupole MS (Agilent Santa Clara, USA) with an electron impact (EI) ion source was used to analyze samples with an injection volume of 1 μL for each derivatized sample. Instrumental settings were adapted from (Dias et al. 2015). Calibration and quantification of polar metabolites was done using serial concentrations of calibration authentic standards.

3.2.7 LCMS untargeted analysis for lipid analysis

A modified extraction method was used from Shiva et al., 2018 (Shiva et al. 2018). Thirty (30 ± 1) mg of fresh frozen root tissue was transferred to cryomill tubes and their weights were recorded. 400 μ L of cold isopropanol with 0.01% butylated hydroxytoluene (BHT) was added to the tubes. The samples were homogenized using a cryomill (Bertin Technologies; Program #2 (6,100–3 $\times$ 45 $\times$ 45) at -10°C). Each tube was then incubated at 75°C while shaking on a thermomixer at 1400 rpm (170.8 $\times$ g) for 15 minutes. After 15 minutes, tubes were cooled to room temperature and a 1200 μ L of solvent mixture containing chloroform:methanol:water (30/41.5/3.5, v/v/v) was added, to make the final solvent mixture with chloroform: isopropanol:methanol:water (30/25/41.5/3.5, v/v/v/v). Tubes were then kept for shaking on a thermomixer at 300 rpm (36.6 $\times$ g) for 24 hours maintained at 25°C . After 24 hours of shaking, tubes were subjected to centrifugation at 13,000rpm (15,860 $\times$ g) for 15 minutes. Supernatant was separated and dried overnight in vacuo. Before lipid analysis, dried extract was resuspended with 150 μ L of butanol:methanol (1:1, v/v) with 1% ammonium formate. Samples were then analyzed using LC-ESI-QqTOF-MS.

1 μ L aliquots were injected onto a Poroshell 120, EC-C8, 2.1 $\times$ 150 mm (2.7 μ m particle size) column (Agilent Technologies) at 15°C using an Agilent LC 1290 (Agilent Technologies, Mulgrave, Australia). Lipids were eluted at 0.26 mL/min over 30 min with a binary gradient of ACN-water (60:40, v/v) and IPP:water (90:10, v/v) containing 10 mM ammonium formate as described by Hu et al., 2008 (Hu et al. 2008). Tandem mass spectra and MS data were acquired on a TripleTOF[®] 6600 (Sciex) equipped with an ESI source in positive ion mode. For all the experiments performed, Information Dependent Acquisition (IDA) criteria were employed in order to automatically trigger the acquisition of full-scan MS/MS spectra for any compounds that were detected by the survey scans. The MS data presented corresponds to six pooled biological replicates for each treatment group.

3.2.8 Data preprocessing and statistical analysis

For GC-MS data analysis, in silico data pre-processing for mass spectral deconvolution and aided peak picking and identification was done using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) software [National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA]. A target component library (TCL) was generated from the PBQC file. Agilent MassHunter Qualitative Analysis B.05.00 (Agilent Technologies, Inc. 2011) was used to confirm peak identification. Agilent MassHunter Quantitative Analysis software (for GCMS) Version B.07.00/Build 7.0.457.0 (Agilent Technologies, Inc. 2008) was used to create batch and method files which were subsequently exported to Microsoft Excel. Manual integration was done to correct for baseline drift using the latter software. The exported peak area data from the batch file was normalized to internal standard [$^{13}\text{C}_6$] sorbitol, and fresh weight of each root sample.

Further, data were log transformed and the Student's t-test p-values were determined. The p-values were further subjected to Benjaminie Hochberg False Discovery Correction (Benjamini and Hochberg 1995).

MetaboAnalyst 3.0 (Xia et al. 2015) software was then employed for multivariate data analysis of the four biological replicates. Pattern recognition was determined through unsupervised learning algorithms such as principal component analysis and hierarchical cluster analysis. Log data transformation and mean centering were applied for the principal component analysis. Hierarchical cluster analysis was performed using Euclidean distances with ward clustering algorithm. Fold-change for univariate analysis and bar plots were generated using GraphPad Prism 8.0 (GraphPad Software, Inc., USA; Full Version).

For LC-MS data analysis, raw HPLC-MS data was visually inspected for integrity using PeakView Sciex Software (ver. 2.2), the raw LC-TripleTOF-MS data containing m/z_RT (mass to charge_retention time) and associated peak intensities were converted to ABF (analysis base file) format using the Reifycs file converter for processing using MS-Dial 3.98 (http://prime.psc.riken.jp/Metabolomics_Software/MS-DIAL/index2.html, accessed 18 September 2019) (Tsugawa et al. 2015). Statistical analysis was performed using MetaboAnalyst 3.0 (<http://www.metaboanalyst.ca/>, accessed 10 September 2019) (Xia et al. 2015). MS-Dial export was done using the default parameters for a Lipidomics omics project. The MS peak filter threshold was established after manual inspection of the raw data. Additionally, adduct ion setting was set to search for $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ and $[M+NH_4]^+$, adducts and alignment parameters were established considering a retention time tolerance of 0.5 min and a MS1 tolerance of 0.1 Da. A matrix containing tentatively identified m/z_RT and associated intensity peaks was exported as a csv file. The level of identification was carried out based on the Metabolomics Standards Initiative requirements (Sumner et al. 2007). Relative quantities were determined as given in **Supplementary Table S1**.

The processed lipid data was subjected to multiple comparison statistical analyses using Analysis of Variance (ANOVA), with a false discovery rate (FDR)-adjusted p value of 0.05 and using the Benjamini–Hochberg method. Additional statistical analysis (Student's t-tests) comparing each treatment in both genotypes was also performed using MetaboAnalyst to generate a list of features (m/z_RT) with a false discovery rate corrected p-value ($P < 0.01$) fold change of two or more in peak intensity relative to the respective controls. All bar plots were generated using GraphPad Prism 8.0 (GraphPad Software, Inc., USA; Full Version).

3.3 Results

3.3.1 Fungal association within roots confirmed using microscopy

Barley roots of cv. Gairdner were inoculated with *Trichoderma* strain T-22 and ink-vinegar was used for staining to confirm the association of the fungus with roots. The method allowed colonized roots to be easily distinguished from noncolonized roots. Roots were viewed daily for 7 days after inoculation of fungal spores (**Figure 2.4**). No fungal spores were observed within and around roots on day 1. From day 2 until day 4, fungal spores were observed surrounding the roots and on day 7, individual hyphae in heavily and partially colonized roots were clearly visible. Based on these results all analyses in this study used roots that were maintained in spore suspension for seven days to ensure successful colonization. Our results with ink-vinegar solutions show that association of fungal hyphae within roots can be observed after an adequate clearing time, good staining and destaining of roots.

3.3.2 Plant growth and root development

Two barley cultivars, cv. Vlamingh and cv. Gairdner, were selected for study based on their known differences in germination phenology and salinity tolerance. Vlamingh has good germination in high salt conditions while Gairdner is less tolerant of these conditions (Gupta et al. 2019). Roots of control plants (uninoculated) or those inoculated with *Trichoderma* strain T-22 were treated with 0 or 200 mM NaCl and average total root length (TRL) of each plant was analysed using WinRHIZO root-scanning (**Figure 2.5**).

Uninoculated Salt treatment (S) reduced TRL in both genotypes, but the reduction was more marked for Gairdner (**Figure 2.6**). In the control with inoculum (CF) treatment, TRL increased by 6.04% ($P < 0.05$) in cv. Vlamingh and 9.87% ($P < 0.01$) in cv. Gairdner compared to the uninoculated roots of the same cultivar not treated with salt (C). In the inoculated salt (SF) treatment, TRL increased by 14.85% ($P < 0.05$) in cv. Vlamingh and 35.07% ($P < 0.05$) in cv. Gairdner compared to treatment S.

The decrease in TRL was 6.83% ($P < 0.01$) in cv. Vlamingh and 23.73% ($P < 0.01$) in cv. Gairdner when treatment S was compared to the same cultivar not treated with salt (C). However, for inoculated plants, TRL of Vlamingh increased 0.91% ($P < 0.01$), while that of Gairdner decreased by only 6.24% ($P < 0.01$) after salt treatment.

3.3.3 The effect of inoculation with *T. harzianum* and salt stress to polar metabolites in roots

A total of 110 metabolites, of which 73 were identified, were detected in roots of both barley genotypes using GC-MS. These detected metabolites are categorized according to compound classes and are given in **Supplementary Table S1**. Of the known metabolites, twenty-eight were

amino acids and organic acids and twenty-seven were sugars. The average normalized responses of all metabolites in all treated barley roots can be found in **Supplementary Table S2**.

A principle component analysis (PCA) of all metabolites showed a well-defined separation among samples from the different treatments in both genotypes (**Figure 3.1**). The first and second principal components accounted for 53.5% and 50.3% of the variation in Vlammingh and Gairdner, respectively. The PCA scatterplot divided the samples into four main groups-C, CF, S and SF. In Vlammingh, metabolite profiles of treatment SF clustered on the right quadrant along PC1, fully separated from all other treatment groups contributing to the clear separation on component 1 (PC1), which described 35.7% of the variability. Along PC2, treatment S was separated from all other treatments contributing to 17.8% of the variability. In Gairdner, salinity contributed to separation on PC1, which described 35.5% of the variability, whereas fungal inoculation contributed to separation on PC2, describing 14.8% of the variability.

A heatmap representing the changes in metabolite levels in different treatments for both genotypes provided an integrated view of the effects of inoculation of fungus under salt stress (**Figure 3.1**). The results show a strong separation between the treatments for both genotypes. In Vlammingh, two major clusters were identified, C, CF and S fall in the same major class but SF formed a separate class. Further clustering was identified forming a subclass between C and CF separating S. This implies fungal inoculation under salt stress had the strongest effect on the root metabolite profiles compared to all other treatments. In cv. Gairdner, two major clusters were identified, C and CF fall in the same major class whereas S and SF formed a separate subclass. Further clustering separated all treatments showing distinct differences from one another. Further detailed analysis on the heatmap revealed similarities and differences in metabolite abundances between treatments for both genotypes due to groups of metabolites with similar patterns. The highest number of metabolites with the greatest variability amongst treatments were sugars.

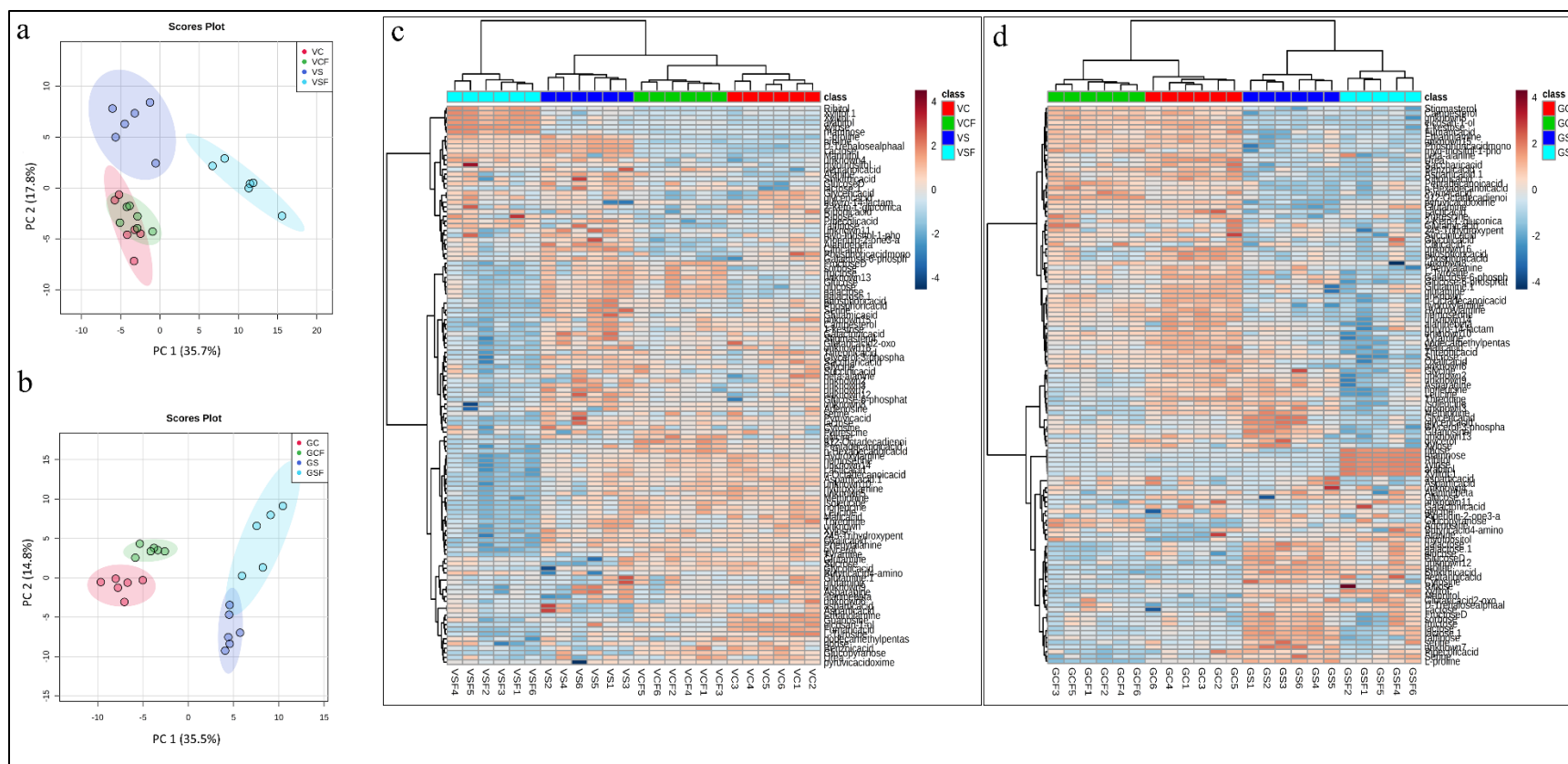


Figure 3.1 Principal Component Analysis (PCA) and heatmaps of four treatments in cv. Vlamingh and cv. Gairdner. a- PCA of Control- C, Control + fungus- CF, Salt- S and Salt + fungus- SF in cv. Vlamingh; b- PCA of Control- C, Control + fungus- CF, Salt- S and Salt + fungus- SF in cv. Gairdner; c and d- heatmap obtained from hierarchical cluster analysis (HCA) of all identified metabolites in cv. Vlamingh and cv. Gairdner, respectively. The legend on the bottom indicates the sample groups and legends on the right are the identified metabolites. The column indicated in **3.1c** and **3.1d** with same colors indicate biological replicates.

Analysis of a 2-fold change or greater ($P < 0.05$) was performed to compare the profiles of amino acids, organic acids and sugars in different treatment groups in both genotypes. **Figure 3. 2** shows X-fold graphs for treatment groups S and CF compared to treatment C as well as SF compared to treatment S. In Vlamingh S, trehalose (+10.50-fold), arabitol (+5.30-fold), xylitol (+5.60-fold) and proline (+12.39-fold) showed the greatest increases compared to Vlamingh C. In Vlamingh CF, arabitol had the greatest increase, 3.04-fold, compared to Vlamingh C. In Vlamingh SF, sucrose (+3.13-fold), inositol (+6.60-fold), xylitol (+39.50-fold), ribitol (+15.60-fold), arabitol (+40.70-fold), rhamnose (+43.12-fold), ribose (+2.43-fold) and pipecolic acid (+2.20-fold) were strongly increased compared to S.

In Gairdner S, 22 metabolites were increased with +2.0-fold or greater when compared to C and the highest ones were raffinose (+12.0-fold), trehalose (+4.67-fold), lactose (+8.60-fold), glucopyranose (+8.20-fold), shikimic acid (+5.52-fold), guanosine (+5.21-fold), and proline (+10.20-fold). In Gairdner CF, the metabolites that were increased most compared to C were trehalose (+2.40-fold), glucopyranose (+15.81-fold), adenosine (+3.14-fold) and butyric acid (+2.50-fold). In Gairdner SF, ribitol (+39.25-fold), arabitol (+91.20-fold), rhamnose (+73.80-fold), xylitol (+7.80-fold), ribose (+5.30-fold) and glutamine (+5.20-fold) were increased compared to S.

In Vlamingh SF, glucopyranose (-2.72-fold) decreased significantly compared to S. However, in Gairdner many more metabolites were decreased in SF compared to S, including raffinose (-2.79-fold), lactose (-3.44-fold), sucrose (-4.35-fold), guanosine (-5.23-fold), tyramine (-2.02-fold), asparagine (-2.73-fold), proline (-2.82-fold), methionine (-2.12-fold) and leucine (-2.70-fold).

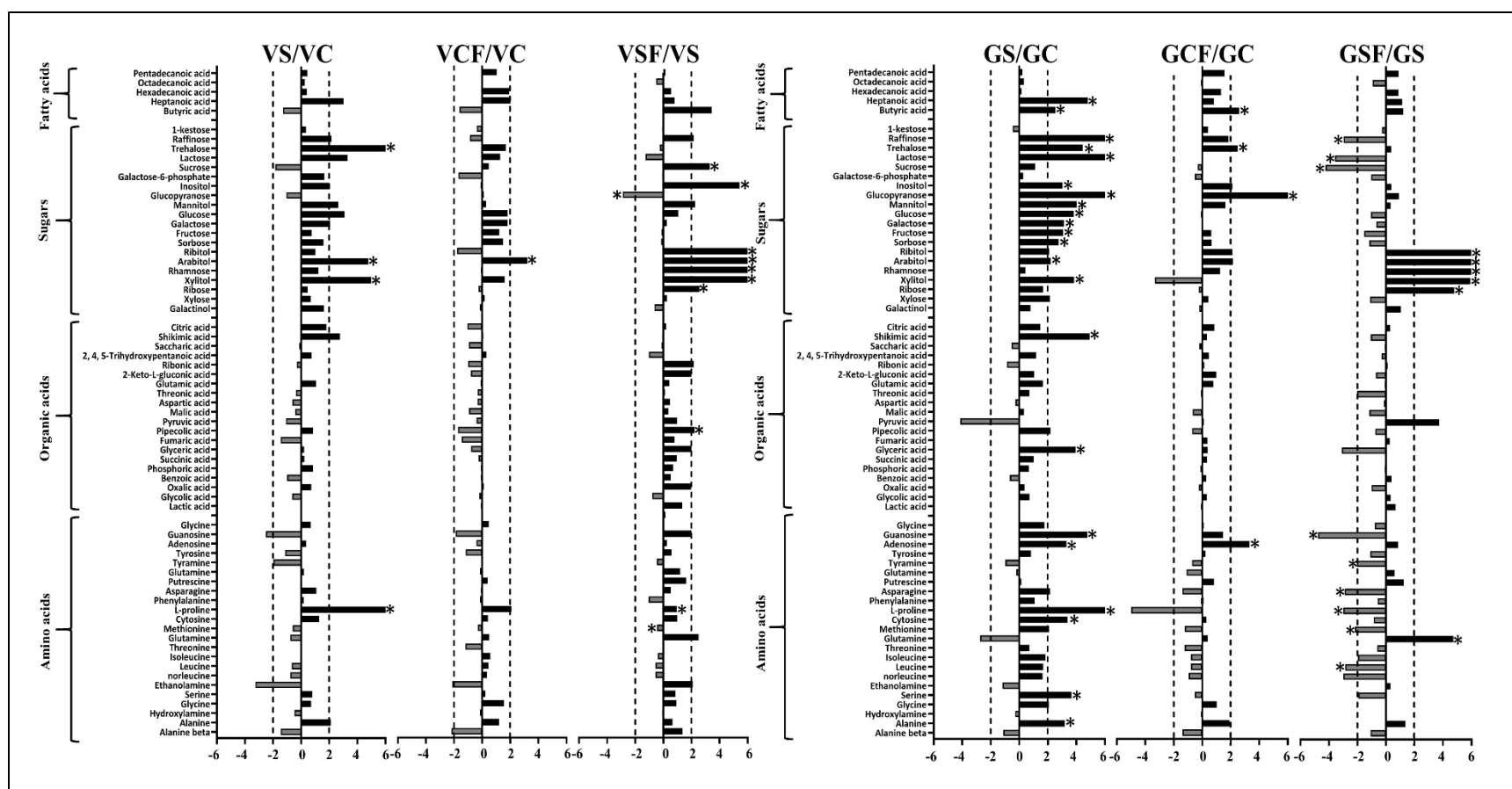


Figure 3.2 Logarithmic ratios of representative sugars, organic acids and amino acids in roots of barley cv. Vlammingh (V) and cv. Gairdner (G) of salt grown compared to control grown (VS/VC and GS/GC); control with fungus compared to control grown (VCF/VC and GCF/GC); and salt with fungus compared to salt grown (VSF/VS and GSF/GS) as analysed by Student's t -test. Values that are significantly higher ($P < 0.05$, FDR) are indicated by *. A threshold of ± 2 -fold change is indicated by a dashed line.

It is worth mentioning that in cv. Gairdner SF, raffinose (-0.36-fold), lactose (-0.30-fold) and sucrose (-0.23-fold) significantly decreased as compared to S and glucopyranose (-0.40-fold) decreased significantly in Vlamingh SF as compared to S. Among identified amino acids, guanosine (-0.20-fold), tyramine (-0.49-fold), asparagine (-0.36-fold), proline (-0.35-fold), methionine (-0.47-fold) and leucine (-0.37-fold) decreased in cv. Gairdner SF as compared to S. No significant reduction in identified amino acids was observed in cv. Vlamingh.

To elucidate the metabolic pathways involved and affected, identified metabolites were analysed for their involvement in metabolism and a pathway enrichment analysis using MetaboAnalyst was carried out. This analysis is based on the compound concentration values as compared to the overall compound lists. **Figure 3.3a** show an example of the metabolome view of all matched pathways arranged by p values on the Y axis and pathway impact values on the X-axis. The node colour is based on its p value and the node radius is determined based on their pathway impact values. **Figure 3. 3b** shows an example of a pathway view for alanine, aspartate and glutamate metabolism. The pathway given here is a simplified KEGG pathway and the matched metabolites are shown in red colour. Further detailed analysis was done to identify biochemical pathways involved in this interaction with fungus in both genotypes. To achieve this, the number of identified metabolites were divided by the total number metabolites in a pathway and a pathway coverage percentage was obtained. **Figure 3.4** demonstrates the top ten biochemical pathways affected in this study

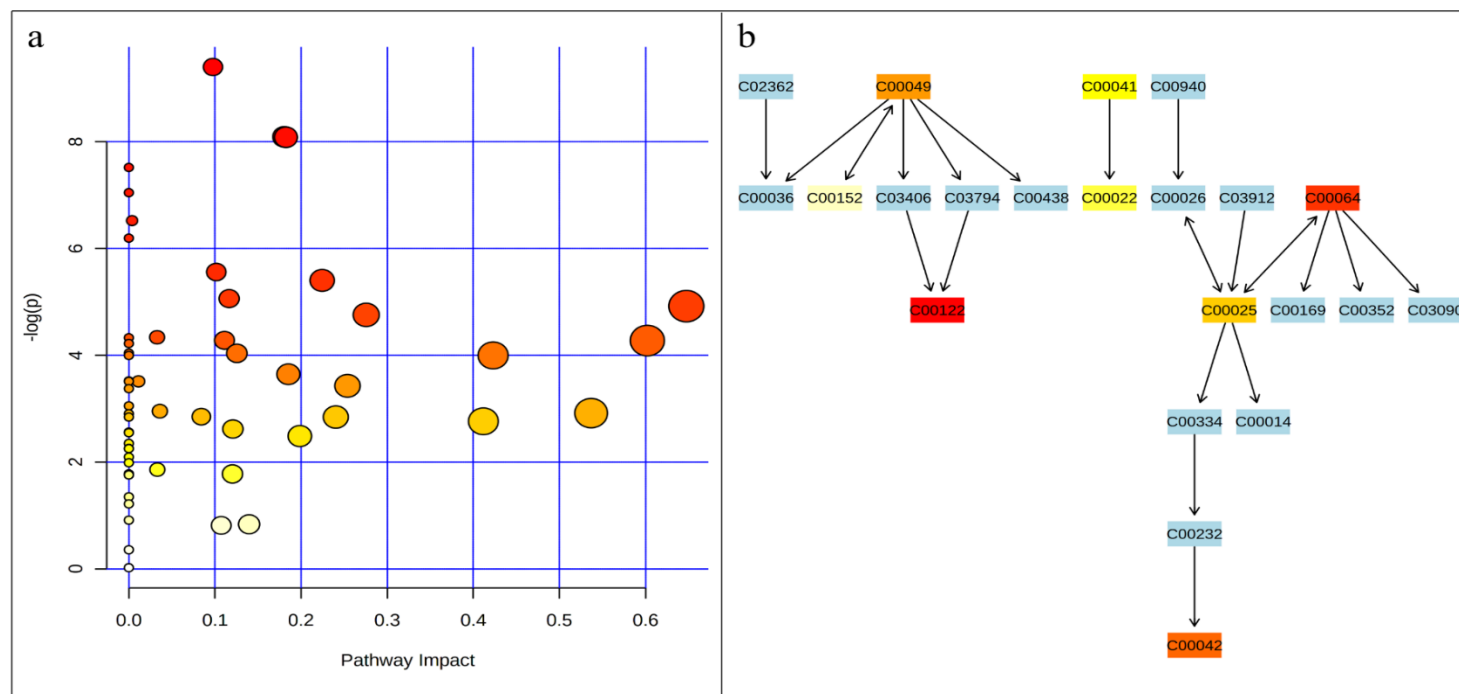


Figure 3.3 Metabolome and pathway view of alanine, aspartate and glutamate metabolism for metabolites in roots of barley cv. Vlammingh under salt and salt + fungus treatments. 3a- Metabolome view showing all matched pathways arranged by p values on Y-axis and pathway impact values on X-axis. The node colour is based on its p value and the node radius is determined on their pathways impact values. 3b- Pathway view showing current metabolic pathway achieved by clicking the corresponding node on the metabolome view as given in 3a. Blue colour indicates default node and matched nodes are given in red colour. The compound names are given in nodes as explained- C02362- 2-oxosuccinamate, C00049- L-Aspartate, C00041- L-Alanine, C00940- 2-Oxoglutaramate, C00036- Oxaloactetae, C00152- L-Asparagine, C03406- M- (L-Arginino)succinate, C03794- N6-(1,2-Dicarboxyethyl)-AMP; N6-(1,2-Dicarboxyethyl)AMP. C00438- N-Carbomyl-L-aspartate, C00022-Pyruvate, C00026- 2-Oxoglutarate, C03912- (S)-1-Pyrroline-5-carboxylate, C00064- L-Glutamine, C00122- Fumarate, C00025- L-Glutamate, C00169- Carbomyl phosphate, C00352- D-Glucosamine 6-phosphate, C03090- 5-Phosphoribosylamine, C00334- 4-Aminobutanoate, C00014- Ammonia, C00232- Succinate semialdehyde, C00042- Succinate

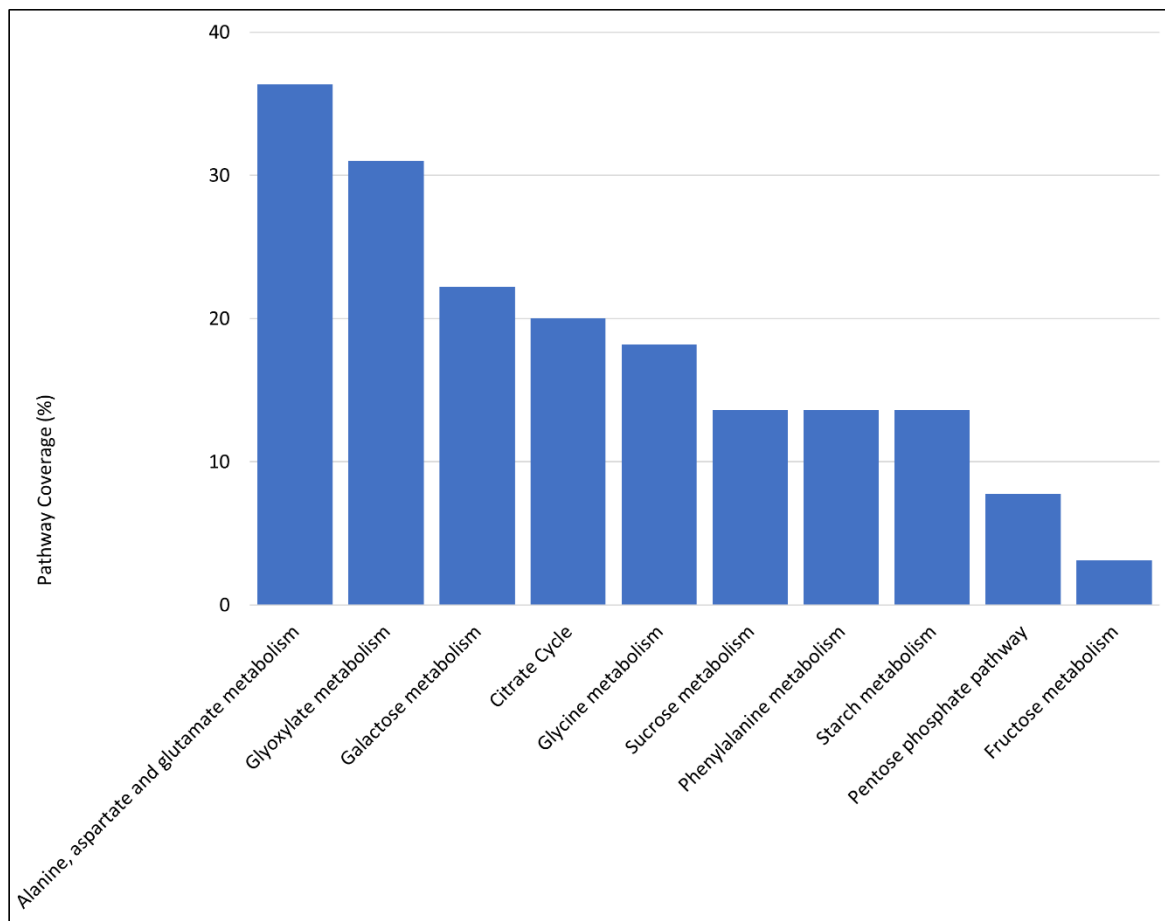


Figure 3.4 Pathway coverage graph for metabolites with 2-fold change or greater in roots of barley cv. Vlammingh and cv. Gairdner under four treatments. Top ten biochemical pathways that were affected are shown on X-axis and percentage of coverage is given on Y-axis

3.3.4 Global untargeted lipid profiling of barley roots

An untargeted LC-MS based study was performed to identify detailed changes in the lipidome of two barley genotypes in four treatments, C, CF, S and SF. Processing of data, identification of peaks (m/z values) with their corresponding retention times (RT) and alignment of the detected features was performed using MS-DIAL v3.98 (Tsugawa et al. 2015) that allowed the analysis of the raw mass spectra for all samples. This resulted in 3468 m/z _RT mass features (**Supplementary Table S3**). Further, detected m/z were subjected to multivariate analyses using the Metaboanalyst webtool (<https://www.metaboanalyst.ca/>, accessed 10/11/2019) (Xia et al. 2015). This was done to identify discriminate changes between the four treatments.

A hierarchical clustering analysis (HCA) represented by a heatmap (**Figure 3.5**) with changes in the detected mass features (m/z _RT) across four treatments in two barley genotypes, showed a clear separation between four treatments in both genotypes. A major clustering was found to be between control and salt treatment. Further sub-clusters were found specific to genotypes

where GSF clustered with GS and VSF clustered with VS. Under control treatment, VCF formed a separate cluster and VC, GC and GCF clustered together. Hence, clustering of the mass features was predominantly determined by the salt treatment then fungal treatment.

Similarly, an unsupervised chemometric analysis of the datasets by principal component analysis (PCA) revealed clear separation between the treatments in both genotypes (**Figure 3.6**) In cv. Vlamingh and cv. Gairdner, the first and second components accounted for 72.3% and 63.6% of the variability between 24 samples, respectively. The salt treatment clearly contributed to the separation observed in Component 1 in cv. Vlamingh, which describes 55.9% of the variability primarily caused due to Vlamingh roots grown in control conditions and Vlamingh roots inoculated with fungus under control conditions being different from the salt treatment with and without fungus. The lipid profiles in control roots inoculated with fungus exhibited a much stronger separation from the other three treatments. On the other hand, in cv. Gairdner, Component 2 clearly separated control (with and without fungus) and salt treatment (with and without fungus), representing 19.9% of the variability.

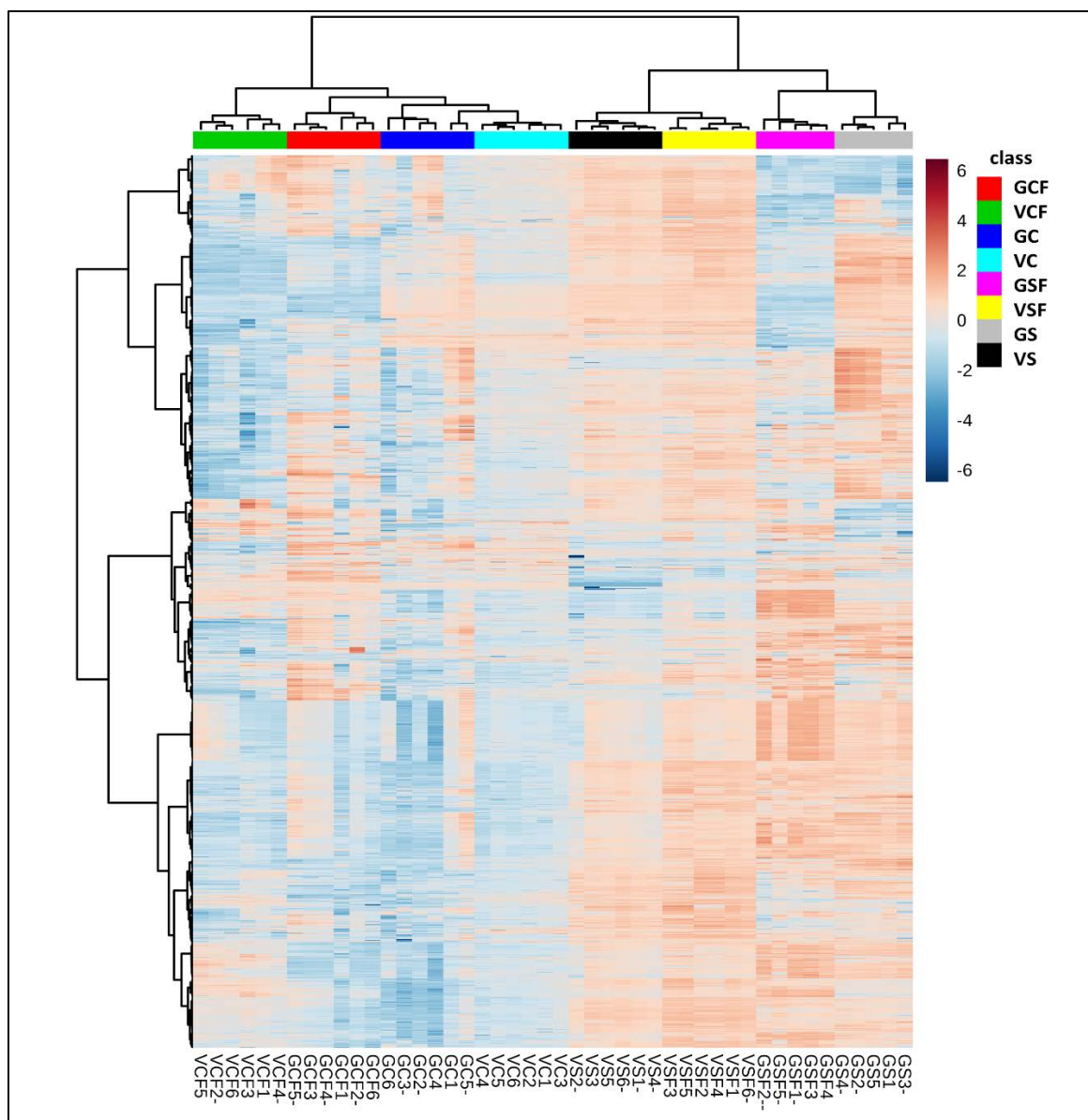


Figure 3.5 Heatmap showing the relative abundances of detected lipid species in samples from four treatments in two barley genotypes. Heatmap represents unsupervised hierarchical clustering of lipid species (rows). The row displays identified lipid species and the column represents the samples. Relative lower abundance of lipids in samples was displayed in blue, whilst relative higher abundance of lipids was displayed in red. Clustering was performed using Euclidean distance matrices on in the R package heatmap. VC- control treated cv Vlammingh; VCF- control and fungus treated cv Vlammingh, VSF- salt and fungus treated cv Vlammingh; GC- control treated cv Gairdner; GCF- control and fungus treated cv Gairdner, GSF- salt and fungus treated cv Gairdner. n = 6 for all treatments. salt- 200mM NaCl.

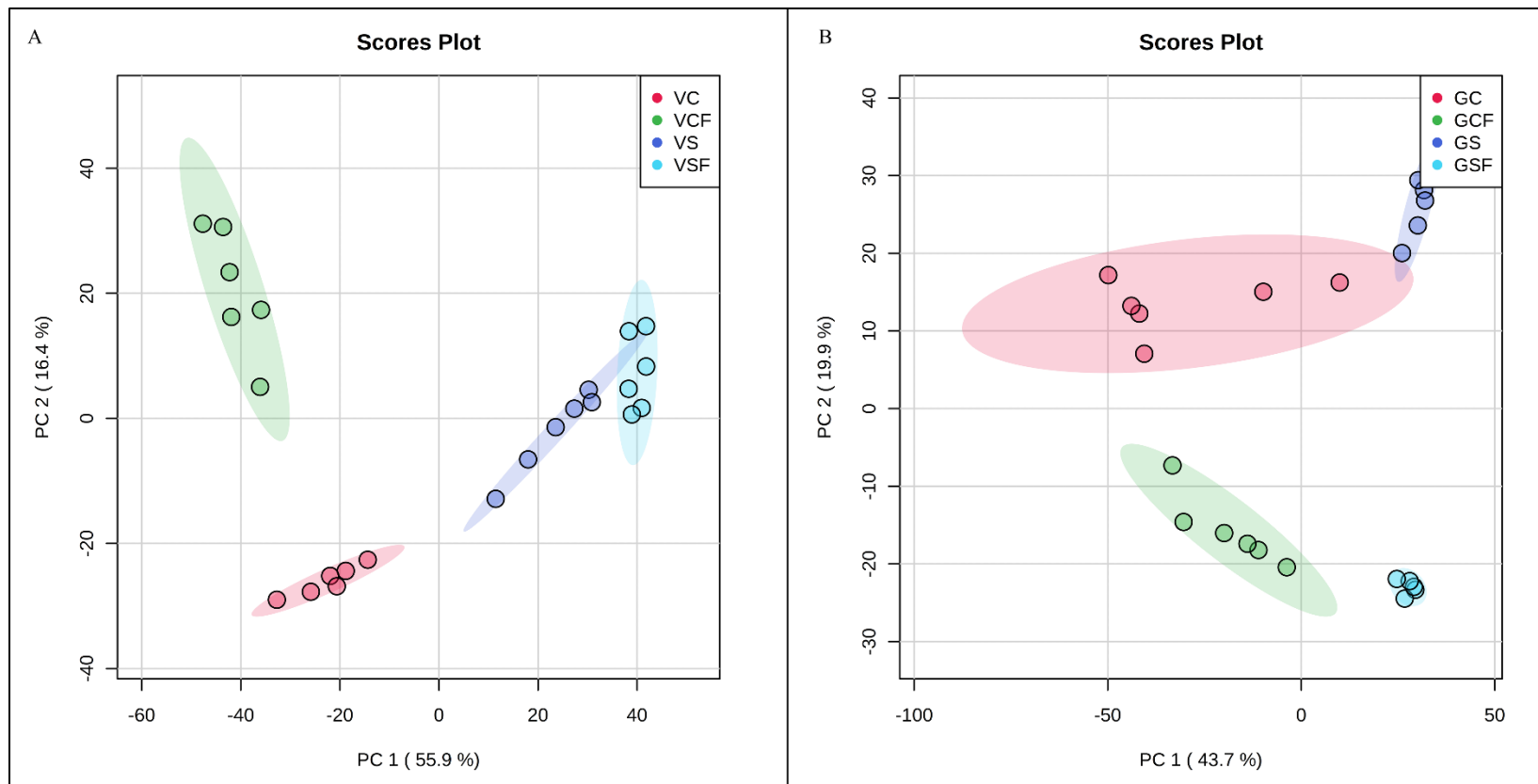


Figure 3.6 Principle component analysis (PCA) of the mass features (m/z_RT) extracted from lipid profiles of dissected roots of two barley genotypes grown under control and saline conditions with and without fungus. PC 1 versus PC 2 displays the separation between control and salt treatment for cv. Vlammingh (A) and cv. Gairdner (B). VC- control treated cv. Vlammingh; VCF- control and fungus treated cv. Vlammingh, VSF- salt and fungus treated cv. Vlammingh; GC- control treated cv. Gairdner; GCF- control and fungus treated cv. Gairdner, GSF- salt and fungus treated cv. Gairdner. n = 6 for all treatments. salt= 200 mM NaCl

3.3.5 Significant differences in the lipid profiles under saline conditions

The analysis of the effect of a short-term salt stress with and without fungal inoculation on the lipid profiles in roots of cv. Vlamingh and cv. Gairdner showed that in cv. Vlamingh 436 unique features showed significant changes while only 134 features were changes in cv. Gairdner under saline conditions when compared to control. Of those, 107 were common features in both genotypes.

It was found that fungal inoculated roots of cv. Vlamingh under saline conditions exhibited only 58 significantly changed features while 176 were found in fungal inoculated roots of cv. Gairdner after salt stress. And both genotypes shared 103 common features in inoculated roots under saline conditions. It is interesting to note that only four features were found to be common in inoculated and uninoculated roots of both genotypes under saline conditions. However, it is worth mentioning that 259 features were common between roots of cv. Gairdner and inoculated roots of cv. Vlamingh after salt stress. On the other hand, only 57 features were common to roots of cv. Vlamingh and inoculated roots of cv. Gairdner (**Figure 3.7**). These results have shown that cv. Vlamingh and cv. Gairdner exhibited a larger number of significantly different ($P < 0.05$) features (607 and 548 respectively) which were found after short-term salt stress without inoculation of fungus. This suggests that the number of differences in the lipid profiles of barley roots might be primarily salt stress dependent rather than due to the effect of fungus.

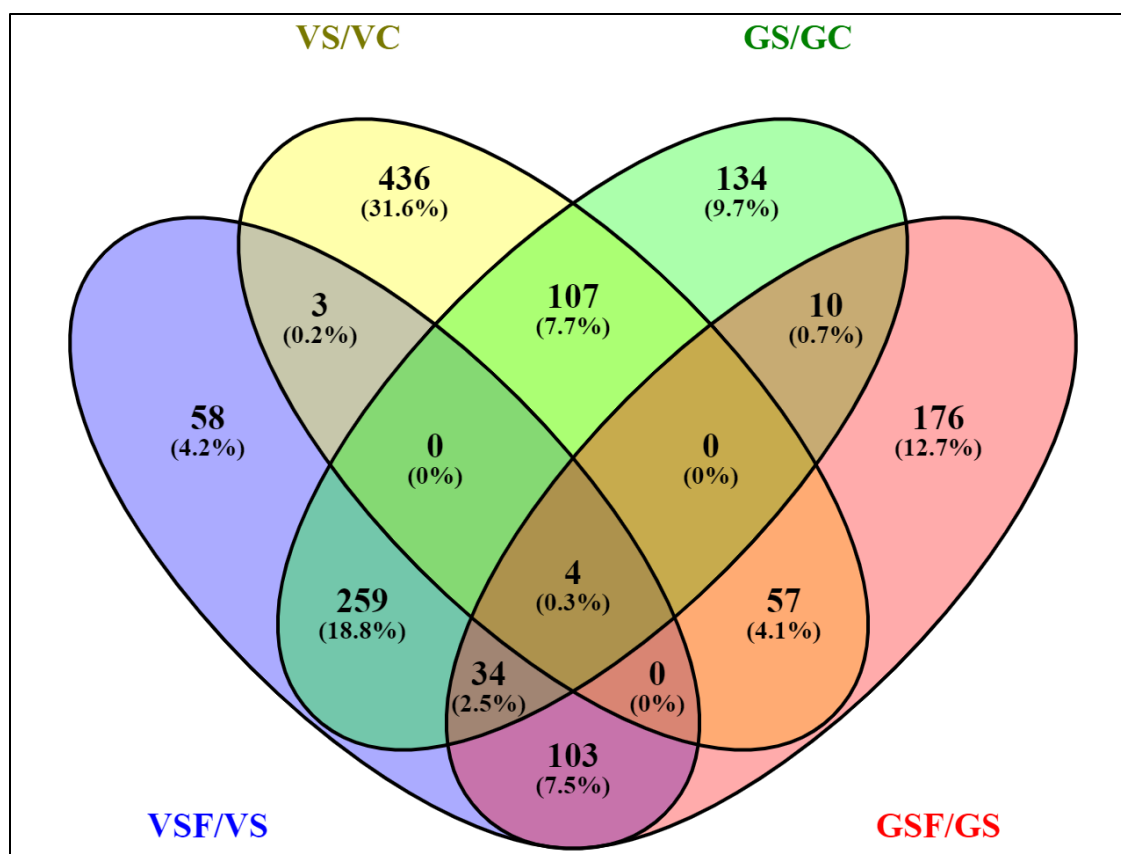


Figure 3.7 Venn diagram summary of the number of significantly altered features identified by one-way ANOVA and post-hoc analysis of the lipid profiles from roots of two barley genotypes under salt stress and with and without fungal inoculation. The number represent significantly altered features regardless of their directionality (upregulation and downregulation). Numbers appearing in overlapped sections are common between treatments. Four analysis performed are Vlamingsh salt (VS) compared to Vlamingsh control (VC), Vlamingsh salt and fungus (VSF) compared to Vlamingsh salt (VS), Gairdner salt (GS) compared to Gairdner control (GC) and Gairdner salt and fungus (GSF) compared to Gairdner salt (GS).

3.3.6 Targeted MS/MS lipid analysis

MS-DIAL's in silico LipidBlast database and an in-house plant lipid database (Cheka Kehelpannala, personal communication) was further used to identify lipids by the similarity calculation of retention time, precursor m/z, isotopic ratios, and MS/MS spectrum. False positives and true positives in the results of peak identifications were manually checked, curated and modified where needed. Out of 3468 m/z_RT mass features, 2675 m/z_RT mass features were identified as unknown that did not return with any match, 522 m/z_RT mass features were annotated without MS2 spectra (**Supplementary Table S4**) and 271 lipid molecular species were identified with MS2 spectra (**Supplementary Table S5**). An unsupervised chemometric analysis of the datasets by principal component analysis (PCA) revealed clear separation between treatments in both genotypes. These results indicate a substantial difference in the root lipidome between

treatments in both genotypes. **Figure 3.8a** show that in cv. Vlamingh, the first and second components accounted for 56.8% of the variability between all samples. Clearly, short-term salt stress contributed to the separation observed in Component 1, described by 41.9% of the variability which is primarily due to the roots inoculated (VSF) and uninoculated (VS) under salt stress being different from the roots inoculated (VCF) and uninoculated (VC) under control conditions. Component 2 separates the samples based on fungal treatment showing 14.9% of the variability between inoculated (VCF and VSF) and uninoculated (VC and VS) roots. On the other hand, **Figure 3.8b** shows that in cv. Gairdner, the first and second components accounted for 57.9% of the variability between all samples. Similar to cv. Vlamingh, Component 1 in cv. Gairdner separates samples primarily based on salt treatment accounting for 35.5% of the variability. Here Component 2 failed to show any clear separation based on either salt treatment or fungal effect on roots (**Figure 3.8b**).

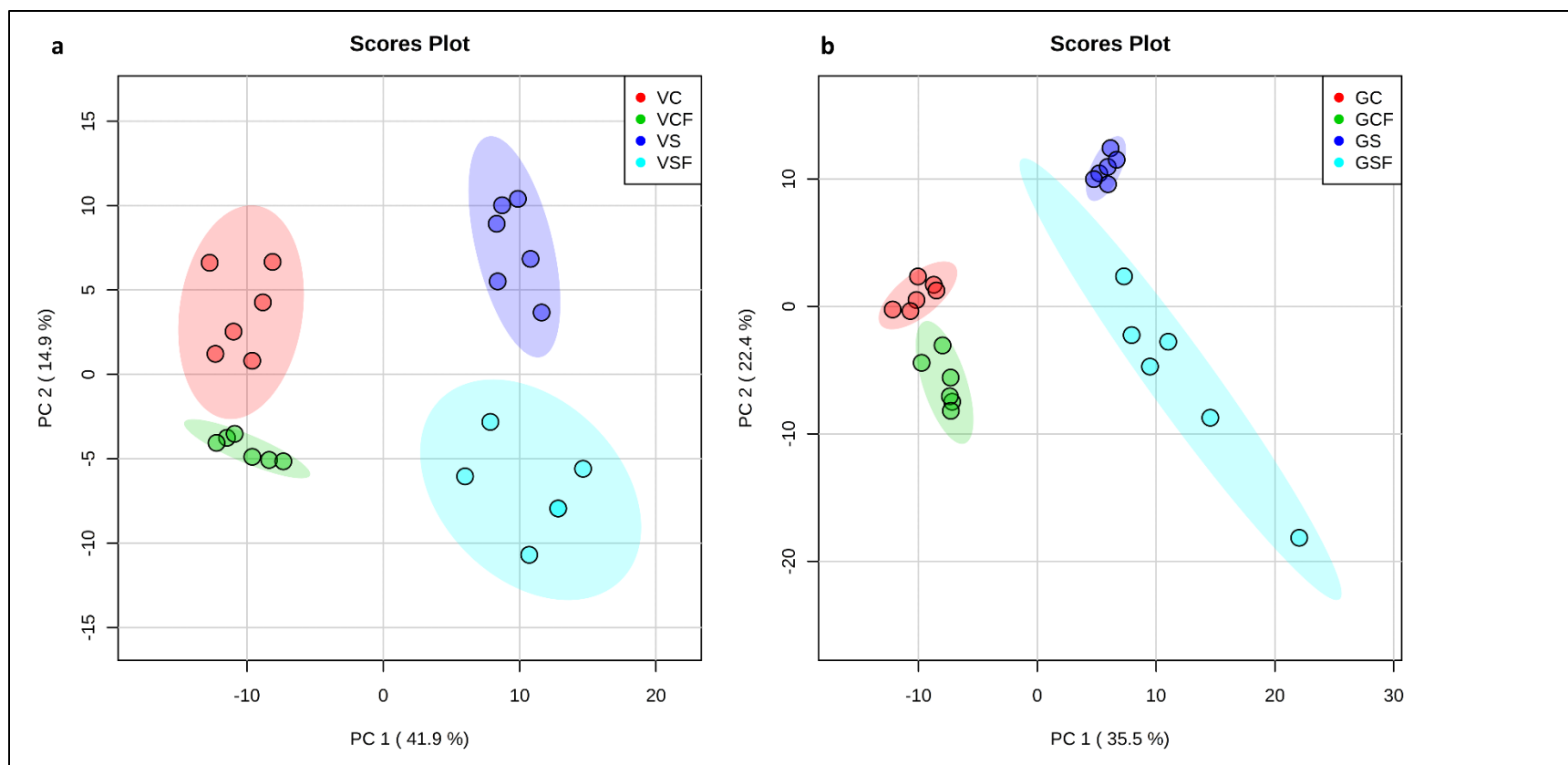


Figure 3.8 Principle component analysis (PCA) of the identified lipids from dissected roots of two barley genotypes grown under control and saline conditions with and without fungus. PC 1 versus PC 2 displays the separation between control and salt treatment for cv. Vlammingh (A) and cv. Gairdner (B). VC- control treated cv. Vlammingh; VCF- control and fungus treated cv. Vlammingh, VSF- salt and fungus treated cv. Vlammingh; GC- control treated cv. Gairdner; GCF- control and fungus treated cv. Gairdner, GSF- salt and fungus treated cv. Gairdner. n = 6 for all treatments. salt =200 mM NaCl.

A total of 271 lipids identified in both genotypes under four treatments were categorised into different lipid classes (**Table 3.1**). Major classes identified were phospholipids-phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS), lysophospholipids-lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), storage lipids triacylglycerol (TAG), lipid intermediates diacylglycerol (DAG), galactoglycerolipids/ membrane glycolipids including monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG), acyl diacylglycerol glucuronide (ADGGA) and sphingolipids (SL).

Table 3.1 Summary of the number of identified lipid species from roots of two barley genotypes (cv. Vlamingh and cv. Gairdner) from four treatment groups control C, control inoculated with fungus CF, salt (200 mM NaCl) S and salt inoculated with fungus SF. Analysis was performed using LC-TripleTOF-MS (positive ionization mode).

Lipid class	Lipid subclass	Species identified
Glycerophospholipids (GP) (total = 111)	PC/LPC	37
	PE/LPE	40
	PG	10
	PI	14
	PS	8
Glycerolipids (GL) (total = 128)	MAG	1
	MGDG	7
	DAG	18
	DGDG	17
	TAG	76
	ADGGA	9
Sphingolipids (total = 33)	Cer-AP	14
	Cer-NDS	1
	Cer-NS	3
	HexCer-AP	12
	PI-Cer	3

Abbreviations: PC- phosphatidylcholine, LPC- lysophosphatidylcholine, PE- phosphatidylethanolamine, LPE- lysophosphatidylethanolamine, PG- phosphatidylglycerol, PI- phosphatidylinositol, PS- phosphatidylserine, DAG- diacylglycerol, TAG- triacylglycerol, MAG- monoacylglycerol, MGDG- monogalactosyl diacylglycerol, DGDG- digalactosyl diacylglycerol, CE- ceramides, ACer- acyl carnitines, ADGGA- Acyl diacylglycerol glucuronide, Cer-AP- Ceramide alpha-hydroxy fatty acid-phytospingosine, Cer-NDS- Ceramide non-hydroxyfatty acid-dihydrosphingosine, Cer-NS- Ceramide non-hydroxyfatty acid-sphingosine, HexCer-AP- Hexosylceramide alpha-hydroxy fatty acid-phytospingosine, PI-Cer- ceramide phosphoinositol

Total percentage of individual species detected in each lipid classes in roots of both genotypes is given in (**Figure 3.9**). The largest numbers of species were identified in the Glycerolipids (GLs) and Glycerophospholipids (GPs) classes in both genotypes.

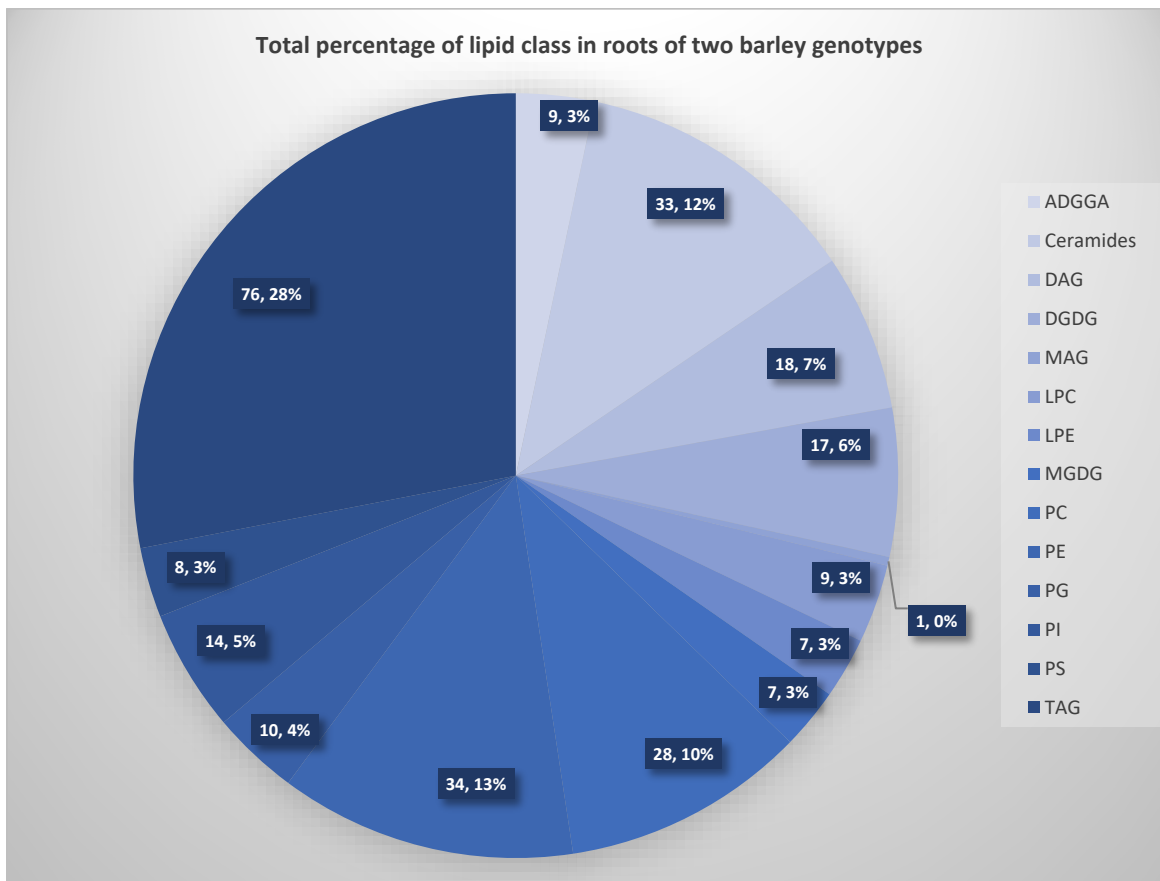


Figure 3.9 Total percentage of identified lipid species in each lipid class in roots of two barley genotypes grown under control and saline conditions with and without fungus. Major classes identified were the phospholipids- phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), triacylglycerol (TAG), diacylglycerol (DAG), monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), acyl diacylglycerol glucuronide (ADGGA) and ceramides. A total of 271 lipids were identified and number for each class is given with the corresponding percentage

A more rigorous statistical analysis based on pairwise comparisons between the four treatments in both genotypes was carried out for GPs and GLs in order to adequately understand the directionality of the changes in the identified features. **Table 3.2** and **Table 3.3** summarises the number of significantly altered lipids found in each treatment for several pairwise comparisons. In class GP, the directionality of these changes showed a larger number of increased lipids under S as compared to control in cv. Vlammingh where 35 lipids were significantly increased showing +2-fold ($P < 0.05$) or more as opposed to cv. Gairdner where only 23 lipids increased significantly.

In treatment CF compared to C, 20 and 23 lipid species showed significant increase in inoculated roots as compared to uninoculated in cv. Vlamingh and cv. Gairdner, respectively. However, in SF compared to S, cv. Gairdner showed a significantly larger number of increased lipids (35 lipids species increased) with +2-fold or more ($P < 0.05$) as compared to cv. Vlamingh where zero lipids were increased. But it is worth noting that inoculation of fungus resulted in greater reductions in GP class in cv Vlamingh where 11 lipids were reduced significantly while in cv Gairdner only 7 lipids from GP class showed significant reductions.

Table 3.2 Number of Glycerophospholipids as defined by Student's t-test ($P < 0.05$, FDR) with a 2-fold change or greater, found for two barley genotypes in three treatments

Lipid class	Treatment specific		Vlamingh	Gairdner
Glycerophospholipids (GP)	Salt vs Control	Higher	35	23
		Lower	2	5
	Control+ fungus vs Control	Higher	20	23
		Lower	0	3
	Salt + fungus vs Salt	Higher	0	35
		Lower	11	7

Table 3.3 Number of Glycerolipids as defined by Student's t-test ($P < 0.05$, FDR) with a 2-fold change or greater, found for two barley genotypes in three treatments

Lipid class	Treatment specific		Vlamingh	Gairdner
Glycerolipids (GL)	Salt vs Control	Higher	47	55
		Lower	0	3
	Control+ fungus vs Control	Higher	24	23
		Lower	9	24
	Salt + fungus vs Salt	Higher	10	39
		Lower	34	16

In class GL, cv. Gairdner showed a larger number of increased lipids (55 glycerolipids) with +2-fold or more ($P < 0.05$) under treatment S as compared to treatment C. However, in treatment CF, cv. Vlamingh showed a slightly greater number of lipids exhibiting significantly increased fold changes as compared to cv. Gairdner (24 in cv. Vlamingh and 23 in cv. Gairdner). It is interesting to note that the fungus significantly reduced 24 lipids from GL class in cv Gairdner while only 9 lipids were reduced in cv Vlamingh. An opposite trend was observed in treatment SF where fungal inoculation resulted in a drastic effect in cv. Gairdner roots under saline conditions, significantly increasing 39 lipids as compared to only 10 lipids in cv. Vlamingh. The largest number of

significantly reduced lipids was observed in inoculated roots of cv. Vlamingh from treatment SF (34 lipid species) which were majorly from the GL class.

In the following paragraphs, lipid nomenclature follows the “Comprehensive Classification System for Lipids” given by the International Lipid Classification and Nomenclature Committee (ILCNC) (Fahy et al. 2009). For example, the nomenclature PC (38:n) indicates a PC species with a fatty acyl sum composition of 38 carbons containing all identified double bonds in this study which is given as “n”. The information on the number of double bonds in each individual lipid species can be found in **Supplementary Tables S5 (refer to the attached hyperlink given in Chapter7)**.

3.3.7 Salt stress induced changes in GPs and GLs in cv. Vlamingh and cv. Gairdner

Out of 111 GPs, PC and PE were the classes with the most lipid species identified in both genotypes. Further analysis on the trend of species in these two classes was performed to easily compare lipid levels in different treatments and genotypes.

3.3.7.1 Salt stress strongly affects GP lipids in cv. Vlamingh

Salt stress triggered the statistically significant increase of a large number of PC species in cv. Vlamingh when compared to cv. Gairdner. Out of 28 PCs, nineteen PC species with higher number of unsaturation in their molecular compositions increased in cv. Vlamingh with fold changes greater +2-fold or more. Of these, PC (36:n) exhibited the strongest significant increase in cv. Vlamingh showing up to +9.1-fold change after salt stress. On the contrary, there was a reduction in cv. Gairdner in PC (36:n) with -1.3-fold to -0.025-fold decrease after salt stress although not significant. And only four PCs (42:1, 42:3, 42:4 and 42:5) increased significantly in cv. Gairdner. Two species in the LPC (18:1, 18:3) class showed reduction in cv. Vlamingh but in cv. Gairdner five out of nine identified LPCs showed a significant increase under salt stress ranging between +2-fold to +5.3-fold (**Figure 3.10** and **Supplementary Table S6**).

In both genotypes, salt stress significantly increased a large number of PE species. Sixteen PE species exhibited significant increases ranging between 2-fold to 11.5-fold after salt stress in cv. Vlamingh. In cv. Gairdner, fourteen PE species increased significantly ranging between 2-fold to 5.12-fold after salt stress. PE (40:n, 41:n, 42:n and 43:n) increased significantly under salt stress as compared to control conditions in both genotypes. PE (44:n) showed an increase in cv. Vlamingh ranging between +2-fold to +7.3-fold. However, an opposite trend was observed in cv. Gairdner where PE (44:n) reduced significantly. No significant reductions in PE class were observed in both genotypes and salt stress did not affect the levels of LPE species in both genotypes (**Figure 3.11** and **Supplementary Table S7**).

3.3.7.2 Salt stress strongly affects GL levels in both genotypes

Under saline conditions, GL species exhibited significant increases ranging between 2-fold to 9.5-fold in both genotypes. Out of 128 identified glycerolipids, seventy-five and eighty-three increased with +2-fold or more under salt stress in cv. Vlamingh and cv. Gairdner, respectively. Out of these, 47 and 55 lipid species from GL class were significant in cv. Vlamingh and cv. Gairdner, respectively. These were identified as species from the DAG, TAG, MGDG, DGDG and ADGGA classes. Further analysis was performed to determine genotype specific changes in subclasses of lipids in the GL class, and it was found that cv. Gairdner showed more fold changes in comparison to cv. Vlamingh under salt stress in diacylglycerols. In cv. Vlamingh, only three DAGs (33:3, 34:1 and 34:2) showed significant increases ranging between +2-fold to +10.8-fold. On the contrary, nine DAGs exhibited significant increases in cv. Gairdner under salt stress as compared to control roots with +2-fold to +9.3-fold increase. It is interesting to note that out of nine identified species of ADGGA, five increased significantly in cv. Vlamingh with fold changes ranging between +2-fold to +6.3-fold while no significant increases were observed in cv. Gairdner for ADGGA (**Figure 3.12** and **Supplementary Table S8**).

Among all identified lipids in GL class, the most affected lipid species were in the TAG class. It was found that twenty-seven TAGs exhibited significant increases in cv. Vlamingh with +2-fold to +9.3-fold increase in salt stressed roots as compared to control. These belonged to family 45:n, 46:n, 47:n, 49:n, 50:n, 54:n and 58:n. In cv. Gairdner, forty-two TAGs belonging to 45:n, 46:n, 47:n, 48:n, 49:n, 51:n, 53:n, 54:n, and 60:2, 60:3, 60:4 family increased significantly ranging between +2-fold to +5.0-fold (**Figure 3.13** and **Supplementary Table S9**).

Out of 17 DGDG species, seven DGDG showed significant increases in roots of cv. Vlamingh ranging between +2-fold to +7.5-fold as compared to roots grown in control conditions. And only four DGDG increased significantly in cv. Gairdner with fold changes ranging from +2-fold to +6.9-fold. DGDG (36:n) increased significantly in both genotypes ranging between +2-fold to +6.9-fold. It is worth mentioning that out of seven identified MGDGs, five increased significantly in cv. Vlamingh whereas all identified MGDGs showed no significant changes in cv. Gairdner (**Figure 3.12** and **Supplementary Table S8**). No significant reductions were observed in GL class under salt stress in both genotypes.

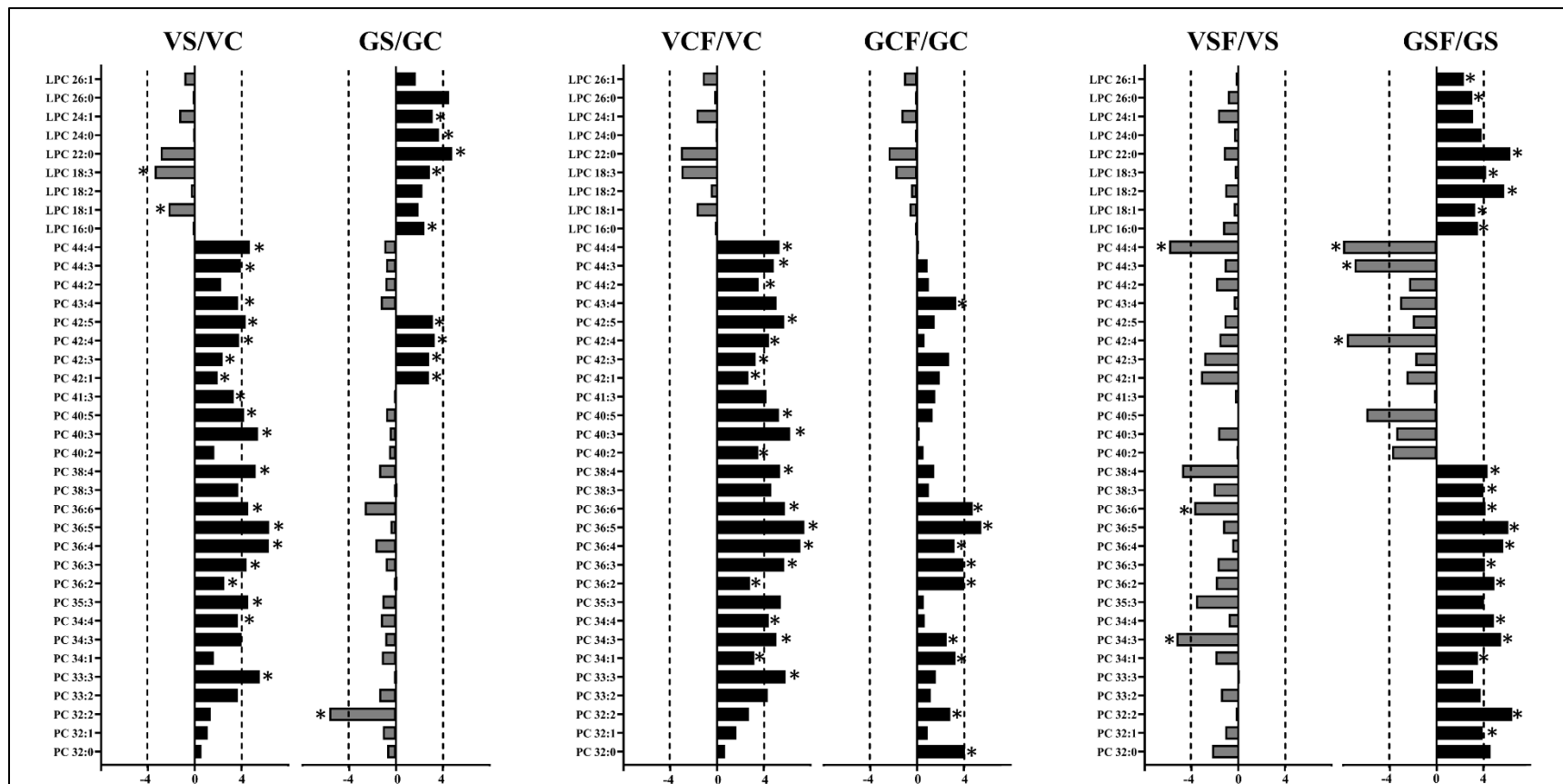


Figure 3.10 Logarithmic ratios of representative phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) content in roots of barley cv. Vlammingh and cv. Gairdner of salt grown (200 mM NaCl) compared to control grown; control with fungus grown compared to control grown; salt and fungus grown compared to salt grown. Values that are significantly higher ($P < 0.05$, FDR) are indicated by *. A threshold of ± 4 -fold change is indicated by a dashed line. X-axis represents the geometric progression with common ratio 4.

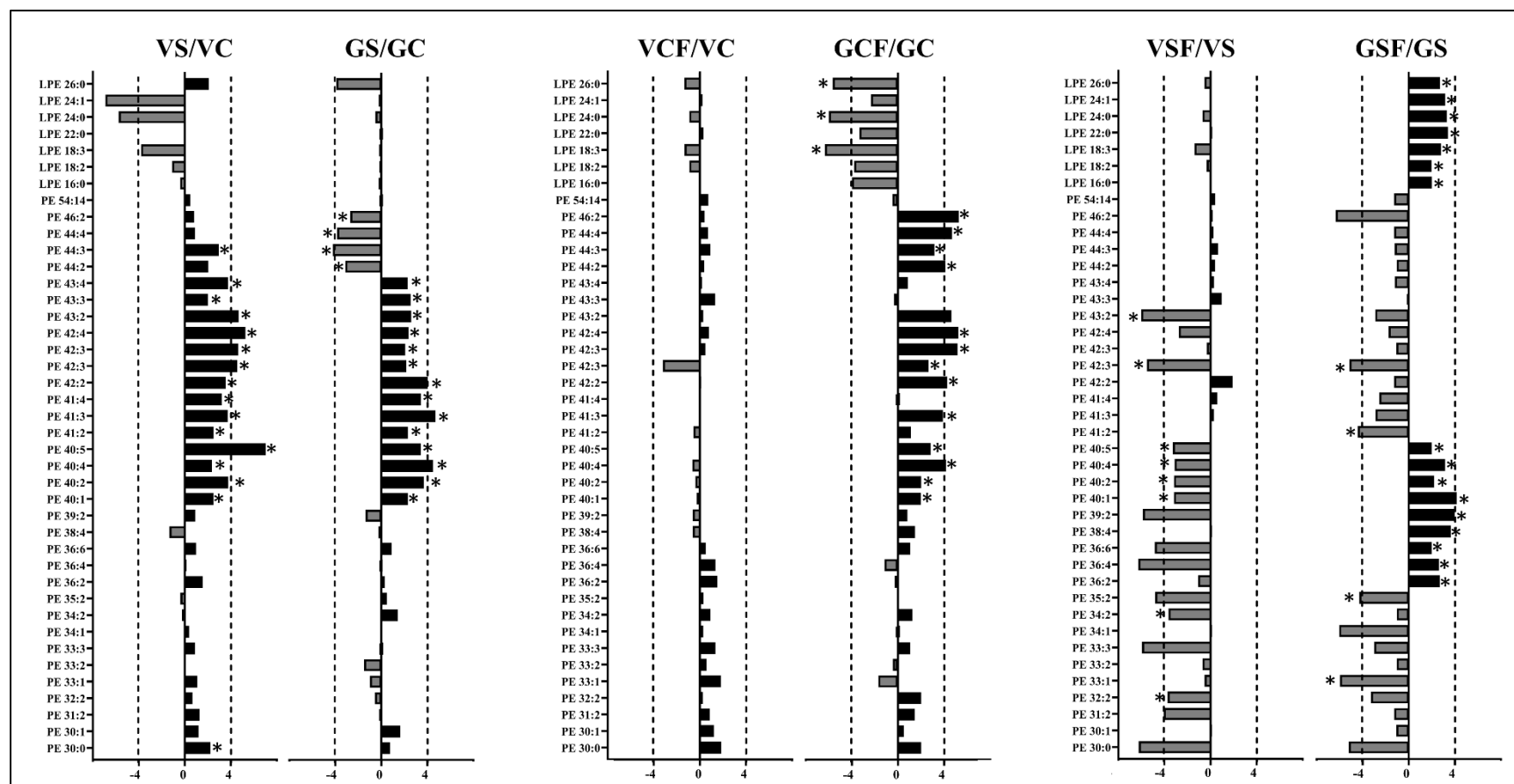


Figure 3.11 Logarithmic ratios of representative phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (LPE) content in roots of barley cv. Vlamingham and cv. Gairdner of salt grown (200 mM NaCl) compared to control grown; control with fungus grown compared to control grown; salt and fungus grown compared to salt grown. Values that are significantly higher ($P < 0.05$, FDR) are indicated by *. A threshold of ± 4 -fold change is indicated by a dashed line. X-axis represents the geometric progression with common ratio 4.

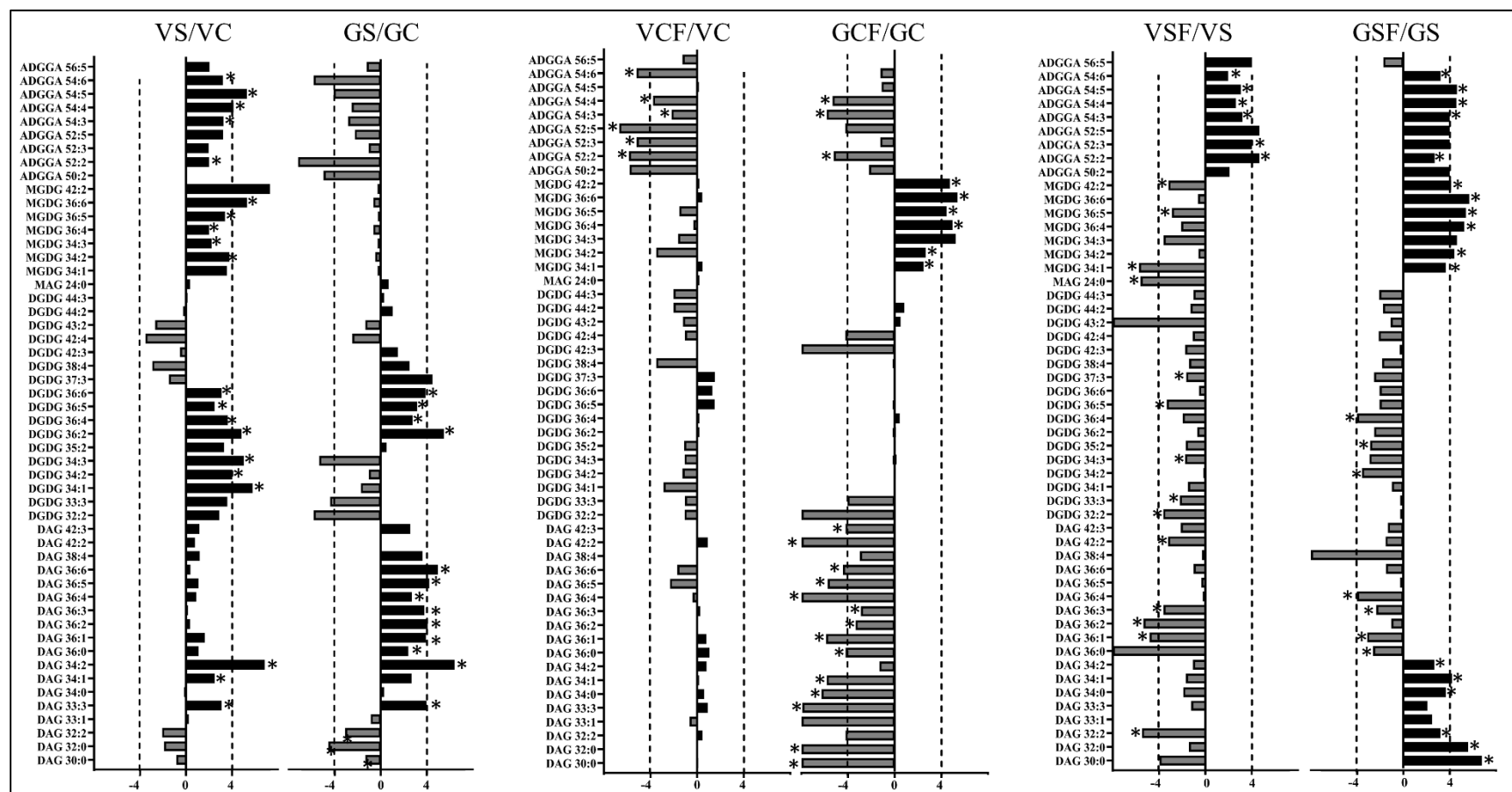


Figure 3.12 Logarithmic ratios of representative acyl diacylglyceryl glucuronide (ADGGA), monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), monoacylglycerol (MAG) and diacylglycerol (DAG) content in roots of barley cv. Vlammingh and cv. Gairdner of salt grown (200 mM NaCl) compared to control grown; control with fungus grown compared to control grown; salt and fungus grown compared to salt grown. Values that are significantly higher ($P < 0.05$, FDR) are indicated by *. A threshold of ± 4 -fold change is indicated by a dashed line. X-axis represents the geometric progression with common ratio 4.

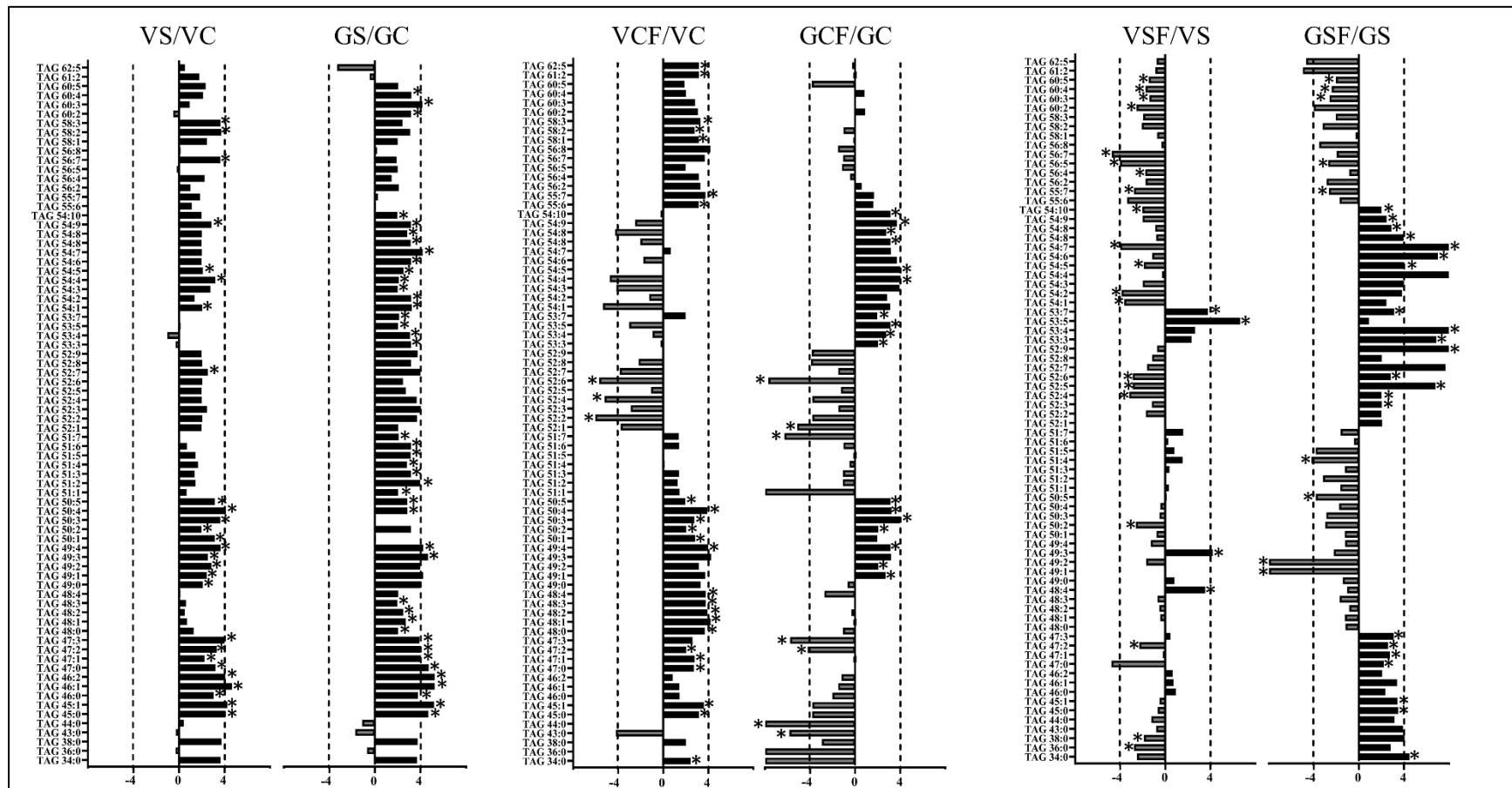


Figure 3.13 Logarithmic ratios of Triacylglycerol (TAG) content in roots of barley cv. Vlammingh and cv. Gairdner of salt grown (200 mM NaCl) compared to control grown; control with fungus grown compared to control grown; salt and fungus grown compared to salt grown. Values that are significantly higher ($P < 0.05$, FDR) are indicated by *. A threshold of ± 4 -fold change is indicated by a dashed line. X-axis represents the geometric progression with common ratio 4.

3.3.8 Changes in Glycerophospholipids (GP) and Glycerolipids (GL) in fungal inoculated roots of cv. Vlamingh and cv. Gairdner under control conditions

Here, lipid species identified in GP and GL class were compared in inoculated roots to uninoculated roots to observe the effect of fungal inoculation in control conditions.

3.3.8.1 *T. harzianum* affected more PCs in cv. Vlamingh and more PEs in cv. Gairdner

It is interesting to note that inoculation with fungus in roots significantly increased a large number of PC species in cv. Vlamingh when compared with uninoculated roots. Out of 30 identified phosphocholines, twenty and only ten PCs increased significantly in cv. Vlamingh and cv. Gairdner, respectively. The greatest increase was observed in PC (36:2, 36:3, 36:4, 36:5 and 36:6) with up to +13-fold in inoculated roots as compared to uninoculated roots in cv. Vlamingh. *T. harzianum* was also successful in significantly increasing PC (36:n), as mentioned above, in cv. Gairdner ranging between +2-fold to +6.7-fold. It is worth noting here that these were the same species which also decreased in saline conditions in cv. Gairdner. No significant changes in LPCs were observed in both genotypes (**Figure 3.10** and **Supplementary Table S6**).

Inoculation of roots with *T. harzianum* affected phosphatidylethanolamines in cv. Gairdner as compared to cv. Vlamingh. It was observed that no significant changes occurred in inoculated roots of cv. Vlamingh. But in cv. Gairdner, thirteen PEs increased significantly with fold changes ranging between +2-fold to +6.2-fold. These were PE (40:n, 41:3, 42:n and 44:n). It is interesting to note that inoculation with fungus increased PE (44:n) in inoculated roots of cv. Gairdner which were also seen to be significantly decreased under saline conditions. No significant changes in LPEs were observed in cv. Vlamingh. However, three LPEs (18:3, 24:0, 26:0) decreased significantly in cv. Gairdner with fold changes of -0.1-fold (**Figure 3.11** and **Supplementary Table S7**).

3.3.8.2 Increased glycerolipids in inoculated roots of cv. Gairdner under control conditions

T. harzianum showed stronger effects in roots of cv. Gairdner by significantly changing more DAG, MGDG and TAG as compared to cv. Vlamingh under controlled conditions. These changes were also due to significant reductions in GLs found in cv. Gairdner as compared to cv. Vlamingh. Out of 130 identified glycerolipids, 24 GL lipids increased significantly in cv. Vlamingh and 23 in cv. Gairdner. The increases in cv. Vlamingh were observed only in triglycerols ranging between +2-fold to +4.3-fold in inoculated roots. The fungus affected 24 TAGs belonging to lipids with total fatty acyl chain length of 45, 47, 48, 49, 50, 55, 56, 58, 61, and 62 with unsaturated double bonds in cv. Vlamingh. On the other hand, in cv. Gairdner, 17 TAGs from family 49:n 50:n, 53:n and 54:n were significantly increased significantly following inoculation with fungus ranging between +2-fold to +4-fold (**Figure 3.13** and **Supplementary Table S9**). It is interesting that fungal inoculation

significantly increased six out of seven identified MGDGs in roots of cv. Gairdner while no significant changes in MGDGs were observed in cv. Vlamingh.

As mentioned in the previous paragraph, fungal inoculation caused significant reductions in more lipid species from the GL class in inoculated roots of cv. Gairdner (24 species) as compared to cv. Vlamingh (9 species). Amongst those 24 species that reduced in cv. Gairdner, 14 species were from DAG class. However, in cv. Vlamingh, most lipid species that reduced significantly belonged to ADGGA. (**Figure 3.12** and **Supplementary Table S8**).

3.3.9 Effect of salt stress and fungal inoculation on GP and GL profiles in cv. Vlamingh and cv. Gairdner

The inoculation of *T. harzianum* under salt stress caused changes in the root lipid profiles of both genotypes. GP and GL were found to be significantly increased in inoculated roots of cv. Gairdner under salt stress. On the contrary, salt stress caused a significant decrease in most of the species from GP and GL class in inoculated roots of cv. Vlamingh.

3.3.9.1 *T. harzianum* changed PCs and PEs under saline conditions

Under saline conditions, *T. harzianum* significantly increased 12 PCs from 32:n, 34:n, 36:n and 38:n family ranging between +2-fold to +9.5-fold in roots of cv. Gairdner. On the contrary, the fungal inoculation did not result in any significant increases in cv. Vlamingh under saline conditions. However, three unsaturated PCs (34:3, 36:6, 44:4) reduced significantly with fold changes between -0.27-fold and -0.13-fold in cv. Vlamingh and three PCs (42:4, 44:3 and 44:4) reduced significantly in cv. Gairdner under saline conditions with fold changes ranging between -0.088-fold to -0.009-fold. It is worth mentioning that out of nine identified LPCs, seven increased significantly in cv. Gairdner and no increases in LPCs were observed in inoculated roots of cv. Vlamingh after salt stress (**Figure 3.10** and **Supplementary Table S6**). Similar to PCs, nine PEs were significantly increased in inoculated roots of cv. Gairdner as compared to none in cv. Vlamingh following salt stress. It was found that nine PE species (36:2, 36:4, 36:6, 38:4, 39:2, 40:1, 40:2, 40:4 and 40:5) significantly increased between +2-fold to +4.2-fold in cv. Gairdner. However, it is interesting to note that eight PEs exhibited significant decrease in cv. Vlamingh while in cv. Gairdner fungal inoculation reduced four PEs significantly under saline conditions. Similar to LPC, fungus increased all identified LPEs in cv. Gairdner significantly after salt stress, but no significant changes were observed in cv. Vlamingh (**Figure 3.11** and **Supplementary Table S7**).

3.3.9.2 Triglycerols - the class of lipids most affected by *T. harzianum* under saline conditions

The inoculation of *T. harzianum* under saline conditions drastically affected glycerolipids as compared to uninoculated roots in both genotypes. However, the fungal inoculation increased a

greater number of triglycerol species with greater fold changes in saline conditions in cv. Gairdner. It was observed that 39 lipids from the GL class increased significantly between +2-fold to +36.2-fold in cv. Gairdner while only 10 increased in cv. Vlamingh. Six DAGs (30:0, 32:0, 32:2, 34:0, 34:1 and 34:2), six MGDG from families 34:n, 36:n and 42:2, five ADGGA from families 54:n and 52:2, and 22 TAGs from families 34:0, 45:0, 45:1, 47:n, 52:n, 53:n, 54:n were significantly increased in cv. Gairdner ranging between +2-fold to +36-fold. On the other hand, six ADGGA from families 52:n and 54:n and four TAGs (48:4, 49:3, 53:5 and 53:7) increased significantly in cv. Vlamingh between +2-fold to +10.12-fold (**Figure 3.12** and **Figure 3.13** and **Supplementary Table S8 and S9**).

T. harzianum significantly reduced 34 and 16 lipid species from GL class in cv. Vlamingh and cv. Gairdner, respectively. Out of 34 lipids that reduced in cv Vlamingh, 20 belonged to TAGs and in cv. Gairdner, 9 TAGs were reduced significantly.

3.4 Discussion

Increase of salts in soils is detrimental to plants and often results in a reduction in overall shoot and root biomass accumulation (Julkowska and Testerink 2015). The deleterious effects of salinity affect several physiological and metabolic processes of plants (Rahneshan et al. 2018). To counter the problems of salinity, plants exhibit growth plasticity, enhanced photosynthesis, accumulation of osmolytes, detoxification of ROS and induction of phytohormones. However, these adaptive strategies become insufficient to cope with increasing levels of salinity.

At the plant organ level, shoots are more sensitive to salinity than roots. However, the roots are often reported to play a key role in salt tolerance mechanisms of plants as they represent the first organs that control the uptake and translocation of nutrients and salts throughout the plant (Munns 2002). Besides playing these roles, roots are associated with a large number of microbes (Van Der Heijden et al. 2008) that can have profound effects on seed germination, seedling vigour, plant growth and development and imparting tolerance against several abiotic stresses (Kumar and Verma 2019).

In this study, the influence of the beneficial endophytic fungus *Trichoderma* strain T-22 was examined to study the physiological and biochemical changes in roots of two barley genotypes (cv. Vlamingh and cv. Gairdner) with contrasting tolerance capabilities grown on agar plates under salt stress. The recent advances in metabolomics and multivariate analysis contribute to a wider profiling of roots inoculated with microbes, thus enabling a deeper understanding of the below-ground interactions (van Dam and Bouwmeester 2016). Although the full characterization of the metabolome of roots is still technologically challenging, a great diversity of compounds can now be annotated through metabolomics. This study illustrates the potential of broad-based

metabolite and lipid profiling in the analysis of metabolic changes associated with *T. harzianum* T-22 symbiosis in barley roots.

3.4.1 Total Root length is a good indicative root trait to measure plant growth

To determine treatment effects, Total Root Length (TRL) was measured as a means of analysing plant development. Salinity reduced root length of both genotypes in this study. According to Acosta-Motos et al. (2017) and Shelden et al. (2016), this could be due to inhibitory impacts of salts and by restriction of cell division and cell expansion in the root apical meristem. The effect of salinity was more severe in the sensitive genotype cv. Gairdner as compared to the tolerant genotype cv. Vlamingh, confirming the greater salt tolerance potential of the latter genotype. Fungal inoculation of control roots resulted in considerable increases in root lengths of both genotypes with a greater effect in cv. Gairdner suggesting growth-promoting characteristics of the fungus consistent with previous reports (Altomare et al. 1999; Björkman et al. 1998; Contreras-Cornejo et al. 2009). When combined, salinity and *T. harzianum* T-22 inoculation resulted in an increase in TRL in both genotypes of 14.84% (cv. Vlamingh) and 35.06% (cv. Gairdner) compared to uninoculated seedlings, respectively.

Furthermore, TRL decrease between S and C showed higher values than TRL decrease between SF and CF. This suggests that the fungus is able to help plants cope with the adverse effects of salt stress by increasing root length. Therefore, inoculation of *T. harzianum* T-22 in combination with NaCl mitigated the negative effects of NaCl and these results are consistent with the findings of Mastouri et al. (2012).

3.4.2 Fungus changes osmolytes in roots as a protective adaptation to salt stress

Plants have ubiquitously evolved mechanisms to adapt to salinity that involve cations, such as sodium, ionically bonding to anions, like chloride, and partitioning them into the vacuole while synthesising and accumulating osmolytes in the cytosol (Annunziata et al. 2017). These osmolytes are also termed, compatible compounds. In addition to the fundamental role of compatible compounds to balance the cytosol osmotically, osmolytes also protect macromolecular subcellular structures and mitigate oxidative damages (Shabala and Munns 2012). Most compatible solutes are low molecular weight metabolites such as amino acids, amines, and sugars (Annunziata et al. 2017; Woodrow et al. 2017). In this study, a global analysis of the GC-MS dataset using PCA and HCA indicated substantial metabolic differences between the two inoculated and uninoculated barley genotypes, cv. Vlamingh and cv. Gairdner, under control and saline conditions. Global analysis facilitated the construction of a data matrix composed of 110 metabolites detected in all six biological replicates from all treatments in both genotypes. The tight clustering between biological replicates in both genotypes confirms the robustness and reproducibility of the experimental protocol.

Sugars provide carbon and energy for normal functioning of cellular metabolism and regulate growth and development of plants. Roles of sugars and sugar alcohols have been widely accepted as osmoprotectants, which regulate osmotic adjustment, provide membrane protection, and scavenge toxic ROS under various kinds of stress (Singh et al. 2015). Higher numbers of sugars changed significantly in cv. Gairdner as compared to cv. Vlamingh after salt stress suggesting the higher need for stabilizing the cell membrane and protoplast in the more sensitive genotype. Similar results were shown by Cao et al. (2017) where varying changes in sugar metabolism after salinity stress were detected for several barley genotypes. Sugar alcohols with linear structures such as mannitol, xylitol and ribitol enhance tolerance potential of plants against drought and salinity (Williamson et al. 2002). Increased synthesis of these in plants help to quench the most toxic hydroxyl radical (OH) generated via the Fenton reaction (Gill and Tuteja 2010). In this study, higher numbers of significant changes were observed in polyols in roots of cv. Gairdner after salt stress. This suggests the increased requirement of water adjustment in the cytoplasm, Na⁺ sequestration into the vacuole or apoplast, and protection of cellular structures in cv. Gairdner. Sherameti et al. (2005) reported that one of the major starch-degrading enzymes, glucan-water dikinase, is activated by the fungus in colonized roots. This supports our findings where compatible solutes did not increase with the inoculation of *T. harzianum* under control conditions after seven days of inoculation. It can be presumed that the faster growth of colonized plants requires the breakdown of starch, which is deposited in the root and shoot amyloplasts. Ghabooli (2014) also observed the same results in fungal inoculated plants. One of the most common form of a sugar, trehalose, which is an oxidized form of disaccharide, exists in some microbes including bacteria and fungi (Singh et al. 2015). *T. harzianum* increased trehalose significantly in cv. Gairdner under control conditions suggesting its increased role as an osmoprotectant in the more sensitive genotype.

Several abiotic stresses including salinity lead to major changes in carbohydrate metabolism, and the sugar signalling pathways also interact with stress pathways to modulate metabolism. Thus, sugars indirectly play an important role during plant growth and development under abiotic stress by regulating carbohydrate metabolism (Ghabooli 2014). In this study, fungal inoculation resulted increased levels of sugars in both genotypes under saline conditions. This may help plants to protect against the severe effects of salinity and thus increase the growth rate of inoculated plants.

Another class of metabolites that have been shown to accumulate under salt stress are amino acids (Ahanger and Agarwal 2017). According to (Widodo et al. 2009), salt sensitive genotypes accumulate more free amino acids than tolerant ones upon stress. This aligns with our study where the salt sensitive genotype, cv. Gairdner, showed larger significant increases in amino acid levels compared to cv. Vlamingh under salt stress. According to Munns (2002), increases in amino

acid concentrations following salt stress have been attributed to arising from the decrease in growth rate and therefore were suggested as a secondary consequence of reduced growth. The results from this study show a greater reduction in root length in cv. Gairdner which supports findings from (Munns 2002). Increased accumulation of amino acids in stressed plants could also be caused by increased protein degradation, inhibition of protein synthesis, decreases in amino acid and amide export, or growth inhibition (Parihar et al. 2015). It can be hypothesized with results from this study that increased protein degradation was required in providing amino acids for synthesis of new proteins suited for growth or survival under the stress conditions and also provided substrates for energy metabolism in the less tolerant genotype cv. Gairdner.

Among increased amino acids, proline has been largely shown to act as an osmoprotectant in plants affected by salinity as previously reported in both barley roots and leaves (Munns 2002; Shelden et al. 2016; Wu et al. 2013). Interestingly, cv. Vlamingh shows higher increase in proline accumulation in response to salt stress as compared to cv. Gairdner. Mattioli et al. (2009) showed role of proline in increased cell division in *Arabidopsis* under salt stress. This aligns with our study where lesser reduction in root length was observed in cv. Vlamingh after salt stress as compared to cv. Gairdner (**Chapter 2, Figure 2.6**), showing lower concentration of proline under salt stress.

Under control conditions, *T. harzianum* inoculation failed to show major differential responses in metabolite levels compared to uninoculated roots in either genotype. But under salt stress, the fungus inoculated roots showed significantly decreased amino acid levels in cv. Gairdner. This is in alignment with past research in this field where Li et al. (2017b) found that *Aspergillus aculeatus* inoculated roots of ryegrass plants showed decreased amino acid concentrations following salt stress. Here we can speculate that the fungus may enhance the capacity of cv. Gairdner to withstand salt stress through coordinating with amino acids exerting a protective mechanism. Amino acids can be taken up directly by the roots and are transported between different organs through xylem and phloem. These are essential for plant growth. In this study, fungus causes significant reduction in amino acid levels which can indicate their contribution to growth in cv. Gairdner under saline conditions. Proline was also significantly reduced following salt stress in inoculated roots of cv. Gairdner. It has been suggested that proline can act as a stress marker and thus its decline may indicate that the toxic effects of salinity are reduced following colonization of fungus. Similar results are reported by (Abdelaziz et al. 2019) where the authors attributed the decrease in proline to the decrease in abscisic acid (ABA) levels. ABA controls proline biosynthesis (Verslues and Bray 2005) to regulate the potential cytoplasmic osmotic stress caused by increasing NaCl in the root zone (Cao et al. 2014).

3.4.3 Alterations in the lipid profiles in response to salt stress

Untargeted lipidome analyses were performed on roots of both genotypes in order to elucidate the role of lipid species in adaptive mechanisms of roots following short-term salt stress. The untargeted data strongly suggests that salt treatment has a much bigger impact on the root lipidome than the effect of fungus in both genotypes. To cover a wide range of lipid species, an untargeted LC-MS lipidomic analysis was applied to elucidate the dynamic changes in inoculated and uninoculated roots of both genotypes, under control and saline conditions. Here, a moderate alteration of root lipidome was triggered by salt stress in two barley genotypes. Several interesting lipid classes and specific lipid species were discovered in the responses of roots to salinity stress, and the potential roles of these lipids in mediating tissue tolerance upon salinity stress in cv. Vlamingsh and cv. Gairdner are further discussed.

A plethora of studies have reported alterations in plant membrane lipids in the presence of NaCl (Guo et al. 2019a; Sarabia et al. 2019; Yu et al. 2020). Plant membrane lipids belong to three major groups: sphingolipids, sterols, and glycerolipids (Borrell et al. 2016). Membrane glycerolipids have glycerol backbones with two fatty acid molecules bound to sn-1 and sn-2 and either a phosphorous (phospholipid) or sugar (glycolipid) molecule at position sn-3. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the dominant membrane phospholipids, which have been extensively studied. On the other hand, glycolipids including monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG), together with the phospholipids, phosphatidylglycerol (PG), make up the plastid membrane (Kalisch et al. 2016).

3.4.3.1 Changes in membrane phospholipids in response to salt stress

Membrane phospholipids serve as structural and signalling molecules in plant cells (Furt et al. 2011; Xue et al. 2009) and reports have shown alterations in lipid levels in a number of plant species subjected to salinity stress. In this study, salt stress increased PC lipids in cv. Vlamingsh compared to cv. Gairdner. Similar results were observed in callus culture of the halophyte *Spartina patens* (Wu et al. 2005), epidermal bladder cells of the halophyte *M. crystallinum* (Barkla et al. 2018), roots of salt-tolerant *Plantago* cultivars (ERDEI et al. 1980) and salt-acclimated tomato calli (Kerkeb et al. 2001). Another study using *Arabidopsis thaliana* L. reported increase in PCs concentration after exposure to salt and cold stress, which was suggested to be an important membrane-mediated cell signalling mechanism (Pical et al. 1999; Tasseva et al. 2004). Increases in PC could also be due to net production of PC from DAG through DP-choline:diacylglycerol cholinephosphotransferase (CPT) as explained in details in **Figure 3.14**. Further, in support of the positive role of PC lipids in salt tolerance, the addition of choline, a key substrate for PC biosynthesis, improved salt tolerance of wheat as shown by Mansour et al. (2002). In contrast, decreases in PC species have been observed in more salt-sensitive plants such

as maize, oats and wheat (Magdy et al. 1994; Norberg et al. 1991; Salama and Mansour 2015; Salama et al. 2007); this may suggest that a plant's ability to maintain PC levels is an adaptive mechanism for salt tolerance (Sarabia et al. 2019). Van Meer et al. (2008) also reported that maintenance and increase of highly unsaturated PC species is considered to potentially be linked to increased membrane fluidity. This study showed that PC species containing a higher number of unsaturations are either synthesized, as seen in cv. Vlamingh, and preferentially maintained after salt stress, as observed in cv. Gairdner (**Figure 3.10**).

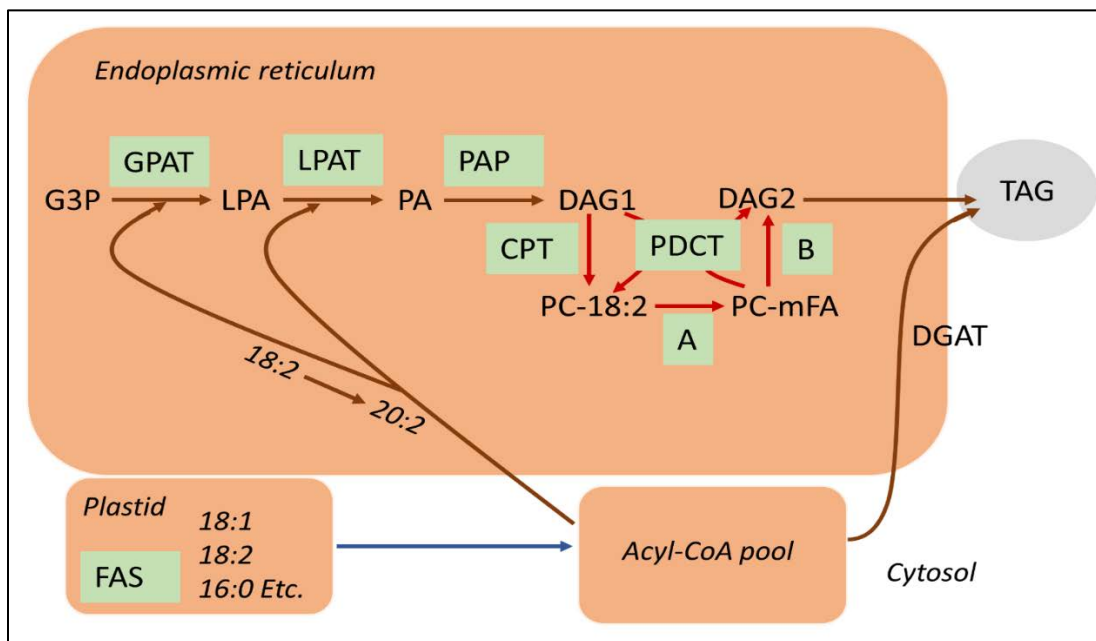


Figure 3.14 PC-derived TAG synthesis. Brown arrows indicate reactions involved in de novo DAG1 synthesis. Red arrows indicate reactions involved in PC-derived DAG2 synthesis. Enzymatic reactions are embedded in green boxes: A- modification of FA esterified to PC, B- reversible CPT or phospholipase C or phospholipase D/PAP DAG production. CPT- CDP-choline:DAG cholinephosphotransferase, DGAT- acyl-CoA:DAG acyltransferase, FAS- fatty acid synthesis, LPAT- acyl-CoA:LPA acyltransferase, GPAT- acyl acyl-CoA:G3P acyltransferase, PDCT- PC:DAG cholinephosphotransferase, PAP- PA phosphatase. Substrates are DAG- diacylglycerol, G3P- glycerol-3-phosphate, LPA- lyso-phosphatidic acids, PA- phosphatidic acid. Figure adapted from Bates (2012)

3.4.3.2 Changes in membrane phospholipids in inoculated roots of both genotypes under control and saline conditions

Glycerophospholipids are major components of cellular membranes and they play important roles in various cellular functions including signal transduction, vesicle trafficking and membrane fluidity (Hishikawa et al. 2014). *T. harzianum* showed similar increases in glycerophospholipids in roots of both genotypes under control conditions. To help with root penetration, the endophyte uses a variety of cellulolytic and ligninolytic enzymes such as oxidases, cellulases and laccases to

break down the cellulose, hemicellulose, pectin and lignin in the cell wall. Increased biomass in inoculated roots of both genotypes compared to uninoculated roots suggests the successful penetration of fungus into the roots and therefore, can be assumed as the reason for increased accumulation of membrane lipids in both genotypes. Similar results were shown by Wei et al. (2016) where Ericoid mycorrhizal (ERM) fungi *Oidiodendron maius* var. increased expression of genes involved in glycerophospholipid metabolism in inoculated roots of *Rhododendron fortunei* Lindl though the actual levels of these lipids were not determined.

Several reports indicate that *Trichoderma* spp. enhances tolerance to salt stress during plant growth, in part due to improved root growth, improvement in water-holding capacity of plants, or enhancement in nutrient uptake (Brotman et al. 2013; Contreras-Cornejo et al. 2014; Gangwar and Singh 2018; Lei and Zhang 2015; Mohamed and Haggag 2006; Rawat et al. 2011; Yasmeen and Siddiqui 2018). According to Magdy et al. (1994), decreases in PC species have been observed in more salt-sensitive barley plants which was suggested to result in enriched non-lamellar-forming domains, a more rigid membrane and phase separation of non-lamellar-forming lipids. However, they also observed increased free sterols that counterbalanced the formation of non-bilayer structures. In this study, it is interesting to note that under saline conditions, *T. harzianum* either reduced or resulted in no change in most PC and PE profiles in roots of cv. Vlammingh. Since free sterols were not measured in this study, function of PCs cannot be ascertained. Thus, further analysis is required to confirm the role of PC in inoculated roots of cv. Vlammingh.

An opposite trend was observed in cv. Gairdner where significant increases in highly unsaturated PC lipids were observed following inoculation under salt stress. Increased PC lipids have been reported in salt-sensitive species such as in cell cultures of *Catharanthus roseus* (Elkahoui et al. 2004) and *Arabidopsis thaliana* (Tasseva et al. 2004). This suggests a role of the endophyte in maintaining membrane structure and function as a response to salt stress in the sensitive genotype cv. Gairdner. Our findings of a significant increase in TRL as observed in inoculated roots of cv. Gairdner under salt stress compared to uninoculated roots also align with these results confirming the positive role of the fungus in imparting salt tolerance to the sensitive genotype.

3.4.3.3 Alterations in membrane glycolipids in response to salt stress

Plastid envelope membranes are major site of glycolipids biosynthesis (Joyard et al. 1991) which comprise of plastidial membrane lipids monogalactosyldiacyloglycerol (MGDG) and digalactosyldiacyloglycerol (DGDG) (Douce et al. 1987). Plastid morphology and glycolipid distribution differ between leaves and roots as leaves harbor chloroplasts with thylakoid membranes rich in MGDG and DGDG, but roots contain plastids without internal membranes and therefore contain few glycolipids (Wewer et al. 2014). Wilson et al. (2014) showed that osmotic

imbalance in the plastid can induce a cellular osmoregulatory response similar to that induced by environmental stresses, such as drought or salinity stress.

MGDG forms a reversed hexagonal phase with water, while DGDG adopts a lamellar phase. Changes in the ratio of these two types of lipids thus induce different physical properties in the membranes. The two glycolipid types might therefore play different roles in regulation mechanisms under salinity. However, there are only a limited number of reports on glycolipids changes under salinity stress. A small number of studies have found non-uniform changes in roots of barley and salt-sensitive wheat with non-significant effect (Magdy et al. 1994) whereas a salt-tolerant maize cultivar shows a decrease in MGDG and DGDG content following salt stress (Brown and DuPont 1989; Salama et al. 2007). In this study, the salt tolerant genotype cv. Vlammingh showed higher contents of these lipid species as compared to cv. Gairdner after salt stress. From the research so far, the change in the glycolipids is not uniform and varies with species or tissue type. Therefore, further intensive research in order to elucidate the glycolipid participation in salt tolerance is required.

3.4.3.4 Alterations in membrane glycolipids in inoculated roots of both genotypes under control and saline conditions

In exchange for mineral nutrients, e.g., phosphorus, the plant supplies the fungal partner with reduced carbon (Behie and Bidochka 2014). This carbon is stored primarily as lipids (Bago et al. 2000; Yang et al. 2014), with smaller amounts of carbohydrates stored as glycogen (Bago et al. 2003; Bonfante and Perotto 1995). For example, up to 70% of the dry weight of *Glomus caledonius* is composed of lipids, predominantly triglycerides (Beilby and Kidby 1980). Here, under controlled conditions, *T. harzianum* failed to show any significant changes in profiles of DGDG from inoculated roots of both genotypes. Decreased levels of this species was found under saline conditions of both genotypes. It can therefore be assumed that these lipids may have been taken up into the fungus body to be metabolised as an energy source. Further studies are required to test this hypothesis. However, upregulation of MGDG was observed in inoculated roots of cv. Gairdner. MGDG is located in non-green plastid membranes and the higher level of glycolipids may result from the presence of more plastids in roots of cv. Gairdner. Moreover, the maximum state of plastid development is achieved in the cortical cells of primary roots which are the same cells where fungal hyphae penetrate and accumulate. Therefore, it can be presumed that an increase in MGDG in roots of cv. Gairdner is from the fungus although further confirmation is required. To the best of our knowledge, little is known about the accumulation of membrane glycerolipids in host plants by endophytic fungi and thus requires future studies for validation.

3.4.3.5 Changes in membrane neutral lipids after salt stress

Diacylglycerol is known to be an intermediate in the synthesis of membrane lipids and is involved in phospholipid signalling in plant cells (Dong et al. 2012). Plants can utilize at least two metabolic pathways to produce different molecular species of the immediate precursor to TAG, diacylglycerol (DAG): (1) *De novo* DAG synthesis (termed as DAG1 here), and (2) conversion of the membrane lipid phosphatidylcholine (PC) to DAG (termed as DAG2 here). Here, since there was no significant increase or change in PC species in cv. Gairdner after salt stress, it can be postulated that DAG and subsequent TAG production was through utilization of the products of FA synthesis exported from the plastid directly for *de novo* DAG/TAG synthesis through the Kennedy pathway (Weiss and Kennedy 1956; Weiss et al. 1960) (**Figure 3.15**). Conversely, in cv. Vlamingh, TAG synthesis was presumably through PC-derived DAG/TAG synthesis (**Figure 3.14**). The pathway of PC-derived DAG2 synthesis starts with the production of PC lipids from *de novo* DAG1. Here, PC is synthesized through CDP-choline:diacylglycerol cholinephosphotransferase (CPT) (Li-Beisson et al. 2013) leading to a net production of PC lipids from DAGs. Further, phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) (Lu et al. 2009) activity transfers the phosphocholine head group from PCs to DAGs generating new molecular species of DAGs and PCs, which does not lead to net accumulation of PCs or DAGs. This supports our findings where only three DAGs increased after salt stress in cv. Vlamingh as compared to nine DAGs in cv. Gairdner. Once PC lipids are formed from *de novo* DAG1 by either CPT or PDCT, the FAs esterified to PCs are available for FA modification and acyl editing (Bates et al. 2009; Bates et al. 2007; Li-Beisson et al. 2013) to generate new molecular PC species. And the DAG substrate for TAG synthesis is subsequently derived from PCs by removal of the phosphocholine headgroup (Bates 2012). This eventually results in TAG production.

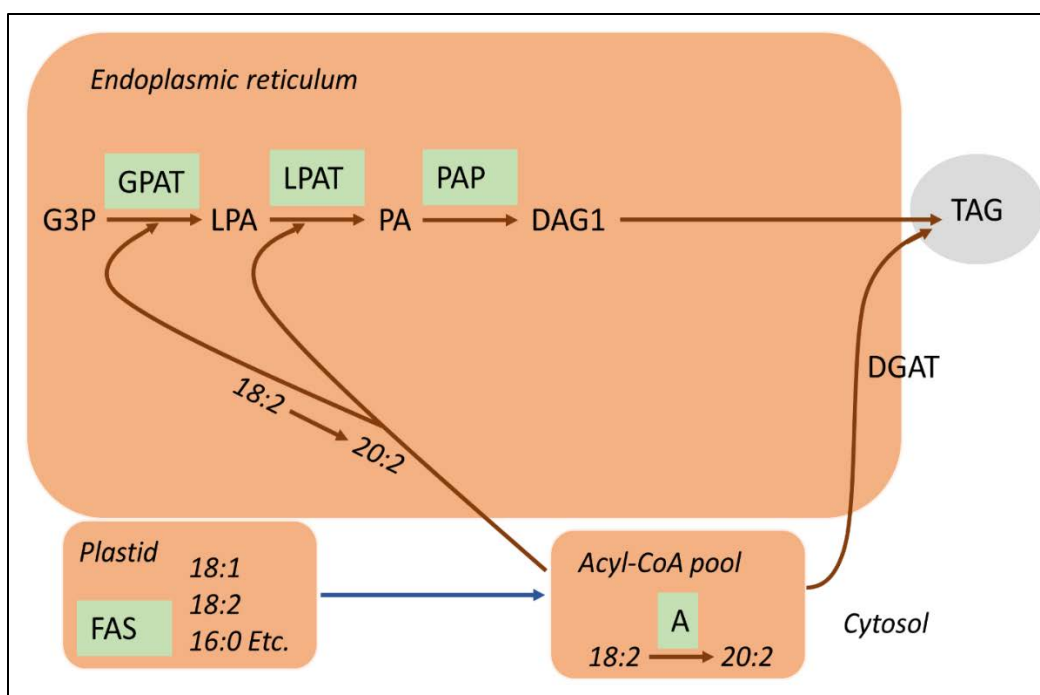


Figure 3.15 *De novo* TAG synthesis using the Kennedy pathway. Brown arrows indicate reactions involved in *de novo* DAG1 synthesis. Enzymatic reactions are embedded in green boxes: A- FA elongation, DGAT- acyl-CoA:DAG acyltransferase, FAS- fatty acid synthesis, LPAT- acyl-CoA:LPA acyltransferase, GPAT- acyl acyl-CoA:G3P acyltransferase, PAP- PA phosphatase. Substrates are DAG- diacylglycerol, G3P- glycerol-3-phosphate, LPA- lyso-phosphatidic acids, PA- phosphatidic acid. Figures adapted from Bates (2012)

3.4.3.6 Changes in membrane neutral lipids in inoculated roots of both genotypes under control and saline conditions

The amount of neutral lipids (for energy storage) is usually higher than that of phospholipids (membrane constituents) in endophytic fungi, since these fungi store a large proportion of their energy carbon as neutral lipids (Olsson and Johansen 2000). Amongst neutral lipids, TAGs are the main type of neutral lipids found in large amounts in endophytic fungal spores and hyphae (Beilby and Kidby 1980; Cooper and Losel 1978; Nagy et al. 1980). The intraradical mycelium takes up hexoses from the host, which are metabolized to yield neutral lipids (Pfeffer et al. 1999). Another study by Serrano-Carreón et al. (1992) also showed that *T. harzianum* accumulated more neutral lipids than *T. viride*. Neutral lipids thus have a central role in carbon metabolism and transport in endophytic fungi and are probably the main respiratory substrate in the extraradical mycelium (Bago et al. 2000).

Under controlled conditions, *T. harzianum* inoculation did not change the DAG profile in roots of cv. Vlammingh. But a significant increase in TAGs in inoculated roots of cv. Vlammingh suggests the ability of the fungus to synthesize fatty acids *de novo* and incorporation into TAGs through the

Kennedy pathway as shown in **Figure 3.15** (Weiss and Kennedy 1956). On the other hand, a significant reduction in DAGs in inoculated roots of cv. Gairdner can be presumed as a role of DAGs in the synthesis of TAGs which were strongly increased after inoculation in cv. Gairdner. An interesting pattern with reduction in higher numbers of neutral lipids was observed in inoculated roots of cv. Vlamingh after salt stress. Downregulation of TAGs was proposed to be an adaptive feature of salt-tolerant plants by Barkla et al. (2018) and may provide precursor lipid molecules such as DAG and FA for phospholipid synthesis. Therefore, it can be suggested that fungus imparts tolerance to cv. Vlamingh by reducing TAGs under saline conditions.

Under saline conditions, TAGs accumulated in inoculated roots of cv. Gairdner. Similar results were observed by Mueller et al. (2015) where salt stress triggered accumulation of TAGs in *Arabidopsis* seedlings. Higher accumulation of TAGs may reflect lipid remodelling, i.e. channelling of fatty acids from constitutive membrane lipids to TAGs. Further, as TAGs predominantly contain polyunsaturated fatty acids, it is likely that the fungus helps in the release of polyunsaturated fatty acids from structural lipids and use for transient assembly of TAGs during membrane remodelling.

3.5 Conclusion

This study investigated the role of *Trichoderma harzianum* T-22 on two barley genotypes, cv. Vlamingh and cv. Gairdner, based on their known differences in germination phenology and salinity tolerance. Total root length was measured on both genotypes after the final harvest. From the results, it is clear that salt treatment has an effect TRL but, the fungus restores growth close to or better than normal confirming the positive role of the fungus to help plants cope with the adverse effects of salt stress. Further, to investigate the metabolite and lipid changes in roots of both genotypes grown in soil, untargeted GC-MS and LC-MS approaches were used. This combined approach provides unique insights into the metabolome and lipidome in barley roots influenced by the inoculation of endophytic fungi identifying metabolic processes that are induced during this interaction. Based on GC-MS results, it was found that the fungus employs two mechanisms in both genotypes. The osmolytes were found to be decreased in cv. Gairdner and the fungus caused less changes in tolerating genotype cv. Vlamingh. changes higher number of metabolites in cv. Vlamingh after salt stress. LC-MS results revealed that the fungus accumulates higher levels TAGs in cv. Gairdner, reflecting lipid remodelling, i.e. channelling of fatty acids from constitutive membrane lipids to TAGs.

Based on our results, the fungus modifies the metabolome and lipidome of each genotype in a specific manner, improving their ability to cope with salt stress. And thus, this calls for future studies to confirm the genotypic preference of the fungus. Fungal-plant interactions and their effect on plant growth and metabolism is an important question which our study aims to address.

The fungus was unable to promote equivalent levels of tolerance in both genotypes although it provided significant improvement in growth of the sensitive genotype Gairdner. Due to time limitations, only positive ionization mode analysis was performed on lipids which could detect GP species such as PCs, PEs. However, negatively charged species like PIs and PSs could not be detected and thus future studies must focus on determining the role of those lipids in plant-fungal interaction.

Chapter 4

Biochemical changes in two barley genotypes inoculated with a beneficial fungus *Trichoderma harzianum* Rifai T-22 grown in saline soil

Abstract

Soil salinity is one of the most significant environmental factors threatening the productivity of crop plants. Fungal endophytes have been characterised as biocontrol agents that induce resistance responses to several abiotic stresses, including salinity, and were used in this study to colonize two genotypes of the salt-tolerant cereal crop, barley (*Hordeum vulgare* L. cv. Vlamingh and cv. Gairdner). These genotypes were selected based on their tolerance levels to saline conditions. The salt-tolerant genotype was cv. Vlamingh and salt-sensitive was cv. Gairdner. *Trichoderma harzianum* strain T-22 was used to treat these plants under control and saline (200 mM NaCl) conditions. For both genotypes, endophytic-colonized plants exposed to NaCl had greater root and shoot biomass, and better chlorophyll content than non-colonized plants with colonized-cv. Vlamingh performing even better than uninoculated control plants. Metabolome analyses was performed using gas chromatography mass spectrometry (GC-MS) for polar metabolites and liquid chromatography mass spectrometry (LC-MS) for lipids. The metabolome dataset identified using GC-MS consisted of a total of 93 metabolites of which 74 were identified in roots of both barley genotypes. The metabolites identified by GC-MS were categorized into organic acids, sugars (sugar acids and sugar alcohols), amino acids and amines, and a small number of fatty acids. LC-MS analysis resulted in the detection of 186 lipid molecular species, which via the use of MS/MS spectra could be classified into three major lipid classes - glycerophospholipids, glycerolipids, and sphingolipids, in roots of both genotypes. Cultivar Vlamingh showed an increase in both identified metabolites and lipids with fungus and salt treatment as compared to cv. Gairdner where these were found to decrease. The results from this study suggest the role of fungus in helping both genotypes cope with salt stress via varying metabolic pathways.

4.1 Introduction

Soil salinity is one of the most major global problems that negatively affects crop productivity. Salinity impairs plant growth and development via water stress, cytotoxicity due to excessive uptake of ions such as sodium (Na^+) and chloride (Cl^-), and nutritional imbalance. FAO 2018 recognises this issue as a high priority which requires several approaches to reclaim and manage the affects to ensure agricultural production. This is further exacerbated by the increasing number of people to feed with the human population predicted to exceed more than 9 billion by the year 2030 (Hannah et al. 2017).

In an attempt to overcome these limitations and improve agricultural productivity, modern agricultural production is being strongly intensified through the use of huge quantities of agro-chemicals in the form of synthetic fertilizers and pesticides (Duan et al. 2016). However, the intensification of agriculture adversely affects ecological balance, reduces the fertility of soil, contaminates the food chain, pollutes the groundwater, reduces microbial diversity, reduces soil pH, and leads to increased microbial resistance (Uphoff and Dazzo 2016). Therefore, sustainable methods are necessary for the achievement of an increase in agricultural productivity and maintenance of the ecological balance. This will translate into higher crop yields owing to improvements in plant resilience and the ability of plants to adapt to changing climatic conditions, as well as to biotic and abiotic stress.

A sustainable and more eco-friendly means of achieving agricultural intensification and improving productivity is by adopting the use of microbial inoculants to enhance the availability and use of vital soil nutrients. Additionally, these inoculants are extremely useful in mitigating both abiotic and biotic stresses in growing crops (Jambon et al. 2018). There are several reports highlighting the importance and beneficial properties of microbe-based formulations in enhancing crop development and productivity (Bulegon et al. 2019; Evelin et al. 2019; Gangwar and Singh 2018; Huang et al. 2014).

In recent years, research has focused on the plant microbiome that colonizes the internal tissues of the host plant without causing any disease symptoms. These are termed endophytes. Endophytic microbes can enter and colonize plants through the vertical seeding method or through horizontal transmission from the soil to the plants (Huang et al. 2016; Verma and White 2018). This results in beneficial mutualistic interaction. The endophytes interact with their plant host, elicit positive responses to abiotic environmental stresses in their host plants, and also synthesize important bioactive metabolites (Khan et al. 2016). Several reports have described endophytic microorganisms with beneficial traits/functions to their plant host (Bulegon et al. 2019; Evelin et al. 2019; Gangwar and Singh 2018; Huang et al. 2014).

Fungal endophytes exist in close, mutually beneficial association with their plant host. They provide ecological support to their host plants by allowing them to survive adverse biotic and abiotic stresses. In return, the endophytes derive nutrients and protection from the plant (Suryanarayanan 2017). Endophytes accomplish this through inducing and expressing genes that are responsive to stress, synthesizing metabolites that act against stress, and also producing scavengers for reactive oxygen species to take care of free radicals (Lata et al. 2018).

Trichoderma species are one group of fungal endophytes that impart beneficial effects on plants. They are widespread and can be easily found in soil, on decaying wood, or on other fungi (Carreras-Villaseñor et al. 2012). Soil is an important substrate for *Trichoderma* and studies focusing on soil-inhabiting species of the genus have been carried out by several researchers around the world (Chen and Zhuang 2017). *Trichoderma* have been long recognized as agents for the control of plant disease and for their ability to increase plant growth and development. However, recently it has become clearer that certain species also have substantial direct influence on conferring stress tolerance in plants (Brotman et al. 2013; Contreras-Cornejo et al. 2014; Velázquez-Robledo et al. 2011). For example, *Trichoderma spp.* resulted in the accumulation of proline in wheat plants under salt stress conditions (Rawat et al. 2011), and microarray analysis of *Arabidopsis* and cucumber roots exposed to salt stress and inoculated with *Trichoderma spp.* revealed an increased expression of genes related to salt-tolerance, osmoprotection processes, and ascorbic acid (AA) production.

In Chapter 3, several metabolites and lipids were measured that were correlated with the positive effects of *Trichoderma harzianum* strain T-22 inoculation in roots of two barley genotypes under control and saline conditions when grown on agar medium. However, soil is an important substrate for *Trichoderma*. Therefore, for this study, we examined the role of *Trichoderma* strain T-22 on two barley genotypes (cv. Vlamingh and cv. Gairdner) grown in saline soil. Biomass results described in Chapter 2 of this thesis clearly show the positive effect of this fungus on both genotypes under control and saline conditions as measured using several physiological parameters. Here, the aim of this part of study was to determine the metabolites and lipids modified in roots grown in saline soil following endophyte inoculation that are involved in conferring positive effects on barley plants as mentioned in Chapter 2. We employed gas chromatography and liquid chromatography both coupled to mass spectrometry to analyse metabolites and lipids in inoculated and uninoculated roots of both genotypes under control and saline conditions.

4.2 Materials and Methods

4.2.1 Plant Material and growth conditions

Two barley genotypes were used in the study, cv. Vlamingh as a salt tolerant cultivar and cv. Gairdner as a salt sensitive cultivar, based on their known differences in germination phenology and salinity tolerance (Gupta et al. 2019). Cultivar Vlamingh seed had a 95% germination rate after four days treatment with 200 mM NaCl whereas for cv. Gairdner, the germination rate was 75% (Gupta et al., 2019). In a glasshouse study (data not shown) comparing control and saline conditions (200 mM NaCl) cv. Vlamingh had higher biomass production than other tested cultivars and cv. Gairdner the lowest. All seeds were sourced from the University of Adelaide, Australia.

4.2.2 Fungal isolates and growth conditions

For this part, refer to **Chapter 3, Section 2.2.2**

4.2.3 Material and chemicals

Analytical or mass spectrometric grades solvents and reagents were purchased from Merck Millipore (Bayswater, VIC, Australia). 18.2 Ω deionized water was obtained from a Synergy UV Millipore System (Millipore, USA). Chemicals such as 2,5-dihydroxy benzoic acid (DHB), elemental red phosphorus and Supra pure[®] nitric acid (70%) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Embedding and freezing supplies were purchased from Section-Lab Co. Ltd (Tokyo, Japan). Sectioning supplies were purchased from Grate HDS (Ringwood, Australia). Lysing Matrix Tubes with 0.5 g Lysing Matrix D (1.4 mm ceramic spheres) were purchased from MP Biomedicals (Seven Hills, NSW, Australia). PDA was purchased from Sigma-Aldrich.

4.2.4 Plant growth conditions and inoculation with *T. harzianum*

To initiate fungal association with barley roots, seeds from both genotypes were sterilized by immersion in 70% ethanol for 1 min and rinsed 4-5 times in sterile 18.2 Ω deionized water followed by immersion in 1.0% (v/v) household bleach for 10 min and rinsed thoroughly in sterile 18.2 Ω deionized water 6–7 times. Seeds were then imbibed overnight (~16 h) in sterile 18.2 Ω deionized water with continuous aeration. Sterilized seeds were transferred to cylindrical PVC plastic pipes filled with a sandy loam soil (Fultons, Australia). Soil was pasteurised by steam for 1 h at 85°C and rapidly cooled by blowing air through the mix. The cylindrical plastic pipes were placed in a growth cabinet maintained at 17°C constant temperature with no light (**Chapter 2, Figure 2.3**) to allow germination of the sown seeds. Simultaneously to seed preparation, a fresh conidial suspension was prepared by flooding a PDA plate containing observable conidia, with sterile 18.2 Ω deionized water, the subsequent suspension was collected and passed through a filter to separate conidia from hyphae. The suspension was diluted to yield 1×10^8 CFU mL⁻¹ conidia.

After 48 h, the conditions of the growth cabinet were adjusted to 17°C for 16 h light and 10°C for 8 h dark cycles for the remainder of the experiment. The average water holding capacity of soil in each pot was 300 mL, with approximately 20-30 mL water loss per day, whether taken up by the plant or evaporated to the environment. Prior to salt treatment, all plants were given an equal amount of water (200 mL) every alternate day.

After 7 d, 24 seedlings from each genotype were inoculated with a conidial suspension of 25 mL of 1×10^8 CFU g⁻¹ of seedling and 24 seedlings from each genotype received an equal amount of sterile 18.2 Ω deionized water. After 7 d post inoculation, salt treated seedlings received 50 mM NaCl and 50 mL of sterile 18.2 Ω deionized water (to prevent salt precipitation) until the NaCl concentration in each pipe reached 200 mM NaCl. A total of twelve replicates were allocated for each condition and each condition was assigned as follows, 'C' (control), uninoculated seeds with no salt stress; 'CF' (no salt + fungus), fungal inoculated seeds with no salt stress; 'S' (negative control), uninoculated seeds with salt stress; and 'SF' (salt + fungus), fungal inoculated seeds with salt stress. Plants were harvested 48 h after the 200 mM NaCl treatment reached its final level.

4.2.5 Shoot and root length, weight and chlorophyll content determination

The roots from six harvested plants from each condition and genotype were immersed in water and scanned using a flatbed scanner followed by analysis using WinRHIZO software (Regent Instruments, Canada) to determine total root length (TRL). Shoot lengths were determined at harvest by measuring above ground shoot length with a ruler. The aerial parts of the plants were separated and dried in an oven (75°C) for 48 h and weighed using a weighing scale. The chlorophyll content was measured as a SPAD value (SPAD-502Plus, Konica, Europe). The remaining six plants were harvested, and roots were immediately frozen in liquid nitrogen and stored in a -80°C freezer until extraction for metabolomics analyses.

4.2.6 GC-MS untargeted analysis for polar metabolites

Fifty (50 ± 2) mg of fresh frozen root tissue harvested from both varieties of barley were extracted in 500 µL of 100% MeOH (Scharlau, Barcelona, Spain), containing 4% v/v of [¹³C₆] Sorbitol/[¹³C₅ ¹⁵N] Valine (0.5 mg mL⁻¹). The tissue was homogenized using a cryomill (Bertin Technologies, France) using the following program (6100-3 x 45 x 45) at -10°C. Homogenized tissue was then shaken at 800 rpm for 15 min at 30°C on a thermomixer (Eppendorf, Hamburg, Germany), centrifuged for 15 min at 13,000 rpm (15860 x g). The supernatant was then transferred into a fresh 2 mL Eppendorf tube and 500 µL MilliQ water was added to the remaining pellet. The mixture was then vortexed and centrifuged at 13,000 rpm (15860 x g). The supernatants were combined and 50 µL of the supernatant for each sample was completely dried down using a speed vacuum dryer (John Morris Scientific Pty. Ltd, Australia). Samples were then

transferred to crimp vials and sealed with metal caps prior to analysis. Pooled Biological Quality Control (PBQC) samples were prepared by pooling 50 μL aliquots from each sample solution.

The dried extracts were derivatized with methoxyamine hydrochloride in pyridine and bis-(trimethylsilyl)-trifluoroacetamide (BFTFA) as described by (Dias et al. 2015). Briefly, 30 mg mL^{-1} methoxyamine hydrochloride in pyridine was added to all samples and derivatized at 37 °C for 120 minutes with mixing at 500 rpm. The samples were further incubated on a Thermomixer (500 rpm, maintained at 37°C for 30 minutes by adding 20 μL N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA). Each sample was then allowed to rest for 60 minutes prior to injection. A GC-MS system comprising of a Gerstel 2.5.2 autosampler, a 7890A Agilent gas chromatograph, and a 5975C Agilent quadrupole mass spectrometer (Agilent, Santa Clara, USA) with an electron impact (EI) ion source was used to analyse samples with an injection volume of 1 μL for each derivatized sample. Instrumental settings were adapted from (Dias et al. 2015). Calibration of polar metabolites was done using serial concentrations of calibration authentic standards.

4.2.7 LC-MS untargeted analysis for lipid analysis

For this part, refer to **Chapter 3, Section 3.1.7**

4.2.8 Data preprocessing and statistical analysis

For GC-MS data analysis, in silico data pre-processing for mass spectral deconvolution and peak picking and identification was done using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) software [National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA]. A target component library (TCL) was generated from the PBQC file.

Agilent MassHunter Qualitative Analysis B.05.00 (Agilent Technologies, Inc. 2011) was used to confirm peak identification. Agilent MassHunter Quantitative Analysis software (for GCMS) Version B.07.00/Build 7.0.457.0 (Agilent Technologies, Inc. 2008) was used to create batch and method files which were subsequently exported to Microsoft Excel. Manual integration was done to correct for baseline drift using the former software. The exported peak area data from the batch file was normalized to internal standard [$^{13}\text{C}_6$] sorbitol, and fresh weight of each root sample. Further, data were log transformed and the Student's t-test p-values were determined. The p-values were further subjected to Benjamini-Hochberg False Discovery Correction (Benjamini and Hochberg 1995).

MetaboAnalyst 3.0 (Xia et al. 2015) software was then employed for multivariate data analysis of the six biological replicates. Pattern recognition was determined through unsupervised learning algorithms such as principal component analysis (PCA) and hierarchical cluster analysis (HCA).

Log data transformation and mean centering were applied for the principal component analysis. Hierarchical cluster analysis was performed using Euclidean distances with Ward's clustering algorithm. Further, the construction, interaction, and pathway analysis of significantly changed metabolites in both genotypes was performed with MetaboAnalyst 3.0 (<https://www.metaboanalyst.ca/MetaboAnalyst/upload/PathUploadView.xhtml>) based on database sources including KEGG (<http://www.genome.jp/kegg/>) to identify the top altered pathways analysis and for visualization. Fold-change for univariate analysis and bar charts were generated using GraphPad Prism 8.0 (GraphPad Software, Inc., USA; Full Version).

For LC-MS data analysis, raw HPLC-MS data was visually inspected for integrity using PeakView Sciex Software (ver. 2.2), the raw LC-TripleTOF-MS data containing m/z_RT (mass to charge_retention time) and associated peak intensities were converted to ABF (analysis base file) format using the Reifycs file converter for processing using MS-DIAL 3.98 (http://prime.psc.riken.jp/Metabolomics_Software/MS-DIAL/index2.html, accessed 18 September 2019) (Tsugawa et al. 2015). Statistical analysis was performed using MetaboAnalyst 3.0 (<http://www.metaboanalyst.ca/>, accessed 10 September 2019). MS-DIAL export was done using the default parameters for a Lipidomics 'omics project. The MS peak filter threshold was established after manual inspection of the raw data. Additionally, adduct ion setting was set to search for ([M+H]⁺), ([M+Na]⁺), ([M+K]⁺) and ([M+NH₄]⁺), adducts and alignment parameters were established considering a retention time tolerance of ± 0.5 min and a MS1 tolerance of 0.1 Da. A matrix containing tentatively identified m/z_RTs and associated intensity peaks was exported as a csv file. The level of identification was carried out based on the Metabolomics Standards Initiative requirements (Sumner et al. 2007). Absolute quantities were determined, and the concentration unit was expressed as picomole/mg of fresh weight.

The processed lipid data was subjected to multiple comparison statistical analyses using Analysis of Variance (ANOVA), with a false discovery rate (FDR)-adjusted p value of 0.05 and using the Benjamini–Hochberg method. Additional statistical analysis (Student's t-tests) comparing each treatment in both genotypes was also performed using MetaboAnalyst to generate a list of features (m/z_RT) with a false discovery rate corrected p-value ($P < 0.01$) fold change of two or more in peak intensity relative to the respective controls. All bar charts were generated using GraphPad Prism 8.0 (GraphPad Software, Inc., USA; Full Version).

4.3 Results

4.3.1 Effect of fungus and salt stress on seminal root length of cv. Vlamingh and cv. Gairdner

Nodal roots could not be distinguished from seminal roots due to soil aggregates in both genotypes. Therefore, average total seminal root length was measured. This method of measurement provided a reasonably fast and accurate alternative for phenotyping of seedlings.

Average seminal root length (SRL) was measured for all conditions in both genotypes. When grown in control conditions, the sensitive genotype cv. Gairdner had higher SRL than the tolerant genotype cv. Vlamingh. The patterns of growth for salt and fungal treated seedlings grown in soil were similar to those for seedlings grown on agar plates. Although SRL was increased in CF compared to C in both genotypes, the increase was greater in cv. Gairdner (3.50%) than in cv. Vlamingh (0.35%)

(**Figure 2.7** and **Table 2.2**).

Salt treatment reduced the SRL for both genotypes in control seedlings (14.0% ($P < 0.01$) for cv. Vlamingh and 23.0% ($P < 0.01$) for cv. Gairdner) but when seedlings were grown with fungus and treated with salt (SF), SRL increased significantly by 20.0% ($P < 0.01$) in cv. Vlamingh and 28.50% ($P < 0.05$) in cv. Gairdner. It was also observed that SRL in seedlings grown with fungus and treated with salt recovered better than control (C) seedlings in cv. Vlamingh (3.21% ($P < 0.05$)). However, the SRL for seedlings grown with fungus and treated with salt in cv. Gairdner was slightly reduced as compared to control seedlings (-0.9%).

4.3.2 Root and shoot dry weight

The effects of fungal inoculation on root and shoot dry weight under control and saline conditions were measured (**Table 2. 3**). Root dry weight in uninoculated S compared to C was significantly reduced in cv. Vlamingh with $P < 0.01$ (from 0.24 g in C to 0.16 g in S) and in cv. Gairdner with $P < 0.05$ (from 0.24 g in C to 0.16 g in S) (**Chapter 2, Figure 2.8**). In CF compared to C, root dry weight increased in both genotypes. However, these were not significant. Root dry weight in cv. Vlamingh increased from 0.24 g in C to 0.27 g in CF, and in cv. Gairdner it increased from 0.21 g in C to 0.30 g in CF when compared to inoculated seedlings without salt. In fungal treatments with salt, root dry weight significantly increased from 0.16 g in S to 0.28 g in SF ($P < 0.01$) in cv. Vlamingh. Similarly, in cv. Gairdner, root dry weight increased significantly from 0.16 g in S to 0.22 g in SF ($P < 0.01$).

Shoot dry weight was measured to determine the above ground plant biomass (**Chapter 2, Table 2. 3**). It was found that shoot dry weight reduced significantly in cv. Vlamingh (from 0.40 g in C to 0.31 g in S) but not significantly in cv. Gairdner under salt stress. In treatment CF, shoot dry weight increased in cv. Vlamingh but reduced in cv. Gairdner. However, the shoot dry weight in treatment CF was not significantly changed in either genotype. With fungal inoculation under salt stress, a significant increase in shoot dry weight in cv. Vlamingh (from 0.31 g in S to 0.51 g in SF, $P < 0.05$) was observed. Although the shoot dry weight increased from 0.38 g in S to 0.50 g in SF in cv. Gairdner, it was not statistically significant due to high variation between samples.

4.3.3 Root to shoot ratio

The root to shoot ratio was measured to assess the overall health of the plants in all treatments (**Chapter 2, Table 2. 3**). When grown in control conditions, root to shoot ratio was higher in the tolerant genotype cv. Vlamingh as compared to the sensitive genotype cv. Gairdner. Under salt stress, the root to shoot ratio of plants decreased in cv. Vlamingh from 0.6 in C to 0.52 in S and in cv. Gairdner, there was a slightest difference between control and salt treated seedlings (from 0.43 in C to 0.42 in S) (**Chapter 2, Figure 2.8**). The effect of fungal inoculation without salt (CF) resulted in decreased root to shoot ratio in cv. Vlamingh (0.53) as compared to uninoculated seedlings under no salt (C) (0.60). However, the root to shoot ratio increased in inoculated seedlings in cv. Gairdner (0.68) when compared to uninoculated seedlings (0.43) under no salt conditions. But when grown with fungus and treated with salt, root to shoot ratio was increased in both genotypes (from 0.52 to 0.55 in cv. Vlamingh and from 0.42 to 0.44 in cv. Gairdner) with the effect better observed in cv. Gairdner where seedlings showed better root to shoot ratio than seedlings grown under control conditions without fungus.

4.3.4 Chlorophyll content

Leaf chlorophyll content was measured to examine the effect of fungal inoculation with *T. harzianum* T-22 on the photosynthetic potential of both genotypes. Under control conditions, the chlorophyll content was higher in the tolerant genotype cv. Vlamingh as compared to the sensitive genotype cv. Gairdner. But when seedlings were grown with the fungus and without salt (CF), the chlorophyll content increased in both genotypes and the effect was higher in cv. Gairdner (**Table 2. 3 and Figure 2.9**).

Salt stress greatly reduced the chlorophyll content in both genotypes, but the difference was greater in cv. Gairdner where the chlorophyll content reduced from 32.42 SPAD units in C to 18.09 SPAD units in S as opposed to the effect observed in cv. Vlamingh where the chlorophyll content was 34.32 SPAD units in C and reduced to 26.14 SPAD units in S. But when seedlings were grown with the fungus and treated with salt, the chlorophyll content was significantly increased in both genotypes making it equivalent to or better than those for untreated control seedlings (**Figure 2.9**).

4.3.5 The effect of inoculation with *T. harzianum* and salt stress on polar metabolites in roots

The untargeted metabolomics approach aims to analyse hundreds of metabolites some of which are identified while many remain unknown. A total of 93 metabolites, of which 74 were identified, were detected in roots of both barley genotypes using GC-MS. The identified metabolites were categorized according to compounds classes and are listed in **Supplementary Table S10**. Among the identified metabolites, twenty-eight were organic acids, twenty-two were sugars, twenty were amino acids, seven were fatty acids and nineteen were unknowns.

Unknowns were categorised as compounds that did not return any mass spectral library match. The average normalized responses of all metabolites in all treated barley roots can be found in **Supplementary Table S10**.

Data dimension reduction performed by principal component analysis (PCA) plots show clear clustering between replicates from all treatments (**Figure 4.1**). The pink circles in the upper left quadrant represents pooled biological quality control samples clustering together. This indicates good reproducibility and reliable detection by the method and therefore data can be used for subsequent analysis and interpretation.

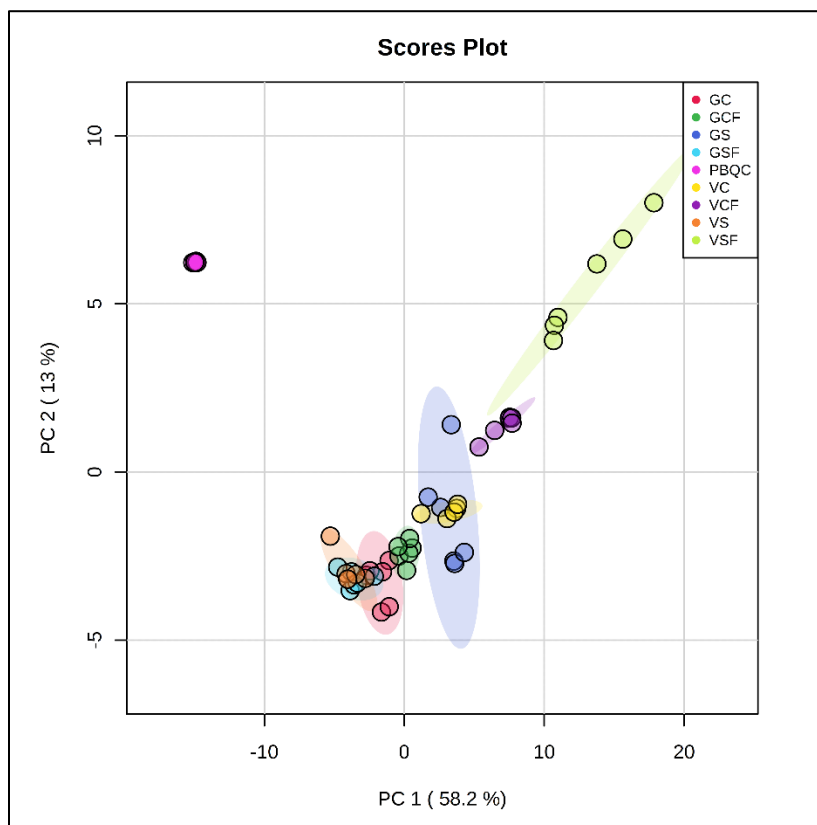


Figure 4.1 Principal Component Analysis (PCA) score plot for two barley genotypes - cv. Vlamingh and cv. Gairdner from four treatments - control, control + fungus, salt, and salt+ fungus - and quality control samples. Abbreviations: G- cv. Gairdner, V- cv. Vlamingh, C- control treated roots, S- Salt treated roots (200 mM NaCl), CF- control + fungus, SF- salt + fungus, PBQC- pooled biological quality control samples

Further PCA plots were generated for the individual genotypes under the four treatments. **Figure 4.2a** and **Figure 4.2b** shows a well-defined separation among samples from the different treatments in both genotypes. The first and second principal components accounted for 84.9% and 64.1% of the variation in cv. Vlamingh and cv. Gairdner, respectively. The PCA scatterplot

divided the samples into four main groups- C, CF, S and SF in cv. Vlamingh. The fungal inoculated plants clearly separate from the uninoculated plants in cv. Vlamingh on component 1 (PC1), describing 75.1% of the variability. The salt treatment contributed to distinct separation on PC2 giving 9.8% of the variability in cv. Vlamingh. In cv. Gairdner, PC1 clearly separates treatment S from all three treatments along PC1 contributing to 48.2% of the variability. PC2 failed to show any clear separation among treatments in all samples from cv. Gairdner. **Figure 4.2c** and **Figure 4.2d** illustrates a heatmap showing altered levels of metabolites in both genotypes under the four different treatment conditions. The results show a strong separation between the treatments in both genotypes. In cv. Vlamingh, two major clusters were identified, separating salt treatment from the other treatment groups. Further clustering was identified forming a subclass which grouped C and CF together whereas treatment SF clustered separately. In cv. Gairdner (**Figure 4.2d**), two major clusters grouped treatments SF and C together and treatments S and CF together. Further sub classes clearly separated between four treatments in cv. Gairdner.

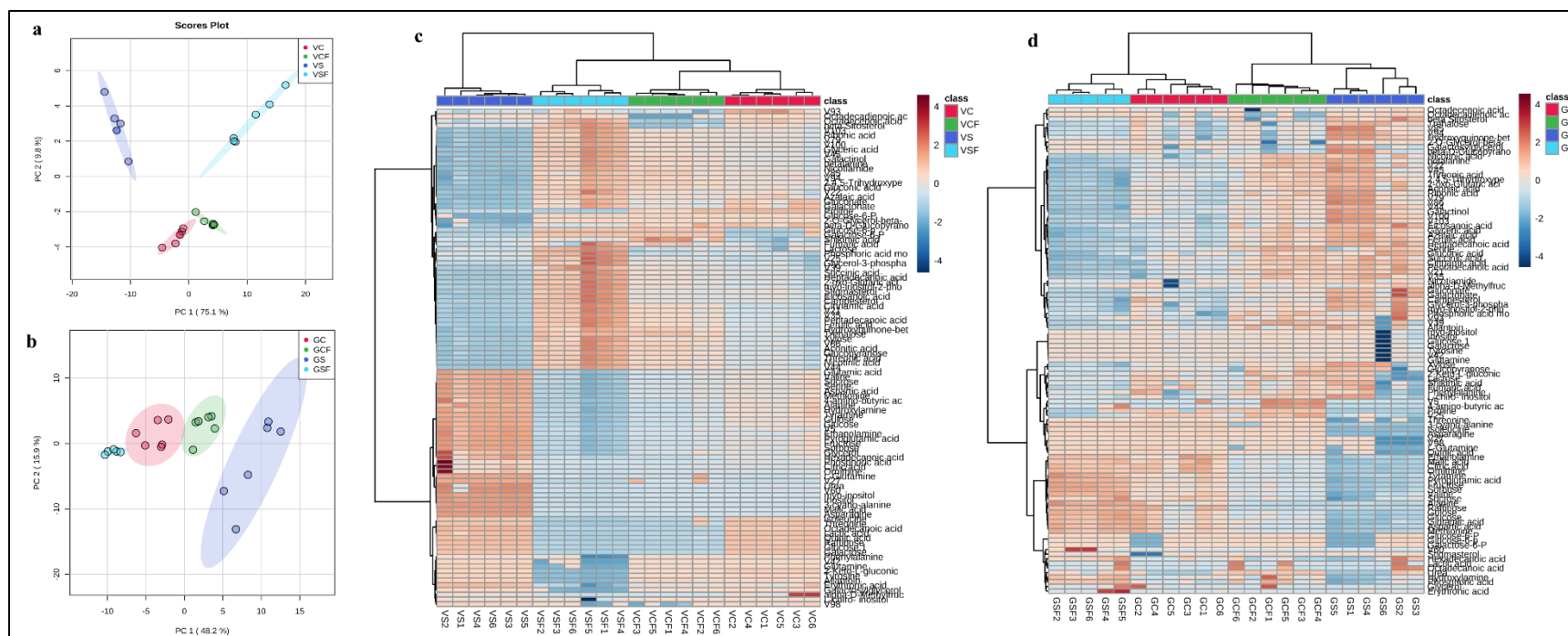


Figure 4.2 Principal Component Analysis (PCA) score plot and Heatmaps for cv. Vlamingh (a and c) and cv. Gairdner (b and d) under four treatments- C- control treated roots, S- Salt treated roots (200 mM NaCl), CF- control + fungus, SF- salt + fungus. The fungal inoculated samples (VCF and VSF) separate from the uninoculated samples (VC and VS) in cv. Vlamingh on component 1 (PC1). The salt treatment contributed to distinct separation on PC2 in cv. Vlamingh. In cv. Gairdner, PC1 clearly separates treatment GS from the other three treatments (GCF, GSF and GC). The heatmaps represent unsupervised hierarchical clustering of lipid species (rows). The row displays identified lipid species and the column represents the samples. Relative lower abundance of metabolites is displayed in blue, whilst relative higher abundance of metabolites are displayed in red. Clustering was performed using Euclidean distance matrices on in the R package heatmap.

A detailed analysis of the heatmap identified similarities and differences in metabolite abundances between treatments for both genotypes. Therefore, analysis of a 2-fold change or greater ($P < 0.05$) was performed to compare the profiles of amino acids, organic acids and sugars in different treatment groups from both genotypes. **Figure 4.3** and **Figure 4.4** represent a graphical view of X-fold changes for treatment groups S and CF compared to treatment C as well as SF compared to treatment S in cv. Vlamingh and cv. Gairdner, respectively.

In cv. Vlamingh S compared to C (**Figure 4.3**), galactinol (+7.14-fold) and proline (+6.15-fold) increased significantly ($P < 0.05$), whereas seventeen metabolites decreased significantly with $P < 0.05$. When treatment CF was compared to C (**Figure 4.3**), cv. Vlamingh showed significant increases ($P < 0.05$) in twenty-four metabolites as given in **Figure 4.3**, with highest increase in raffinose (+15.19-fold), gulose (+5.80-fold), tyramine (+8.30-fold), alanine (+15.41-fold), octadecenoic acid (+6.50-fold), n-octadecanoic acid (+4.19-fold), 9,12-octadecadienoic acid (+4.29-fold), whereas glucopyranose (-0.24-fold), trehalose (-0.30-fold), n-pentadecanoic acid (-0.28-fold), nicotinic acid (-0.28-fold), n-heptadecanoic acid (-0.51-fold), ferulic acid (-0.26-fold) and cinnamic acid (-0.26-fold) decreased significantly ($P < 0.05$) in cv. Vlamingh when treatment CF was compared to treatment C.

In cv. Vlamingh SF compared to S (**Figure 4.3**), of the twenty-two identified sugars, seventeen sugars increased significantly ($P < 0.05$) by +2-fold or greater as shown in **Supplementary Table S11**, amongst twenty identified amino acids, sixteen increased significantly ($P < 0.05$) showing greater than or equal to +2-fold (**Supplementary Table S11**), and seventeen out of twenty-eight organic acids increased significantly ($P < 0.05$) showing +2-fold increase (**Supplementary Table S11**). No significant increase in fatty acids were found and no significant decreases were observed in inoculated roots of cv. Vlamingh as compared to uninoculated roots under saline conditions.

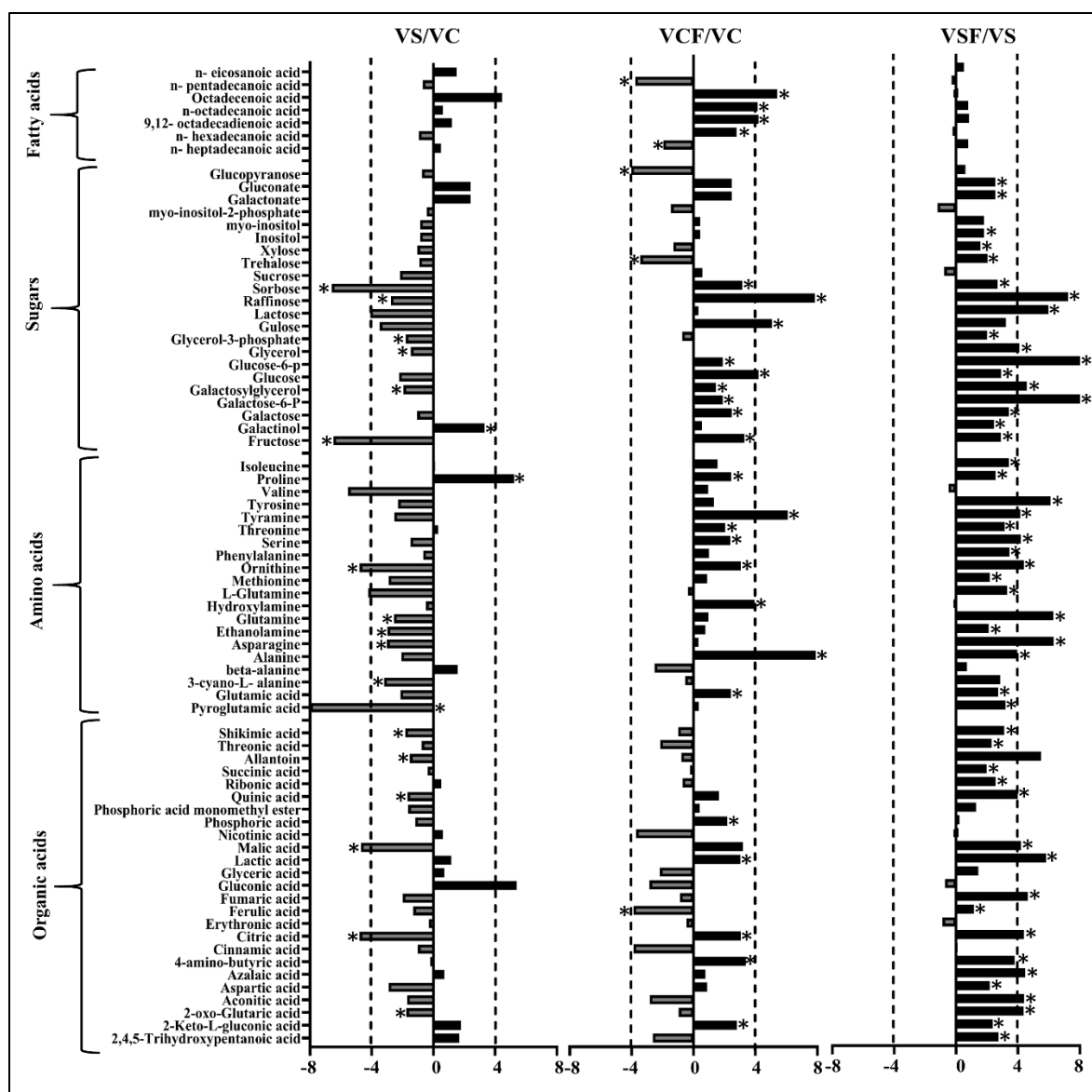


Figure 4.3 Logarithmic ratios of representative sugars, organic acids and amino acids in roots of barley cv. Vlammingh (V) of salt grown compared to control grown (VS/VC); control with fungus compared to control grown (VCF/VC); and salt with fungus compared to salt grown (VSF/VS). Values that are significantly different ($P < 0.05$, FDR) are indicated by *. A threshold of ± 4 -fold change is indicated by a dashed line.

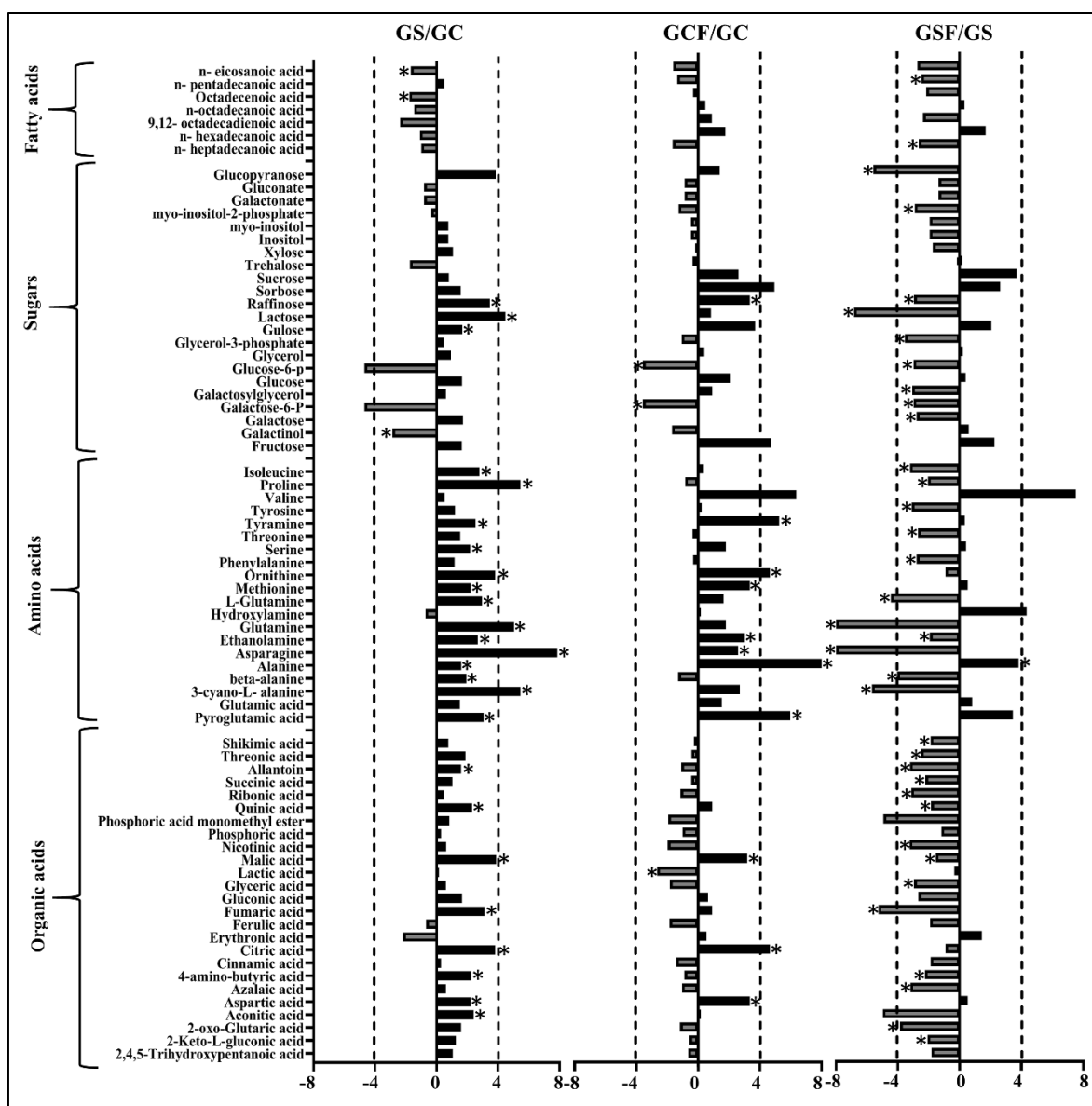


Figure 4.4 Logarithmic ratios of representative sugars, organic acids and amino acids in roots of barley cv. Gairdner (G) of salt grown compared to control grown (GS/GC); control with fungus compared to control grown (GCF/GC); and salt with fungus compared to salt grown (GSF/GS). Values that are significantly higher ($P < 0.05$, FDR) are indicated by *. A threshold of ± 4 -fold change is indicated by a dashed line.

X-fold analysis performed in cv. Gairdner S compared to C (**Figure 4.4**) showed significant increases with +2-fold or greater in sugars such as raffinose (+3.32-fold) and lactose (+4.75-fold). Among twenty amino acids, fourteen increased significantly in cv. Gairdner S as compared to C and these were isoleucine (+2.64-fold), proline (+6.64-fold), tyramine (+2.41-fold), serine (2.14-fold), ornithine (+3.76-fold), methionine (2.16-fold), L-glutamine (+2.80-fold), ethanolamine (+2.53-fold), asparagine (+15.21-fold), pyroglutamic acid (+2.90-fold), alanine (+2.11-fold), beta-alanine (+2.16-fold) and 3-cyano-L-alanine (+6.65-fold). Treatment S as compared to C showed

significant increases in cv. Gairdner among organic acids such as allantoin (+2.70-fold), quinic acid (+2.24-fold), malic acid (+3.84-fold), fumaric acid (+3.1-fold), citric acid (+3.76-fold), 4-amino-butyric acid (+2.20-fold), aspartic acid (+2.16-fold) and aconitic acid (+2.31-fold). Only three metabolites, galactinol (-0.30-fold), n-eicosanoic acid (-0.57-fold) and octadecenoic acid (-0.54-fold), decreased significantly in treatment S as compared to C in cv. Gairdner. Treatment CF compared to C showed (**Figure 4.4**) relatively lesser significant changes in cv. Gairdner. The significantly increased metabolites showing +2-fold or greater were raffinose (+3.20-fold), tyramine (+6.19-fold), ornithine (+5.03-fold), methionine (+3.21-fold), ethanolamine (2.87-fold), asparagine (+2.44-fold), alanine (+30.22-fold), pyroglutamic acid (+7.88-fold), malic acid (+3.01-fold), citric acid (+5.03-fold) and aspartic acid (+3.21-fold) whereas glucose-6-phosphate (-0.29-fold), galactose-6-phosphate (-0.29-fold) and lactic acid (-0.40-fold) decreased significantly. **Figure 4.4** shows X-fold changes between treatment SF as compared to S in cv. Gairdner. It is worth noting that out of total ninety-three detected metabolites, only asparagine (+8.88-fold) increased significantly and a total of thirty-six metabolites showed significant decreases after salt stress as listed in **Supplementary Table S11**.

To better understand the relevant pathways involved or being affected in the three comparisons made from four treatment groups in both genotypes, pathway analyses was performed using MetaboAnalyst. The metabolite levels were used to generate graphical outputs of pathways involved in each treatment in both genotypes. **Figure 4.5** shows an example of the comparison between the metabolome view with pathway impact on X-axis and log (p value) on Y-axis. The pathway impact is calculated as the sum of the importance measures of the matched metabolites normalized by the sum of the importance measures of all metabolites in each pathway. The node colour is based on its p value and the node radius is determined based on their pathway impact values. Further, the corresponding 'pathway view' was used to visualize the pathways affected for a specific treatment comparison. The analysis was applied to all treatment groups in both genotypes (**Figure 4.6**). It was found that the top ten pathways that were affected in both genotypes between the three treatment group comparisons were Arginine biosynthesis, Galactose metabolism, Nicotinate and nicotinamide metabolism, Tyrosine metabolism, Nitrogen metabolism, Citrate acid cycle (TCA cycle), Glyoxylate and dicarboxylate metabolism, Aminoacyl-tRNA biosynthesis, Alanine, aspartate and glutamate metabolism, and Isoquinoline alkaloid biosynthesis.

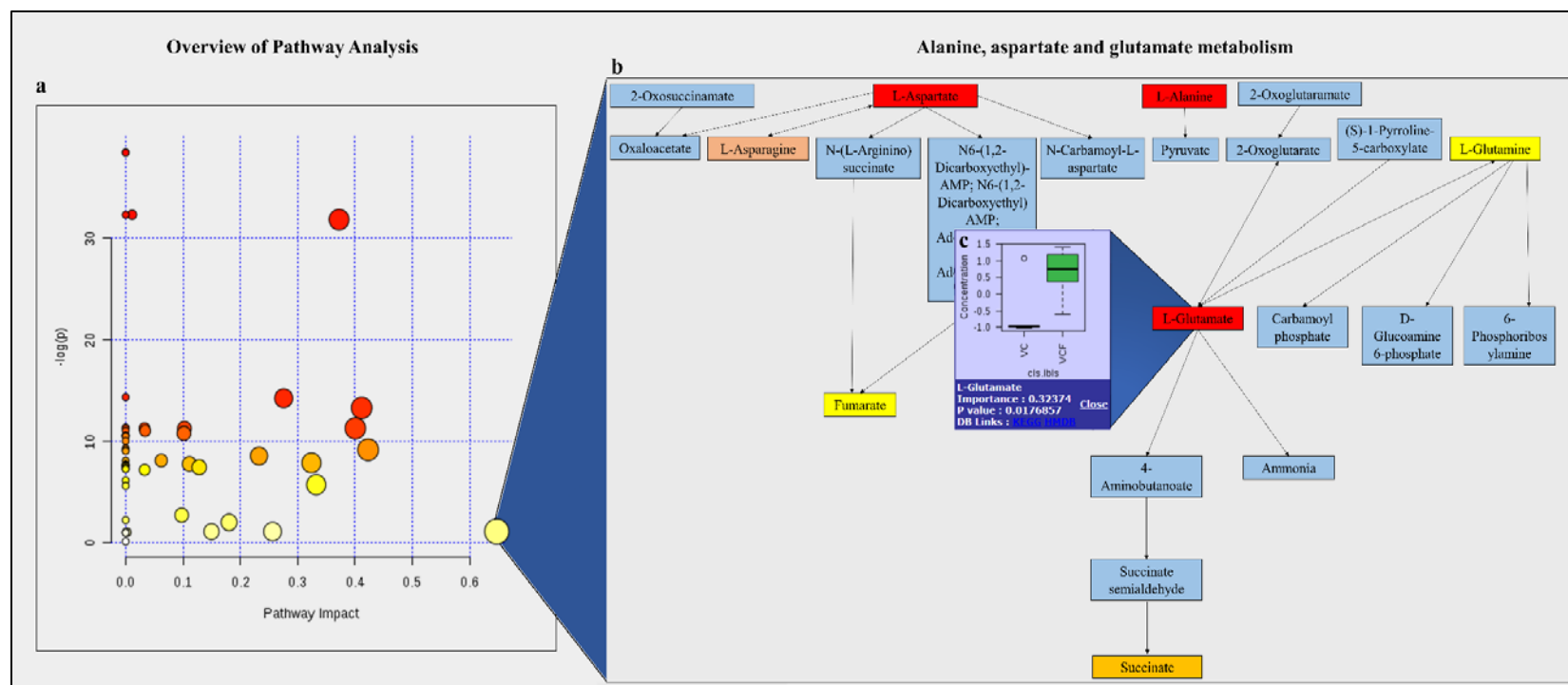


Figure 4.5 Overview of pathway analysis, corresponding pathway view and significantly changed compound - L-glutamate in cv. Vlammingh treatment control + fungus (CF) compared to control (C). (a): metabolome view- pathway impact is given on X-axis and log (p value) on Y-axis. The pathway impact is calculated as the sum of the importance measures of the matched metabolites normalized by the sum of the importance measures of all metabolites in each pathway. The node colour is based on its p value and the node radius is determined based on their pathway impact values. Red colour indicates the most significant pathway and light-yellow colour indicates least significant pathway. (b): Corresponding pathway view for node colour given in yellow. Alanine, aspartate and glutamate metabolism is highlighted by pointing and clicking on hyperlinked nodes (yellow colour above in 'a'). (c): Single metabolite node. L-glutamate is highlighted as an example to compare its concentration between CF and C in cv. Vlammingh.

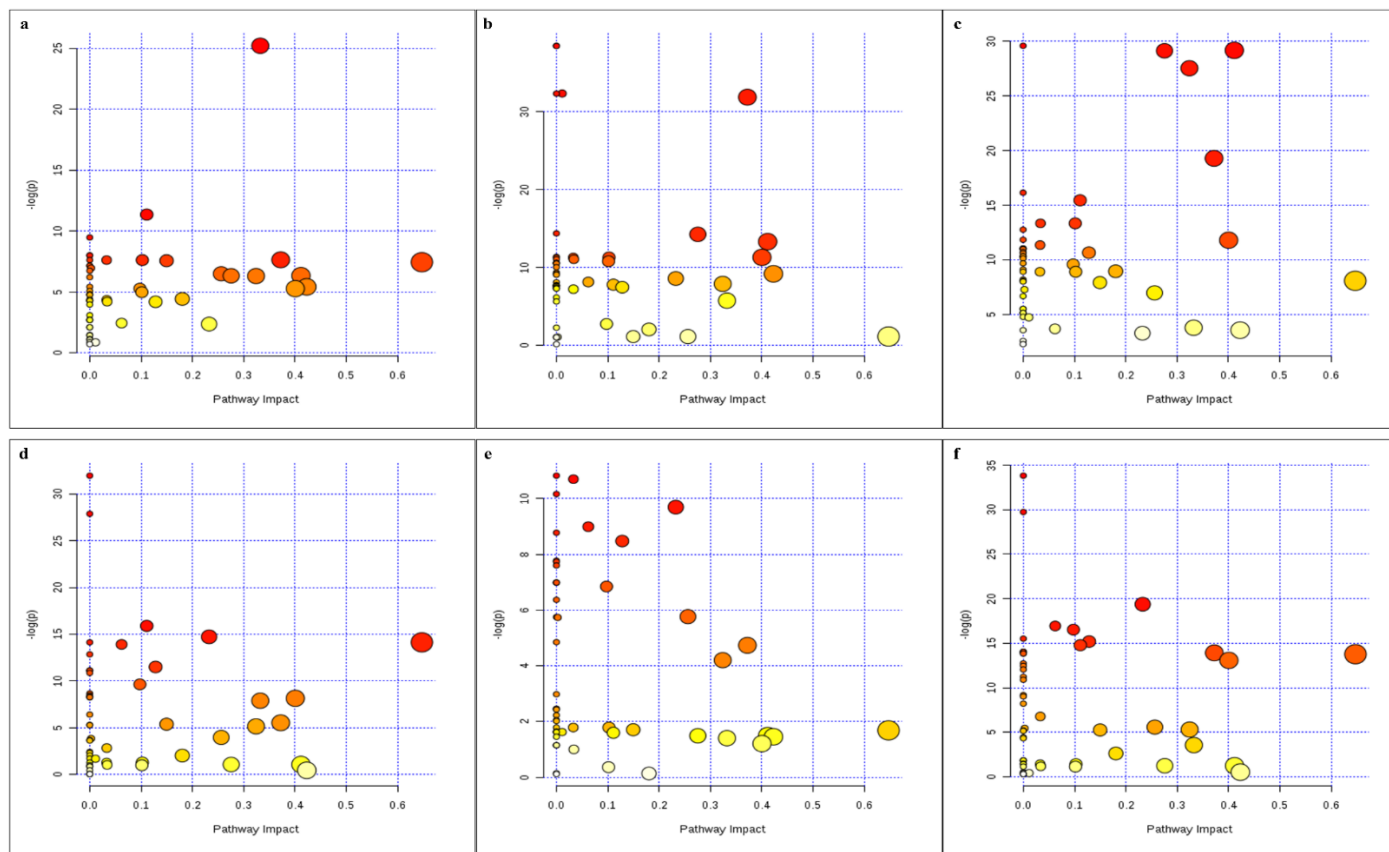


Figure 4.6 Metabolome view for all identified metabolites of barley cv. Vlammingh (V) and cv. Gairdner (G) of salt grown compared to control grown (VS/VC-a and GS/GC-d); control with fungus compared to control grown (VCF/VC-b and GCF/GC-e); and salt with fungus compared to salt grown (VSF/VS-c and GSF/GS-f). Metabolome view shows all matched pathways arranged by p values on Y-axis and pathway impact values on X-axis. The node colour is based on their p value and the node radius is determined on their pathways impact values.

4.3.6 Untargeted lipid profiling of barley roots

An untargeted strategy was followed to extract the information from the large data sets obtained in LC-ESI-QqTOF-MS analyses in positive ionization mode. A total of 3645 m/z_RT features were detected and quantified from roots of cv. Gairdner and cv. Vlamingh under the four treatments (**Supplementary Table S12**) using MS-DIAL. PCA analysis was performed to identify the effect of salt stress and Trichoderma inoculation in roots of cv. Gairdner and cv. Vlamingh under controlled and saline conditions. The first and second principal components accounted for 25.8% of the variation for both genotypes. **Figure 4.7a** separate all treatments into two groups based on the genotypic variation. PC1 failed to show any clear clustering based on either treatments or genotypes. However, PC2 separates the two genotypes contributing to 11.2% of the variation.

Similarly, a hierarchical clustering analysis (HCA) represented by a heatmap showing the changes in the detected mass features (m/z_RT) from roots of both genotypes under control and saline conditions revealed a clear separation between two genotypes (**Figure 4.7b**). No distinct further clustering was introduced by the inoculation of fungus in both genotypes. Therefore, untargeted analyses showed clustering of the mass features predominantly based on the barley genotype demonstrating a strong genetic impact on the root lipidome.

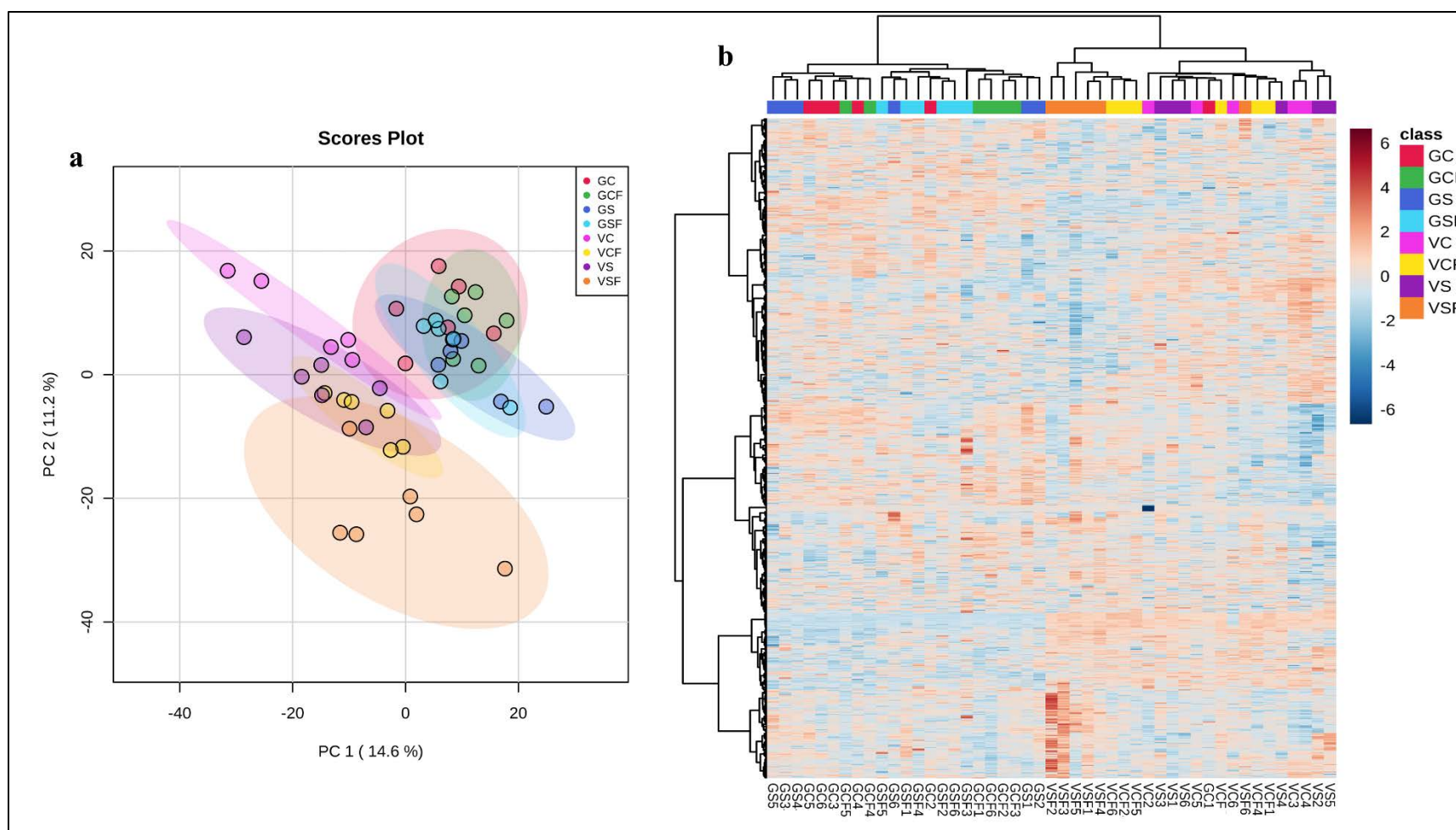


Figure 4.7 Principle Component Analysis (PCA) and heatmap of the mass features (m/z_{RT}) extracted from lipid profiles from roots of two barley genotypes (cv. Vlamingham and cv. Gairdner) grown under control and saline conditions, with and without fungus. 11a- PCA showing scores plot for detected m/z_{RT} for Control- C, Control + fungus- CF, Salt- S and Salt + fungus- SF in cv. Vlamingham and cv. Gairdner; 11b- heatmap obtained from hierarchical cluster analysis (HCA) of all detected m/z_{RT} features in cv. Vlamingham and cv. Gairdner. The labels on the bottom indicate the sample groups.

A one-way analysis of variance (ANOVA) with an adjusted p-value (FDR-false discovery rate) of <0.05 and Turkey's HSD (honestly significant difference) test were carried out on all datasets to discover discriminate features that showed significant changes between uninoculated and inoculated roots under control and saline conditions. A Venn diagram in **Figure 4.8** shows that in cv. Vlamingh 67 unique features changed significantly, while only nine features were changed in cv. Gairdner under saline conditions when compared to control. Of those, only one feature was common to both genotypes.

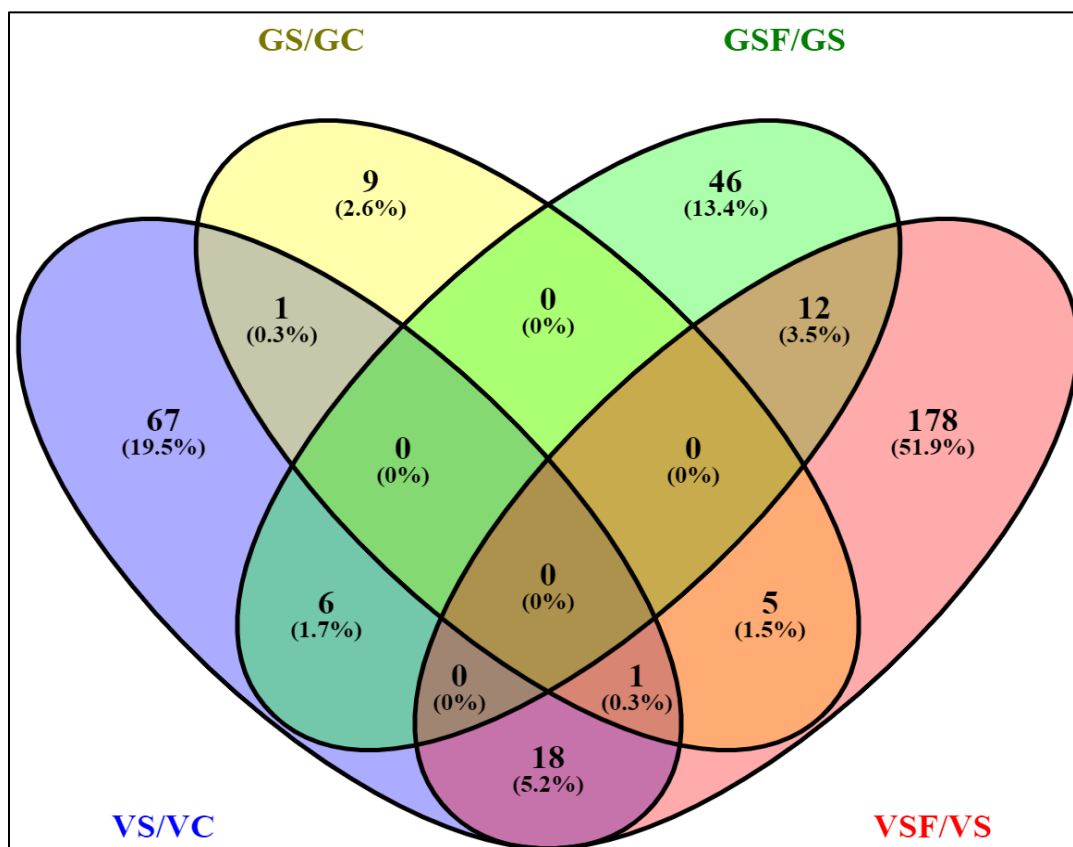


Figure 4.8 Venn diagram summary of the number of significantly altered features identified by one-way ANOVA and post-hoc analysis of the lipid profiles from roots of two barley genotypes under salt stress and with and without fungal inoculation. The number represent significantly altered features regardless of their directionality (upregulation and downregulation). Numbers appearing in overlapped sections are common between treatments. Four analysis performed are cv. Vlamingh salt (VS) compared to cv. Vlamingh control (VC), cv. Vlamingh salt and fungus (VSF) compared to cv. Vlamingh salt (VS), cv. Gairdner salt (GS) compared to cv. Gairdner control (GC) and cv. Gairdner salt and fungus (GSF) compared to cv. Gairdner salt (GS).

Out of 3645 m/z_{RT} features, treatment SF showed 178 unique features that were significantly changed in cv. Vlamingh, while 46 significantly changed features were found in cv. Gairdner when compared to uninoculated roots under saline conditions (treatment S). Twelve common features

were shared between both genotypes from inoculated roots under saline conditions. It is interesting to note that 18 features were common in inoculated and uninoculated roots of both genotypes under saline conditions. These results have shown that inoculated roots of cv. Vlamingh exhibited a larger number of significantly different ($P < 0.05$) features as compared to cv. Gairdner after salt stress.

4.3.7 Targeted MS/MS lipid analysis

MS-DIAL's in silico LipidBlast database and an in-house plant lipid database (Cheka Kehelpannala, personal communication) were further used to identify lipids by the similarity calculation of retention time, precursor m/z , isotopic ratios, and MS/MS spectrum. False positives and true positives peak identifications were manually checked, curated and modified where needed. Out of 3645 m/z _RT features, 186 lipid molecular species were identified with MS/MS spectra, 1733 were annotated without MS/MS spectra and 1716 m/z _RT mass features remain as unknown they did not return with any match (**Supplementary Table S13**). Further analyses were performed on the identified lipids in both genotypes and therefore following sections will discuss the significant changes of identified lipids only. An unsupervised chemometric analysis of the identified lipids datasets by principal component analysis (PCA) separated treatment SF of cv. Vlamingh from the other groups along PC1 representing 43.9% of the variation (**Figure 4.9**). This indicates a substantial difference in the lipidome of inoculated roots of cv. Vlamingh under salt stress. A similar plot was observed when both genotypes were separately analysed using PCA as given in **Figure 4.10a**.

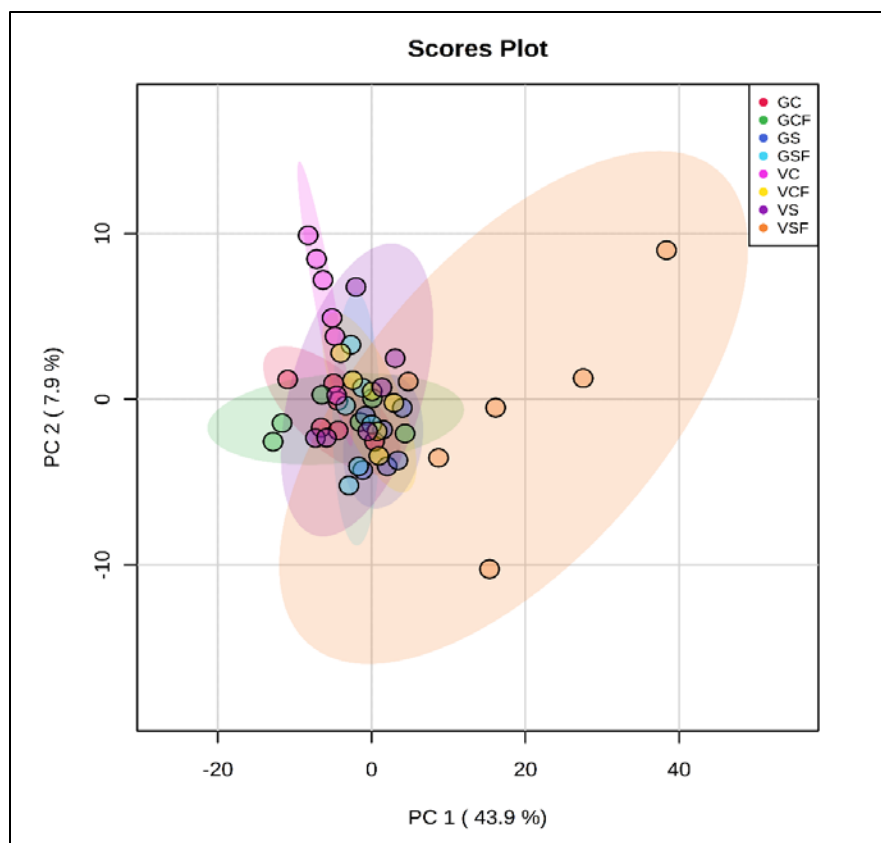


Figure 4.9 Principle component analysis (PCA) of the identified lipids from roots of two barley genotypes grown under control and saline conditions, with and without fungus. VC- control treated cv. Vlamingh; VCF- control and fungus treated cv. Vlamingh, VSF- salt and fungus treated cv. Vlamingh; GC- control treated cv. Gairdner; GCF- control and fungus treated cv. Gairdner, GSF- salt and fungus treated cv. Gairdner. PC 1 versus PC 2 shows separation between VSF from other groups. n = 6 for all treatments. PC2 failed to show any separation between treatments or genotypes

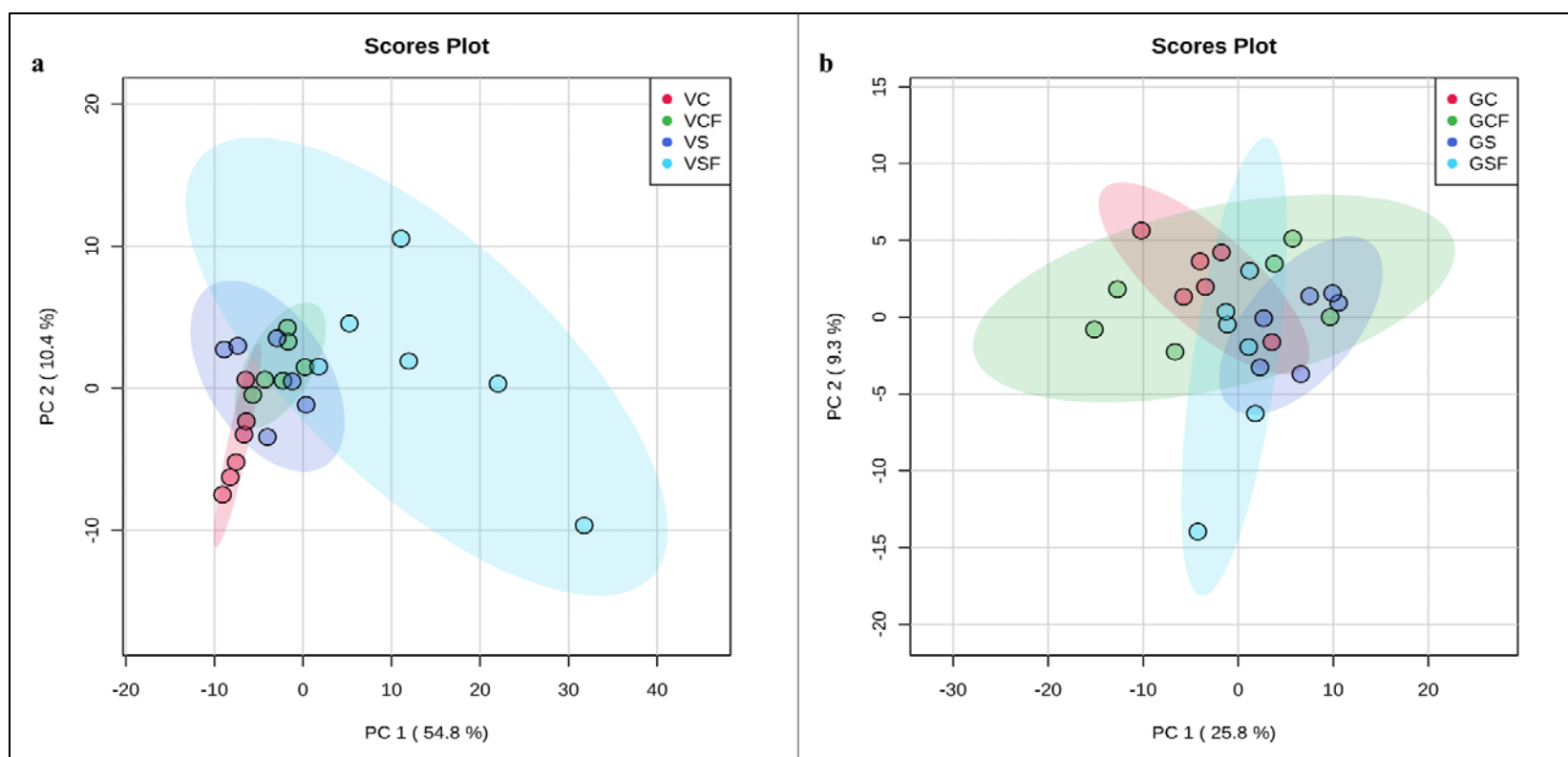


Figure 4.10 Principle component analysis (PCA) of the identified lipids from roots of two barley genotypes grown under control and saline conditions, with and without fungus. VC- control treated cv. Vlammingh; VS- salt treated cv. Vlammingh; VCF- control and fungus treated cv. Vlammingh; GC- control treated cv. Gairdner; GS- salt treated cv. Gairdner; GCF- control and fungus treated cv. Gairdner, GSF- salt and fungus treated cv. Gairdner. PC 1 versus PC 2 shows the separation between SF and other groups in cv. Vlammingh (a). PCA failed to show any clear separation in cv. Gairdner (b). n = 6 for all treatments.

A total of 186 lipids (**Supplementary Table S13**) identified in both genotypes under four treatments were categorised into different lipid classes. **Table 4.1** shows the number of lipid species in each class and the total percentage of individual species detected in each lipid class from roots of both genotypes. Major classes identified were phospholipids- phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS), lysophospholipids- lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), storage lipids triacylglycerol (TAG), lipid intermediates diacylglycerol (DAG), acyl carnitines (ACar) and ceramides (CE).

Table 4.1 Summary of the number of identified lipid species from roots of two barley genotypes (cv. Vlamingh and cv. Gairdner) from four treatment groups- control C, control inoculated with fungus CF, salt (200 mM NaCl) S and salt inoculated with fungus SF. Analysis was performed using an LC-TripleTOF-MS (positive ionization mode).

Lipid class	Lipid subclass	Species identified
Glycerophospholipids (GP) (total = 109)	PC	35
	LPC	23
	PE	23
	LPE	6
	PG	7
	PI	3
	PS	12
Glycerolipids (GL) (total = 65)	DAG	24
	TAG	41
Sphingolipids (SL)	CE	5
Fatty acyls (FA)	ACar	7

Abbreviations: PC- phosphatidylcholine, LPC- lysophosphatidylcholine, PE- phosphatidylethanolamine, LPE- lysophosphatidylethanolamine, PG- phosphatidylglycerol, PI- phosphatidylinositol, PS- phosphatidylserine, DAG- diacylglycerol, TAG- triacylglycerol, CE- ceramides, ACar- acyl carnitines

Further univariate analysis was performed in roots of cv. Vlamingh and cv. Gairdner to identify important lipids with a focus on those that changed due to a) salt stress in both genotypes, b) the interaction of fungus with roots under controlled conditions and c) the effect of salt stress in roots inoculated with the fungus. Univariate analysis examines each variable separately and for this, volcano plots were constructed. Volcano plots are used to compare the size of the fold change (+1.5-fold or greater for this study) to the statistical significance level $P < 0.05$ as shown in **Figure 4.11**. The horizontal axis plots the fold change between the two groups (on a log scale), while the vertical axis represents the p -value for a t-test of differences between samples (on a negative log scale).

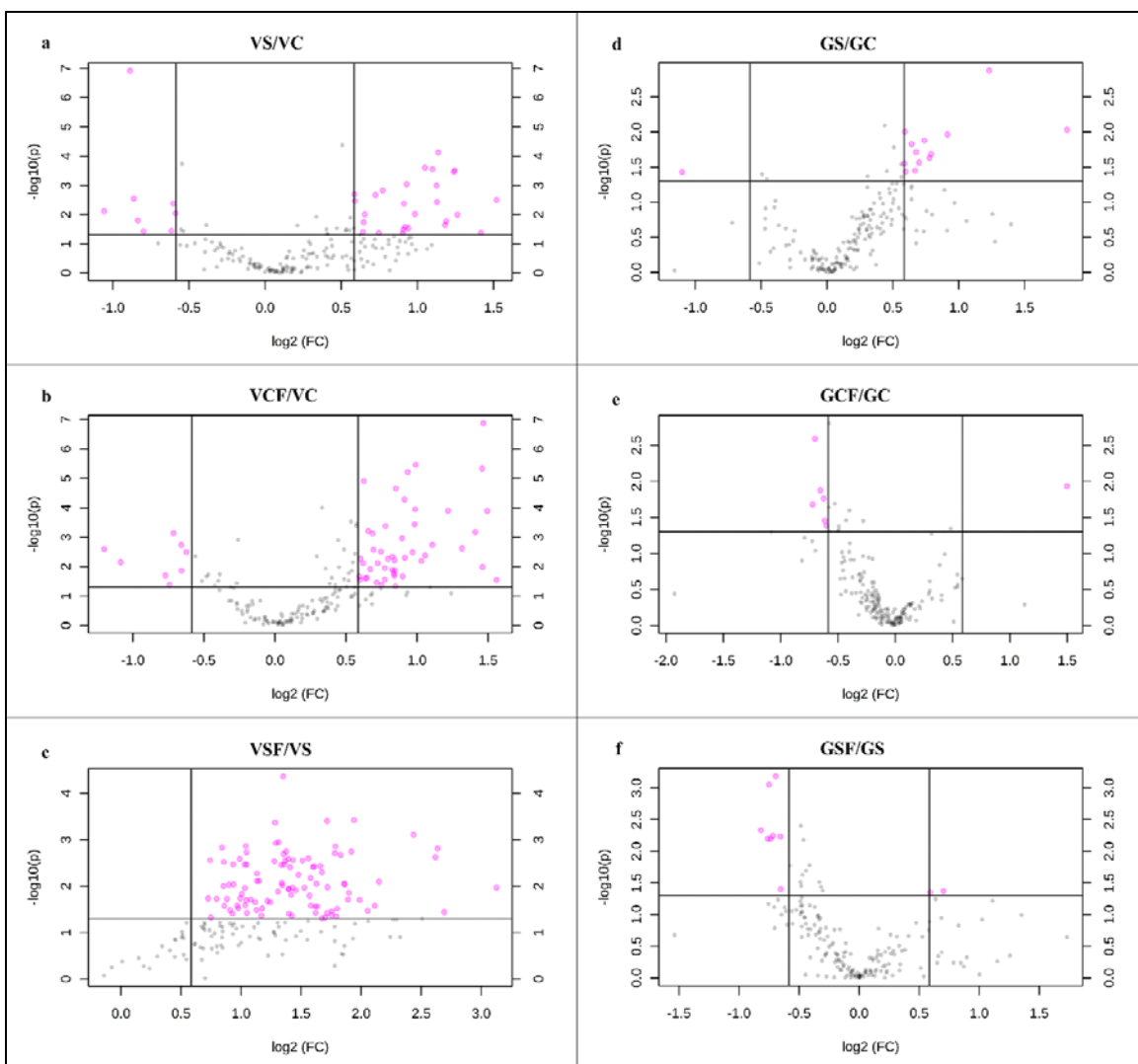


Figure 4.11 Volcano plots showing the three treatment group comparisons from roots of two barley genotypes, cv. Vlamingh and cv. Gairdner grown under control and saline conditions, with and without fungus. VC- control treated cv. Vlamingh; VS- salt treated cv. Vlamingh; VCF- control and fungus treated cv. Vlamingh, VSF- salt and fungus treated cv. Vlamingh; GC- control treated cv. Gairdner; GS- salt treated cv. Gairdner; GCF- control and fungus treated cv. Gairdner, GSF- salt and fungus treated cv. Gairdner. The horizontal axis plots the fold change between the two groups (on a log scale), while the vertical axis represents the $P < 0.05$ for a t-test of differences between samples (on a negative log scale). The black dots represent non-significant lipids whereas pink dots represent significantly changed lipids.

Table 4.2 gives the directionality on changes of lipid species as identified using volcano plots, in three treatment group comparisons in cv. Vlamingh and cv. Gairdner, respectively. When salt (S) treatment was compared to control (C) in cv. Vlamingh, it was found that 14 lipid species increased by +1.5-fold or more with $P < 0.05$ and in cv. Gairdner, 13 lipid species increased by +1.5-fold or more. On the other hand, 15 lipid species decreased by -1.5-fold in cv. Vlamingh and

only one lipid species decreased by -1.5-fold in cv. Gairdner when salt treatment was compared to control. When treatment control + fungus (CF) was compared to control (C), 47 lipid species increased with +1.5-fold in cv. Vlamingh and only one lipid species increased in cv. Gairdner with +1.5-fold. It was also found that eight lipid species decreased with -1.5-fold in cv. Vlamingh and six lipid species decreased in cv. Gairdner when CF was compared to C. It is worth noting that 102 lipid species increased with +1.5-fold in cv. Vlamingh and only two increased in cv. Gairdner when salt+ fungus (SF) was compared to salt (S). However, none decreased in cv. Vlamingh and eight decreased in cv. Gairdner when the same comparison was performed. Detailed fold changes of all identified lipid species is given in **Supplementary Table S14** for the three treatment group comparisons in both genotypes.

Table 4.2 Number of significantly altered lipid species ($P < 0.05$) with ≥ 1.5 fold-changes in relative abundance between the three treatment group comparisons for two barley genotypes.

Treatment specific		Number of lipid species increased or decreased by ≥ 1.5 -fold	
		Vlamingh	Gairdner
Salt vs Control	Higher	14	13
	Lower	15	1
Control + fungus vs Control	Higher	47	1
	Lower	8	6
Salt + fungus vs Salt	Higher	102	2
	Lower	0	8

Similar to results from **Chapter 3**, the largest number of lipid species that changed significantly in both genotypes were members of the Glycerolipids (GLs) and Glycerophospholipids (GPs) classes. Therefore, a more rigorous statistical analysis based on pairwise comparisons between the four treatments in both genotypes was carried out for GPs and GLs in order to adequately understand the directionality of the changes in the identified features. In the following paragraphs, lipid nomenclature follows the “Comprehensive Classification System for Lipids” given by the International Lipid Classification and Nomenclature Committee (ILCNC). For example, the nomenclature PC (38:n) indicates a PC species with a fatty acyl sum composition of 38 carbons containing all identified double bonds in this study which is given as “n”. The information on the number of double bonds in each individual lipid species can be found in Supplementary data (**Supplementary Table S14**).

4.3.7.1 Salt stress induced changes in cv. Vlamingh and cv. Gairdner

The effect of NaCl as compared to controlled conditions showed more significant changes in cv. Vlamingh as compared to cv. Gairdner. The order of significance was considered for lipid species

with ± 1.5 -fold or greater and with $P < 0.05$. It was found that nine lipid species (LPE 20:2, PC 32:3, PC 38:n, PC 41:7, PE 36:2, PG 36:5, PS 34:3 and PS 42:4) from the GP class (**Figure 4.12a** and **Figure 4.13a**) and five lipid species (DAG 34:3, TAG 34:0, TAG 41:0, TAG 51:7 and TAG 56:9) from the GL class (**Figure 4.14a**) increased significantly in cv. Vlamingh. The fold changes were between +1.5-fold to +2.53-fold for GPs and between +1.5-fold to +2.11-fold for GLs in cv. Vlamingh. In cv. Gairdner, eight lipid species (LPC 15:0, LPC 27:0, LPC 28:0, LPE 20:2, PC 40:5, PE 37:4, PE 40:5 and PI 33:3) from the GP class (**Figure 4.12b** and **Figure 4.13b**) increased significantly and five lipid species (DAG 36:1, DAG 40:3, TAG 50:n and TAG 56:4) from the GL class (**Figure 4.14b**) ranging from +1.5-fold to +3.54-fold increase. Salt stress significantly reduced nine lipid species in cv. Vlamingh from the GP class ranging between -1.5-fold to -2.1-fold decrease, six of which were lysophospholipids (LPC 18:2, LPC 20:4, LPC 22:n, LPC 25:0, LPC 28:1 and LPE 20:2) (**Figure 4.12a** and **Figure 4.13a**). Three lipid species (DAG 36:5, TAG 50:0 and TAG 52:1) from the GL class also reduced in cv. Vlamingh after salt stress as compared to control treated roots (**Figure 4.14a**). On the other hand, only one lipid species, DAG 36:5, reduced significantly in cv. Gairdner with -2.14-fold after salt stress (**Figure 4.14b**).

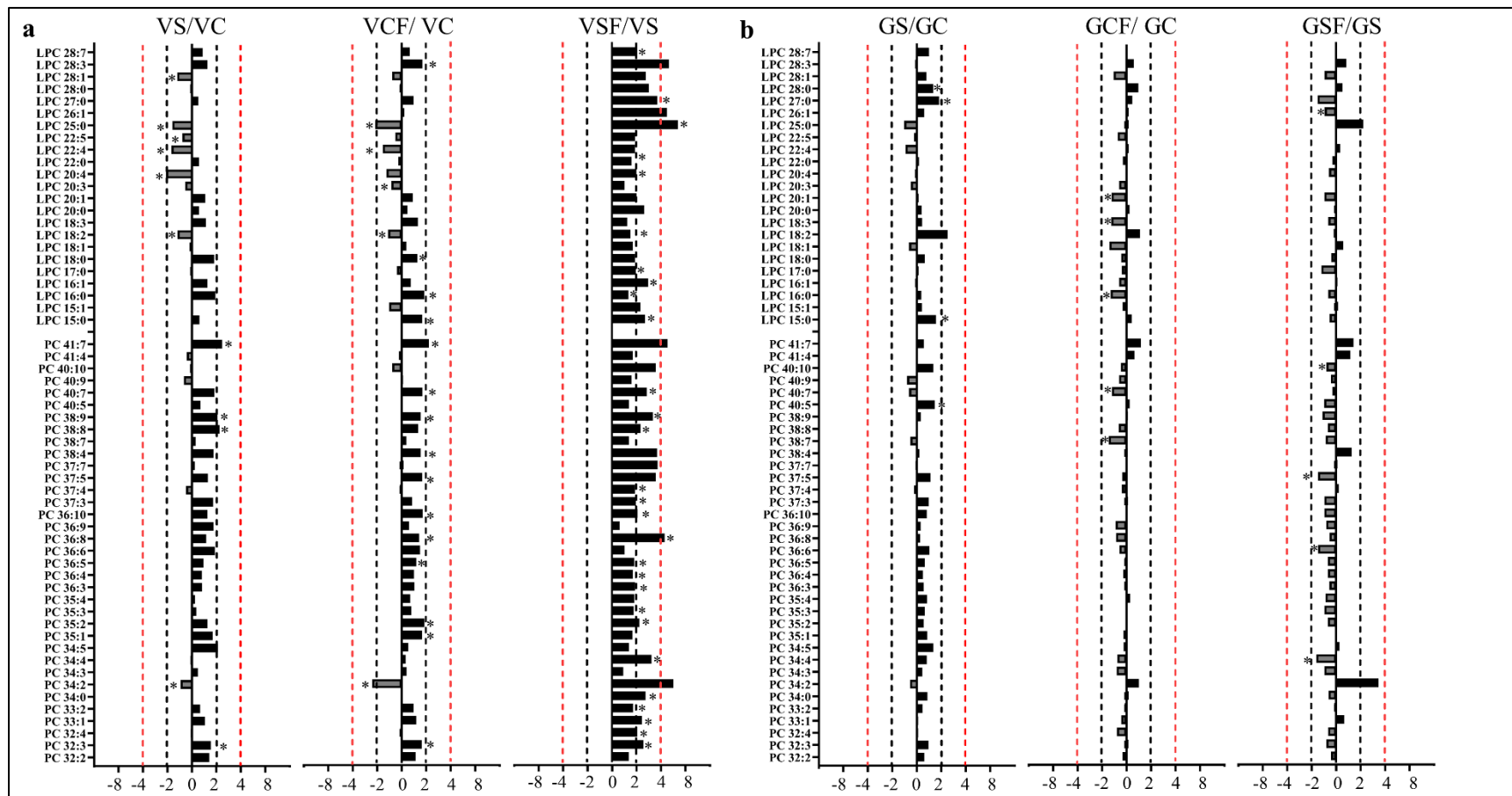


Figure 4.12 Logarithmic ratios of representative phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) content in roots of barley cv. Vlammingh and cv. Gairdner of salt grown (200 mM NaCl) compared to control grown; control with fungus grown compared to control grown; salt and fungus grown compared to salt grown. Values that are significantly different ($P < 0.05$) are indicated by *. A threshold of ± 2 -fold change is indicated by a black dashed line and a threshold of ± 4 -fold change is indicated by a red dashed line. $n = 6$ for all treatments. X-axis represents the geometric progression with common ratio 4.

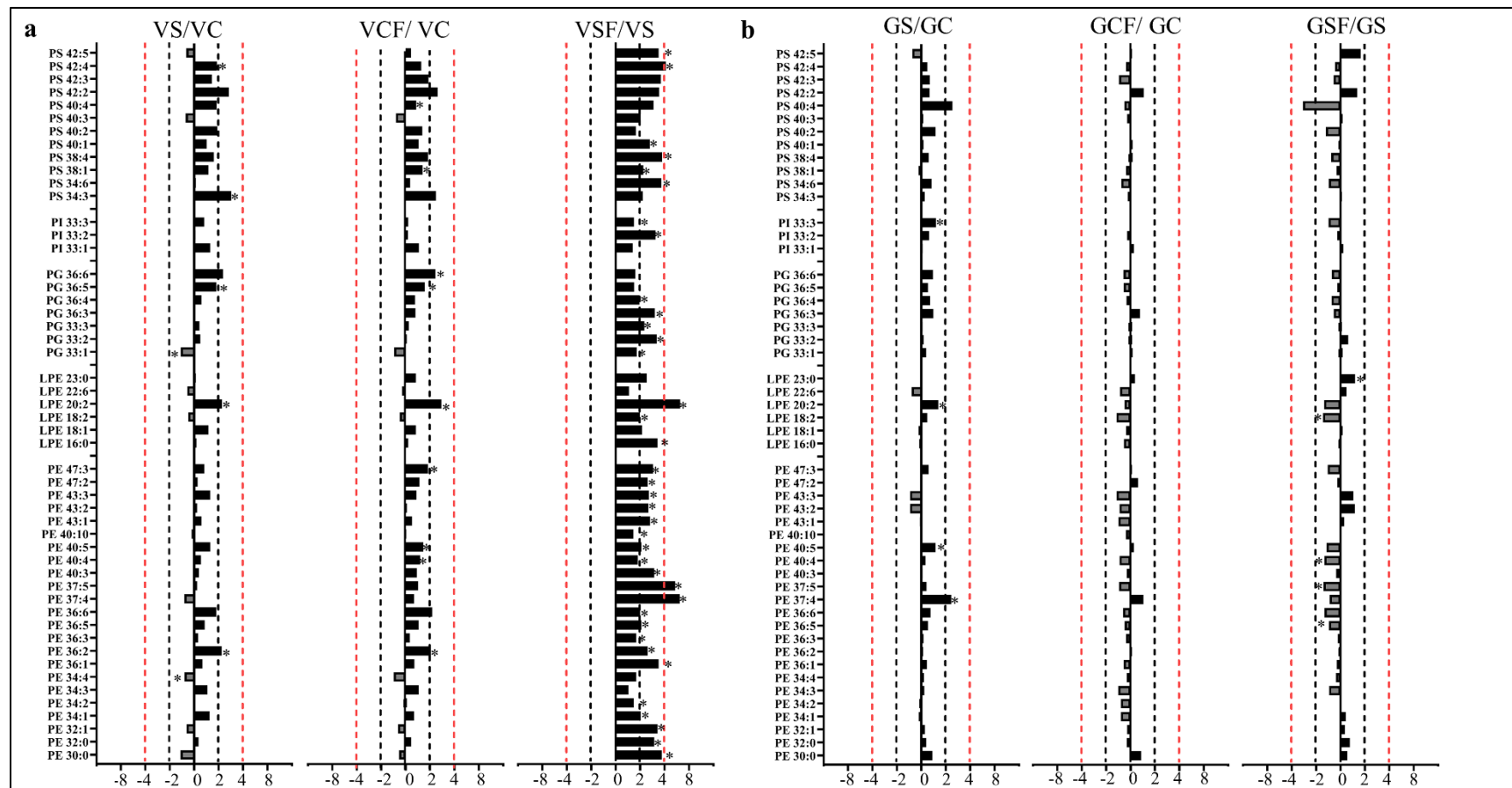


Figure 4.13 Logarithmic ratios of representative phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS) content in roots of barley cv. Vlammingh and cv. Gairdner of salt grown (200 mM NaCl) compared to control grown; control with fungus grown compared to control grown; salt and fungus grown compared to salt grown. Values that are significantly different ($P < 0.05$) are indicated by *. A threshold of ± 2 -fold change is indicated by a black dashed line and a threshold of ± 4 -fold change is indicated by a red dashed line. $n = 6$ for all treatments. X-axis represents the geometric progression with common ratio 4.

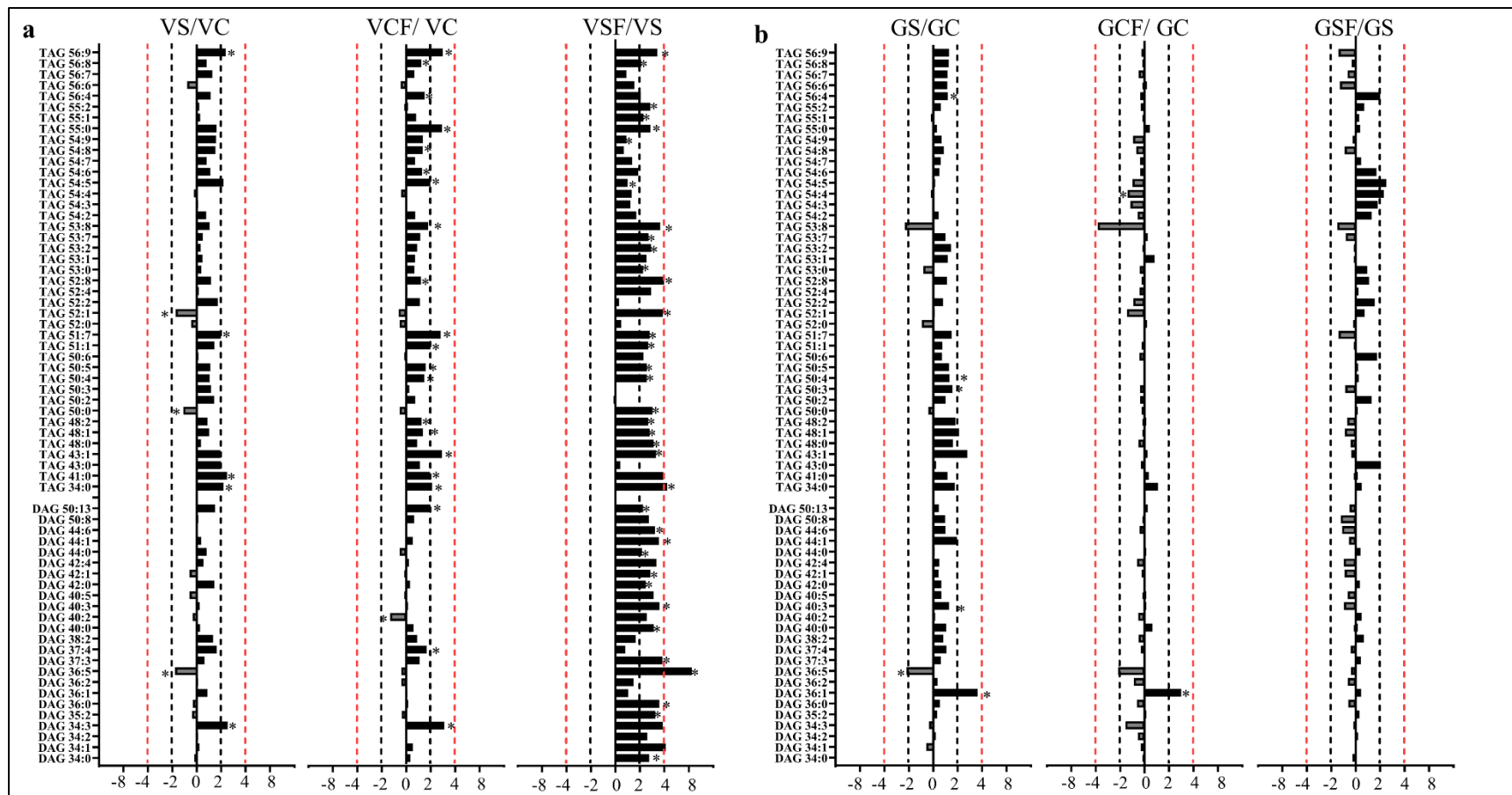


Figure 4.14 . Logarithmic ratios of representative diacylglycerol (DAG) and triacylglycerol (TAG) content in roots of barley cv. Vlamingsh and cv. Gairdner of salt grown (200 mM NaCl) compared to control grown; control with fungus grown compared to control grown; salt and fungus grown compared to salt grown. Values that are significantly different ($P < 0.05$) are indicated by *. A threshold of ± 2 -fold change is indicated by a black dashed line and a threshold of ± 4 -fold change is indicated by a red dashed line. $n = 6$ for all treatments. X-axis represents the geometric progression with common ratio 4.

4.3.7.2 The effect of fungus in roots of cv. Vlamingh and cv. Gairdner under control conditions

For this part, lipid species identified in the GP and GL classes were compared in inoculated roots to uninoculated roots to observe the effect of fungal inoculation in control conditions. The effect of inoculation of *T. harzianum* in roots of cv. Vlamingh significantly increased twenty-five lipid species from GP class showing fold change increases between +1.5-fold to +2.76-fold and twenty-two lipid species from the GL class under controlled conditions with increase in fold changes ranging between +1.5-fold to +3.0-fold (**Figure 4.12a** and **Figure 4.13a**). On the contrary, the fungus increased only one lipid species, DAG 36:1 (+2.82-fold), in roots of cv. Gairdner under control conditions (**Figure 4.14a**). Among the GP and GL classes, the fungus reduced four lipid species (DAG 40:2, LPC 20:4, LPC 22:4, LPC 25:0 and PC 34:2) in cv. Vlamingh under non-saline conditions with fold changes between -1.5-fold to -2.30-fold (**Figure 4.12a**, **Figure 4.13a** and **Figure 4.14a**) and in cv. Gairdner, the fungus reduced six lipid species (LPC 16:0, LPC 18:3, LPC 20:1, PC 38:7, PC 40:7 and TAG 54:4) from the GP and GL classes (**Figure 4.12b**, **Figure 4.13b** and **Figure 4.14b**). The decrease in fold changes in cv. Gairdner was between -1.5-fold to -1.63-fold for all significantly reduced lipid species.

4.3.7.3 Changes in lipids in roots of cv. Vlamingh and cv. Gairdner due to the inoculation of fungus under saline conditions

The inoculation of *T. harzianum* under salt stress caused changes in the root lipid profiles of both genotypes. However, contradictory to our results from **Chapter 3** where GPs and GLs were found to be significantly increased in inoculated roots of cv. Gairdner under salt stress, in soil study, cv. Vlamingh showed significantly increased changes in GP and GL class. Instead, in inoculated roots of cv. Gairdner, only two lipid species increased significantly. It is also interesting to note that the fungus caused reduction in only eight lipid species in cv. Gairdner under salt stress and zero significant reductions in cv. Vlamingh.

4.3.7.3.1 *T. harzianum* changed glycerophospholipids and glycerolipids under saline conditions

Under saline conditions, *T. harzianum* significantly increased 18 PCs from the 32:n, 34:n, 35:n, 36:n, 37:n and 38:n family with changes ranging between +1.5-fold to +4.43-fold in roots of cv. Vlamingh. The fungus also increased eight LPCs with fold changes from +1.5-fold to +6.5-fold in inoculated roots of cv. Vlamingh (**Figure 4.12a**). On the other hand, the fungus reduced four PCs (37:5, 34:4, 36:6 and 41:7) and one LPC (27:0) in inoculated roots of cv. Gairdner after salt stress (**Figure 4.12b**). The fold changes for PCs and LPC was between -1.5-fold to -1.76-fold in cv. Gairdner.

Like PCs, 21 PEs with fold changes ranging from +1.5-fold to +5.41-fold, and three LPE with fold changes between +1.5-fold to +6.22-fold were significantly increased in inoculated roots of cv.

Vlamingh under saline conditions. In addition to these, inoculated roots of cv. Vlamingh also increased five PGs (33:n and 36:n family), two PIs (33:n) and six PSs (34:6, 38:1, 38:4, 40:1, 42:4 and 42:5) under saline conditions (**Figure 4.13a**). The fold changes for PGs, PIs and PSs ranged between +1.5-fold to +4.16-fold. In inoculated roots of cv. Gairdner, three PEs (37:5, 40:4 and 36:6) and two LPEs (18:2 and 23:0) decreased significantly with fold changes between -1.5-fold to -1.6-fold decrease after salt stress (**Figure 4.13b**). No other GPs changed in inoculated roots of cv. Gairdner under saline conditions.

The inoculation of *T. harzianum* under saline conditions drastically affected glycerolipids as compared to uninoculated roots in cv. Vlamingh. It was found that thirteen DAGs ranging from the 36:n family to 44:0 family increased significantly, showing fold changes of between +1.5-fold to +8.74-fold. The inoculation of fungus also resulted in twenty-three increased TAGs with fold changes ranging from +1.5-fold to +1.33-fold in cv. Vlamingh after salt stress (**Figure 4.14a**). On the other hand, it is interesting to note that *T. harzianum* failed to show to any major changes in lipid species of GL class in roots of cv. Gairdner after salt stress except for few TAG species which were increased after salt stress.

4.4 Discussion

Trichoderma spp. are common inhabitants of the rhizosphere and are well recognized as elicitors to improve plant tolerance against abiotic stress, such as salinity, mineral nutrient deficiency and drought. They have been long recognised as agents for the control of plant disease and for their ability to increase plant growth and development. Research on several *Trichoderma* spp. has been performed proving their benefits to alleviate detrimental effects of abiotic stresses on plants. In the previous chapter, salt treatment of uninoculated seedlings caused a reduction in TRL that was larger for the sensitive cultivar Gairdner. However, seedlings inoculated with *T. harzianum* T-22 had TRL longer than control untreated seedlings and almost the same as CF seedlings suggesting that the fungus was able to mitigate the negative effects of NaCl. These results are similar to those for tomato treated with *T. harzianum* T-22 (Mastouri et al. (2012).

In this Chapter, various parameters were assessed to analyze the effect of fungal inoculation in both barley genotypes grown in sandy loam soil. Like other cereals, barley plants have a typical fibrous root system consisting of seminal roots and post embryonic nodal roots. Seminal root lengths were greatly reduced in both genotypes under salt stress. However, the reduction was greater in cv. Gairdner as compared to cv. Vlamingh. The results are in alignment with our glasshouse study where cv. Vlamingh grew better than cv. Gairdner under saline conditions. This study further confirms the ability of cv. Vlamingh to adapt physiologically to the early impact of salinity stress and confirms greater salt tolerance potential of cv. Vlamingh as compared to cv. Gairdner.

For plants grown in soil and inoculated with fungus, the protective effects of fungal inoculation were similar to those seen in the agar grown seedlings. Salt treatment of uninoculated seedlings caused a significant reduction in SRL, root and shoot dry weight and chlorophyll content. In the same salt treatment, these parameters for seedlings inoculated with *T. harzianum* T-22 were equivalent to or better than those for untreated control seedlings and in most cases very similar to those for CF seedlings. In this experiment the effects of salt on cv. Gairdner were more marked than for cv. Vlamingh but the result of inoculation with fungus were similar.

Cultivar cv. Gairdner with fungal inoculation showed increase in seminal root length as compared to cv. Vlamingh under controlled and saline conditions. The patterns of growth and effects of the fungus were similar in both agar plates and soil. Though the preference of the fungus to colonize one genotype over the other needs to be further validated, it can be proposed that the use of *T. harzianum* T-22 in salinity affected crops may assist in reducing the effects of salt stress. Salt stress decreased root to shoot ratio in cv. Vlamingh. There was also a slight reduction in seminal root length in salt stressed seedlings as compared to control seedlings and overall root biomass was also reduced significantly. This suggests that while cell elongation was maintained,

the roots were possibly exhibiting reduced radial expansion. Similar results were observed in maize and barley in other studies (Lemcoff et al. 2006; Sharp et al. 1988; Sheldon et al. 2013). In contrast, root to shoot ratio in cv. Gairdner under saline conditions was almost similar to control seedlings. This may be attributed to the reduction in above ground while maintaining the below ground biomass (determined by root and shoot fresh and dry weight measurements in salt compared to no salt treatments). This characteristic favors the retention of toxic ions in roots, thereby controlling their translocation to the aerial parts. This response is a typical mechanism of plant resistance under saline conditions (Cassaniti et al. 2009; Cassaniti et al. 2012).

In CF, root to shoot ratio was observed to be higher in cv. Gairdner as compared to cv. Vlamingh. This was mainly due to the increase in root biomass which is supported by the higher seminal root length in cv. Gairdner in fungal treatment compared to cv. Vlamingh. In treatment SF as compared to S, it is worth mentioning that although there was an increase in seminal root length in cv. Gairdner as compared to cv. Vlamingh, root to shoot ratio decreased in the former genotype. It is possible that the fungus promoted the production of nodal and lateral roots under salt stress in the sensitive genotype post inoculation. This could be an adaptive mechanism of the sensitive genotype for better nutrient and water absorption needed for resistance against salt stress. From the above results, it is clear that salt treatment has an effect on root and shoot weight (reducing it) but growth with the fungus restores growth close to or better than normal confirming the positive role of the fungus to help plants cope with the adverse effects of salt stress.

Damage to the chloroplast is an important aspect in the effect of salinization on leaf cells. Salinity reduced the chlorophyll content in both genotypes in this study. Ashraf and Foolad (2005) and Roy and Srivastava (2000) reported the reduction in chlorophyll contents in rice and wheat under saline conditions to be associated with disturbances in membrane stability. Khan et al. (2009) also reported a significant decrease in sensitive genotypes of wheat in comparison to tolerant genotypes, which is aligned with results obtained in this study where the sensitive genotype cv. Gairdner had lower chlorophyll content than the tolerant genotype cv. Vlamingh under salt stress.

In treatment CF, a higher chlorophyll content was found in both genotypes when compared to C suggesting increased photosynthetic capacity in these plants. The photosynthetic capacity and carbon production directly relate to growth parameters. This is also supported by the findings of greater biomass in both genotypes in fungal inoculated seedlings. In treatment SF when compared to S, chlorophyll content was higher in both genotypes. Presumably, fungal association with plants improves the plant's capacity of gas exchange and the efficiency of photochemistry and non-photochemistry of photosystem II. Similar results were observed in soybean plants

(Khan et al. 2011) and Bermudagrass (Xie et al. 2017) inoculated with an endophyte under saline conditions.

Further, the goal of the study was to characterise the response of barley roots to salt stress at the metabolite level. An untargeted metabolomics approach using GC-MS analysis was adopted to identify metabolites responsive to salt stress. Global analysis of the GC-MS dataset using PCA indicated clustering between biological replicates proving good reproducibility and reliable detection by the method for subsequent analysis and interpretation. A total of 94 metabolites, of which 75 were identified, were detected and analysed in roots of both barley genotypes (cv. Vlamingh and cv. Gairdner).

Three comparisons were performed; (i) to understand the effect of salt stress in roots of both genotypes, (ii) to measure the beneficial effects of the fungus in roots of both genotypes under control conditions, and (iii) to determine the changes in polar metabolites brought about by the fungus in roots under saline conditions.

4.4.1 Changes in osmolytes in roots of cv. Vlamingh and cv. Gairdner under salt stress

Upon exposure to saline environments, plants undergo a reduction in water absorbing capacity from the soil which disrupts cell water relations and inhibits cell expansion. In order to negate these effects, plants employ osmoregulation as a mechanism to tolerate salt stress (Munns and Tester 2008). This is achieved by accumulation of osmolytes which are found in four main classes of compatible solutes in plant cells. These are N-containing compounds (i.e. proline), sugars (i.e. sucrose and raffinose), straight-chain polyhydric polyols such as mannitol and sorbitol, and cyclic polyhydric alcohols (Chen and Murata 2002).

The accumulation or decrease of these osmolytes in response to salt stress differ in conservation and divergence among species or genotypes within the same species (Gong et al. 2005; Sanchez et al. 2008). In this study, cv. Vlamingh is salt-tolerant whereas cv. Gairdner is less tolerant to salinity. Interestingly, under salt stress, cv. Gairdner has higher levels of raffinose, lactose, gulose and organic acids and higher levels of amines and amino acids such as isoleucine, serine, tyramine, alanine and asparagine as compared to cv. Vlamingh when normalised to fresh weight. These metabolites are commonly considered as compatible solutes, which are involved in osmotic adjustment, protecting membranes from the damage by reactive oxygen species (ROS) (Wang et al. 2008). Higher contents of these metabolites in cv. Gairdner suggest a greater need for stabilising the cell membrane and protoplast in the more sensitive genotype. Similar results were shown by (Cao et al. 2017) where varying changes in sugar metabolism after salinity stress were detected for several barley genotypes with differing salt tolerance levels.

Proline is the only amino acid which accumulated in higher contents in cv. Vlamingh as well as in cv. Gairdner. The physiological significance of proline accumulation is controversial, while some researchers have reported that it is a sign of stress (Hernandez et al. 2000b; Rai et al. 2003), others suggest that it acts as solute intercellular osmotic adjustment when found in high concentration (Kaur and Asthir 2015; Silveira et al. 2003). In addition to acting as an osmoprotectant, proline has been reported to have antioxidant properties and to act as a molecular chaperone to protect the structure of biological macromolecules during dehydration, thereby conferring tolerance to environmental stresses (Ashraf et al. 2007; Tang et al. 2015). In this study, proline content increased by more than +4-fold in both genotypes. Similar substantial increases of proline contents were reported by (Widodo et al. 2009), (Chen et al. 2007) and (Wu et al. 2013).

4.4.2 Changes in polar metabolites in inoculated roots of cv. Vlamingh and cv. Gairdner under non-saline conditions

Trichoderma harzianum T-22 inoculation caused more changes in organic acid profiles in cv. Vlamingh as compared to cv. Gairdner under control conditions. It has been reported that *Trichoderma* spp. promote nutrient uptake by secreting organic acids to dissolve minerals and activate nutrients in the soil, leading to the circulation and utilization of nutrients in the soil. A preference for particular genotypes is yet to be explored but it might be assumed that *T. harzianum* has strong ability to colonize cv. Vlamingh as compared to cv. Gairdner in sandy loam soil under normal conditions.

Amino acids are precursors of several secondary metabolites. The elevated levels of amino acids in both genotypes suggest that the fungus enhanced activity of enzymes involved in secondary metabolite synthesis helping plants growth in a more efficient manner. Amino acids are also the building blocks of proteins. The protein content is influenced by genetics, environment, nitrogen (N) fertilization and application of plant growth promoting microbes, including fungi (Akladios and Abbas 2012). Thus, it seems that the fungus may potentially enhance the N accumulation in both barley genotypes and subsequently the amino acid composition. Similar results were obtained by Mishra et al. (2018) where *Trichoderma* spp. increased the relative abundance of amino acids in chickpeas.

4.4.3 Changes in polar metabolites in inoculated roots of cv. Vlamingh and cv. Gairdner after salt stress

Under saline conditions, the fungus appears to activate two different mechanisms in the two different genotypes to alleviate the effects of salt stress. A significant increase in relative abundances of identified osmolytes was observed in inoculated cv. Vlamingh and an opposite trend was seen in cv. Gairdner. It is widely accepted that osmolytes, including soluble sugars, are

important indicators in response to abiotic stress and the increased accumulation of glucose and sucrose usually indicates a highly protective mechanism against oxidative damage caused by high salinity. The fungus protects cv. Vlamingh by accumulating higher content of sugars when compared to uninoculated seedlings after salt stress. However, in cv. Gairdner, it can be presumed that the fungus helps in translocation of osmolytes in the leaves, to mitigate the effects of salt stress. This is also supported by our physiological results where shoot dry weight and chlorophyll content was increased in inoculated seedlings following salt stress, though further confirmation to measure shoot metabolite content is needed to validate these results.

Maeda et al. (2015) reported that *Trichoderma* spp. can decompose nitrogen compounds into available nitrogen and release less NO₂. In this study, cv. Vlamingh showed significant increase in contents of amino acids suggesting the role of the fungus to convert nutrients into effective bioavailable nutrients to increase circulation in the soil, enabling the reduction of nitrogen fertilizer applications (Harman 2011). However, an opposite trend was observed in inoculated roots of cv. Gairdner where a significant decrease in the content of amino acids was observed. This is in alignment with previous research in the field where Li et al. (2017b) found that *Aspergillus aculeatus* inoculated roots of ryegrass plants showed decreased amino acid concentrations following salt stress. Here we can speculate that the fungus may enhance the capacity of cv. Gairdner to withstand salt stress through coordinating with amino acid homeostasis exerting a protective mechanism. Amino acids can be taken up directly by the roots and are transported between different organs through the xylem and phloem. These are essential for plant growth. In this study, fungus causes significant reduction in amino acid levels which can indicate their contribution to growth in cv. Gairdner under saline conditions. Proline was also significantly reduced following salt stress in inoculated roots of cv. Gairdner as opposed to what was seen in salt treated uninoculated roots. It has been suggested that proline can act as a stress marker and thus its decline may indicate that the toxic effects of salinity are reduced following colonization of fungus. Similar results were reported by Abdelaziz et al. (2019) where the authors attributed the decrease in proline to the decrease in abscisic acid (ABA) levels. ABA controls proline biosynthesis (Verslues and Bray 2005) to regulate the potential cytoplasmic osmotic stress caused by increasing NaCl in the root zone (Cao et al. 2014).

Organic acids play vital roles in lowering soil electrical conductivity and increasing the availability of N, P and K in soil (Sheng et al. 2011). They are important osmolytes in plant vacuoles, and the regulation of their metabolism plays an important role in providing tolerance to salt stress (Guo et al. 2010). Fungal endophytes have been reported to induce the release of organic compounds by the roots (Yang et al. 2015; Zhang et al. 2014), thus influencing the concentrations and profile of organic acids in plants. One of the major plant nutritional disorders associated with increased salinity in soil is iron (Fe) deficiency. Endophytes can enhance Fe acquisition by their host through

their ability to secrete organic acids which chelate and solubilise iron in the soil (Chen et al. 1998; Khan et al. 2006). A study by Zhao et al. (2014a) demonstrated that the release of organic acids from endophytes, resulted in ferric solubilization to form organic ferric salts that can be assimilated directly by plants under saline conditions. It has also been shown that fungal inoculated plants have better nutrient uptake capacity and distribution within plant tissues due to modulation of the root architecture and nutrient availability in the soil. These benefits are imparted by increases in organic acids produced by endophytic colonized plants (Samolski et al. 2012; Zhao et al. 2014a). In this study, the contents of organic acids increased significantly in cv. Vlamingh and a decrease in content of organic acids in inoculated roots of cv. Gairdner was observed. Very little information on the role of fungus in changing organic acids profile in salt stressed plants is available in literature and thus, this calls for further investigation.

4.4.4 Changes in the lipid profiles in response to salt stress

To elucidate the role of lipids in adaptive mechanisms of inoculated and uninoculated roots of both genotypes after short-term salt stress, untargeted lipidome analyses were performed on roots of both genotypes. The untargeted data suggests a strong genetic impact onto the root lipidome based on the barley genotype which overrides the effect of salt or the fungus on the root lipids measured. Here a moderate alteration of root lipidome was triggered by genetic variation between the two genotypes under the different treatment scenarios. Several interesting lipid classes and specific lipid species were discovered in the responses of roots to salinity stress, and the potential roles of these lipids in mediating tissue tolerance upon salinity stress in cv. Vlamingh and cv. Gairdner are further discussed.

Several studies have reported alterations in plant membrane lipids in the presence of NaCl (Guo et al. 2019a; Sarabia et al. 2019; Yu et al. 2020). Plant membrane lipids belonging to three major groups: sphingolipids, glycerophospholipids, and glycerolipids (Borrell et al. 2016) were identified in roots of both genotypes under control and saline conditions. Membrane glycerolipids have glycerol backbones with two fatty acid molecules bound to sn-1 and sn-2 and either a phosphorous (phospholipid) or sugar (glycolipid) molecule at position sn-3 (Kalisch et al. 2016). According to their molecular structure, glycerolipids can be divided into cylindrical shape bilayer forming lipids (e.g., PC, PA, PI, PG, PS, DGDG) and cone-shape non-bilayer forming lipids (e.g., PE, MGDG). Bilayer lipids are involved in maintaining the stability of membranes, whereas non-bilayer lipids are important for mediating proteolipid interactions and increasing morphological plasticity of lipid bilayers (Frolov et al. 2011). Among the diacyl membrane glycerolipids with phosphorus as the headgroup on the glycerol backbone, phosphatidylcholine (PC); phosphatidylethanolamine (PE); phosphatidylglycerol (PG); phosphatidylinositol (PI) and phosphatidylserine (PS) were identified in this study. On the other hand, due low abundance of some lipid classes found in the root samples or their presence majorly in thylakoid membranes,

glycolipids including monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG), were not detected.

Studies focusing on the salt-induced changes in glycerolipids have ignored the importance of neutral lipids. However, recent evidence has shed light on the role of di- and tri-glycerides (DAGs and TAGs) in plant stress tolerance. These studies have proposed that neutral lipids accumulation in vegetative tissues and roots can serve as an energy reservoir under stress conditions (Hernández et al. 2012; Hung et al. 2016; Legeret et al. 2016; Li et al. 2010; Moellering et al. 2010). Several neutral lipids such as DAGs and TAGs were identified in this study and their potential roles are further discussed.

4.4.5 Salt stress resulted in higher changes in membrane phospholipids in cv. Vlamingh as compared to cv. Gairdner

Lipid peroxidation is the most ascribed symptom to oxidative damage, and it is often used as a marker of oxidative stress (Hernandez et al. 2000a; Khan and Panda 2008; Yu et al. 2020). The results reported in this study showed that NaCl treatment led to a significant increase in membrane phospholipids in cv. Vlamingh as compared to cv. Gairdner. Membrane phospholipids serve as structural and signalling molecules in plant cells (Furt et al. 2011; Xue et al. 2009) and reports have shown alterations in lipid levels in a number of plant species subjected to salinity stress. A study on the callus culture of the halophyte *Spartina patens* (Wu et al. 2005), epidermal bladder cells of the halophyte *M. crystallinum* (Barkla et al. 2018), roots of salt-tolerant *Plantago* cultivars (ERDEI et al. 1980) and salt-acclimated tomato calli (Kerkeb et al. 2001) showed similar results where levels of membrane phospholipids elevated in response to salt stress.

Among the identified diacyl membrane glycerolipids, the highest increased lipid subclass was PCs in cv. Vlamingh. Phosphatidylcholine (PC) is one of the most abundant phospholipids in extraplastidic membranes and is also a precursor of glycerolipids that play key roles in maintenance of membrane structure and function (Tasseva et al. 2004). Thus, increases in PC could also be due to net production of PC from DAG through DP-choline:diacylglycerol cholinephosphotransferase (CPT) as explained in detail in **Chapter 3, Figure 3.14**. Further, a study using *Arabidopsis thaliana* L. reported increase in PC concentrations after exposure to salt and cold stress, which was suggested to be an important membrane-mediated cell signalling mechanism (Pical et al. 1999; Tasseva et al. 2004). In support of the positive role of PC lipids in salt tolerance, the addition of choline, a key substrate for PC biosynthesis, also showed improved salt tolerance in wheat as reported by (Mansour et al. 2002). In contrast, decreases in PC species have been observed in more salt-sensitive plants such as maize, oats and wheat (Magdy et al. 1994; Norberg et al. 1991; Salama and Mansour 2015; Salama et al. 2007); this may suggest that

a plant's ability to maintain PC levels is an adaptive mechanism for salt tolerance as shown in barley roots (Sarabia et al. 2019).

Unlike PC, which can be synthesized via alternative pathways, PS is only produced from decarboxylation of PE, so the changes to PS in plants exposed to salinity stress should largely rely on phosphatidylserine synthase (PSS) and phosphatidylserine decarboxylase (PSD). However, to the best of our knowledge, no studies have investigated this pathway under salt stress. The only evidence of increased activity of PSS and non-regulated PSD was observed in root cells of oat during drought acclimation, where it was shown that the levels of PE are increased relative to PC resulting in increased drought tolerance by increasing the fluidity of the membrane (Larsson et al. 2006). No changes in the fold changes of PE levels in cv. Vlamingh were found in this study following salt stress which would support the presumption that increase in PS could be due to decarboxylation of PE. Upregulation of PS was also found in root plasma membrane of salt-treated wheat (Salama and Mansour 2015) and callus culture of *S. patens* (Wu et al. 2005). Another study by Lin and Wu (1996) reported that PS was increased in salt tolerant buffalo grass while sensitive buffalo grass cultivars displayed a significant reduction in PI and PS levels. Similar results were found in this study where the salt-tolerant genotype cv. Vlamingh showed increases in PS whereas the salt-sensitive genotype cv. Gairdner showed decrease in PS levels after salt stress. It was assumed by (Lin and Wu 1996) that the root plasma membrane of the buffalo grass would have higher affinity for Ca^{2+} due to the higher amounts of negatively charged PS and as a second messenger in stress signalling, Ca^{2+} can alleviate the negative effects of NaCl by activating Na^+ efflux (Zhu 2002).

4.4.6 Changes in membrane phospholipids in inoculated roots of both genotypes under control and saline conditions

In order to penetrate the root tissue, the endophyte uses a variety of cellulolytic and ligninolytic enzymes such as oxidases, cellulases and laccases to break down the cellulose, hemicellulose, pectin and lignin in the plant cell wall. Increased biomass in inoculated roots of cv. Vlamingh compared to uninoculated roots suggests the successful penetration of fungus into the roots and therefore, it can be assumed as the reason for increased accumulation of membrane lipids under controlled conditions. Glycerophospholipids are major components of cellular membranes and they play important roles in various cellular functions including signal transduction, vesicle trafficking and membrane fluidity (Hishikawa et al. 2014). Increase in GPs in inoculated roots of cv. Vlamingh suggest that the fungus increases membrane fluidity to move inside the membranes of root tissue. Similar results were shown by Wei et al. (2016) where *Ericoid* mycorrhizal (ERM) fungi *Oidiodendron maius* var. increased expression of genes involved in glycerophospholipid metabolism in inoculated roots of *Rhododendron fortunei* Lindl though the actual levels of these lipids were not determined. On the other hand, in cv. Gairdner, no change was observed in

identified GPs species under controlled conditions. Therefore, it can be assumed that other macromolecules are responsible for increasing the biomass and growth of cv. Gairdner under controlled conditions.

A plethora of studies have reported that *Trichoderma* spp. enhances tolerance to salt stress during plant growth, in part due to improved root growth, improvement in water-holding capacity of plants, or enhancement in nutrient uptake (Brotman et al. 2013; Contreras-Cornejo et al. 2014; Gangwar and Singh 2018; Lei and Zhang 2015; Mohamed and Haggag 2006; Rawat et al. 2011; Yasmeen and Siddiqui 2018). Since lipid peroxidation is associated with cellular membrane damage elicited by salinity stress, the rate of lipid peroxidation can be used to characterize how effectively *T. harzianum*- treated plants cope with salt stress. Significant increases in highly unsaturated PC species were observed in cv. Vlamingh following salt stress suggesting the role of the endophyte in maintaining membrane structure and function as a response to salt stress in the tolerant genotype cv. Vlamingh. Our findings of a significant increase in biomass as observed in inoculated roots of cv. Vlamingh under salt stress compared to uninoculated roots also align with these results confirming the positive role of the fungus in imparting salt tolerance.

In contrast, the inoculated roots showed either reduced or no change in membrane phospholipids in cv. Gairdner after salt stress. Decreases in PC species have been observed in more salt-sensitive plants such as oats, wheat and barley (Magdy et al. 1994; Norberg et al. 1991), suggesting a plant's ability to maintain PC levels as an adaptive mechanism for salt tolerance. Therefore, it can be suggested that the fungus imparts salt tolerance to the sensitive genotype by maintaining the levels of membrane phospholipids under saline conditions. Another study by Magdy et al. (1994) showed that decreases in PC species resulted in enriched non-lamellar-forming domains, a more rigid membrane and phase separation of non-lamellar-forming lipids in salt-sensitive barley. However, they also observed increased free sterols that counterbalanced the formation of non-bilayer structures. Since free sterols were not measured in this study, the function of PCs cannot be ascertained. Thus, further analysis is required to confirm the role of PC in inoculated roots of cv. Gairdner.

4.4.7 Changes in membrane neutral lipids after salt stress

The diacylglycerol (DAG) content of the plant cell is low, but its presence is necessary for certain developmental processes and the response to environmental stimuli. DAG is rapidly converted to PA in plant cells, but some reports have suggested that the decrease in DAG content is not accompanied by any increase in that of PA. DAG is also known to be an intermediate in the synthesis of membrane lipids and is involved in phospholipid signalling in plant cells (Dong et al. 2012).

Plants can utilize at least two metabolic pathways to produce different molecular species of the immediate precursor to TAG, diacylglycerol (DAG): (1) *De novo* DAG synthesis (termed as DAG1 here), and (2) conversion of the membrane lipid phosphatidylcholine (PC) to DAG (termed as DAG2 here) (**Chapter 3, Figure 3.14 and Figure 3.15**).

In this study, it can be postulated that in cv. Vlamingh, DAG, and subsequent TAG, production was through PC-derived DAG/TAG synthesis as there was an increase in PC species under saline conditions. The pathway of PC-derived DAG2 synthesis starts with the production of PC lipids from *de novo* DAG1. Here, PC is synthesized through CDP-choline:diacylglycerol cholinephosphotransferase (CPT) (Li-Beisson et al. 2013) leading to a net production of PC lipids from DAGs. Further, phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) (Lu et al. 2009) activity transfers the phosphocholine head group from PCs to DAGs generating new molecular species of DAGs and PCs, which does not lead to net accumulation of PCs or DAGs. This supports the current findings where only one DAG (34:3) increased after salt stress in cv. Vlamingh. Once PC lipids are formed from *de novo* DAG1 by either CPT or PDCT, the FAs esterified to PCs are available for FA modification and acyl editing (Bates et al. 2009; Bates et al. 2007; Li-Beisson et al. 2013) to generate new molecular PC species. And the DAG substrate for TAG synthesis is subsequently derived from PCs by removal of the phosphocholine headgroup (Bates 2012). This eventually results in TAG production.

In contrast, DAG and subsequent TAG production in cv. Gairdner can be presumed through utilization of the products of FA synthesis exported from the plastid directly for *de novo* DAG/TAG synthesis through the Kennedy pathway (Weiss and Kennedy 1956; Weiss et al. 1960) since there was no significant increase in PC species in cv. Gairdner. These results align with the findings from Chapter 3 where both genotypes showed similar results for membrane neutral lipids after salt stress.

4.4.8 Changes in membrane neutral lipids in inoculated roots of both genotypes under control and saline conditions

The amount of neutral lipids (for energy storage) is usually higher than that of phospholipids (membrane constituents) in endophytic fungi, since these fungi store a large proportion of their energy carbon as neutral lipids (Olsson and Johansen 2000). Amongst neutral lipids, TAGs are the main type of neutral lipids found in large amounts in endophytic fungal spores and hyphae (Beilby and Kidby 1980; Cooper and Losel 1978; Nagy et al. 1980). The intraradical mycelium takes up hexoses from the host, which are metabolized to yield neutral lipids (Pfeffer et al. 1999). Another study by Serrano-Carreón et al. (1992) also showed that *T. harzianum* accumulated more neutral lipids than *T. viride*. Neutral lipids thus have a central role in carbon metabolism and transport in endophytic fungi and are probably the main respiratory substrate in the extraradical mycelium (Bago et al. 2000).

T. harzianum did not result in significant changes in DAGs found in cv. Vlamingh under controlled conditions. However, a significant increase in TAGs in inoculated roots of cv. Vlamingh was observed. This suggests that the fungus synthesizes fatty acids de novo and incorporate into TAGs through the Kennedy pathway. Conversely, there was no significant changes in the levels of DAGs and TAGs in cv. Gairdner under controlled conditions. The preference of fungus to change the neutral lipids in varying genotype needs to be further investigated. Since this study was first of its kind to investigate the changes in lipidome of roots subjected to control and saline conditions with and without fungus, many questions remain unanswered and therefore, this calls for future studies in the field to further explore the potential changes in lipids of both genotypes brought about by the inoculation of fungus.

Under saline conditions, significantly higher accumulation of DAGs and TAGs were observed in cv. Vlamingh. Similar results were observed by Mueller et al. (2015) where salt stress triggered accumulation of TAGs in Arabidopsis seedlings. Higher accumulation of TAGs may reflect lipid remodelling, i.e. channelling of fatty acids from constitutive membrane lipids to TAGs. Further, as TAGs predominantly contain polyunsaturated fatty acids, it is likely that the fungus helps in the release of polyunsaturated fatty acids from structural lipids and use for transient assembly of TAGs during membrane remodelling. On the other hand, the fungus caused no significant changes in roots of cv. Gairdner after salt stress with few increases in TAG species.

Since there was a significant increase in the biomass of both genotypes inoculated with the fungus under saline conditions, the study calls for further detailed investigation on lipids in negative ionization mode, especially in cv. Gairdner.

4.5 Conclusion

This study investigated the role of *Trichoderma harzianum* T-22 on two barley genotypes, cv. Vlamingh and cv. Gairdner, based on their known differences in germination phenology and salinity tolerance. Various physiological parameters were measured as biomass production is an important trait to breed productive plant species in various types of abiotic stress conditions to optimize production practices. From physiological results, it is clear that salt treatment has an effect on root and shoot length and weight (reducing it) and also on chlorophyll content but, the fungus restores growth close to or better than normal confirming the positive role of the fungus to help plants cope with the adverse effects of salt stress. Further, to investigate the metabolite and lipid changes in roots of both genotypes grown in soil, untargeted GC-MS and LC-MS approaches were used. To the best of our knowledge, no previous studies have closely looked at the plant lipidome inoculated with the fungus under saline conditions. Thus, this study provides evidence of the molecular adaptations enacted by metabolites and lipid species in barley roots from genotypes with differing levels of salinity tolerance under these conditions. cv. Vlamingh

displayed significantly high increase of polar metabolites and lipids within roots inoculated with fungus under saline conditions and an opposite pattern was observed in cv. Gairdner. This points to these findings as potential targets for engineering of salt tolerant crops, especially TAGs with highly unsaturated fatty acids.

Based on the physiological results, the fungus proves beneficial to both genotypes under control and saline conditions and the results have given insights into the changes in both genotypes based on the identified metabolites and lipids. However, several questions remain unanswered. Due to time limitations, only positive ionization mode analysis was performed on lipids which could detect GP species such as PCs, PEs. However, negatively charged species like PIs and PSs could not be detected and thus future studies must focus on determining the role of those lipids in plant-fungal interaction.

Chapter 5
Spatio-Temporal Metabolite and Elemental Profiling of Salt Stressed Barley
Seeds During Initial Stages of Germination by MALDI-MSI and μ -XRF
Spectrometry

Spatio-Temporal Metabolite and Elemental Profiling of Salt Stressed Barley Seeds During Initial Stages of Germination by MALDI-MSI and μ -XRF Spectrometry

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Abstract

Seed germination is the essential first step in crop establishment and can be severely affected by salinity stress which can inhibit essential metabolic processes during the germination process. Salt stress during seed germination can trigger lipid-dependent signalling cascades that activate plant adaptation processes, lead to changes in membrane fluidity to help resist the stress, and cause secondary metabolite responses due to increased oxidative stress. In germinating barley (*Hordeum vulgare* L), knowledge of the changes in spatial distribution of lipids and other small molecules at a cellular level in response to salt stress is limited. In this study, mass spectrometry imaging (MSI), liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QToF-MS), inductively coupled plasma mass spectrometry (ICP-MS), and X-ray fluorescence (XRF) were used to determine the spatial distribution of metabolites, lipids and a range of elements, such as K^+ and Na^+ , in seeds of two barley genotypes with contrasting germination phenology (Australian barley varieties Mundah and Keel). We detected and tentatively identified more than 200 lipid species belonging to seven major lipid classes (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, prenol lipids, sterol lipids and polyketides) that differed in their spatial distribution based on genotype (Mundah or Keel), time post-imbibition (0 to 72 hours), or treatment (control or salt). We found a tentative flavonoid was discriminant in post-imbibed Mundah embryos under saline conditions, and a delayed flavonoid response in Keel relative to Mundah. We further employed MSI-MS/MS and LC-QToF-MS/MS to explore the identity of the discriminant flavonoid and study the temporal pattern in five additional barley genotypes. ICP-MS was used to quantify the elemental composition of both Mundah and Keel seeds, showing a significant increase in Na^+ in salt treated samples. Spatial mapping of elements using μ -XRF localised the elements within the seeds. This study integrates data obtained from three mass spectrometry platforms together with μ -XRF to yield information on the localization of lipids, metabolites and elements improving our understanding of the germination process under salt stress at a molecular level.

5.1 Introduction

Barley is a model organism for investigating the cereal germination process (Gorzolka et al. 2016) which commences with the uptake of water by the quiescent dry seed and finishes with the emergence of the radicle through the seed coat. Seed germination requires sufficient moisture and is also affected by temperature. Salinity affects normal seed germination through osmotic stress (Bliss et al. 1986), ion toxicity (Hampson and Simpson 1990) or a combination of both (Huang and Steveninck 1988). High intracellular concentrations of both Na^+ and Cl^- can inhibit the metabolism of dividing and expanding cells (Neumann 1997), restrict mobilization, and hinder seedling emergence (Alencar et al. 2015; Marques et al. 2013). These conditions result in retarded (Zhang et al. 2010) or delayed (Ashraf et al. 2003) germination. Barley genotypes can be classified as either tolerant or sensitive to salt stress depending upon their genetic diversity and the ability to germinate and, survive under these conditions (Shelden et al. 2016; Shelden and Roessner 2013; Shelden et al. 2013). The molecular changes in barley seed during germination that may play a role in tolerance or sensitivity are important in determining a plant's overall response to salinity.

Mature seeds contain a variety of compounds such as proteins, carbohydrates, lipids, vitamins and phenolics. These compounds provide essential nutrients for the seed to germinate and develop into a mature plant, and are synthesized, packaged and stored in specific tissues that are chemically and morphologically distinct from each other (Fulcher 1982). For example, a reserve of carbohydrates (starch) is found in the endosperm of cereal seed whereas a reserve of lipids and proteins is found in the scutellum.

Lipids are vital and abundant cellular constituents responsible for the structure and function of cell membranes, and act as an energy store to allow metabolism to continue during abiotic stress. Recent research has shown that lipids are involved as signal mediators in the initiation of defence reactions (Sarabia et al. 2018). Lipids are also involved in processes to mitigate the effects of stress in plant cells (Hasanuzzaman et al. 2013; Okazaki and Saito 2014) for example through remodelling of glycerolipid levels to maintain membrane integrity and optimal fluidity (Sarabia et al. 2018). Plants also produce a vast array of secondary metabolites such as carotenoids, flavonoids and coumestans (Gry et al. 2007). These compounds have long been known to protect plants from different biotic and abiotic stresses, function as signal molecules and act as antimicrobial agents (Bartwal et al. 2013).

Although changes in profiles of protein (Yang et al. 2007), transcripts (Nakabayashi et al. 2005; Reyes and Chua 2007) and metabolites (Fait et al. 2006; Howell et al. 2009) during germination are well documented, there is currently little information on molecular changes during seed germination in response to abiotic stress including salinity. With the current changes in the global

climate (Karl et al. 2009), understanding these responses to stress will be more important than ever. Information on how an embryo mobilizes its internal reserves during the early stages of germination in saline conditions can provide insights into the metabolic process of germination; and consequently help us understand how certain crop genotypes germinate better under adverse environmental conditions (Achakzai 2014).

To understand the role and function of metabolites and lipids within an organism at the tissue level, spatial information about target compounds is required (Li et al. 2008). Many studies analyze whole tissue responses (Cevallos-Cevallos et al. 2009; Fiehn 2002; Lee et al. 2010; Sumner et al. 2003; Weckwerth 2003), and so information about the localization of small molecules in cell types within the tissue is lost.

Mass Spectrometry Imaging (MSI) provides a platform to detect and localise intra-tissue variation and the spatial distribution of metabolites at the cellular and sub-cellular levels at high resolution (Boughton et al. 2016; Lee et al. 2012). MSI enables visualization of the distribution of individual biomolecules in a tissue section without requiring staining or complicated pretreatment (Cornett et al. 2007). This method of direct analysis of a tissue section (Zaima et al. 2009) allows detection of a wide range of endogenous molecules such as lipids, peptides and secondary metabolites (Boughton et al. 2016; Hansen et al. 2018; Heskes et al. 2018). In plants, MSI has been used to target small molecules in different plant species and tissues. Recent examples include *Arabidopsis* leaves (Shroff et al. 2008), *Medicago* roots (Ye et al. 2013), wheat seeds and stems (Burrell et al. 2006; Robinson et al. 2007), soya leaves (Mullen et al. 2005), rice seeds (Zaima et al. 2010a), different tissues of *Eucalyptus* species (Hansen et al. 2018; dos Santos et al. 2019), and barley roots (Sarabia et al. 2018). Novel metabolites were discovered using Matrix Assisted Laser Desorption Ionization – Mass Spectrometry Imaging (MALDI-MSI) (Gorzolka et al. 2014), further highlighting the benefit of obtaining spatially resolved information.

In this study, we used a combination of four high-end analytical techniques- MALDI-MSI, liquid chromatography-tandem mass Spectrometry (LC-QToF-MS/MS), inductively coupled plasma mass spectrometry (ICP-MS), and micro X-ray fluorescence (μ -XRF), to give new insights into the metabolism of metabolites including lipids, as well as elements including Na^+ and K^+ . These analytical platforms were used to analyse early stages of germination in two barley genotypes that differ in their germination phenology (Mundah-early germinating and Keel-late germinating) under salt stress. Barley was chosen as it is not only an agriculturally and industrially important crop, but also exhibits a typical cereal germination process, allowing the results to be applied to other cereal crops.

This combined approach provides unique insights into the spatially resolved coordination of metabolic processes that are sequestered among the germinating embryo axis, the lipid rich scutellum, the nutritive endosperm, the digestive enzyme secreting aleurone and the outer pericarp cell layers during the early stages of seed germination. LC-QToF-MS provided information on the distribution of a specific flavonoid in five additional barley genotypes to determine potential roles in response to salt stress. On-tissue analysis using MSI-MS/MS further confirmed the identity of a tentative flavonoid resulting in nine fragment ions. Techniques such as ICP-MS and μ -XRF were used to provide data on element levels that are dynamically mobilized during barley seed germination.

5.2 Material and methods

5.2.1. Plant material

Seven varieties of barley were selected based on their importance for crop production, commercially relevant traits, and known difference in germination phenology and salinity tolerance (Kamboj et al. 2015; Sarabia et al. 2018; Tavakkoli et al. 2012; Yu et al. 2018). This selection comprised of two Australian barley feed (Mundah, Keel), one Australian food (Hindmarsh), and three malting cultivars (Gairdner, Vlamingh, and Clipper), as well as one North African landrace (Sahara). All seeds were sourced from The University of Adelaide, Australia.

5.2.2. Seed germination and sample collection

The germination of seven barley varieties was compared under normal and saline conditions. Twenty seeds from each variety were sterilized in 70% ethanol for 1 minute. They were then rinsed 4-5 times in sterile 18.2 Ω deionised water before being treated with 1.0% (v/v) bleach for 10 minutes, followed by thorough washing in sterile 18.2 Ω deionised water 6-7 times. Seeds were then imbibed overnight (~16 hours) in sterile 18.2 Ω deionised water with continuous aeration before being transferred to 1% agar plates containing a range of salt (NaCl) concentrations: 0 mM (control), 50 mM, 100 mM, 125 mM, 150 mM, 200 mM and 250 mM. The plates were then sealed with parafilm, wrapped in aluminium foil and kept at 4°C for 48 hours before being transferred to a growth chamber maintained at 17°C constant temperature with no light. Plates were scored for the number of seeds germinating at different times after transfer to the growth chamber (48, 72, 96 and 120 h post-imbibition). The germination percentage was calculated using the following equation: Germination (%) = $G/X \times 100$, where, G = number of seeds germinated and X = number of seeds sown.

Seeds from barley varieties with the highest and lowest germination frequencies under salinity, respectively, were used in a second experiment to produce germinating seeds for MALDI-MSI, ICP-MS, and μ -XRF. The seeds were sterilized as described above. After 16 hours of overnight aeration, seeds were harvested for the 0 h time point (0h) and the remaining seeds were

aseptically transferred to petri dishes with and without saline agar. These dishes were kept in the dark at 4°C for 48 hours and then transferred into a growth chamber maintained at a constant temperature of 17°C with no light. Germinating seeds were harvested at 8, 16, 24, 48 or 72 h growth during this time so that seeds at different developmental stages could be analysed. These seeds were immediately frozen in Super Cryo Embedding Medium (SCEM, Section Lab) using liquid nitrogen and stored at -80°C into 50 mL Conical Centrifuge Tubes (Thermo Fisher Scientific, USA) until subjected to cryosectioning.

5.2.3. Chemicals

Solvents were purchased from Merck Millipore (Bayswater, VIC, Australia), Chemicals including 2,5-dihydroxy benzoic acid (DHB), elemental red phosphorus and Supra pure[®] nitric acid (70%) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Embedding and freezing supplies including a cryofilm fitting tool set (2.0 cm), embedding container (1.5cm × 2.0 cm), embedding medium (SCEM), and cryofilm 2C(9) (2.0 cm in width), were purchased from Section-Lab Co. Ltd. (Tokyo, Japan). Sectioning supplies including Menzel-Gläser Superfrost Ultra Plus Glass slides, Optimal Cutting Temperature (O.C.T.) compound and Feather[®] C35 tungsten microtome blades were purchased from Grate HDS (Ringwood, Australia). Lysing Matrix Tubes with 0.5 g Lysing Matrix D (1.4 mm ceramic spheres) were purchased from MP Biomedicals (Seven Hills, NSW, Australia). Elemental standards were purchased from PerkinElmer (Melbourne, VIC, Australia). Aqua Regia (concentrated hydrochloric acid and concentrated nitric acid in the ratio 4:1) was prepared fresh immediately prior to use.

5.2.4. MALDI-MSI: Sample preparation and imaging

For MALDI-MSI, samples from six time points during germination (**Supplementary Figure 7-1**) of the two barley varieties were analysed. Prior to sectioning, frozen seed samples were kept in a Reichert Jung 2800 FRIGOCUT M cryostat at -20°C for 30 min. Later, the frozen sample was attached to a cold specimen disk using Optimal Cutting Temperature (OCT) compound and trimmed with a C35 tungsten microtome blade (Feather[®]). Longitudinal sections of 14 µm were stabilized with cryofilm 2C(9) (Section Lab, Japan), attached to a glass slide with electrically conducting double-sided tape according to the Kawamoto's film method with slight modifications (Boughton et al. 2016; Kawamoto 2003; Zaima et al. 2010b), and immediately freeze dried in a Christ Alpha 1-4 LD plus Freeze Drier (John Morris Scientific, Australia) under vacuum (1.0 mbar) for 20 minutes. This step was important in order to eliminate water from seed sections and also to facilitate their manipulation and storage at room temperature before further analysis. The sections were chosen from the centre of the seed so the embryo, endosperm and aleurone layer were included in all sections. In order to account for biological variation present, at least three different seeds from each time point and treatment were analyzed. These were treated as technical and biological replicates.

2,5-dihydroxy benzoic acid (DHB) was used as sublimation matrix for positive mode analyses. For this study, the optimal conditions were 0.11 Torr pressure during sublimation, a hot plate temperature of 140°C, 300 mg DHB as matrix added to the sublimator, and a sublimation period of 4 minutes.

MS imaging of seed sections was performed on a Bruker Solarix 7T hybrid ESI-MALDI-FT-ICR mass spectrometer equipped with a Smartbeam II laser (Nd:YAG laser 355 nm) operating at 2 kHz, using the “minimum” focus setting and smart walk enabled (Bruker, Bremen, Germany). For every run, laser fluence was optimized to obtain the best signal-to-noise (S/N) ratio. The MS was operated in the positive ionisation mode across the mass range m/z 150–2,800. MSI measurements were acquired with a raster of 75 × 75 μm . The measurement of the spots was carried out in random order to eliminate influences of measurement order. An external calibration (quadratic equation) was carried out using elemental red phosphorus obtaining a minimum of seven points of calibration over the mass range. A mass accuracy better than 4 ppm was obtained across a tissue section image. Co-registration between each MSI data set and its optical image was performed with flexImaging software v.4.1 (Bruker Daltonics, Bremen, Germany).

MS/MS spectra of selected ions, previously identified through SCiLS Lab (SCiLS Lab 2017a, SCiLS GmbH) analysis were conducted in positive mode using a laser power of 40%. A total of 5,000 laser shots per sample were fired randomly across the relevant tissue region. The isolation window was set to 5 m/z with the collision RF amplitude set to 1200.0 Vpp and collision voltage set to 20V.

5.2.5. MALDI-MSI: Analysis, data processing, detection and annotations

The raw MALDI-MSI datasets were analyzed using flexImaging software v.4.1 (Bruker Daltonics, Bremen, Germany) and normalized to the Root Mean Square (RMS). Root mean square is a popular normalization method used for normalisation in MALDI-MSI, where each spectrum is divided by the square root of the mean of all squared intensity values. Any m/z peaks that showed spatial distribution across the tissue sections were manually annotated and used to create a list of spatially distributed potential mass features. The generated list was further analyzed to identify protonated ($[M+H]^+$), sodiated ($[M+Na]^+$) and potassiated ($[M+K]^+$) adducts of the mass features that displayed spatial distribution prior to identification of potential metabolites using accurate mass search against the LIPID MAPS (Fahy et al. 2007) databases by accurate precursor mass (< 5 ppm). Further average MS spectra were also exported from flexImaging and imported into SimLipid v.5.50 (Premier Biosoft International, Palo Alto, CA, USA) for accurate mass annotation. Tentative annotations were performed to level two based on accurate mass match

and spectral libraries (Sumner et al. 2007). All potential lipid and metabolites detected with multiple adducts were collapsed into single ID for identification and analysis purposes.

Annotated m/z features were imported into SCiLS Lab (SCiLS Lab 2017a, SCiLS GmbH; (<http://scils.de/>; Bremen, Germany) software from flexImaging and further used to identify discriminant features between control sections and treatments. Sub regions were created to analyze discriminant lipids in embryo and endosperm regions. Normalization of data was performed according to the Total Ion Count (TIC) of all spectra prior to data analysis. Next, discriminative m/z values were identified from all individual spectra with a minimal interval width of ± 5 mDa between control and salt-treated sections. A computer-generated diagram known as a Receiver Operating Characteristic (ROC) curve was used to quantify how well the m/z values discriminated between two treatments (control and salt). Strict ROC Area Under the Curve (AUC) values of ≥ 0.8 and ≤ 0.2 were set as an additional criterion to the Student's t-test p-value of < 0.005 for peaks considered as statistically significant for salt vs control and control vs salt respectively (Rauser et al. 2010). The Intensity Box Plot chart was generated which depicts intensities of a given m/z interval filtered by the visible regions through their quartiles.

Peak lists were then exported and reimported into flexImaging to verify and confirm the spatial distribution of identified discriminant peaks. After analysis, a curated list of spatially distributed peaks was generated for control vs salt-treated images. For MS/MS analysis, raw data was analysed on Bruker Compass DataAnalysis v5.0 using the deconvolution and manual annotation tools.

5.2.6. ICP-MS: Sample preparation and analysis

Dry seeds were ground to a fine powder using a Tube Mill control (IKA Mills, Selangor, Malaysia) at 25,000 rpm ($30500 \times g$) for 4 min. The ground samples were accurately weighed (20 mg) into 10 mL Falcon tubes (Sarstedt, Australia). These samples were then digested in 300 μ L acid solution (Aqua Regia) by heating for 1.5 hours at 80°C. After digestion and cooling to room temperature, deionized water was added to make the final volume to 10 mL. The tubes were then centrifuged at 5,000 rpm ($6,100 \times g$) for 5 min. The supernatant was used for ICP-MS analysis.

A modified method from Callahan et al. (2016) was used for this study. An ICP-MS instrument (NexION 350X, PerkinElmer, USA) was used to measure the concentration of the following elements: P, S, K, Ca, Mg, Mn, Co, Ni, Cu, Zn and Fe. The internal standards Sc (200 ppb) and Rh (20 ppb) in 1% Aqua Regia were used for correction. The internal standard was mixed with the sample stream via a T-piece in a 1:1 ratio giving a final acid concentration of 2% at the source. Two sets of calibration standards were prepared. Set 1 contained P, Na, K and Ca at 500, 1,000,

and 5,000 ppb. The second set contained the other elements at 0.1, 1, 10, 50, 100, 500 ppb. The mass spectrometer was operated in kinetic energy discrimination mode (KED) with 50 ms dwell times, 20 sweeps, one reading and three replicates. The plasma source conditions were: nebulizer gas flow 1.02 L min⁻¹, auxiliary gas flow 1.2 L min⁻¹, plasma gas flow 15 L min⁻¹, ICP RF power 1,500 W.

Data were processed using Syngistix (PerkinElmer) software. Signal responses were normalized to the rhodium (Rh) internal standard, sample weights, and dilution factors.

5.2.7. μ -X-ray fluorescence analysis

An M4 Tornado μ -XRF spectrometer (Bruker, Bremen, Germany) equipped with a Rh anode side window X-ray tube with a polycapillary lens was used to obtain elemental distribution maps in this study. The step size was set to 40 μ m (for whole seed) combined with high excitation intensity. Direct analysis on seed sections prepared similarly as described in **Section 5.2.4** (without matrix application). This was achieved by placing the sections on the μ -XRF platform under 20 mbar vacuum, 50 kV voltage and an anode current of 199 μ A conditions (Sarabia et al. 2018). Detailed summary on the instrumental conditions and operating parameters used to analyze barley seeds in this study is given in **(Supplementary Table S1-refer to the hyperlink given in Chapter 7)**. Spectra acquisition and 2D elemental maps were obtained using the Esprit software (Bruker, Berlin, Germany).

5.2.8. LC-QToF-MS: Sample preparation and analysis for flavonoid detection

Seeds were germinated and harvested as described above (**Section 5.2.2**) followed by immediate freeze drying in a Christ Alpha 1-4 LD plus Freeze Drier (John Morris Scientific, Australia) under vacuum (1.0 mbar) for 48 hours. This facilitated fine powdering of seeds prior to extraction. A modified method from (Routaboul et al. 2006) was used for extracting flavonoids. Briefly, flavonoid extracts were obtained from 30 mg dried seed powder. Powder was transferred into cryo-mill tubes along with 1 mL acetonitrile/water (75:25; v/v) containing the following internal standards, 13C6-Sorbitol (0.02 mg/mL) and 13C5-15N-Valine (0.02 mg/mL). The samples were homogenized using a cryomill (Bertin Technologies; 6,000 rpm (7320 $\times$ g) for 3 repetitions at 45 seconds with a 45 second interval between replicates) at -10°C. The mixture was then sonicated for 20 mins followed by centrifugation at 13,000 rpm (15,900 $\times$ g) for 10 mins at room temperature. The supernatant was separated and transferred into a fresh 2mL tube. The remaining pellet was further extracted with 1 mL acetonitrile/water (75:25; v/v) on a thermomixer, shaking overnight (500 rpm, 4°C). The solution was centrifuged, and the two extracts were pooled, dried in a SpeedVac (Martin Christ Gefriertrocknungsanlagen GmbH, Germany) without heating.

Dried flavonoid extracts were resuspended in 200 μ L acetonitrile/water (75:25; v/v), and 5 μ L (for MS analysis) or 10 μ L (for MS/MS analysis) aliquots were injected onto a Poroshell 120, HILIC-Z, 2.1 $\times$ 100 mm (2.7 μ m particle size) column (Agilent Technologies, Santa Clara, CA, USA) at 25°C, and analysed on a LC system coupled to an Agilent Technologies (Santa Clara, CA, USA) 6545B ESI-QToF-MS. Flavonoids were eluted at 0.250 mL/min over 18 mins with a binary gradient of ACN-water (90:10, v/v) containing 20 mM ammonium formate and formic acid and water containing 20 mM ammonium formate and formic acid as described by Routaboul et al. (2006). MS data were acquired with an ESI source in positive ion mode. The MS data presented corresponds to ten pooled biological replicates for each treatment group. MS/MS was performed to obtain the MS2 spectra of the identified compound.

5.2.9. LC-QToF-MS: Data processing and analysis

LC-MS data was processed using MassHunter Qualitative Analysis and MassHunter Quantitative Workstation v.B.08.00 software (Agilent Technologies, Santa Clara, CA, USA) for flavonoid identification. Peak spacing tolerance was set to 0.5 m/z with a retention time window of 0.1 sec. The acquired data was exported and normalised with sample weight and log transformed. Univariate statistical analysis tests (Student's t-test) was carried out using Minitab software. X-fold changes were determined between control and treated samples of seven genotypes.

5.3 Results

5.3.1. Overview of experimental workflow

A schematic of the experimental workflow is provided in Figure 1. MALDI-MSI analysis was performed for seeds grown under control and saline conditions and harvested at six different time points, namely 0, 8, 16, 24, 48, and 72 hours post imbibition (**Supplementary Figure 7-1**). Sublimation of sectioned tissue with DHB matrix was used for MALDI-MSI positive mode ionisation to image the spatial distribution of a wide range of lipids (m/z 150–2800) in the seed sections. ICP-MS (for total elemental analysis) and μ -XRF spectrometry (to obtain the spatial information of targeted elements) was also performed for three time points (0, 24, and 48 hours post imbibition).

5.3.2. Germination assay: Germination and seedling growth under salt stress

In the initial germination assay, seeds from seven barley genotypes (Vlamingh, Mundah, Clipper, Sahara, Hindmarsh, Gairdner and Keel) were germinated in petri dishes containing different salt concentrations (0–250mM NaCl) and observed for germination from 0–120 hours post imbibition (**Supplementary Table S2-refer to the hyperlink given in Chapter 7**). Seeds were considered to be germinated once emergence of the cotyledon was visible. There was a clear difference in the germination frequency of the seeds germinating in the presence of salt. Keel showed the greatest inhibition (only 65% germinated at highest salt concentration) while all Mundah and Vlamingh

seeds germinated. Other genotypes showed germination frequencies between 75 and 90%. The most tolerant genotypes were viable under saline conditions for at least 7 days. Based on this assay, two Australian barley feed varieties were chosen to investigate the metabolic changes that occur during germination under salinity: Mundah, a very early spring variety from Western Australia with very high early vigour (highest germination rate under salinity), and Keel, an early spring variety from South Australia with moderate early vigour (lowest germination rate under salinity) (Long et al. 2003).

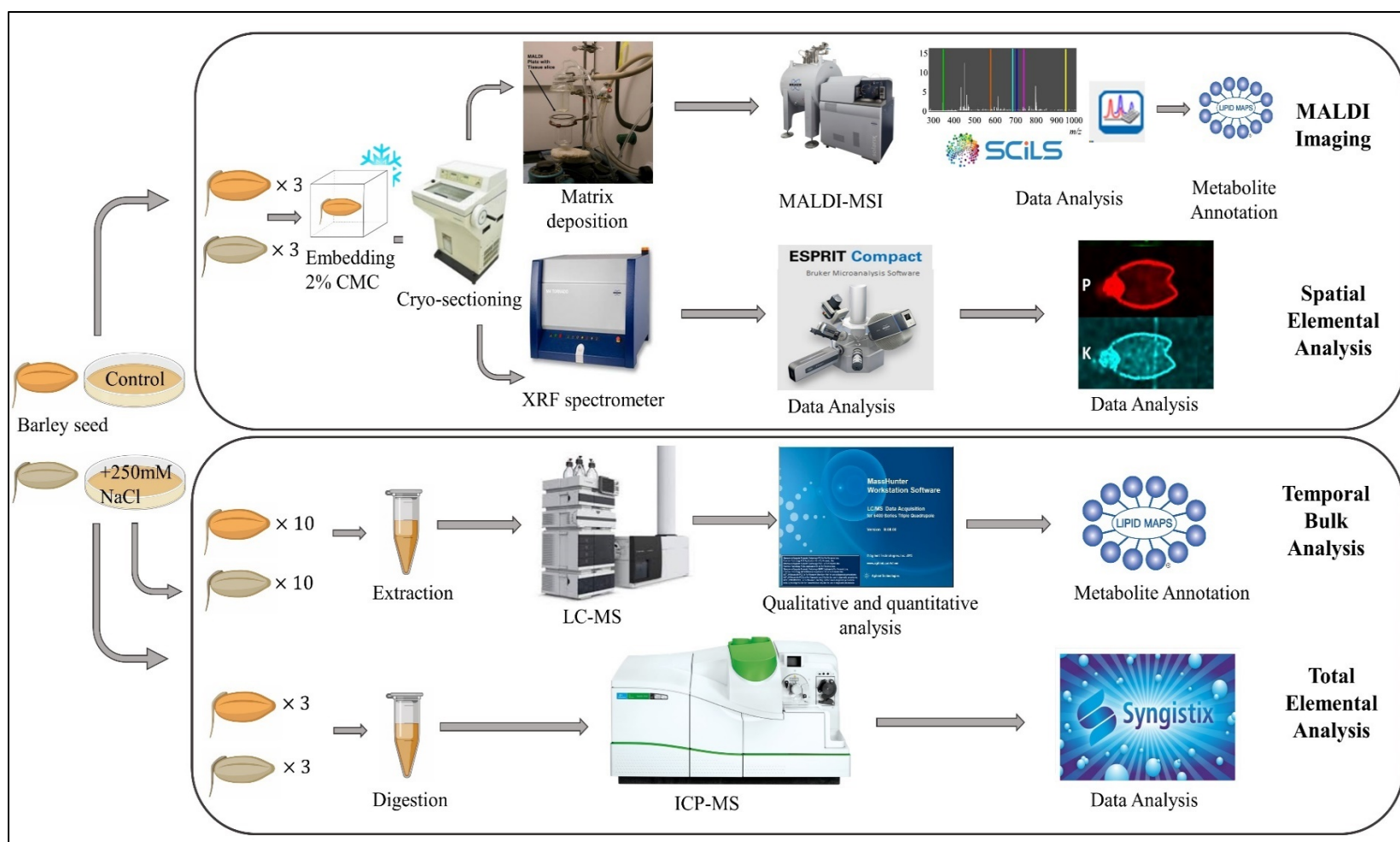


Figure 5.1 Overall workflow for MALDI-MSI, ICP-MS and LC-MS analysis of barley seeds. Figure modified from (Sarabia et al. 2018).

5.3.3. MALDI-MSI of lipids in barley seeds during germination

To identify lipids and other small molecules, the distribution of these compounds at all post imbibition time points in control and saline conditions were compared in both genotypes. Lipids within longitudinal sections of mature barley seeds were profiled by MALDI-MSI at six time points post imbibition (**Supplementary Figure 7-1**). Signals derived from the DHB matrix were excluded from further analyses. A total of 774 peaks in the average MS spectra were found to have a differential spatial distribution in the samples. An example of an average mass spectrum between m/z 780-900 acquired from a Mundah seed after 24 hours post imbibition is presented in **Figure 5.2**, illustrating a specific peak that can be selected to display the distribution of a corresponding lipid across the section. Many low abundance lipids were not reproducibly detected across all sections due to analytical and biological variations and thus, only the presence and tissue distribution of the tentatively identified lipids observed across three biological replicates are discussed (**Supplementary Figure 7-2**).

All peaks showing spatial distribution in the seed sections were selected from flexImaging (**Supplementary Table S3A-D-refer to the hyperlink given in Chapter 7**). Mass precursor ion search (<5 ppm) of the LIPID MAPS database (Fahy et al. 2007) was performed to provide tentative annotations of the features detected and resulted in between 246 to 319 tentatively assigned mass features for each sample. Some of these mass features corresponded to $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ ion adducts of the same lipid and were observed in all samples. Two hundred and sixty-nine lipid species were identified in both the Mundah control and salt treated seeds. Three hundred and nineteen lipid species were identified in Keel control seeds but only two hundred and forty-six in salt treated Keel seeds. Lipids were annotated to the sum level only using the Liebisch notation (Liebisch et al. 2013). In-source fragmentation of more complex lipid species, such as Phosphatidylcholine (PC), during detection, may lead to incorrect identification of fragments belonging to other lipid species such as Phosphatidic Acid (PA), Fatty acyls (FA) and Lysophosphatidylcholines (LPC) (Wang 1999) and this was taken into account during annotation of the lipid species. For example, it was demonstrated that PC, PA, and LPC species had different spatial distribution in seeds (**Figure 5.3**) suggesting that intact lipid species are being detected and not fragments of other lipids.

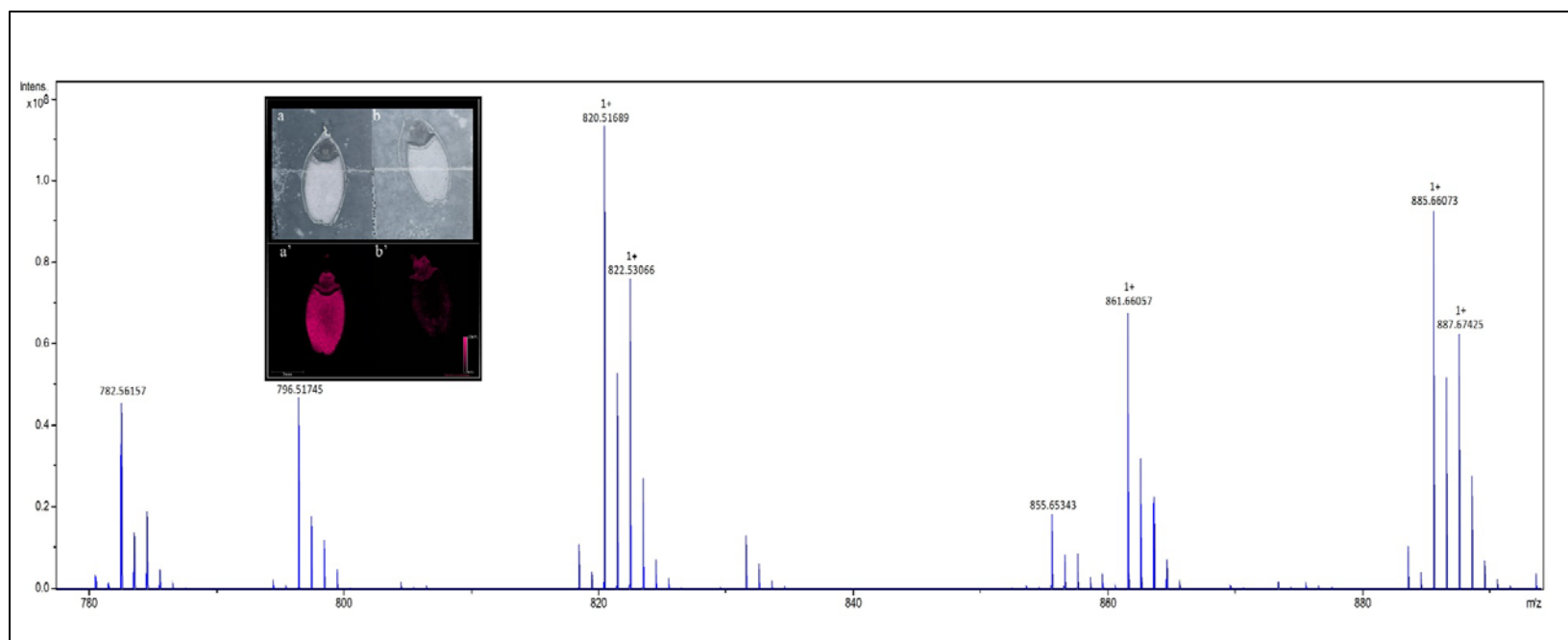


Figure 5.2 Average mass spectrum between m/z 780-890 acquired from barley seed cv. Mundah after 24 hours of germination. Each peak illustrates a specific lipid species (or isotope) that can be selected to display the distribution of the lipid across the section. The images obtained at 50 ×50 μm raster size display the different spatial distribution of the lipids across different timepoints, different treatments and between the two genotypes. To ensure the reproducibility of detection of the lipids, three independent seed sections were analysed for each mass spectrometry imaging experiment. Distribution of the m/z 782.5466 PC(36:4) signal in sections of control (a) and salt treated (b) seeds at 24 hours are shown in the inset panel. Data was normalized using Root Mean Square.

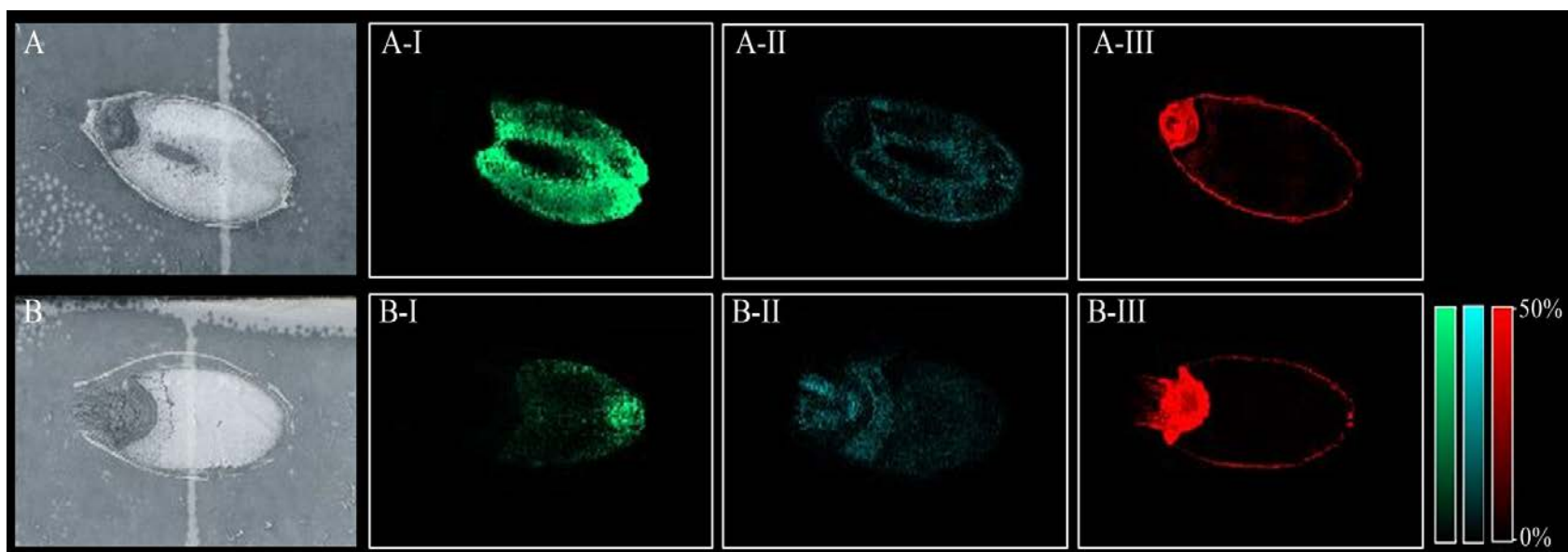


Figure 5.3 Different lipid species displaying different spatial distribution. A: scanned image of Mundah seed at 48 hours in control conditions; B: scanned image of Mundah seed at 48 hours in salt conditions; A-I and B-I: m/z 496.338 LPC(16:0) showing spatial distribution only in endosperm of both seeds with different intensities in control and salt conditions. A-II and B-II: m/z 757.574 PA(40:2) showing spatial distribution in entire seed with different intensities in control and salt treated seed; A-III and B-III: m/z 820.530 PC(36:4) showing spatial distribution in the aleurone layer and embryo of control and salt treated seeds. Intensity scale for both images was set to 0-50%. Data was normalized using Root Mean Square.

Tentatively annotated lipid species with several ion adducts, but the same masses, were collapsed into a single ID. It was noted that the returned matches were filtered to remove tentative annotations from mammalian origins. As a result, 234 lipid species were tentatively identified in Mundah control (**Supplementary Table S4a-refer to the hyperlink given in Chapter 7**), 202 in salt treated Mundah seeds (**Supplementary Table S4b-refer to the hyperlink given in Chapter 7**), 155 in Keel control (**Supplementary Table S4c-refer to the hyperlink given in Chapter 7**) and 145 in salt treated Keel seeds (**Supplementary Table S4d-refer to the hyperlink given in Chapter 7**). The tentatively annotated lipids were categorized into seven major lipid classes: Fatty acyls (FA), Glycerolipids (GL), Glycerophospholipids (GP), Sphingolipids (SP), Prenol lipids (PR), Sterol lipids (ST) and Polyketides (PK). The percentage of each lipid class in both genotypes is given in **Table 5.1**. This was solely based on the number of lipid species matched with the LIPID MAPS database. The distribution of lipid classes at all timepoints in both genotypes are shown in **Supplementary Figure 7-3**. For both genotypes, the number of lipids identified is smaller in the germinated seeds in the presence of salt compared to the control, but in Mundah, the difference between the two treatments is much smaller. The number of lipids identified in each class decreased in Keel after salt treatment, whereas in Mundah, only GL, GP and ST decreased.

Table 5.1 Percentage of individual lipid classes in two treatments of Mundah and Keel. The values are total percentages of annotated lipids at all timepoints

Lipid Classes	Mundah (%)		Keel (%)	
	Control	Salt	Control	Salt
Fatty acyls	18.70	20.00	26.60	26.62
Glycerolipids	14.07	13.85	24.68	23.55
Glycerophospholipids	49.81	44.92	26.28	34.13
Polyketides	7.96	10.15	7.69	6.48
Prenol lipids	1.85	2.15	5.13	3.07
Saccharolipids	0.56	0.00	0.00	0.00
Sphingolipids	3.89	4.31	3.53	2.39
Sterol lipids	3.15	4.62	6.09	3.75

In Mundah seeds, GP was the major class of lipid altered (43% in Mundah control and 40.6% in Mundah salt). In the GP class, PA, Phosphatidylethanolamine (PE), Phosphoglycerols (PG) and PC were tentatively identified as the major lipid subclasses. The GL class decreased in saline conditions but in control conditions, TAGs were detected in high number. The percentage of ST and GP decreased under salinity, but the sterol/phospholipid ratio was unaffected by salt (0.25% in control and 0.24% in salt).

GP is also the major class altered in Keel seeds (26.3% in Keel control and 34.1% in Keel salt). It is worth noting that an increased number of GP lipids in Keel salt treated seeds was detected likely

due to different adducts detected for the same lipid. For example, in the GP class, LPC (16:0), LPC(18:0) and PC(36:5) were found with $[M+H]^+$ and $[M+K]^+$ ion adducts and PC(34:2) with $[M+Na]^+$ and $[M+K]^+$ ion adducts. The percentage of ST decreased significantly under salt stress in Keel with a slight decrease in GP under salt stress and hence the sterol/phospholipid ratio was affected by salt in Keel seeds (0.25% in control and 0.12% in salt). It is noted that PC lipid species of 36:n family have been found to be differentially distributed at all timepoints in both genotypes under control and salt conditions.

Gorzolka et al. (2016) performed MSI to localize metabolites during the first seven days of germination in barley seeds and showed the distribution of 11 lipids in barley seeds at different stages of germination and different regions of seeds. In our study, we confirmed the presence of 10 out of 11 lipids from Gorzolka et al. (2016) in Mundah control, 9 in Mundah salt, 4 in Keel control and 9 in Keel salt treated seeds (listed in **Supplementary Table S5-refer to the hyperlink given in Chapter 7**). Time-dependent changes were also observed in the lipid profile of the endosperm early in germination in Gorzolka et al. (2016). Mono-and diacyl PCs (m/z 496, 520, 758) were highly abundant, and these lipids changed their distribution at later germination stages (Gorzolka et al. 2016). In the current study, the m/z 496 and 758 ions could be detected in both genotypes under both treatments. However, they were not consistently observed across all time points. In contrast, the m/z 520 ion was only detected in salt treated Keel seeds. The distribution pattern for these three PCs is given in **Supplementary Table S6-refer to the hyperlink given in Chapter 7**.

5.3.4. Lipids detected immediately after imbibition and after 8 hours of salt stress

Comparison between the annotated lipid classes at 0 hours and 8 hours post-imbibition in control Mundah seed sections showed 26 lipids species present at both time points whereas only 16 lipids species were found in common when 0-hour and 8-hour salt treated seeds were compared (**Supplementary Table S7a and S7b-refer to the hyperlink given in Chapter 7**). In Keel, 30 lipids species were detected in both 0-hour and 8-hour control sections whereas 20 lipids species were found between 0-hour control and 8-hours salt treated seed sections (**Supplementary Table S7c and S7d-refer to the hyperlink given in Chapter 7**). After 8 hours of salt stress, 65 lipid species were differentially accumulated compared to the 8 hours post imbibition in untreated seeds of Mundah (**Supplementary Table S8-refer to the hyperlink given in Chapter 7**). The major lipid class detected in 8 h salt treated seeds in Mundah was GP. In contrast, 38 lipids were detected after 8 hours of salt stress when compared to the 8 h untreated seeds in Keel (**Supplementary Table S9-refer to the hyperlink given in Chapter 7**). The major lipid class detected in Keel after exposure to salinity stress was GL.

5.3.5. Spatial segmentation of the MALDI-MSI data using an unsupervised clustering approach using SCiLS Lab

Relatively few lipid species changed their spatial distribution in Keel compared to Mundah seeds. In Mundah, out of 225 lipid species that were found to be spatially distributed in salt treated seeds at all time points, 71 (31.5%) were salinity treatment specific. In Keel, out of 163 lipid species spatially distributed in salt seeds at all time points, 48 (29.4%) were specific to salt stress based on LIPID MAPS annotations.

SCiLS Lab was used to determine the discriminative features for both treatments in Mundah and Keel. A univariate measure was used to assess the quality of discrimination for imported m/z values for masses created in flexImaging, which quantified how well the m/z values discriminated between two treatments. A threshold for Area Under ROC (Receiver Operating Characteristic) Curve (AUC) was used to determine the discriminating masses in both treatments for both genotypes. A perfect discrimination was accepted where AUC value was equal to or close to 1 for salt treated seeds and close to or equal to zero for control seeds. The threshold for this analysis was set to 0.8 for salt treated seeds and 0.2 for control seeds. Subregions were created in SCiLS Lab to find m/z values that discriminate between the two treatments for embryo and endosperm in both genotypes. An example of a subregion created for Mundah seeds (m/z 615.494) and its corresponding ROC plot for DAG (34:2) with AUC value of 0.087 is given in **Supplementary Figure 7-4**. The intensity box plot chart that describes a single m/z interval and a single region was also used.

Further discriminant lipids were detected at all timepoints using the above-mentioned threshold values in both genotypes. Two subregions were created - embryo and endosperm. In Mundah embryo, lipids discriminant to control seeds (AUC close to zero) increased in number over time whereas in Keel embryo, the number of discriminant lipids with AUC close to zero decreased over time. In Mundah endosperm, 45 lipids were found to be discriminant between control and salt treated seeds with only nine lipids showing AUC value between 0.8-1.0. In Keel endosperm, only four lipids were discriminant with AUC values between 0–0.2 (**Supplementary Table S10-refer to the hyperlink given in Chapter 7**).

In Mundah embryos, a tentative flavonoid, m/z 365.102 $[M+H]^+$ (AUC for Mundah 16 hours, 0.867; Mundah 24 hours, 0.949; Mundah 48 hours, 0.907) was found to be strongly discriminant at 16, 24 and 48 hours (**Figure 5.4**) whereas the same mass was observed in Keel embryos at 72 hours only (AUC 0.946) (**Figure 5.4D** and **Figure 5.4D'**). Signal intensity was scaled to optimise visualisation of the respective ion. The intensity box plot and ROC plot for the above mentioned timepoints shows the discrimination capabilities of the given m/z for two (control and salt) regions in Mundah at 16, 24 and 48 hours (**Supplementary Figure 7-5**) and Keel at 72 hours

respectively (**Supplementary Figure 7-6**). No common lipids were found in Mundah embryos over all time points. In Keel embryos, diacylglycerols (m/z 602.523) and a fatty alcohol (m/z 576.508) discriminate between control and salt treatments at 16 and 24 hours with an AUC of 0.19 and 0.13 respectively. In Mundah endosperms, it is worth noting that fatty acyls (m/z 339.288, $M+Na^+$) were found in salt treated seeds at 48 hours with an AUC 0.88 whereas at 72 hours, they were found intensively in control seeds with AUC value of 0.17. No discriminant lipids were found in Keel endosperms. Although several lipid species were found distinct at 8 hours salt as compared to 8 hours control as mentioned in section 3.4, no discriminant lipids between these two timepoints in both genotypes were found.

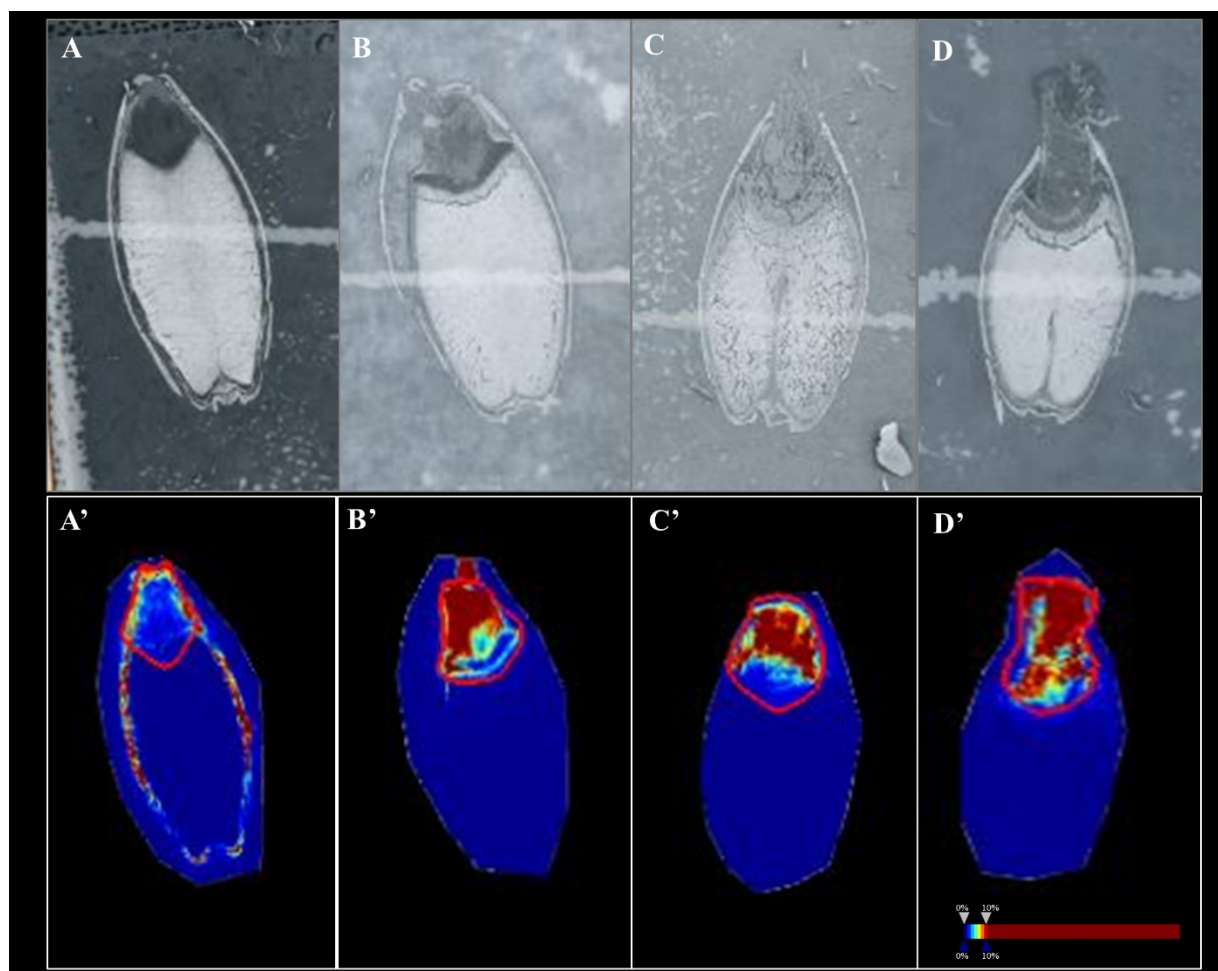


Figure 5.4 A flavonoid (m/z 365.102) with AUC close to 0.8 at 16, 24 and 48 hours in Mundah embryos and at 72 hours in Keel embryos. A, B and C: scanned images of Mundah seeds at 16, 24 and 48 hours of salt treatment; D: scanned image of Keel seed at 72 hours of salt treatment; A', B' and C': Embryo region showing high intensity of the flavonoid in Mundah salt treated seeds at 16, 24 and 48 hours; D': Embryo region showing high intensity of the flavonoid in Keel salt treated seed at 72 hours. Intensity scale was set to 0-10% for all seeds. Data was normalized using Root Mean Square.

5.3.6. Confirmation of tentative flavonoid using MS/MS

5.3.6.1 MALDI-MS/MS

To probe the identity of the tentative flavonoid (m/z 365.102 $[M+H]^+$), on-tissue MALDI-MS/MS spectra was generated and identified fragments were recorded. A 5 m/z window centred around m/z 365.1 was isolated with no collision voltage applied. **Figure 5.5** shows the two distinct peaks with very close mass obtained from MALDI-MS, m/z 365.1024 was matched in the LipidMaps and METLIN (<http://metlin.scripps.edu>) databases to a flavonoid and m/z 365.1058 which was assigned as a dihexose sugar (Calvano et al. 2017). Subsequent MS/MS using the same isolation window and application of 20V collision energy led to observation of nine product ions (**Supplementary Figure 7-7**) that were matched with the in silico predicted spectra of a flavonoid, Gancaonin F (METLIN database) (Smith et al. 2005), corresponding ions associated with the fragmentation of a dihexose sugar were also observed.

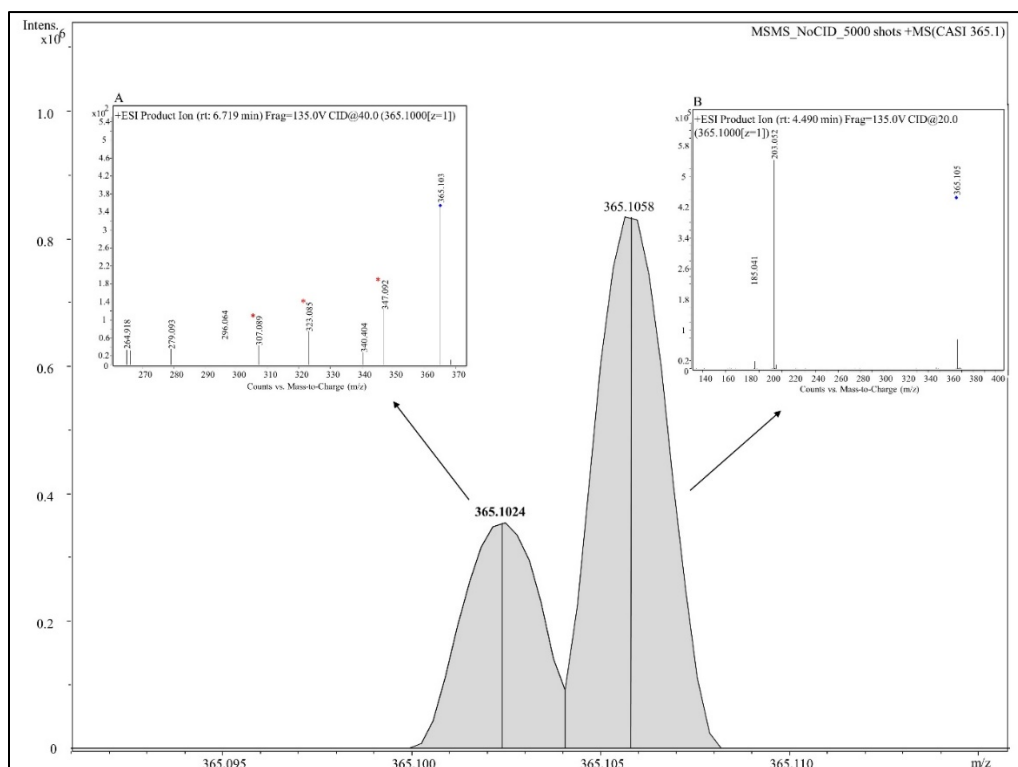


Figure 5.5 Combined MS and MS/MS spectra of m/z 365.1 $[M+H]^+$ derived from on tissue MALDI-MS/MS and LC-MS/MS respectively. A mass spectrum between 365.090-365.115 is shown in the figure. Two peaks, m/z 365.10243 (shown in **bold**) and m/z 365.10582 are clearly shown corresponding to a tentative flavonoid and dihexose respectively. Inset Figure 5A shows the mass spectrum analysed using LC-MS/MS for a tentative flavonoid with retention time of 6.719 mins giving seven fragments. Three fragments (marked with asterisks) match with fragments obtained from MALDI-MS/MS given in Figure S6. Inset Figure 5B displays the mass spectrum for a dihexose sugar analysed using LC-MS/MS with retention time of 4.49 mins and displaying two fragments- m/z 203.052 and 185.041.

5.3.6.2 LC-MS/MS

LC-MS/MS was performed on seed extracts to provide further evidence for annotation of the possible flavonoid and dihexose sugar. Both MS and MS/MS were conducted with the protonated precursor ion m/z 365.1 isolated for MS/MS. The extracted compound chromatogram (ECC) at the MS level showed two separate peaks with retention time of 6.71 mins and 4.49 mins. MS/MS using 40eV and 20eV respectively showed distinct differences in the corresponding spectra between the two compounds. Inset **Figure 5.5A** displays the mass spectrum for one compound with seven fragments, of which three fragments (m/z 347, 323 and 307) match closely with in silico predicted spectra of Gancaonin F (METLIN database) and MALDI-MS/MS data obtained from on-tissue analysis in this study. Inset **Figure 5.5B** shows the mass spectrum of a compound with two fragments (m/z 203 and 185) that match exactly to dihexose sugar fragments Calvano et al. (2017). The mass spectrum along with the database search showed a difference of less than 4 ppm in experimental and database mass identified using LC-MS/MS. **Table 5.2** shows the observed fragments of the tentative flavonoid detected using MALDI-MS/MS and LC-MS/MS that matched with the in silico predicted spectra of Gancaonin F.

Table 5.2 Theoretical and observed m/z for the tentative flavonoid, Gancaonin F from MALDI-MS/MS and LC-MS/MS

In silico predicted fragments of Gancaonin F	Observed m/z by MALDI-MS/MS	MALDI-MSI mass error in ppm	Observed m/z by LC-MS/MS	Mass error in Da
347.091	347.092	2.9	347.092	<0.001, <0.001*
337.107	337.103	11.9	-	0.004
335.091	335.092	2.9	-	0.001
333.076	333.076	<0.001	-	<0.001
323.055	323.055	<0.001	323.085	<0.001, 0.03*
321.112	-	-	-	-
309.039	309.040	3.2	-	0.001
307.060	307.060	<0.001	307.089	<0.001, 0.029*
297.039	297.040	3.4	-	0.001
281.044	281.045	3.5	-	0.001

5.3.7. Level of tentative flavonoid increases in response to salinity stress in four out of seven barley genotypes

The response of the tentative flavonoid was further elucidated in seven barley genotypes (Mundah, Keel, Vlamingh, Gairdner, Hindmarsh, Clipper and Sahara) at five timepoints (8, 16, 24, 48 or 72 hours) in control and saline conditions (250 mM NaCl) using LC-QToF-MS. The aim was to understand the differential expression of the detected tentative flavonoid in germinating seeds of a barley variety panel consisting of a small but representative set of six barley genotypes. **Supplementary Figure 7-8** shows the differential change in the tentative flavonoid over time in seven genotypes. Hindmarsh, Clipper and Gairdner showed no significant difference over time in both conditions. In Sahara, the highest increase was shown at 16 hours under saline conditions. In Keel, a significant increase was observed at 72 hours under saline conditions as compared to control seeds. A similar pattern was observed in Vlamingh where significant increase in the tentative flavonoid was seen at 72 hours in salt treated seeds as compared to control seeds. In Mundah salt treated seeds, there was a decrease in the tentative flavonoid at 72 hours. **Supplementary Table S11 (refer to the hyperlink given in Chapter 7)** shows fold changes in signal response of the flavonoid for seven genotypes over time. Under saline conditions, amongst all genotypes, Vlamingh (+6.3-fold) and Keel (+14.04-fold) show the highest increase after 72 hours salt stress ($p < 0.001$). However, in Mundah the highest increase was observed at 48 hours (+9.0-fold) after salt stress with small decrease at 72 hours (+8.9-fold) after salt stress.

5.3.8. Elemental concentrations in seeds

The elemental composition of total barley seeds was analyzed to determine the effect of 24 hours and 48 hours of salt stress (250 mM NaCl) on the content of eight macro- and micronutrients of both barley genotypes. Table 2 summarizes the concentrations of elements in control and salt treated seeds and **Figure 5.6** shows the change in Na^+ concentrations in both genotypes after 24 hours in control and salt treated seeds. Of all the elements detected, only Na^+ content was significantly altered ($p < 0.001$) after exposure to salt. In Mundah, Na^+ concentrations were $0.5 \pm 0.12 \text{ mg g}^{-1}$ in control and $3.1 \pm 0.48 \text{ mg g}^{-1}$ after salt treatment for 24 hours and $0.5 \pm 0.06 \text{ mg g}^{-1}$ in control and $4.7 \pm 0.06 \text{ mg g}^{-1}$ after salt treatment for 48 hours. In Keel, Na^+ concentrations were $0.4 \pm 0.02 \text{ mg g}^{-1}$ in control and $2.7 \pm 0.21 \text{ mg g}^{-1}$ after salt treatment for 24 hours and $0.4 \pm 0.06 \text{ mg g}^{-1}$ in control and $4.2 \pm 0.11 \text{ mg g}^{-1}$ after salt treatment for 48 hours. This indicated a significant increase in Na^+ content in both Mundah (+9.4-fold) and Keel (+10.1-fold) after 48 hours salt stress.

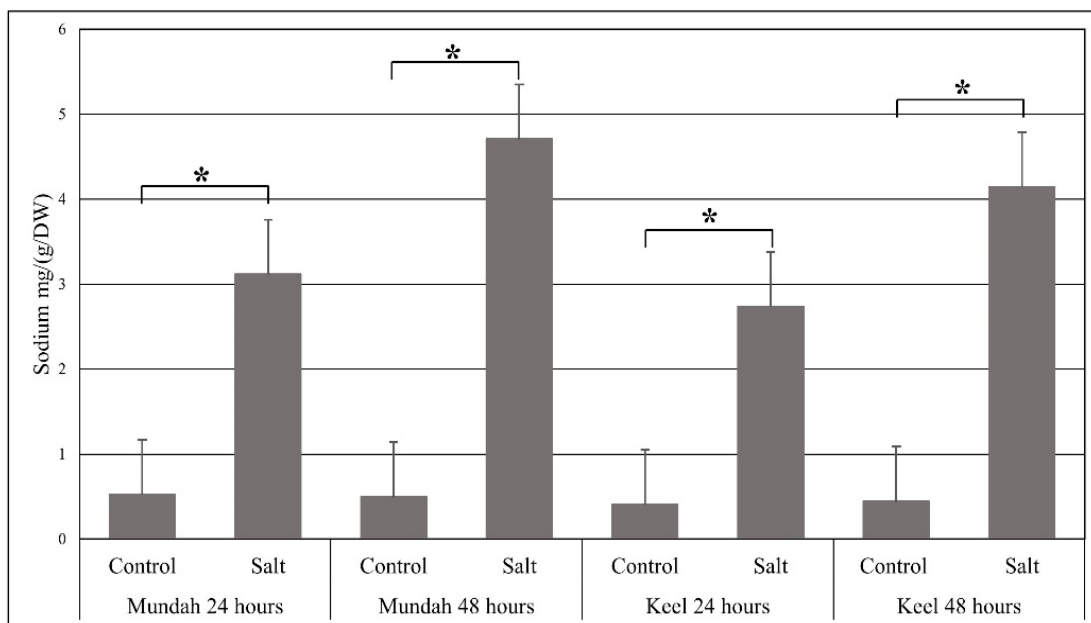


Figure 5.6 Change in the sodium concentrations identified by ICP-MS in Mundah and Keel seeds after 24 hours and 48 hours in control and salt treated conditions. Concentration of sodium mg g⁻¹/dry weight (n = 4). Scales over the bar graph indicates SDs. Asterisks on the top of each bar highlight the significance levels between two treatments at individual timepoints (*, P < 0.001).

The K⁺ content in seeds decreased slightly after salt treatment but this was not statistically significant. None of the other six elements (P, Mg, Ca, Mn, Fe and Zn) showed a statistically significant difference in seeds after salt stress as shown in **Table 5.2**. Since the overall concentration of most elements did not change during germination or in response to salt treatment we examined whether the treatments caused changes in the localization of the elements (S, P, K and Cl) using μ -XRF analysis on seed sections from time point 24 and 48 hours. In Mundah seeds, S accumulated in the entire seed irrespective of treatment. P was distributed in all seeds at moderate concentrations and was highly abundant in the aleurone layer. K was found in higher concentrations in control seeds than in salt treated seeds at all time points, with high abundance observed in the aleurone layer. These results complement the data obtained from ICP-MS. We were unable to observe Na⁺ due to instrumental limitations. However, Cl⁻ was used as an indirect measure of the Na⁺ distribution in seeds (Sarabia et al. 2018). Cl⁻ was highly abundant in salt treated seed sections with a uniform distribution correlating with the increased Na⁺ content as detected by ICP-MS (**Supplementary Figure 7-9**). In Keel, S accumulated across the entire seed similarly to Mundah but was found to be more abundant in sections from salt-treated seeds obtained at 0 and 48 hours post imbibition. P was also found to be highly accumulated in the aleurone layer of all sections and with lower abundance in the endosperm. K⁺ was seen abundantly in samples from the 0 h time point followed by a less intense distribution over time in both treatments. Cl⁻ was prominently abundant in salt treated seed sections

correlating with the increased Na⁺ content detected by ICP-MS data (**Supplementary Figure 7-10**).

Table 5.3 ICP-MS of whole barley seed (Mundah and Keel) and its main tissue fractions (mg g⁻¹ dry weight). Each tissue type was weighed and analysed separately by ICP-MS. Data points represent as mean $\pm$ standard error, n = 3. Scale in mg g⁻¹/DW

*Values with significant differences (p < 0.001).

Element	Mundah (24 h)		Mundah (48 h)		Keel (24 h)		Keel (48 h)	
	Control	Salt	Control	Salt	Control	Salt	Control	Salt
P 31	12.41 $\pm$ 1.69	12.89 $\pm$ 1.99	12.64 $\pm$ 2.89	14.29 $\pm$ 2.13	11.07 $\pm$ 1.9	12.17 $\pm$ 2.6	12.54 $\pm$ 4.43	13.34 $\pm$ 2.12
*Na 23	0.53 $\pm$ 0.12	3.12 $\pm$ 0.48	0.50 $\pm$ 0.06	4.71 $\pm$ 0.06	0.44 $\pm$ 0.02	2.74 $\pm$ 0.21	0.45 $\pm$ 0.06	4.16 $\pm$ 0.11
Mg 24	2.95 $\pm$ 0.22	2.72 $\pm$ 0.74	2.7 $\pm$ 0.61	2.94 $\pm$ 0.62	1.81 $\pm$ 0.5	2.21 $\pm$ 0.61	2.17 $\pm$ 0.95	2.36 $\pm$ 0.63
K 39	8.63 $\pm$ 1.24	8.23 $\pm$ 1.91	8.3 $\pm$ 2.04	8.05 $\pm$ 0.8	7.12 $\pm$ 1.18	7.01 $\pm$ 0.51	7.71 $\pm$ 2.49	7.58 $\pm$ 0.9
Ca 44	1.16 $\pm$ 0.08	0.76 $\pm$ 0.24	1.06 $\pm$ 0.3	0.85 $\pm$ 0.22	0.79 $\pm$ 0.22	0.7 $\pm$ 0.16	0.88 $\pm$ 0.39	0.79 $\pm$ 0.24
Mn 55	0.04 $\pm$ 0.01	0.04 $\pm$ 0.01	0.04 $\pm$ 0.01	0.04 $\pm$ 0.01	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01
Fe-3 57	0.06 $\pm$ 0.01	0.06 $\pm$ 0.02	0.06 $\pm$ 0.02	0.06 $\pm$ 0.02	0.04 $\pm$ 0.01	0.05 $\pm$ 0.02	0.05 $\pm$ 0.03	0.06 $\pm$ 0.02
Zn 66	0.06 $\pm$ 0.01	0.06 $\pm$ 0.01	0.06 $\pm$ 0.02	0.07 $\pm$ 0.02	0.05 $\pm$ 0.01	0.06 $\pm$ 0.03	0.05 $\pm$ 0.03	0.07 $\pm$ 0.02

5.4 Discussion

In recent decades, salinization of agricultural land has resulted in lower crop productivity. Germination of seeds is directly affected by salinity either by the resulting osmotic stress or by toxic effects of the sodium and chloride ions themselves. In particular, salinity perturbs plant hormone balance (Khan and Rizvi 1994) and reduces the utilization of seed reserves (Ahmad 1992). In the past, metabolomics studies have mainly focused on the germination processes in rice and very little has been published on barley or on the effects of saline conditions on the germination process. In addition, these published studies used techniques that do not provide spatial information on metabolic processes. Gorzalka et al. (2016) previously studied the changes in metabolite profiles in barley seeds after 5 days of germination using MALDI-MSI, but changes in the metabolite profiles of barley seeds during the early stages of germination (0 to 72 hours post imbibition) were not explored.

The process of uptake of water by a mature dry seed, which occurs in the initial hours of reintroduction of water to the dry seed, is triphasic (Bewley (1997) and results in the resumption of metabolic activity. These metabolic changes are extremely important to successful germination and, as such, it is vital to document and understand what changes occur (Bewley 1997; Bewley 2001; Bewley and Black 1994). We first conducted a germination assay to establish the germination efficiency of seven barley genotypes under salinity stress. From these results, we selected two genotypes with contrasting germination phenology and salinity tolerance for this study: Mundah (early germinating and salinity tolerant, in agreement with Cao et al. (2017) and Keel (late germinating and sensitive to salinity).

Following the germination test, ICP-MS and μ -XRF were combined to profile the levels and measure the spatial distribution of elements across six time points in the two selected barley genotypes (Mundah and Keel) under control and saline conditions. Munns and Tester (2008) reported that salt stress results in the accumulation of Na^+ in plants and results in an adverse effect on K^+ concentration which causes detrimental effects to plant growth and development. Various physiological studies have demonstrated that Na^+ toxicity is caused due to the ability of Na^+ to compete for K^+ binding sites to disrupt K^+ homeostasis (Hasegawa 2013; Shabala and Cuin 2008; Volkov and Amtmann 2006). Hence, to avoid an ion homeostasis disorder under saline conditions, plant cells need to maintain a low Na^+ concentration and a concurrent high K^+ concentration in the cytosol, where enzymes for metabolism are located (Pardo et al. 2006; Zhu 2003). In this study, we observed a substantial increase in Na^+ content and a non-significant decrease in K^+ content in germinating seeds of both genotypes under saline conditions.

It is apparent from this result that accumulation of excessive amounts of Na^+ in germinating seeds are likely to be responsible for inhibition of germination under salt stress in Keel as compared to

Mundah which was still able to germinate under these conditions. This view is supported by a previous report where increased accumulation of Na^+ in wheat seeds under salinity stress inhibited the rate of germination (Begum et al. 1992). The K^+/Na^+ ratio decreased to greater extent in both Mundah and Keel after 48 hours compared to 24 hours salt stress. This result is in alignment with previously reported reduction of the K^+/Na^+ ratio in barley roots after salt stress (Shelden and Roessner 2013). Salinity tolerance also depends on limiting Na^+ accumulation and maintaining K^+ content in the cytosol in order to achieve the preservation of ion homeostasis (Flowers et al. 1977; Hasegawa et al. 2000). Cl^- was observed to increase but no change in K^+ was observed using $\mu\text{-XRF}$ after salt stress. Increased Cl^- distribution obtained from $\mu\text{-XRF}$ analysis is an indirect measure of Na^+ as Cl^- is likely associated with Na^+ as a counterion (**Supplementary Figure 7-9** and **Supplementary Figure 7-10**). The prevailing higher concentrations of these elements after salt stress are likely to be affecting metabolism and further impacting the observed levels of lipids and other metabolites in a germinating seed. It is worth noting that elemental composition in embryo region of both genotypes change after salt stress. It was observed that the embryo size in both conditions of two genotypes were increased under salt treatment. This is potentially due to role of osmoregulators in plants that exhibit more abscisic acid (ABA) under salt stress. And increase in production of ABA induces the accumulation of more elements to be retained in those regions post-salt treatment (Sun et al. 2015).

Lipids are one of the major components stored exclusively in seed embryos and aleurone layer. They are vital and abundant cellular constituents responsible for cell membrane functionality as well as acting as an energy store to allow metabolism to continue during abiotic stress. The content of lipids in most cereals is relatively low (about 3%) compared to starch and protein. However, their contribution toward the nutritional value, as well as storage stability, of cereal-based food is important (Liu 2011). Several studies have reported on the content and composition of lipids in whole grains (Mehmood et al. 2008; Osman et al. 2000; Price and Parsons 1975; Welch 1975; Zhou et al. 1998) without investigating spatial distributions. In this study, we investigated the effect of salt on the germination of two barley genotypes and on lipid composition and distribution using MALDI-MSI. High quality images taken at a 75 μm raster step size allowed observation of the spatial distribution of a high number of lipids and metabolites in barley seeds. The choice of matrix (DHB) also allowed tentative identification, and profiling, curation and annotation of a large number of lipids. This allowed observation of major lipid species showing differential spatial distributions across seed sections at all time points under control and saline conditions. Despite the higher proportion of lipid species in the embryo (20% lipids (Newman and Newman 2008; Price and Parsons 1979)), the largest number of lipids were detected in the endosperm region and not in the embryo. This could be due to the tissue type, as on-tissue extraction of ions is highly influenced by the properties of the tissue. For example, ion abundances can influence the adduct formation, and highly abundant, co-localized lipids or

lipids bound to tissue structures can lead to ion suppression (Gorzolka et al. 2016). It could also be due to the thin tissue content of the embryo at these developmental stages which is highly hydrated (less dense) whereas the endosperm is still very dense.

Lipids play vital roles in providing structural integrity to cell membranes and are involved in signal transduction and membrane trafficking (Krauß and Haucke 2007). In seeds, lipids serve as a reserve energy source and act as emulsifiers of fat substrates for lipases (Ory et al. 1967). Various environmental stresses including salinity have been shown to be responsible for changes in lipid metabolism and composition (Mansour 2013; Meijer and Munnik 2003; Natera et al. 2016; Sarabia et al. 2018; Wang 2002; Yu et al. 2018). Lipids are rich in olefinic bonds which are susceptible to oxidative attack (Parida and Das 2005; Singh and Sinha 2002) from reactive oxygen species (ROSs) present in membranes during salinity stress (Esfandiari et al. 2007). The numbers of annotated species from the GP and ST classes decrease with increasing salinity in both genotypes. This is in line with findings by Wu et al. (2005) where analysis of changes in lipid composition following salt stress was studied in *Spartina patens*, a member of the Poaceae family. The decrease in the number of lipid species in Keel could also be due to peroxidation of lipids as mentioned by Pérez-López et al. (2009).

Among the detected lipid classes in this study, phospholipids were major species of lipids that were tentatively annotated in both genotypes. This was indirectly supported by μ -XRF data where the distribution of P was found across the entire seed of both genotypes (**Supplementary Figure 7-9** and **Supplementary Figure 7-10**), correlating with regions where higher numbers of phospholipids were also observed using MSI. Presence of P in the aleurone layer from μ -XRF data also correlates with MALDI-MSI data where higher number of lipids were observed over time. Phospholipid metabolism is associated with a large number of biological molecules and plays a crucial role in various signal transduction pathways in higher plants. In response to various biotic and abiotic stresses (Munnik et al. 1998; Pappan and Wang 1999), phospholipase D catalyzes the hydrolysis of structural phospholipids, PC and other phospholipids, resulting in the formation of PA. Ritchie and Gilroy (1998) reported that PA is an important mediator of abscisic acid (ABA) signal transduction in barley aleurone protoplasts. Levels of PA were observed to increase slightly with increasing salinity in both genotypes. Salinity stress dependent activation of PA could be due to activation of phospholipase D (PLD) that hydrolyses structural lipids such as PC and PE (Meijer et al. 2002). Increased levels of PA are also proposed to facilitate vesicular trafficking and to recruit target proteins to the membrane, influencing their activity (Roth 2008; Testerink and Munnik 2005; Wang 2005).

High salt concentrations in the growing medium usually lead to an increase in the plasma membrane sterol content (López-Pérez et al. 2009). This increase facilitates hydrophobic

interactions among the acyl chains, producing greater order in the membrane, and consequently, a more rigid membrane (Silva et al. 2011). In this study, the percentage of sterols decreased under salt stress in Mundah and Keel seeds. A seed usually has a pair of growing tips (apical meristems), that develop into stem (hypocotyl) and root (radicle). Thus, decrease in sterols in seeds of both genotypes could indicate a need for mobilizing lipids into meristematic tissue, providing nutrients during germination to support seedling growth during the early stages of development. It was also observed that sterol/phospholipid ratio was greatly affected after salt stress in Keel. Similar results were found in halophyte Brassicaceae species where a significant decrease in sterol lipids was observed after salt stress (Chalbi et al. 2015). However, the sterol/phospholipid ratio was unaffected by salt in Mundah which align with Wu et al. (2005) where the changes in lipid composition after salt stress in *Spartina patens* were studied.

Although phosphatidylcholine levels remained unchanged in Mundah seeds after salt treatment and increased in Keel after exposure to salinity, PC lipid species of 36:n was found at all timepoints in both genotypes under control and saline conditions. Similar results were found in the embryonic axis of *Camelina* seeds detected by MALDI-MSI under normal conditions (Horn and Chapman 2014; Horn et al. 2013). Increased levels of PCs in response to high salinity was also found by Pical et al. (1999) in *Arabidopsis thaliana*. In plants, PCs are generally produced through a mixed cytidyl diphosphate-choline (CDP-Cho) pathways and methylation pathway (Tasseva et al. 2004). Tasseva et al. (2004) also suggested that enhanced synthesis of PCs is due to an accelerated CDP-Cho pathway. The accumulation of betaine in response to salt has been widely recognised in various plants. In vitro studies have proved the positive correlation between the accumulation of betaine and the acquisition of tolerance to salt in maize (*Zea mays*) (Rhodes et al. 1989) and barley (Kishitani et al. 1994; Strange 1993). Hitz et al. (1981) demonstrated the use of phosphocholine for glycinebetaine synthesis in barley while McDonnell and Jones (1988) also proved that the choline required for oxidation of glycinebetaine is produced via turnover of PC. In our study, increased levels of PC over time could possibly result in the synthesis of glycinebetaine, helping seeds to cope under saline conditions. However, this particular mechanism needs further investigation.

High levels of Na^+ also cause secondary responses in plants due to increased oxidative stress. Cellular damage from oxidative stress within plant cells (Apel and Hirt 2004) induces production of reactive oxygen species (ROSs) (Ashraf 2009; Jaleel et al. 2007). ROSs derived from molecular oxygen can accumulate in the plant cell and cause oxidative damage in cellular components, including proteins and lipids. To prevent the potential cytotoxic effects of ROSs, the stimulation of antioxidant systems can assist in plant protection from oxidative stress (Chutipaijit et al. 2009; Grace and Logan 2000). Polyphenolic compounds such as flavonoids play an important role in stopping the propagation of oxidative chain reactions (Ksouri et al. 2007). Their synthesis is

generally stimulated in response to various abiotic stresses including salinity (Zhou et al. 2018) and thus the biosynthesis of such compounds is generally stimulated in salt-exposed plants. Flavonoids are also potential inhibitors of the enzyme lipoxygenase, which converts polyunsaturated fatty acids to oxygen-containing derivatives (Nijveldt et al. 2001). These compounds accumulate in plant tissues protecting them from damaging effects of salt stress and inhibiting lipid peroxidation (Potapovich and Kostyuk 2003). In our MSI results, a tentative flavonoid (m/z 365.102 $[M+H^+]$) was found to be discriminant in Mundah and Keel embryos after exposure to salinity. Analysis using LC-MS/MS and MALDI-MS/MS confirmed the compound to be a flavonoid, tentatively annotated as Gancaonin F. Flavonoid profiling data from LC-QToF-MS supported our MALDI-MSI data (refer to Figure S7 and Table S11). This is in alignment with Chutipaijit et al. (2009) who described a significant increase in total flavonoid content in salt stressed rice seedlings and Taïbi et al. (2016) where a significant increase in flavonoid content was seen in *Phaseolus vulgaris* under salt stress.

Under normal conditions, the quiescent embryo is able to germinate after imbibition and breaking dormancy (McDonald and Copeland 2012). However, in salt stress conditions, the radicle has to avoid oxidative damage for successful germination. To detoxify or scavenge the severe effects of stress, the presence of antioxidants in embryos may play a crucial role in protecting the emerging radicle and help in a successful germination. This is also supported by Shirley (1998), who described the accumulation of flavonoids in the embryos of all plant species. Our observed detection of a tentative flavonoid in the embryos of Mundah and Keel supports the view of flavonoids playing potential roles in the protection of seeds under salt stress. Keel displayed a significantly delayed flavonoid response relative to Mundah as shown in the LC-MS data where highest X-fold increase in Keel was observed at 72 hours. This depicts its lesser ability to deal with salt leading to poorer germination efficiency. However, in Mundah, flavonoid content was highest at 48 hours displaying faster responses to salinity correlating with the higher germination efficiency. These results indicate a possible advantage for the Mundah genotype, making it a better germinator under salt stress. Gradual increase in the number of lipids detected over time from MALDI-MSI in Keel compared to Mundah under salt conditions also support our findings of Keel showing slow germination.

Analysis of the flavonoid profile of the five other barley genotypes showed varied and contrasting responses under salt stress over time. Vlammingh, which showed a germination efficiency between that of Keel and Mundah, also showed an increase in the Gancaonin F flavonoid under salt stress to a level between that of Keel and Mundah. These observations point to Gancaonin F providing some advantage to specific genotypes in the response to salt stress through possible antioxidant scavenging of ROS species and protection of the vulnerable embryo during germination. The other four genotypes showed a variety of differing patterns in Gancaonin F profiles. There are a

multitude of possible response mechanisms that the germinating seed is able to employ, and the varying responses observed are most likely due to the wide range in genetic diversity of the selected barley varieties.

5.5 Conclusions

This study investigated the changes in levels and distribution of metabolites and elements in germinating seeds of Mundah and Keel, two barley genotypes that have contrasting germination rates in response to salt stress. To compare and contrast the lipid and metabolite profiles of these varieties during the early stages of seed germination under control and saline conditions, several orthogonal approaches were combined using MALDI-MSI, elemental composition analysis using ICP-MS, spatial distribution analysis using μ -XRF spectrometry and confirmation of compounds using LC-QToF-MS. Use of discriminative MALDI-MSI analysis as an exploratory and qualitative technique allowed visualization of larger lipid differences within different seeds structures as well as detection of a tentative flavonoid that showed discriminate behaviour between the two genotypes at different time points. Further analysis across genotypes to determine the roles of flavonoids in barley germination under salt stress conditions is required. However, these initial results indicate flavonoid as a strong candidate metabolite biomarker for detecting salinity tolerant varieties.

5.6 Acknowledgements

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5.7 Author Contributions Statements

S.G carried out the experiments, data analysis and interpretation of the results. S.G wrote the manuscript with support from B.B, S.N, P.S, C.H, U.R. LCMS was conducted and analyzed by S.G. and T.R. ICP-MS was conducted and analyzed by S.G. and D.C. μ -XRF imaging was conducted by J.B. and B.B. Both B.B. and U.R. supervised the project. All authors provided critical feedback, helped shape the research and authorized the final manuscript.

5.8 Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5.9 Contribution to the Field Statement

Salt stress around the world has significantly reduced crop productivity in recent decades. The germinating seed relies upon reserves of molecules to respond to the environment until it has begun photosynthesis. Germination is greatly affected by salinity and in turn affects the metabolism of stored molecules within the seed. This research examines early crucial timepoints in germination and development through measuring changes in metabolites and lipids under salt stress in different barley varieties in a spatially resolved manner. We used two high-end metabolomics technologies with supporting information on elemental composition to describe salinity response in the early stages of seed germination. We found a flavonoid to be a strong candidate metabolite biomarker for detecting salinity tolerant varieties. These results contribute to understanding the biochemical basis of how plants respond to abiotic stress and provide a possible avenue to develop tolerant plant varieties for future food security.

5.10 Addendum

5.10.1. Introduction

Salinity is one of the most important abiotic stresses in the world affecting almost all developmental stages during the plant life-cycle, including seed germination, seedling establishment and development, vegetative and reproductive growth, and crop survival and yield (Singh and Nautiyal 2012; Zhu 2016). Adverse effects of salinity on plant growth are either due to ion cytotoxicity (mainly due to Na^+ , Cl^- , SO_4^{2-}) and osmotic stress, which result in nutritional deficiencies and metabolic imbalance (Zhu 2016). Seed germination is one of the most critical stages in the plant cycle, as it directly determines the failure or success of the subsequent growth stages (El-Hendawy et al. 2011; Liu et al. 2018). The effects of salinity on seed germination is more pronounced in the salt-sensitive genotype than in the salt-tolerant ones (El-Hendawy et al. 2019; Gupta et al. 2019).

Apart from affecting the growth and development of plants, salt stress also impacts the microbial ecosystem of soil (Poosapati et al. 2014). This is because higher salinity in soil results in higher alkalinity ($\text{pH} > 8.5$) and high exchangeable sodium percentage ($\text{ESP} > 15$) that renders inhospitable rhizosphere environments for normal crop production (Qureshi et al. 2017). In such a scenario, the utilization of salt-affected soil for agriculture has become necessary to meet the rise in food demand. One of the possible strategies to counteract the adverse effect of salinity is by exploitation of the avenues of bio-agents or bio-inoculants (Egamberdieva 2012). One of the bio-control agents, *Trichoderma*, has been used for seed biopriming in many cereal and vegetable crops and has resulted in improved seed performance under adverse soil conditions and increased levels of plant growth hormones (Singh and Nautiyal 2012). *Trichoderma* is well studied for its properties to induce resistance responses to biotic and abiotic stresses (Harman et al. 2004a; Harman 2006; Harman et al. 2004c; Harman and Uphoff 2019).

Biopriming is a process of biological seed treatment that refers to a combination of seed hydration and seed inoculation with beneficial organisms to protect seeds. This technique helps seeds to evenly germinate even under adverse soil conditions (Rawat et al. 2011). In the first half of **Chapter 5**, we studied the germination efficiency of seven barley genotypes (Vlamingh, Mundah, Clipper, Sahara, Hindmarsh, Gairdner, and Keel) with varied germination phenology. The results showed that Mundah, a very early spring variety from Western Australia with very high early vigor had highest germination rate under salinity, and Keel, an early spring variety from South Australia with moderate early vigor showed the lowest germination rate under salinity. However, a glasshouse study (data not shown) performed on these varieties showed that Vlamingh had the highest yield and Gairdner had the lowest yield after three months of growth under saline conditions. Therefore, Vlamingh and Gairdner were used for plant-fungal interaction studies as mentioned in **Chapters 2, 3 and 4**.

The objective of this study was to investigate the effects of seed biopriming of these four barley genotypes- Mundah, Keel, Vlamingh and Gairdner with the salinity tolerant strain *Trichoderma harzianum* T-22, upon seed germination. Barley is one of the most important crops and shows moderate resistance towards heat and salt. It is significant due to its ability to grow in less fertile areas which are limited by drought, low temperatures and salinity (Sarabia et al. 2019; Sarabia et al. 2018; Yu et al. 2020). In this study, we calculated the germination percentage of four barley genotypes at 48h, 72 h, 96 h and 120 h post-imbibition following priming with the fungus. This study is important as it reveals the role of *Trichoderma harzianum* T-22 in imparting stress resistance for barley seeds before seedling establishment.

5.10.2. Materials and methods

5.10.2.1 Plant Material

Four varieties of barley were selected based on their importance for crop production, commercially relevant traits, and known differences in germination phenology and salinity tolerance (Kamboj et al. 2015; Sarabia et al. 2018; Tavakkoli et al. 2012; Yu et al. 2018). This selection comprised of two Australian barley feed (Mundah and Keel) and two malting cultivars (Gairdner and Vlamingh).

5.10.2.2 Seed germination and Sample Collection

The germination rate of the four barley varieties primed with *Trichoderma* was compared under normal and saline conditions. Twenty seeds from each variety were sterilized in 70% ethanol for 1 min. They were then rinsed 4–5 times in sterile 18.2 Ω deionized water before being treated with 1.0% (v/v) bleach for 10 min, followed by thorough washing in sterile 18.2 Ω deionized water 6–7 times. Seeds were then imbibed overnight (~16 h) in sterile 18.2 Ω deionized water with continuous aeration. Seeds were then imbibed in 1×10^8 CFU ml⁻¹ conidia of *Trichoderma harzianum* T-22 in 2 mL Eppendorf tubes for 1 hour before being transferred to 1% agar plates containing either 0 (control) or 250 mM salt (NaCl) concentrations (**Figure 5.7**). The plates were then sealed with parafilm, wrapped in aluminium foil and kept at 4°C for 48 h before being transferred to a growth chamber maintained at 17°C constant temperature with no light. Plates were scored for the number of seeds germinating at different times after transfer to the growth chamber (48, 72, 96, and 120 h post-imbibition). The germination percentage was calculated using the following equation: Germination (%) = $G/X \times 100$, where, G = number of seeds germinated and X = number of seeds sown.

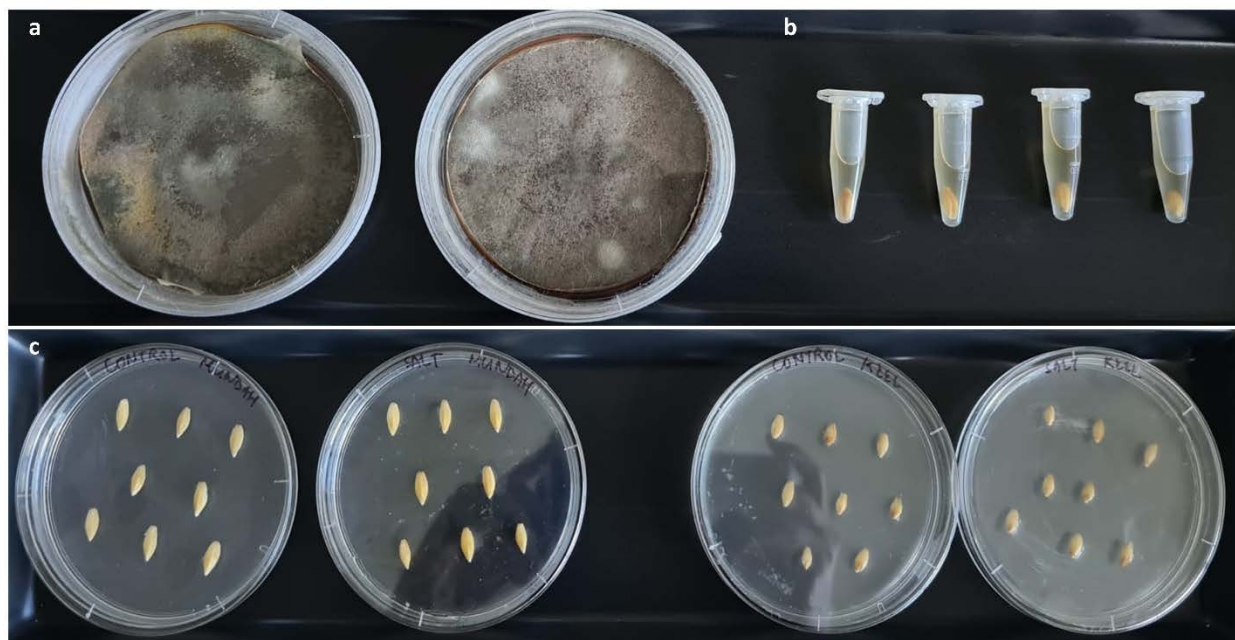


Figure 5.7 A protocol for seed priming with *Trichoderma harzianum* T-22. a – *Trichoderma harzianum* T-22 grown for seven days to obtain conidiophores, b – Priming of barley seeds with 1×10^8 CFU ml⁻¹ conidia in 2 mL Eppendorf, c – Seeds germinating on petri plates with control and saline (250 mM NaCl) agar.

5.10.2.3 Fungal inoculant preparation

Simultaneously to seed preparation in **Section 5.10.2.2** a fresh conidial suspension was prepared by flooding a PDA plate, with observable conidia, with sterile 18.2 Ω deionized water. The subsequent fungal suspension was collected and passed through a miracloth (Sigma-Aldrich, Australia) filter to separate conidia from hyphae. The suspension was diluted to yield 1×10^8 CFU ml⁻¹ conidia.

5.10.3. Results

All the observations were recorded at 48, 72, 96, and 120 h post-imbibition (**Table 5.4**). Seeds were germinated once emergence of the cotyledon was visible. There was a clear difference in the germination frequency of the seeds germinating in the presence of salt and inoculated with the fungus as compared to uninoculated seeds exposed to salt conditions. Keel showed the greatest inhibition (only 65% germinated under salt stress) followed by Gairdner showing 70% germination rate, while all Mundah and Vlamingh seeds germinated. However, seeds imbibed and treated with the fungus prior to transferring to salt conditions showed an increase in the germination rate. The salt-tolerant genotypes, Mundah and Vlamingh showed similar results as observed in control seeds. However, it is interesting to note that in Keel, seed germination percentage increased from 65% to 90% after 120 h of salt exposure and in Gairdner, the germination percentage increased from 70% to 95% after 120 h of salt conditions.

Table 5.4 Germination assay of four barley genotypes at four time points under different salt (NaCl) concentrations. Twenty seeds were germinated for each time point and treatment. The number shown is the number of seeds that germinated (Color gradients represent the decline in the number of seeds germinated- black is twenty seeds germinated, darker grey represents nineteen seeds germinated, dark grey represents eighteen seeds germinated, grey represents seventeen seeds germinated and light grey represents sixteen seeds germinated)

Genotypes	Mundah					Keel					Vlamingh					Gairdner			
Hours (post imbibition)	48h	72h	96h	120h		48h	72h	96h	120h		48h	72h	96h	120h		48h	72h	96h	120h
Control (0 mM)	20	20	20	20		18	19	19	19		18	20	20	20		18	20	20	20
Salt (250 mM)	18	20	20	20		13	13	13	13		15	18	18	20		11	14	14	14
Salt (250 mM) + fungus	20	20	20	20		14	16	16	18		18	19	20	20		11	15	17	19

5.10.4. Discussion

Salt stress is a well-known abiotic factor responsible for limiting the crop productivity. Numerous practices have been made to create genetically transformed commercial crops to fight against the adverse effects of increasing salinity in soil. However, as the complex nature of salt stress tolerance is regulated by many diverse genes, handling multiple genes simultaneously requires efficient performance to utilize effective stress resilience. Further, industrializing those products and releasing in the markets are also limited considering the social and/or legal legislations. Chemical priming is another approach when used with the right and effective doses (Duan et al. 2016). However, overuse of chemicals pose threat to the rhizosphere microbiota (Uphoff and Dazzo 2016). Therefore, sustainable methods are necessary for the achievement of an increase in agricultural productivity and maintenance of the ecological balance. This will translate into higher crop yields owing to improvements in plant resilience and the ability of plants to adapt to changing climatic conditions, as well as to biotic and abiotic stress.

The use of organic fungal bio-control agents instead of synthetic commercials through physical interaction with the seeds could be an effective and easily adaptive strategy. *Trichoderma* have been long recognized as agents for the control of plant disease and for their ability to increase plant growth and development. However, recently it has become clearer that certain species also have substantial direct influence on conferring stress tolerance in plants (Brotman et al. 2013; Contreras-Cornejo et al. 2014; Velázquez-Robledo et al. 2011).

Several studies have reported that seed biopriming with *Trichoderma* spp. improves seed performance and helps seeds to germinate even under adverse soil conditions (Singh et al. 2003). A study by Pehlivan et al. (2017) reported that a positive correlation was observed in maize seed biopriming with *Trichoderma lixii* ID11D (TXD) in increasing salt concentrations. It was also observed that TXD seedlings from bioprimered seeds outperformed in terms of photosynthetic pigment content and water status in salt conditions as compared to uninoculated seedlings. In this study, salt stress caused a significant decrease in the germination rate in the salt-sensitive genotypes, Keel and Gairdner. However, it is clear that seeds primed with *Trichoderma* proved to be effective as compared to salt (uninoculated) in tolerating saline conditions at the germination stage where germination percentage was increased from 65% to close to 100% as observed in the sensitive genotypes. Similar results were obtained by Khomari et al. (2018) where the effect of biopriming with *Trichoderma harzianum* on growth of soybean seedlings under salt stress was evaluated and it was found that the *Trichoderma* isolate was more effective in improving the emergence rate of seedlings with an increase in higher shoot and root length as well as leaf greenness than the control group at different salinity concentrations. In another glasshouse trial on sunflower seedlings, it was found that seed priming of sunflower with

Trichoderma isolate Ta-N13, seedlings produced a higher vigour index and better germination than non-primed seeds (Qureshi et al. 2017).

5.10.5. Conclusion

The results for this study offer a new approach to alleviate salinity stress in barley through seed biopriming with *Trichoderma harzianum* T-22. It could be concluded that seed biopriming with the fungus increased the ability of barley to germinate successfully under saline conditions.

Chapter 6

General Discussion

6.1 Introduction

The main aim of this thesis was to study the beneficial effects of the endophytic fungus *Trichoderma harzianum* T-22 in two barley genotypes under control and salt conditions and to map the biochemical changes using state-of-the-art metabolomics technologies such as gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS). Two barley genotypes, cv. Vlamingh and cv. Gairdner, were selected for this study based on their known differences in germination phenology and salinity tolerance. Cultivar Vlamingh has been shown to have better germination in high salt conditions while cv. Gairdner is less tolerant of these conditions (Gupta et al. 2019). Barley was chosen as it is not only an agriculturally and industrially important crop but is also the most salt tolerant cereal crop (Hill et al. 2016).

Chapter 2 of this thesis investigates the physiological changes in uninoculated and inoculated roots of both barley genotypes under control and salt conditions. Two experimental setups were used for this study. In the first experiment, agar was used as the growth medium for plants. This was performed to observe and determine the effect of fungus on the growth of plants under controlled conditions. In the second experiment, sandy loam soil was used as the matrix to grow plants and to more closely emulate agricultural land scenarios. Light microscopy was used to confirm the association of fungus within roots. The results confirm the positive effect of this fungus under control and saline conditions in both experiments. Various parameters were measured to confirm the positive effects of salt and fungus on both genotypes under control and saline conditions. Both the agar and soil studies suggest that inoculation of salt sensitive plants with *T. harzianum* T-22 may ameliorate the effects of salinity and improve plant growth.

In **Chapter 3**, a metabolomics and lipidomics approach was used to analyse polar metabolites and lipids from uninoculated and inoculated roots grown on agar in control and saline conditions, to provide insights into the biochemical changes in barley roots treated with fungus during the early stages of interaction. The results from this study showed distinct changes in polar metabolite profiles identified using GC-MS. A global analysis of the GC-MS dataset using unsupervised clustering methods such PCA and HCA indicated substantial metabolic differences between the two inoculated and uninoculated barley genotypes, cv. Vlamingh and cv. Gairdner, under control and saline conditions. Global analysis facilitated the construction of a data matrix composed of 110 metabolites detected in all six biological replicates from all treatments in both genotypes. The tight clustering between biological replicates in both genotypes confirms the robustness and reproducibility of the experimental protocol. The identified metabolites were grouped into four major categories - sugars and sugar alcohols, amines and amino acids, organic acids, and fatty acids. It was found that sugars and amino acids were majorly affected due to the inoculation of the fungus in both genotypes. To cover a wide range of lipid species, an untargeted LC-MS lipidomic analysis was applied to elucidate the dynamic changes in inoculated and

uninoculated roots of both genotypes, under control and saline conditions. . The untargeted data strongly suggests that salt treatment has a much bigger impact on the root lipidome than the effect of fungus in both genotypes. Here, a moderate alteration of root lipidome was triggered by salt stress in two barley genotypes. Plant membrane lipids belonging to three major groups: sphingolipids, sterols, and glycerolipids, were the major identified lipids from this study. The fungus played different roles in the two genotypes to confer the growth promoting benefits by changing relative concentrations of identified lipids. The main affected classes were glycerophospholipids and glycerolipids.

Similar analyses were performed for roots harvested from sandy loam soil as described in **Chapter 4**. Here, the metabolome dataset identified using GC-MS consisted of a total of 93 metabolites of which 74 were identified in roots of both barley genotypes. The metabolites identified by GC-MS were categorized into organic acids, sugars (sugar acids and sugar alcohols), amino acids and amines, and a small number of fatty acids. LC-MS analysis resulted in the identification of 186 lipid molecular species from a total set of detected lipids of 3645, which via the use of MS/MS spectra could be classified into three major lipid classes - glycerophospholipids, glycerolipids, and sphingolipids, in roots of both genotypes. Cultivar Vlammingh showed an increase in both identified metabolites and lipids with fungus and salt treatment as compared to cv. Gairdner where these were found to decrease. The results from this study suggest the role of the fungus in helping both genotypes cope with salt stress is via varying metabolic pathways.

In the last study presented in **Chapter 5**, the effect of inoculation of *T. harzianum* T-22 was studied on the germination of barley genotypes. Here, eight varieties of barley were first screened for their germination efficiency under control and saline conditions (increasing salt concentrations between 0 and 250 mM NaCl) and cv. Mundah and cv. Keel were selected based on their most contrasting germination results with cv. Keel showing 65 % germination rate while cv. Mundah germinated at 100%. Further, the spatial distribution of metabolites, lipids and a range of elements, such as K^+ and Na^+ was determined using mass spectrometry imaging (MSI), LC-MS, ICP-MS and XRF under control and saline conditions. Lastly, the effect of inoculation of *T. harzianum* T-22 was analysed on the germination of all four contrasting germinating barley genotypes used in **Chapter 2, 3, 4 and 5** – cv. Mundah, cv. Vlammingh, cv. Gairdner and cv. Keel. It was found that the fungus had positive effects on the sensitive genotypes. The germination percentage was increased from 65% to 90% in cv. Keel and from 70% to 95% in cv. Gairdner after 120 h post-imbibition. From the present investigation, it is quite clear that seeds primed with *Trichoderma harzianum* T-22 proved to be effective as compared to salt (uninoculated) in tolerating saline conditions at the germination stage in the selected barley genotypes.

Considering the results presented in this thesis into consideration, the application and importance of mapping the biochemical changes involving metabolites and lipids, imparted due to the inoculation of the fungus in roots of barley seedlings are discussed in the following two (Section 5.2 and Section 5.3) sections. The last section of this chapter (Section 5.4) provides a view on future perspectives of plant-fungal interactions for plant growth promotion which can be useful for sustaining agricultural productivity.

6.2 *Trichoderma harzianum* T-22 – a bioagent for seed priming and growth of cereal crops under salt stress

Seed germination is the essential first step in crop establishment and can be severely affected by salinity stress which can inhibit essential metabolic processes during the germination process and further growth of plants. Barley is a model organism for investigating the germination process in cereals (Gorzolka et al. 2016) which commences with the uptake of water by the quiescent dry seed and finishes with the emergence of the radicle through the seed coat. Salt stress during seed germination can trigger lipid-dependent signaling cascades that activate plant adaptation processes, leads to changes in membrane fluidity to help resist the stress, and causes secondary metabolite responses due to increased oxidative stress. However, incorporation of *Trichoderma* during seed biopriming treatments in many cereal and vegetable crops has resulted in increased plant growth and improved seed performance (Harman et al. 2004b; Howell 2003).

Chapter 5 describes the potential of seed biopriming with *Trichoderma harzianum* T-22 in two barley genotypes. Several studies have reported that seed biopriming with *Trichoderma* spp. improves seed performance and helps seeds to germinate even under adverse soil conditions (Singh et al. 2003). A study by Pehlivan et al. (2017) reported that a positive correlation was observed in maize seed biopriming with *Trichoderma lixii* ID11D (TXD) in increasing salt concentrations. It was also observed that TXD seedlings from bioprimed seeds outperformed in terms of photosynthetic pigment content and water status in salt conditions as compared to uninoculated seedlings. Another study by Khomari et al. (2018) evaluated the effect of biopriming with *Trichoderma harzianum* on growth of soybean seedlings under salt stress and it was found that the *Trichoderma* isolate was more effective in improving the emergence rate of seedlings with an increase in higher shoot and root length as well as leaf greenness than the control group at different salinity concentrations. In another glasshouse trial on sunflower seedlings, it was found that seed priming of sunflower with *Trichoderma* isolate Ta-N13, seedlings produced a higher vigour index and better germination than non-primed seeds (Qureshi et al. 2017).

The positive effects on growth and development of seedlings inoculated with *Trichoderma* spp. have been well reported (Altomare et al. 1999; Björkman et al. 1998; Contreras-Cornejo et al. 2009; Mastouri et al. 2012). In-keeping with these prior studies, **Chapter 2** demonstrated the

beneficial effect of *T. harzianum* T-22 on two barley genotypes cv. Vlammingh and cv. Gairdner with contrasting germinating phenology and tolerance to salinity under control and salt stress conditions. Various physiological parameters were measured demonstrating different potential roles of the fungus in improving the ability of plants to grow under saline conditions. The patterns of growth and effects of the fungus were similar for plants grown both on agar plates and in soil, confirming that the fungal treatment allowed the seedlings to grow normally even when grown under salt stress.

The results from **Chapter 2 and 5** have provided new insights in the effective use of *Trichoderma harzianum* T-22 under salt conditions. Previous studies have performed physiological analyses using the same strain of fungus on different plants. However, this study expands the knowledge by examining the effect of the fungus under controlled environmental conditions using agar plates as the growing matrix and then replicating the same experimental conditions in soil which is a good representative of actual field scenarios. Barley as a plant model was chosen for this study as it is the most salt-tolerant cereal crop. Selection of two different genotypes with contrasting yield constructed a good experimental set-up for comparison of tolerant and sensitive responses to salt stress.

To summarize, potential roles of the fungus in improving the ability of plants to grow under saline conditions were measured and validated using various physiological parameters. Therefore, this study provides further validation for the effectiveness of *Trichoderma* spp. in mitigating salt stress in cereal crops.

6.3 Osmolytes are greatly affected due to inoculation of the fungus after salt stress

Plants have ubiquitously evolved mechanisms to adapt to salinity that involve cations, such as sodium, ionically bonding to anions, like chloride, and partitioning them into the vacuole while synthesising and accumulating osmolytes in the cytosol (Annunziata et al. 2017). This mechanism is termed as osmoregulation. The osmolytes accumulated in response to salt stress are classified into four main classes of compatible solutes in plant cells. These are N-containing compounds (i.e. proline), sugars (i.e. sucrose and raffinose), straight-chain polyhydric polyols such as mannitol and sorbitol, cyclic polyhydric alcohols (Chen and Murata 2002), amines and amino acids, and organic acids.

The accumulation or decrease of these osmolytes in response to salt stress differ in conservation and divergence among species or genotypes within the same species (Gong et al. 2005; Sanchez et al. 2008). **Chapter 3** and **Chapter 4** describes changes in polar metabolites from roots harvested from agar plates and soil.

In **Chapter 3**, salt stress tolerance per se could not be examined, as the scope of this study was limited to analysing the early hyperosmotic stress response in roots of 48-hour salt stress. But the effect of individual treatments could be measured using agar as the growing matrix, as all the parameters were controlled resulting in no contamination. Furthermore, in vitro analysis provides a quick and reproducible way to study plant development and interaction with microbes under controlled and different stress conditions that can be easily be imposed upon the developing plants to measure phenotypical changes and screen large plant populations with desired characteristics. Here, under control conditions, *T. harzianum* inoculation failed to show major differential responses in metabolite levels compared to uninoculated roots in either genotype. But under salt stress, the fungus inoculated roots showed significantly decreased amino acid levels in cv. Gairdner. This is in alignment with past research in this field where Li et al. (2017b) found that *Aspergillus aculeatus* inoculated roots of ryegrass plants showed decreased amino acid concentrations following salt stress. Here we can speculate that the fungus may enhance the capacity of cv. Gairdner to withstand salt stress through coordinating with amino acids exerting a protective mechanism. Amino acids can be taken up directly by the roots and are transported between different organs through xylem and phloem. These are essential for plant growth. In this study, fungus causes significant reduction in amino acid levels which can indicate their contribution to growth in cv. Gairdner under saline conditions. Proline was also significantly reduced following salt stress in inoculated roots of cv. Gairdner. It has been suggested that proline can act as a stress marker and thus its decline may indicate that the toxic effects of salinity are reduced following colonization of fungus. This suggests that the fungus prefers to modify amino acid levels to increase salt tolerance in the more sensitive genotype cv. Gairdner.

Repeating the same experiment using soil as the growing matrix (refer to **Chapter 4**) for analysis of polar metabolites from uninoculated and inoculated roots under control and saline conditions enabled us to mimic field conditions on a small scale. Moreover, soil is an important substrate for *Trichoderma* and studies focusing on soil-inhabiting species of the genus have been carried out by several researchers around the world (Chen and Zhuang 2017). Although using soil as another matrix to study the effects of the fungus is a good platform for future soil studies, further experiments are required to confirm the findings presented in this thesis as our model was based on analysis of short-term salt stress. The results presented in **Chapter 4** showed that in response to short-term salt stress there is genotype specific response from the fungus to confer salt tolerance. The fungus increases osmolytes in tolerant genotype cv. Vlamingh suggesting its role in protective mechanisms against oxidative damage. However, in the sensitive genotype Gairdner, osmolytes were decreased after salt stress in the inoculated roots suggesting the role of fungus in their translocation to aboveground plant parts for better growth. Similarly, two

patterns were observed for organic acids where organic acids were increased in inoculated roots of Vlamingh after salt stress while the opposite was observed in Gairdner.

A targeted analysis of the metabolome or identified metabolites from this study would confirm description of metabolic networks regulating specific biological processes. However, identified metabolites, mainly sugars and amino acids as identified in **Chapter 3** and **Chapter 4**, from these studies can provide metabolites that can be used as biomarker for salt stress tolerance for future targeted studies.

6.4 Lipid modifications in inoculated barley roots in response to salt stress

Lipid peroxidation is the most ascribed symptom to oxidative damage, and it is often used as a marker of oxidative stress (Hernandez et al. 2000a; Khan and Panda 2008; Yu et al. 2020). A plethora of studies have reported alterations in plant membrane lipids in the presence of NaCl (Guo et al. 2019a; Sarabia et al. 2019; Yu et al. 2020). Plant membrane lipids belong to three major groups: sphingolipids, sterols, and glycerolipids (Borrell et al. 2016). Membrane glycerolipids have glycerol backbones with two fatty acid molecules bound to sn-1 and sn-2 and either a phosphorous (phospholipid) or sugar (glycolipid) molecule at position sn-3. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the dominant membrane phospholipids, which have been extensively studied. On the other hand, glycolipids including monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG), together with the phospholipids, phosphatidylglycerol (PG), make up the plastid membrane (Kalisch et al. 2016).

The untargeted analysis approach used in this study enabled the detection of over 3000 peaks in both studies. PCA plots from this global lipid analysis presented in **Chapter 3** showed clear clustering between treatments in both genotypes. This comprised of unknowns, annotated and identified and thus annotated and unknowns should be targeted for future studies. **Chapter 3** presented that phosphatidylcholine levels were significantly higher after salt stress, but the levels were either not changed or reduced in inoculated roots of cv. Vlamingh. As the production of TAGs were significantly higher in inoculated roots of Vlamingh following salt stress, it can be suggested that the fungus uses the Kennedy pathway for de novo synthesis of DAG and subsequent TAG species in cv. Vlamingh. However, in cv. Gairdner, phosphatidylcholine levels were increased in inoculated roots after salt stress and thus it could be suggested that TAG production in cv. Gairdner is from PC-derived TAG synthesis pathways used by the fungus as explained in detail in **Chapter 3**.

An interesting opposite pattern was observed when inoculated seedlings were grown in soil. **Chapter 4** shows that the fungus uses PC-derived TAG synthesis pathway in cv. Vlamingh as

significant increases in PC species were observed following salt stress and PC-derived TAG synthesis pathways was presumed in cv. Gairdner as glycerophospholipids showed no change in salt treated inoculated roots of cv. Gairdner. Similar results were observed by Mueller et al. (2015) where salt stress triggered accumulation of TAGs in Arabidopsis seedlings. Higher accumulation of TAGs may reflect lipid remodelling, i.e. channelling of fatty acids from constitutive membrane lipids to TAGs. Further, as TAGs predominantly contain polyunsaturated fatty acids, it is likely that the fungus helps in the release of polyunsaturated fatty acids from structural lipids and use for transient assembly of TAGs during membrane remodelling.

In this study, evidence of the molecular adaptations enacted by lipid species in barley roots from genotypes with differing levels of salinity tolerance under these conditions is provided. Vlammingh displayed significantly higher increases of lipids within roots inoculated with fungus under saline conditions while an opposite pattern was observed in cv. Gairdner. This points to these findings as potential targets for engineering of salt tolerant crops, especially TAGs composed with higher numbers of unsaturated fatty acids.

Based on the physiological results and lipid analyses from **Chapter 3 and 4**, the fungus proves beneficial to both genotypes under control and saline conditions and the results have given insights into the changes in both genotypes based on the identified lipids. However, several questions remain unanswered which are outlined in the next section.

6.5 Conclusion and Future perspectives

This study validated the effects of *Trichoderma harzianum* T-22 in the relatively salt tolerant cereal crop barley grown in controlled environmental conditions and soil in the growth chamber. The results from both studies for GC-MS analyses are summarized in **Figure 6. 1**. Therefore, it would be necessary to validate this study in the field to determine similarities and differences from the results obtained in this study. It would also be necessary to re-evaluate the experimental conditions described in this thesis on other cereals such as maize, rice or wheat in order to ascertain whether similar polar metabolites are affected or similar lipid remodelling processes as found in this study are adaptation mechanisms in salt-tolerant cereals or if they are conserved traits in plants in response to hyperosmotic stress. Since the physiological effects of the fungus increased biomass of the above ground plant parts, it would be important to map the biochemical changes of shoots to correlate the effects of the fungus in response to salt stress.

	Agar study		Soil study	
	cv. Vlamingh	cv. Gairdner	cv. Vlamingh	cv. Gairdner
Salt	Not much significant changes	↑ Sugars- higher need for stabilizing the cell membrane and protoplast	Same as agar study	Same as agar study
	Small reduction or no change	↑ Amino acids after salt stress-due to the decrease in growth rate and suggested as a secondary consequence of reduced growth	Same as agar study	Same as agar study
Salt+ Fungus	↑ Sugars- Further increase suggesting the role of fungus adjustment of water in the cytoplasm, Na ⁺ sequestration into the vacuole or apoplast, and protection of cellular structures		↑ osmolytes after salt stress– suggests role of fungus in protective mechanism against oxidative damage	↓ osmolytes after salt stress- role of fungus in translocation to aboveground plant parts
	Amino acids- No change	↓ Amino acids- Decrease-speculate that the fungus helps to take up amino acids directly by the roots and are transported between different organs through xylem and phloem thereby enhancing the capacity of plant to withstand salt stress.	↑ proline- acts as osmoprotectants ↑ Organic acids- role in lowering soil electrical conductivity and increasing the availability of N, P, and K in soil	↓ proline- indicating the toxic effects of salinity are reduced by the fungus ↓ Organic acids- calls for further investigation

Figure 6. 1 Summary for agar study and soil study from metabolomics results given in **Chapter 3** and **Chapter 4** in salt and salt + fungus treatments in cv. Vlamingh and cv. Gairdner. Arrows pointing up indicates increase in the metabolites whereas arrows pointing downwards indicate decrease in metabolites.

Positive mode ionization is the most common mode in LC-MS because it can effectively ionize a wide range of lipid. However, negative ionization provides superior results for certain lipid classes, such as phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA). These were either detected and identified in small number or not detected using the positive mode analysis mentioned in this study, due to time limitations negative mode ionization was not performed. Therefore, future studies should focus on covering the complete profile of lipids by measuring lipids in negative mode to determine the role of those lipids in plant-fungal interaction.

This study determined the changes in roots inoculated with the fungus. However, metabolic analyses of *Trichoderma harzianum* axenic culture is required to understand the fungal metabolite and lipid profile prior to inoculation. This will guide interpretation of results providing a better understanding on the biochemical responses and changes in roots that occurred due to fungal interaction.

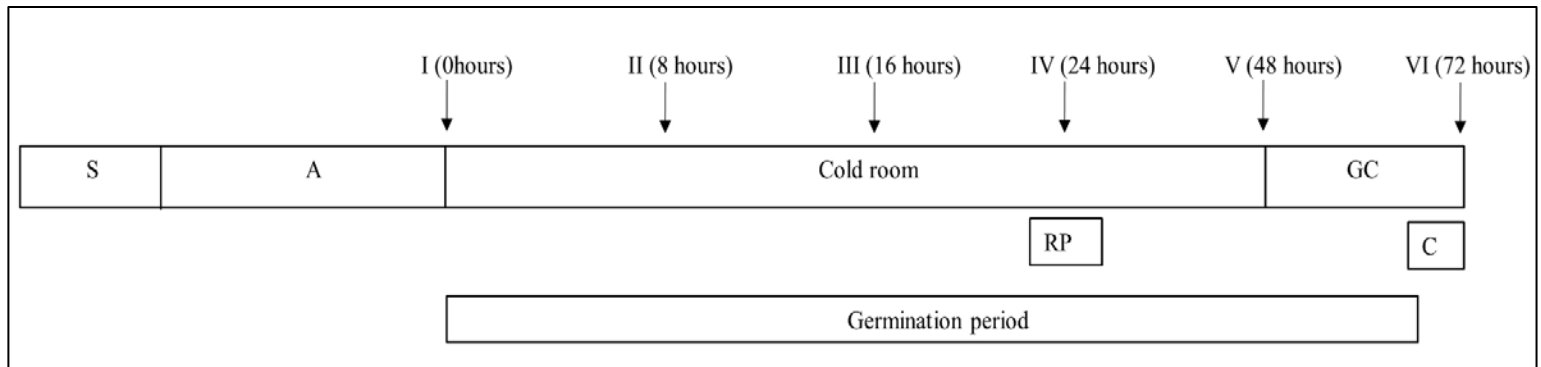
Furthermore, plant metabolites are segregated in highly differentiated and specialized organs, tissues, cells and organelles and are deeply involved in physiological regulation and responses to development and cues from the environment. Therefore, in addition to the high-throughput data generated in metabolomics and lipidomics analyses, there has been an increasing interest in tissue-resolved studies. Spatially resolved metabolomics provides global metabolite analyses with high anatomical, spatial and temporal resolution to describe the complexity of the plant system at a cellular and subcellular level (Sumner et al. 2011). In recent years, development and improvement of Mass Spectrometry Imaging (MSI) platforms for spatial analysis of metabolites and lipids have provided insights into genetic and biochemical aspects of cellular functions. Moreover, MSI will allow elucidation of net metabolic fluxes between cells and discovery of the intricate metabolic networks that control plant responses to phenotypical and environmental changes. MSI has been employed in this study to determine the early stages of germination in barley. However, studies mentioned by (Sarabia et al. 2019; Sarabia et al. 2018), which measured changes in barley roots exposed to salt stress in a spatial manner can be employed to analyse roots inoculated with the fungus used in this study. This will enable us to map the spatial distribution of metabolites and lipids that changed as a response to salt stress in barley genotypes.

Lastly, gene expression studies play significant roles for the discovery of molecular mechanisms in animal, microbial and plant systems (Hua et al. 2014; VanGuilder et al. 2008). RNA-Seq is another powerful tool to disentangle plant-fungal interaction at molecular level. (Balestrini et al. 2017) used RNA-Seq for grapevine roots inoculated with AMF and showed that the fungus altered the expression of genes involved in nutrient transport, transcription factors, and cell wall-related

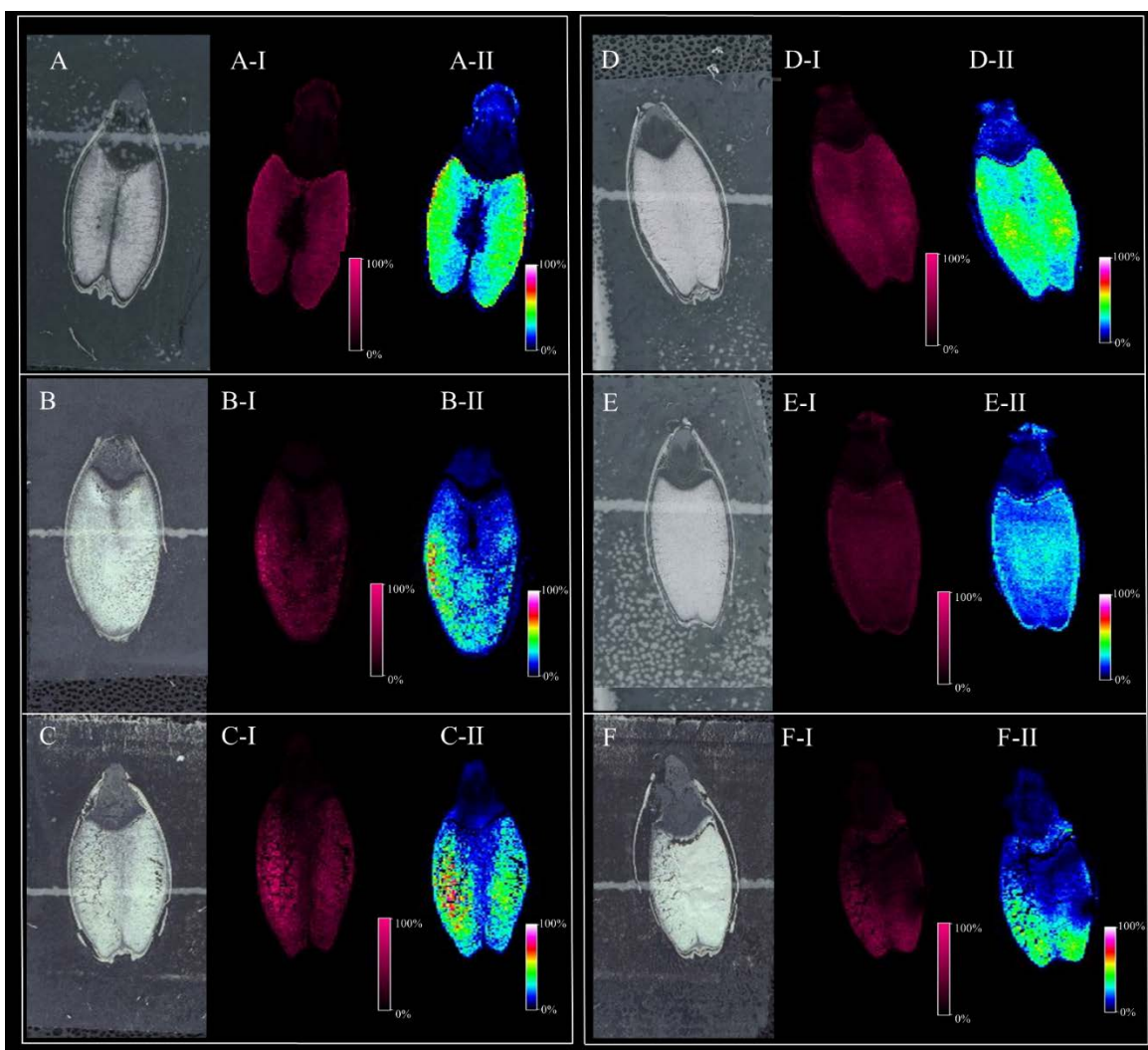
genes. Chen et al. (2017) used this technique to reveal a total of 909 differentially expressed genes (DEGs) in orange seedlings inoculated with *Rhizophagus irregularis*, with 31, 32 and 23 DEGs related to P metabolism, sugar metabolism and plant hormones. These transcriptomic data were further validated using quantitative real time PCR (qRT-PCR). Similarly, it can be suggested that roots from this study can be used for transcriptomic analysis using RNA-Seq to obtain a global view of the pathways by which *Trichoderma harzianum* imparts salt tolerance or activates adaptive mechanism in barley genotypes.

Quantitative reverse transcription PCR (RT-qPCR) is a versatile analytical technique that allows for characterisation and absolute quantitation of transcriptional changes in one or more targeted genes of interest (Jacob et al. 2013). It is also considered as a benchmark molecular diagnostic method that offers reliable information due to its large dynamic range, high specificity, sensitivity, simplicity, costs and high-throughput (Hua et al. 2014; Jacob et al. 2013). RT-qPCR is commonly used for analysis of gene expression in various plant systems (Moraes et al. 2015; Sousa et al. 2015), under different plant developmental stages (Garg et al. 2016; Koo et al. 2010; Li et al. 2016), and determining plant response to adverse environmental conditions (Luan et al. 2015; Makarevitch et al. 2015; Tian et al. 2015), including salinity (Forieri et al. 2016; Zhao et al. 2014b; Ziemann et al. 2013). Results from **Chapter 3 and 4** showed that TAGs production was greatly affected in both genotypes which while other lipid species had varying responses, which indicated potential alternative pathways used for TAG production. Thus, future studies can aim to investigate the changes in the relative gene expression involved in these pathways to confirm our hypothesis generated in this thesis.

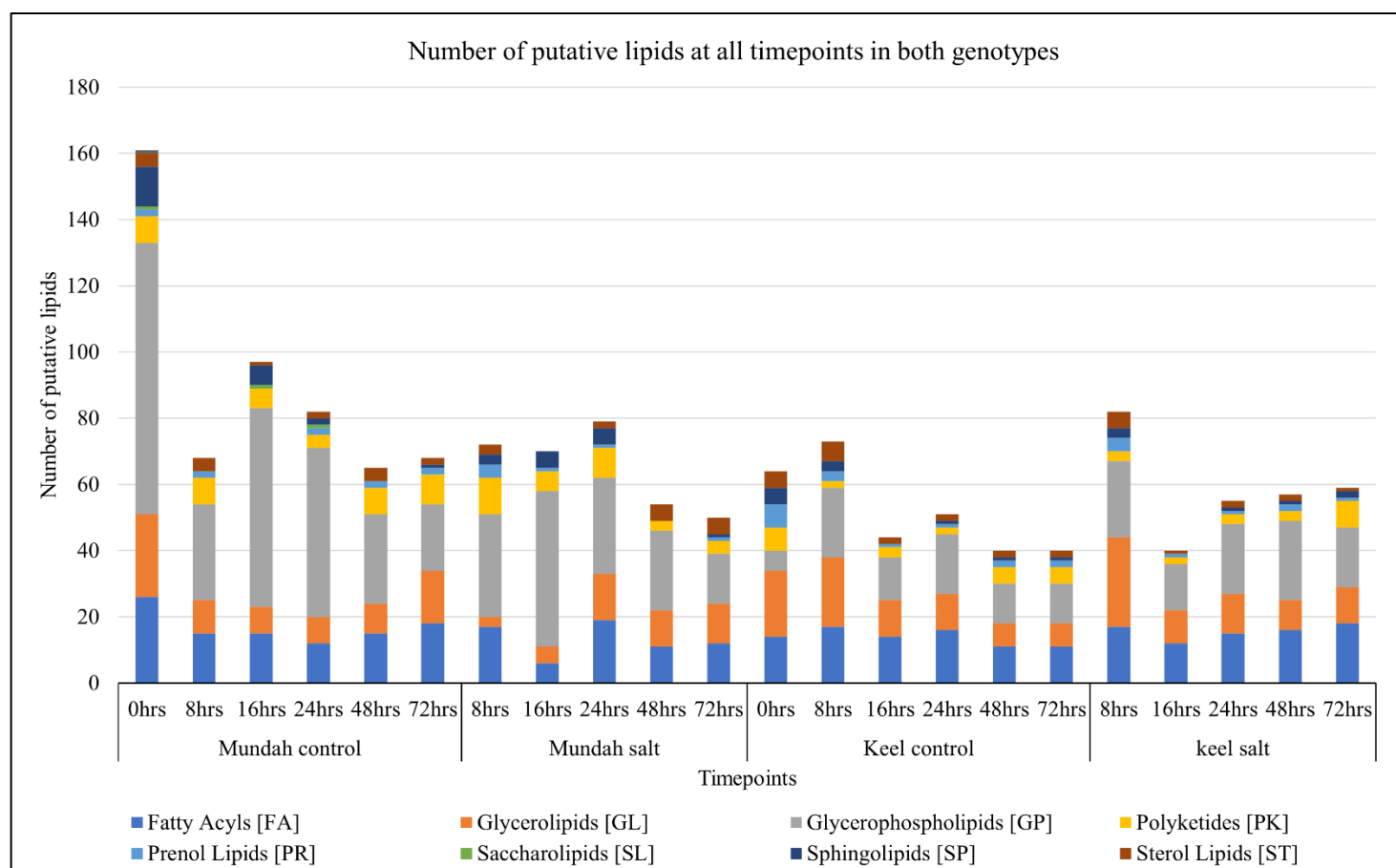
Chapter 7
Appendices



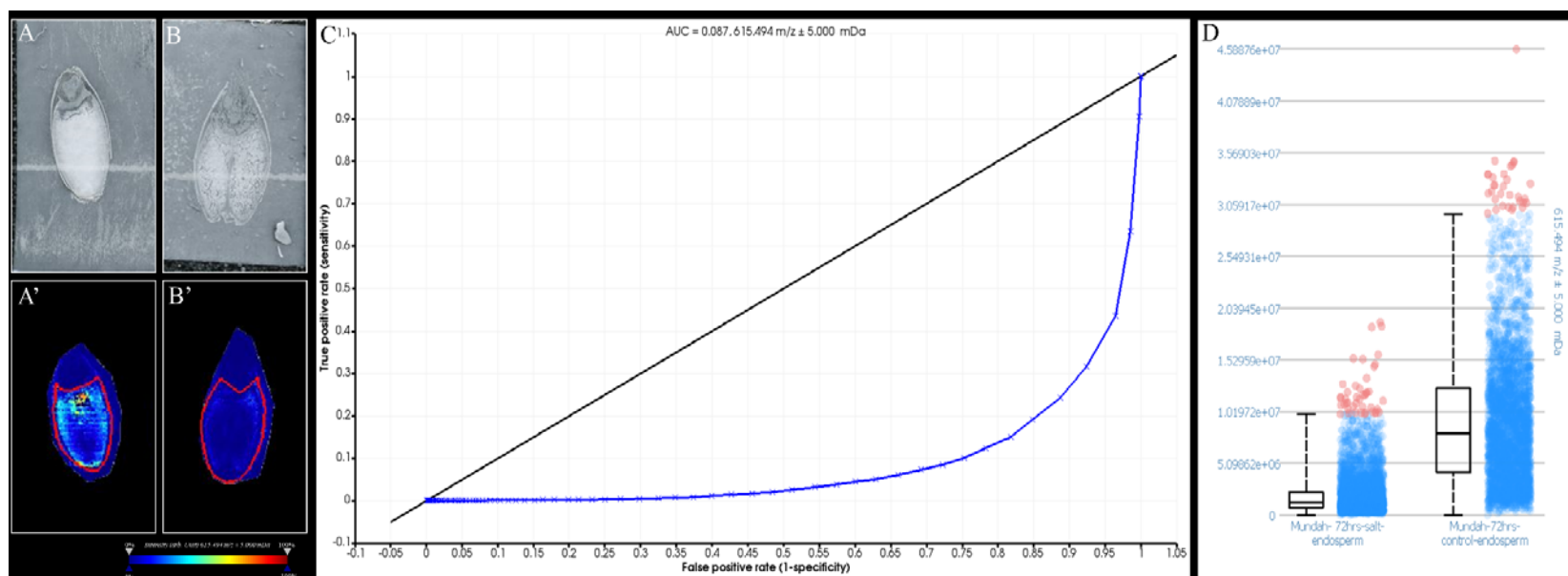
Supplementary Figure 7-2 Timepoints for barley genotypes germinated for the collection of samples. Arrows indicate timepoints for collection of samples. I: 0 hours, II: 8 hours, III: 16 hours, IV: 24 hours, V: 48 hours, VI: 72 hours post aeration. S: sterilization of seeds, A: overnight aeration, GC: growth cabinet (dark, constant 17°C), RP: radicle protrusion, C: coleoptile emergence, Germination period: starts from emergence of radicle (visible germination) until final sample collection (72 hours).



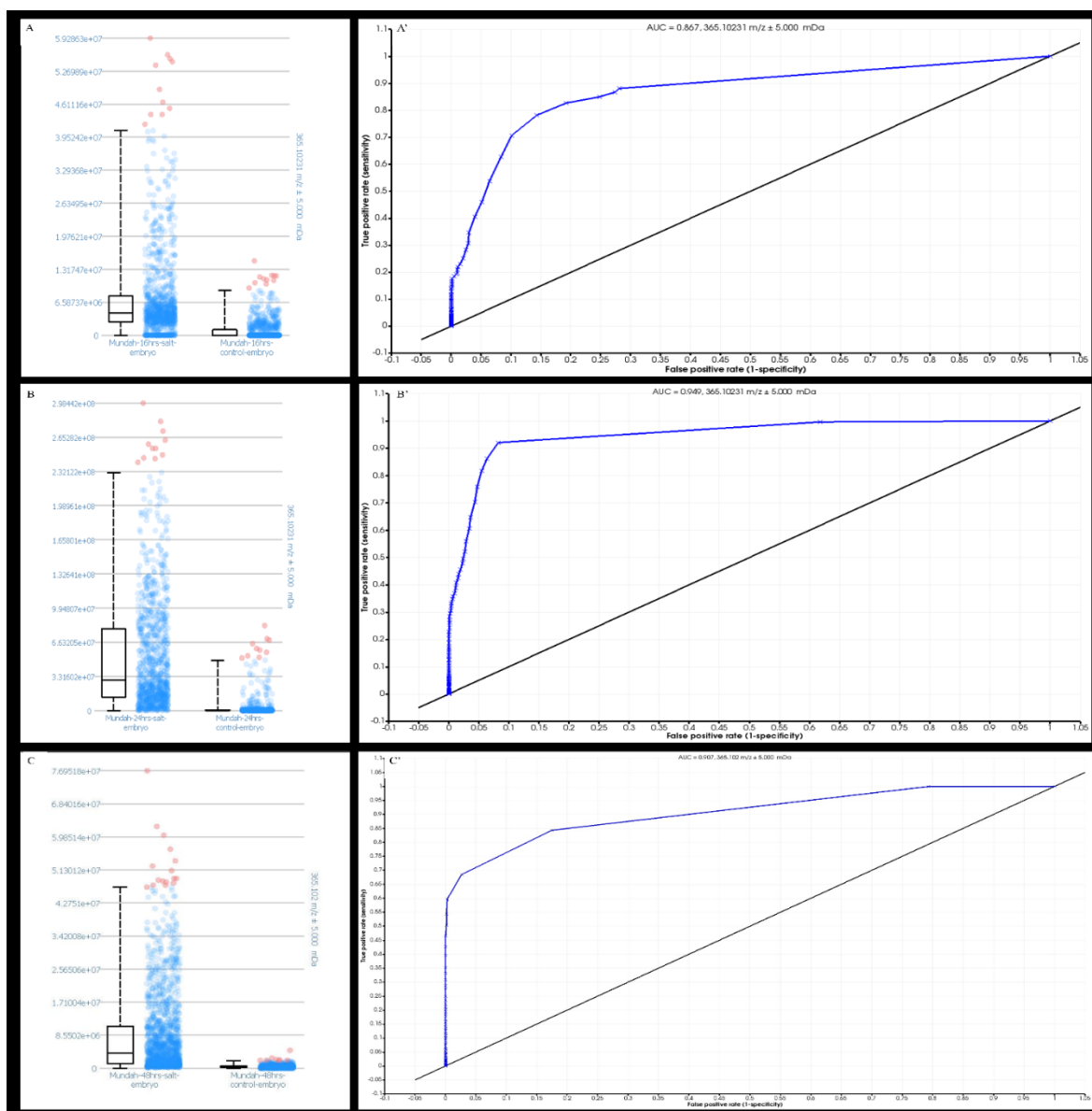
Supplementary Figure 7-3 Tissue distribution of m/z 796.52 PC(34:2) observed in three independent replicates at 16 hours in Mundah control and salt treated seeds. A, B, C: scanned image of Mundah control seed replicates 1,2 and 3; A-I, B-I, C-I: single colour filter for m/z 796.52 [M+K]⁺ showing the intensity scale of 0-100%. A-II, B-II, C-II: colour gradient filter to visualize the distribution of m/z 796.52 [M+K]⁺ with intensity scale of 0-100%. D, E, F: scanned image of Mundah salt treated seed replicates 1, 2 and 3; D-I, E-I, F-I: single colour filter for m/z 796.52 [M+K]⁺ showing the intensity scale of 100%. D-II, E-II, F-II: colour gradient filter to visualize the distribution of m/z 796.52 [M+K]⁺ with intensity scale of 100%.



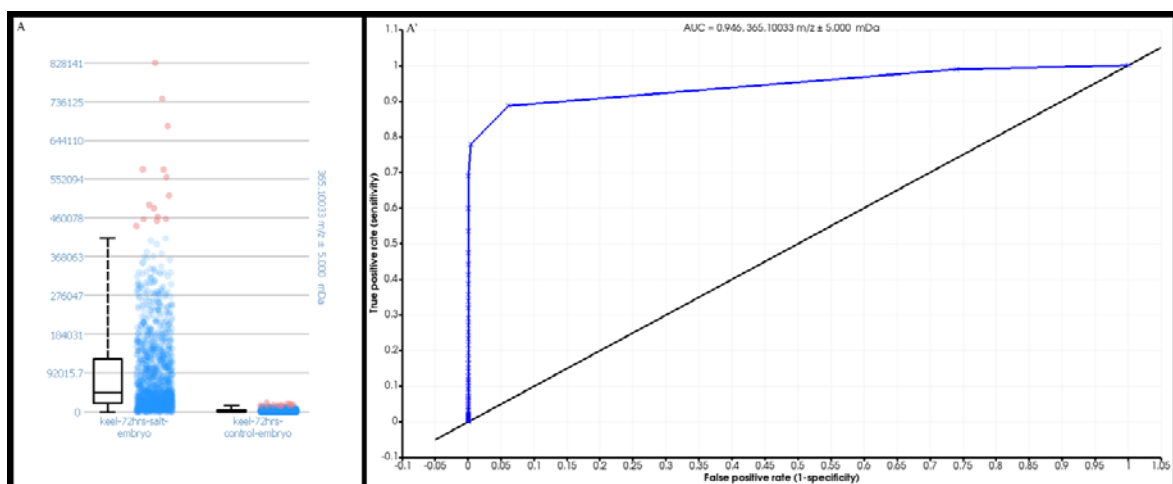
Supplementary Figure 7-4 Distribution of total lipid classes at all time points in cv. Mundah and cv. Keel germinating seed under control and salt-treated conditions. The X-axis shows the six time points for control and 5 time points for salt treated samples. The Y-axis indicates the number of lipids putatively identified at each time point. The putative lipid identities shown in this graph were established using an accurate mass precursor ion search (<5 ppm) of the LIPID MAPS database. Note: These identities are not collapsed into single ID and hence there is repetition of masses at different time points.



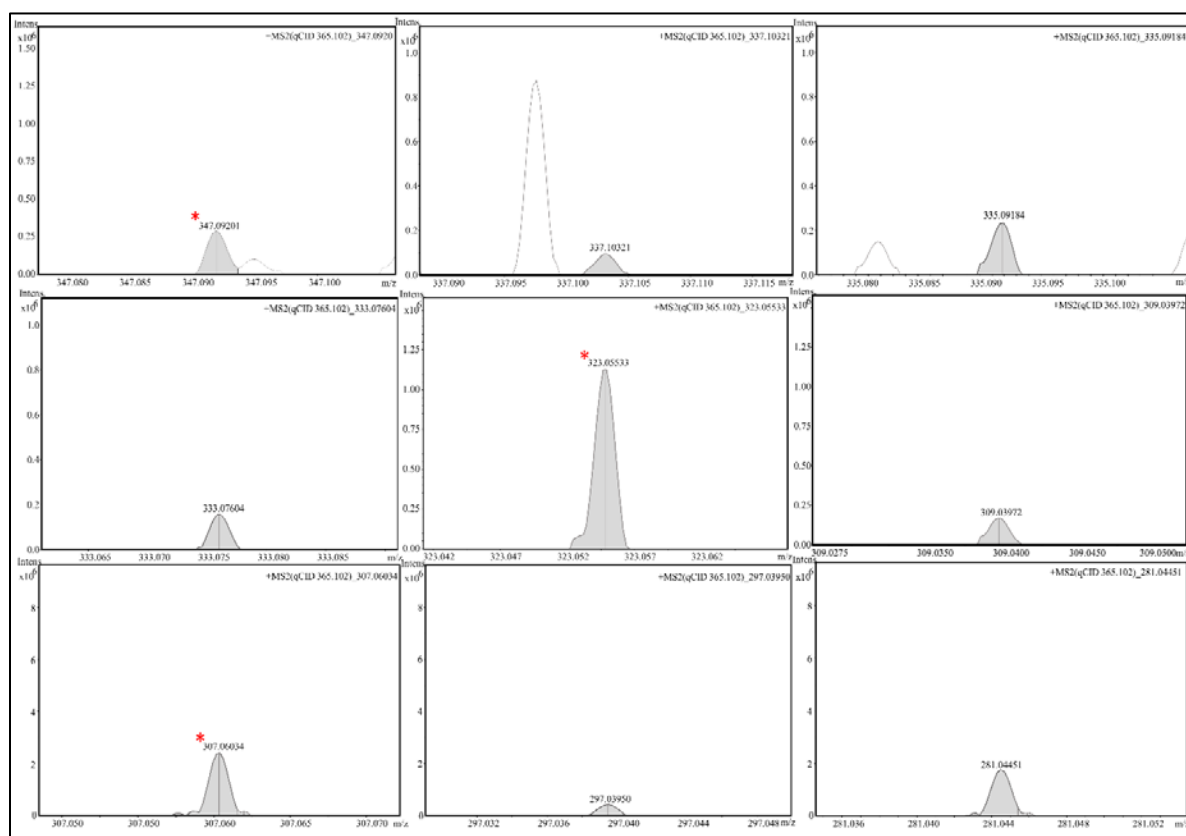
Supplementary Figure 7-5 ROC plot for m/z 615.494 [M+Na]⁺ DAG(34:2) from Mundah endosperm. AUC was 0.087 showing high intensity of this ion in control seed of Mundah at 72 hours. A: Mundah control 72 hours seed scanned image, B: Mundah salt treated 72 hours seed scanned image, A': Mundah control 72 hours showing DAG(34:2) in the endosperm region, B': Mundah endosperm region for salt treated seed at 72 hours. C: ROC plot for m/z 615.494 showing true positive rate higher than 0.8. D: Intensity box plot chart for two selected endospermic regions. Red lines indicate sub regions created in SCiLS Lab. Here, red bordered regions are the endosperms. Intensity scale was set to 0-100%.



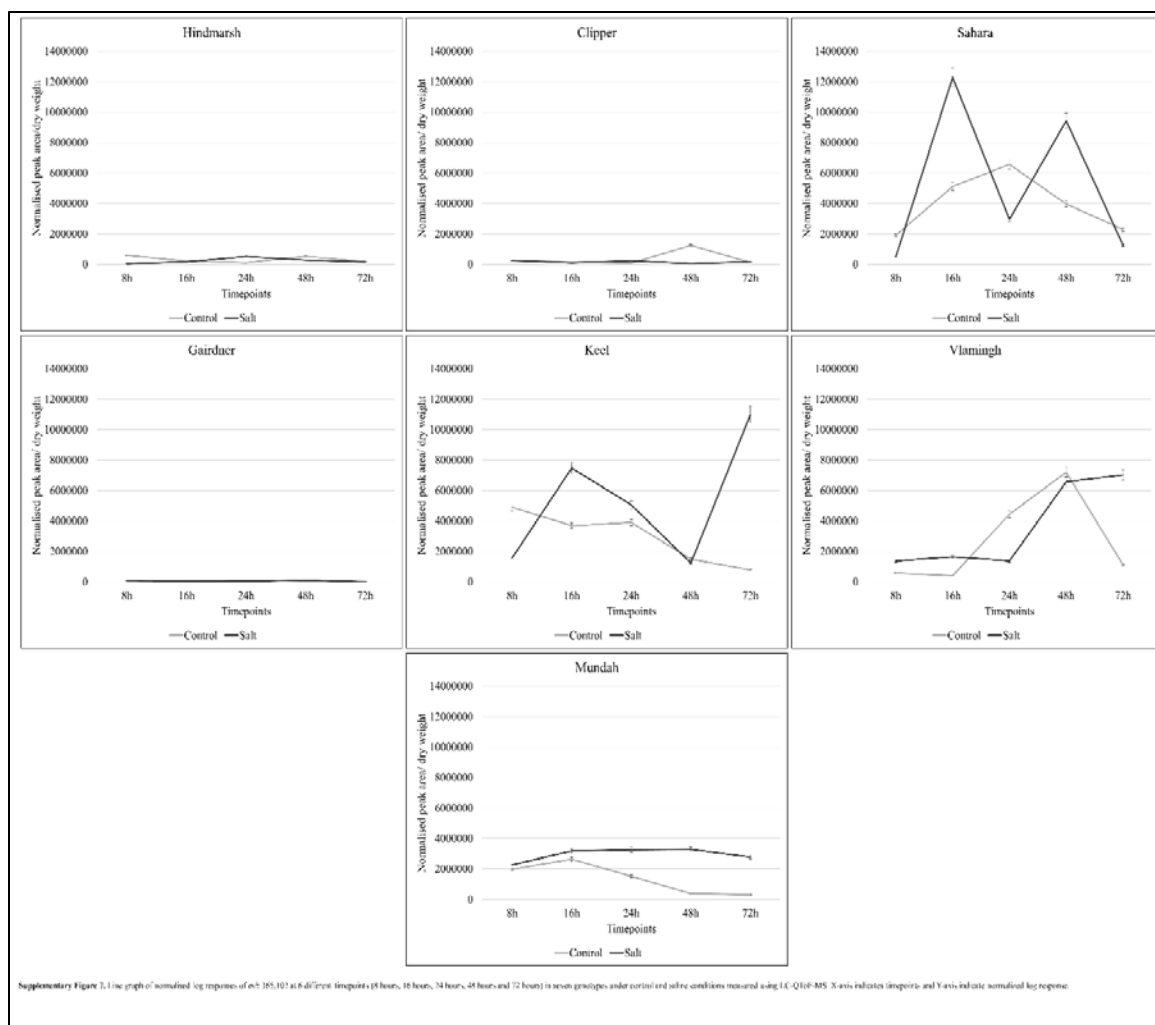
Supplementary Figure 7-6 The intensity box plots and ROC plots for a putative flavonoid (m/z 365.102) observed in Mundah at 16, 24 and 48 hours. A, B, C: The intensity box plots at 16, 24 and 48 hours. The box part contains a rectangle divided by a horizontal line, that represents a median intensity. Lower and upper bounds of the box represent the total number of spectra with intensities below these lines in one quarter and three quarters respectively. The cloud part of the plot shows scattering of spectra of a given region by intensity of a given m/z interval. Blue dots represent the spectra in which intensities of a given m/z interval are between the lower and upper quantiles whereas red dots represent the spectra whose intensities are outliers. A', B', C': ROC plots at 16, 24 and 48 hours showing the discrimination capabilities of given m/z for two (control and salt) regions with AUC values of 0.867, 0.952 and 0.907 respectively.



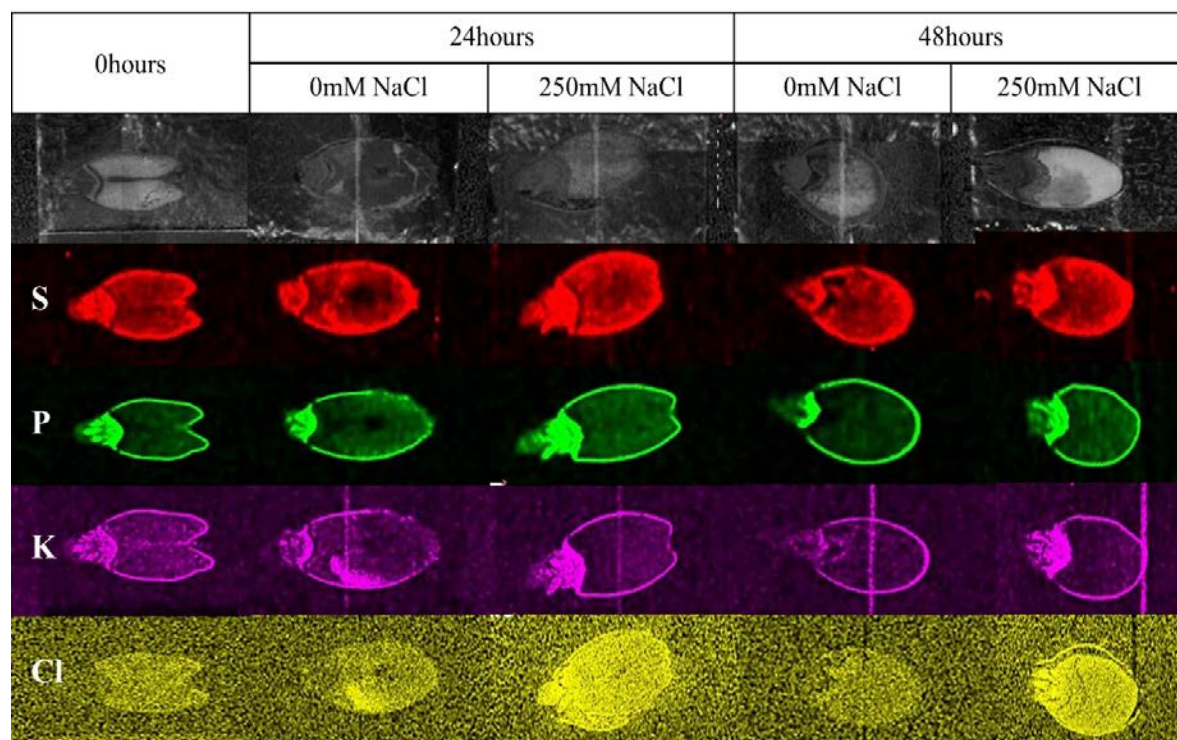
Supplementary Figure 7-7 The intensity box plots and ROC plots (AUC value of 0.953) for a putative flavonoid (m/z 364.0947) observed in Keel at 72 hours.



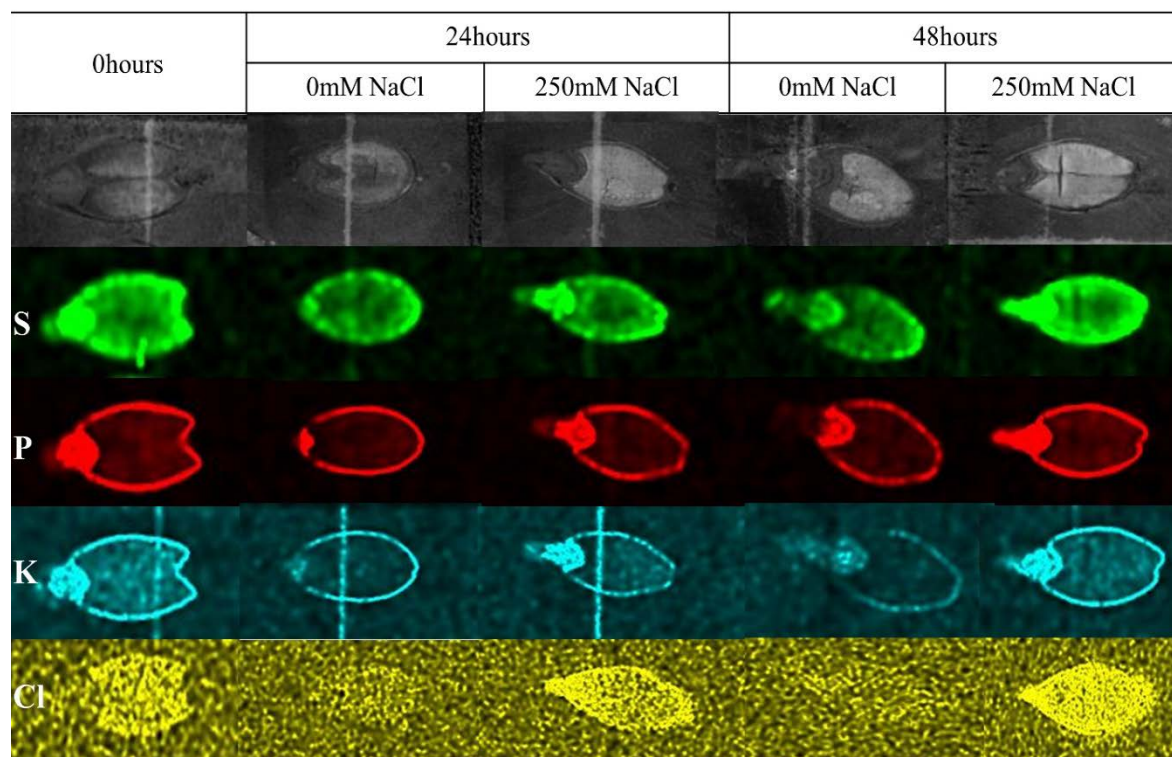
Supplementary Figure 7-8 MS/MS fragments of precursor ion with m/z 365.102 [M+H]⁺. Nine product ions were detected with m/z 347.0920, m/z 337.1032, m/z 335.0918, m/z 333.0760, m/z 323.0553, m/z 309.0397, m/z 307.0603, m/z 297.0395 and m/z 281.0445. Asterisks represent fragments matched with LC-MS/MS analysis shown in figure 5.



Supplementary Figure 7-9 Line graph of normalised log responses of m/z 365.102 at 6 different timepoints (8 hours, 16 hours, 24 hours, 48 hours and 72 hours) in seven genotypes under control and saline conditions measured using LC-QToF-MS. X-axis indicates timepoints and Y-axis indicate normalized log response



Supplementary Figure 7-10 The elemental maps of S, P, K and Cl in germinating barley seeds of Mundah under control and saline conditions. Each image indicates the relative distribution of the specific element captured at 10 ms/pixel.



Supplementary Figure 7-11 The elemental maps of S, P, K and Cl in germinating barley seeds of Keel under control and saline conditions. Each image indicates the relative distribution of the specific element captured at 10 ms/pixel.

To access Supplementary Tables mentioned in Chapter3 and Chapter 4, click on the link- [Supplementary Tables - Chapters 3 and 4 - Sneha Vinay Kumar Gupta PhD thesis](#)

For Supplementary Tables corresponding to Chapter 5, refer to hyperlink- [Supplementary Tables 7-1 to 7-11](#). Click on [Table 1.xlsx](#) under Associated Data Section

Chapter 8

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