FUNGAL ENDOPHYTES THAT CONFER TOLERANCE TO SALT AND DRY CONDITIONS

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfilment of the Requirements

for the Degree of Master of Science

in the Department of Biology

University of Saskatchewan

By

Kumkum Azad

© Copyright Kumkum Azad, 2016. All rights reserved.

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying, publication, or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Biology
University of Saskatchewan
Saskatoon, Saskatchewan
Canada S7N 5E2

ABSTRACT

Fungal endophytes grow symbiotically inside plants, where some strains promote plant growth and survival under particular abiotic stresses. Tomato (*Solanum lycopersicum* Var. Rutgers) seeds were inoculated with systemic (also called class 2) fungal endophytes (*Alternaria* spp and *Trichoderma harzianum*). These endophytes were isolated from plants naturally growing in salinized-soil in Saskatchewan, e.g. Little Manitou Lake shore, Radisson Lake shore, and Mosaic Belle Plaine tailings area. The effects of colonization with systemic fungal endophytes were studied on growth performance of tomato plants under NaCl and drought stress. Endophyte-colonized plants had greater fresh shoot biomass than control plants after 20 d of NaCl stress (300 and 500 mM). They also maintained greater fresh root biomass after 10 d NaCl stress (300 mM). After exposure to chronic 100 mM and 200 mM NaCl stress, there was no remarkable difference in plant biomass (both root and shoot) between endophyte-colonized plants and non-colonized control plants. Exposure to NaCl stress altered different aspects of the plants' physiology such as photosynthetic efficiency, osmolyte adjustment, and reactive oxygen species generation. Photosynthetic efficiency was improved by endophyte colonization during chronic NaCl stress, but decreased significantly during ≥400 mM NaCl stress. Although osmolality of plants increased with the increase of NaCl salinity, there was no effect of endophyte colonization on plant osmolality. On the other hand, reactive oxygen species activity of endophyte-colonized plants was always lower in comparison to non-colonized control plants in response to NaCl stress.

Endophyte-colonized plants growth performance and physiological responses were also determined under drought. Endophyte-colonized plants had significantly higher shoot biomass in comparison to non-colonized control plants after intermittent drought and continuous drought. Physiological responses of plants differed following intermittent and continuous drought stress. Photosynthetic efficiency of endophyte-colonized plants improved significantly after intermittent drought, but there was no effect of endophyte colonization on photosynthetic efficiency of plants after continuous drought. On the other hand, increased proline accumulation and decreased osmolyte concentration were observed in endophyte-colonized plants in response to drought stress. There was also indication of less reactive oxygen species in endophyte-colonized plants upon drought stress. Finally, better fluid use efficiency of endophyte-colonized plants was

observed, which is correlated to drought tolerance in endophyte-colonized plants. These results suggest that our systemic fungal endophytes have the potential to improve agriculture and horticulture on salinized and dry soils which are common phenomenon in semi-arid environments.

ACKNOWLEDGEMENT

First of all I would like to acknowledge my supervisors, Dr. Susan Kaminskyj and Dr. James Basinger. I am grateful to Dr. Kaminskyj for considering me as a member of her research group and then her guidance, support and patience throughout my research project. Then I thank Dr. Basinger for his advice, support, and encouragement till end of my program. It was not possible to complete my research without their continuous support.

I would like to thank my supervisory committee members: Dr. Ken Wilson and Dr. Diane Knight for their suggestion, criticism and patience. I am grateful to Dr. Wilson for his suggestions and guidance during research and thesis writing and editing.

I would like to thank all the members in Biology, my colleagues, and my friends. I would like to acknowledge Dr. Zakia Boubakir and Tim Repas for their support and suggestions. I also would like to thank Marlynn Mierau and Guosheng Liu for technical help. I would like to thank summer students and volunteers: Huimin Zhang, Mariam Goubran, Dominic Olver, Robert Mercado, for their help and time.

I would like to express my gratitude to Solmaz Irani, and Mortuza Reza for their help and support in different stages of my research. I would like to thank Ankur Jamwal, Asad Shah, and Apurba Das for their help in statistics.

Finally, a thank is not enough for my family, my parents, my uncles, and my aunts for holding my hands from thousands miles away, and for their motivation and support to continue my study.

TABLE OF CONTENTS

PERMISSION TO USE I
ABSTRACT II
ACKNOWLEDGEMENTS IV
TABLE OF CONTENTS V
LIST OF FIGURES X
LIST OF TABLES XIII
LIST OF ABBREVIATIONS XIV

Chapter 1: Introduction 1
1.1 Plant response to salt stress 2
1.1.1 Ion toxicity/ Ionic imbalance 3
1.1.2 Nutrient uptake imbalance 3
1.2 Plant response to drought stress 4
1.3 Oxidative stress induced by drought and salt stress 5
1.4 Mechanisms of NaCl and drought stress tolerance 6
1.4.1 Compatible solutes and osmotic adjustment 7
1.4.2 ROS defence mechanism 8
1.4.3 Specific mechanisms for NaCl and drought tolerance 9
1.5 Plant symbionts 10
1.5.1 Fungal symbionts 11
1.5.2 Fungal endophytes 12
1.5.2.1 Functional role of fungal endophytes in plant growth and development 14
1.5.2.1 Fungal endophyte colonization leads to increased plant stress tolerance 15
1.6 Objectives and hypotheses 16
1.6.1 Objectives and hypotheses of Chapter 2 entitled, ‘A fungal endophyte strategy for mitigating the effect of salt and drought stress on plant growth’ 16
1.6.1.1 Objective 16
1.6.1.2 Hypotheses 16
1.6.2 Objectives of Chapter 3 entitled, ‘Characterization of systemic fungal

endophyte-induced abiotic stress-tolerance by alteration of proline accumulation and photosynthetic efficiency' 17
1.6.2.1 Objective 17
1.6.2.2 Hypotheses 17
1.6.3 Objectives of Chapter 4 entitled, 'Protective effects conferred on tomato plants growing in saline or drought conditions by systemic fungal endophytes isolated from plants growing on saline soils in Saskatchewan' 17
1.6.3.1 Objectives 17
1.6.3.2 Hypotheses 17
1.7 References 17

Chapter 2: A fungal endophyte strategy for mitigating the effect of salt and drought stress on plant growth 23
2.1 Introduction 23
2.2 Materials and Methods 26
2.2.1 Endophyte isolation, culture, identification, and storage 26
2.2.2 Plant growth condition 26
2.2.3 Photosynthetic efficiency 28
2.2.4 Localization of H_2O_2 accumulation 29
2.2.5 Statistical analysis 29
2.3 Results 29
2.3.1 Systemic fungal endophytes can mitigate the effect of NaCl stress 29
2.3.2 Systemic fungal endophytes can mitigate the effect of drought stress 30
2.3.3 Systemic fungal endophytes can support and maintain photosynthetic efficiency despite drought stress 32
2.3.4 Systemic fungal endophytes can reduce reactive oxygen species (ROS) accumulation associated with stress 33
2.4 Discussion 34
2.5 References 34

Chapter 3: Characterization of systemic fungal endophyte-induced abiotic stress-tolerance by alteration of proline accumulation and photosynthetic efficiency 38
3.1 Introduction 39
3.2 Materials and Methods 41
3.2.1 Isolation, culture and identification of systemic fungal endophytes 41
3.2.2 Plant growth condition 42
3.2.3 Drought and salinity stress treatment 44
3.2.4 Biomass measurement and assessment of endophyte colonization 44
3.2.4.1 Root-endophytes colony visualization 45
3.2.5 Fluid use efficiency test 46
3.2.6 Photosynthetic efficiency assay 47
3.2.7 Proline accumulation assay 47
3.2.8 Siderophore and indole-type compounds assay 48
3.2.9 Statistical analysis 49
3.3 Results 49
3.3.1 Effects of acute salt stress on biomass 49
3.3.2 Effects of chronic salt stress on biomass 50
3.3.3 Effects of chronic salt stress on photosynthetic efficiency 52
3.3.4 Effects of acute drought stress on biomass 54
3.3.5 Fluid use efficiency 55
3.3.6 Proline accumulation 56
3.3.7 Siderophore and indole production by systemic fungal endophytes 58
3.4 Discussion 59
3.4.1 Systemic fungal endophytes and increased biomass 59
3.4.2 Systemic fungal endophytes and increased photosynthetic efficiency 59
3.4.3 Systemic fungal endophytes and fluid use efficiency 61
3.4.4 Systemic fungal endophytes and proline accumulation under drought 61
3.4.5 Siderophore and indole detection from isolated systemic fungal endophytes 62
3.5 References 63

Chapter 4: 'Protective effects conferred on tomato plants growing in saline or drought conditions by systemic fungal endophytes isolated from plants growing on saline soils in Saskatchewan' 68
4.1 Introduction 69
4.2 Materials and methods 71
4.2.1 Plant growth conditions 71
4.2.2 Salt and drought stress treatments and harvesting 71
4.2.3 Physiological tests 72
4.2.3.1 Detection and estimation of *in vivo* superoxide (O_2^-) generation 72
4.2.3.2 Detection and estimation of *in vivo* hydrogen peroxide (H_2O_2) generation 72
4.2.3.3 Determination of total Osmolyte concentration assay 73
4.2.3.4 Photosynthetic efficiency assay 73
4.2.4 Statistical analysis 73
4.3 Results 74
4.3.1 Maintenance of growth during drought an dsalinity in endophyte-colonized plants 74
4.3.2 *In vivo* localization and estimation of superoxide (O_2^-) accumulation following drought and salinity stress 76
4.3.3 *In vivo* localization and estimation of hydrogen peroxide (H_2O_2) accumulation following drought and salinity 79
4.3.4 Modulated osmolyte concentration following drought and salinity stress 82
4.3.5 Impact of endophyte colonization on PSII photochemical efficiency following drought and salinity stress 83
4.4 Discussion 85
4.4.1 Growth promotion 85
4.4.2 Reduced ROS/oxidative stress 86
4.4.3 Modulated osmolyte concentration 87
4.4.4 Modulated photosynthetic efficiency 88
4.5 References 89

Chapter 5: General Discussion 92
5.1 Tomato plants experimentally colonized with Saskatchewan saline-tolerance endophytes grew better than control plants on saline and/or dry conditions 92
5.2 Plant physiological aspects related to colonization with systemic fungal endophytes 93
5.3 Future studies 96
5.4 References 98

Appendix 1 100
Appendix 2 102
Appendix 3 105

LIST OF FIGURES

Figure 1-1. Schematic illustration of the 'two-phase growth response to salinity' 4

Figure 1-2. ROS generation due to abiotic stresses leads to ultimate cell death 6

Figure 1-3. Antioxidant enzyme activity in ROS scavenging mechanism after abiotic stress 9

Figure 1-4. Association among selected plant symbionts, host plant, and other microbes 11

Figure 1-5. Functional role of fungal endophytes in plant growth promotion 15

Figure 2-1. Experimental method (a-d) and (e) sample results for endophyte-mediated response to NaCl treatment. Seedling establishment (a), growth (b), imposed stress (here, drought; c), recovery (d). Typical response to 15 d NaCl stress (e). 28

Figure 2-2. Effect of 300 mM, and 500 mM NaCl on fresh biomass of non-colonized, positive control (FcRed1), and plants colonized by endophytes collected in this study 30

Figure 2-3. Effect of drought on A) shoot and B) root fresh biomass, comparing non-colonized or negative control plants (blue) with the positive-control FcRed1 and plants colonized by Saskatchewan saline endophytes 31

Figure 2-4. Effect of drought on photosynthetic efficiency of control, FcRed1 and Saskatchewan endophyte-colonized tomato plants 32

Figure 2-5. Localization of H_2O_2 in tomato leaves grown in 300 mM NaCl for 20 d. The H_2O_2-DAB reaction produces a brown pigment that is visible after chlorophyll extraction 33

Figure 3-1. Primary and secondary endophyte cultures 42

Figure 3-2. Magenta box preparation 43

Figure 3-3. Endophyte-colonization test after abiotic stress experiment and reacquisition of endophytes 45

Figure 3-4. Visualization of endophyte colony in plant roots under epifluorescence microscopy using lactofuschin staining method 46

Figure 3-5. Effect of 300 mM NaCl (10 d) on shoot and root biomass of endophyte-colonized and non-colonized plants. 50

Figure 3-6. Effects of chronic NaCl stress (100 mM and 200 mM) on shoot and root biomass of endophyte-colonized and non-colonized plants. 51

Figure 3-7. Effect of chronic NaCl stress (100 mM and 200 mM) on photochemical

efficiency at PsII of endophyte-colonized and non-colonized plants. 53
Figure 3-8. Effects of continuous drought stress (10 d) on shoot and root biomass of endophyte-colonized and non-colonized plants. 55
Figure 3-9. Effect of endophytes on fluid use efficiency (by fresh weight = FW and by dry weight = DW) of endophyte-colonized and non-colonized plants in 21 d. 56
Figure 3-10. Accumulation of proline in well-watered plants (A) compared to plants exposed to 7 d of drought (B) 57
Figure 4-1. Effects 15 d period of 400, 500, and 600 mM NaCl on shoot and root biomass of endophyte-colonized and non-colonized plants. 75
Figure 4-2. Effects of 11 d drought on dry shoot and root biomass of endophyte-colonized and non-colonized plants. 76
Figure 4-3. Qualitative and quantitative estimation of *in vivo* accumulation of superoxide in leaves of endophyte-colonized and non-colonized plants after 15 d NaCl stress. 77
Figure 4-4. Qualitative and quantitative superoxide content using NBT reaction in tomato plants exposed to 11 d drought stress. 78
Figure 4-5. Qualitative (A) and quantitative (B) measure of H_2O_2 content in plants exposed for 15 d to NaCl: 0 mM, 400 mM, 500 mM, or 600 mM. 80
Figure 4-6. Qualitative and quantitative estimation of H_2O_2 content in tomatoes exposed to drought**.** 81
Figure 4-7. Effect of NaCl stress (400 mM, 500 mM, and 600 mM) on shoot osmolyte concentration. 82
Figure 4-8. Effect of 11 d drought on shoot osmolyte concentration for non-colonized and endophyte-colonized tomatoes. 83
Figure 4-9. Effect of NaCl (400 mM, 500 mM, and 600 mM) on photosynthetic efficiency of endophyte-colonized and non-colonized tomato plants. 84
Figure 4-10. Effect of 11 d drought on photosynthetic efficiency of endophyte-colonized and non-colonized tomato plants. 85
Figure 3-11. CAS-agar plates showing siderophores assay for endophytes: *Rhizopus* sp., FcRed1, Hz613, 419, 414, 405. 101
Figure 3-12. Detection of indole-type compounds using Ehrlich reagent. Endophytes were cultured on filter paper disks in tryptophan rich-PDA (10%) medium for 7 days. 102

Figure 3-13. Three-week old plants treated with 300 mM NaCl for 10 d; (A) before stress, (B) after 10 d stress with 300 mM naCl, and (C) after rehydration with RO water for 2 d. 103

Figure 4-14. Three week old plants (A) treaded with 11 d continuous drought (B) and revived with RO water for 2 d (C). 104

LIST OF TABLES

Table 1-1. A comparison of functional characteristics of mycorrhizal fungi and fungal endophytes in plants 12
Table 1-2. Characterization of functional classes of endophytes 13
Table 3-1. Siderophore production by isolated systemic fungal endophytes 58
Table 3-2. Indole-type compounds production by isolated systemic fungal endophytes 58
Table 2-1. Endophytes species identification 100
Table 2-1. Oligonucleotides used in this study for PCR analysis 100
Table 2-3. Sites of plants collection 101

LIST OF ABBREVIATIONS

ABA	Abscisic Acid
AMF	Arbuscular Mycorrhizal Fungi
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
CAS	Chrome-azurol S
DAB	3, 3-Diaminobenzidin
DLAG	Distilled water, Lactic acid, Glycerol
DW	Dry weight
FW	Fresh weight
H_2O_2	Hydrogen peroxide
HTAB	Hexadecyl-trimethyl-ammonium bromide
IAA	Indole Acetic Acid
MBs	Magenta Boxes
MHB	Mycorrhization Helper Bacteria
NaCl	Sodium chloride
NBT	Nitro blue tetrazolium
O_2^-	Superoxide
OD	Optical density
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PGPB	Plant growth promoting bacteria
PGPR	Plant growth promoting rhizobacteria
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
PsII	Photosystem II
PVAG	Plyvinyl alcohol glycerol
RO	Reverse osmosis
ROS	Reactive oxygen species
rpm	Rotation per minute
UV	Ultra violet

CHAPTER 1: INTRODUCTION

Plants survive environmental variability and stress by means of physiological and biochemical adaptation. Symbiotic fungi including arbuscular mycorrhizae and septate endophytes play key roles in plant adaptation to stresses due to growth in terrestrial environments (Rodriguez et al. 2009). The most common abiotic stressors are soil salinity and dryness (Bartels and Sunkar 2007; Chaves et al. 2009; Azad and Kaminskyj 2015). In this thesis, I characterize a number of fungal endophyte strains isolated from plants growing in saline soils in Saskatchewan. I go on to examine if they confer tolerance to the common abiotic stressors: salinity and drought.

Plant growth, development, and yield are determined by biotic and abiotic factors (Bohnert et al. 1995; Hamdia and Saddad 2010; Atkinson and Urwin 2012). Biotic factors include interactions with other organisms, which can be beneficial or harmful. Beneficial symbiotic interactions with some fungi provide nutritional support or contribute to defense against damage from herbivores, but other fungi are pathogens or parasites (Atkinson and Urwin 2012). Abiotic factors that can influence plant growth include temperature, humidity, light intensity, as well as water, mineral and CO_2 availability. Abiotic factors determine plant growth parameters and resources. To achieve maximum growth and yield, plants need optimum levels of abiotic and biotic factors. When abiotic factors or environmental factor(s) are sub-optimal, they impose stressful conditions for plants (Bohnert et al. 1995).

As mentioned above, drought and salinity are two factors that can limit plant growth and development (Hamdia and Saddad 2010). Bartels and Sunkar (2007) stated that more than 10 % of agricultural land is adversely affected by drought and/or salinity. Most plants have similar responses to drought and salt stress, at least in the early stages (Bartels and Sunkar 2007). Because,low water availability and high salt level both induce osmotic stress and eventually cause wilting. Low cell and tissue volumes in non-turgid tissues are associated with generation of reactive oxygen species (ROS) that can damage cell structure and function (Bartels and Sunkar 2007). For example, decreased photosynthetic efficiency and increased abscisic acid production are common plant responses to drought and salt stress (Bartels and Sunkar 2007; Chaves et al. 2009).

Salinity is a major limiting factor for crop growth, development and yield (Gupta and Huang 2014). Salt stress is common in arid and semi-arid lands, where it forms due to high evaporation and low leaching water rate (Jouyban 2012). Over the year, continuous irrigation results in salts or ionic compounds being eventually deposited in the soil surface (Schwabe et al. 2006). Therefore, salinity problems are exacerbated in arid and semi-arid environments due to increased likelihood of irrigation for crop production (Khan and Duke 2001; Schwabe et al. 2006; Chaves et al. 2009). More than 6% of total land area is salt-contaminated (Schwabe et al. 2006) and almost 30 % of irrigated land is reported to suffer with salinity problems (Chaves et al. 2009). The effect of salinity build up due to irrigation can be seen historically in loss of soil fertility, soil compaction, and soil crusting (Dregne 1983). Making things worse, soil texture changes as salts accumulate, decreasing soil porosity leading to poor aeration and low water conductivity. Over time, salt deposition creates a low water potential zone in the soil, so that it becomes increasingly more difficult for plants to take up water and mineral nutrients (Porcel et al. 2012).

1.1 Plant Response to Salt Stress

Chronic salt stress reduces plant growth and development, and increases cell death and early senescence (Zhu 2007). Plants show three salt-specific types of physiological damage. First, high Na^+ and Cl^- levels damage structure and disrupt function of proteins and other macromolecules, leading to organelle damage and metabolic impairment (Evelin et al. 2009). Second, declining osmotic potential in soil solution reduces the amount of water available for uptake, which causes physiological drought (Mahajan and Tuteja 2005). Third, salinity leads to plant nutrient imbalance due to impaired nutrient uptake and transport (Adiku et al. 2001). Thus, salinity impairs processes required for plant growth and development, including photosynthesis, respiration, enzyme and protein synthesis (Ramoliya et al. 2004). Therefore, impacts of salinity on plants include osmotic effects, specific ion-toxicity, and nutritional disorders (Lauchli and Grattan 2007; Jouyban 2012). As plant growth slows, abscisic acid (ABA) levels increase (Jouyban 2012). High ABA causes stomatal closure and leads to reduced photosynthesis and increased photoinhibition which is the result of extreme light-inhibited activity of photosystem II (PSII) (Murata et al. 2007).

1.1.1 Ion toxicity/ ionic imbalance. In addition to osmotic stress, salinity creates ion toxicity for plants (Hasegawa et al. 2000). NaCl stress particularly contributes to ion imbalance at the root surface of plants. Since Na^+ and K^+ have similar chemistry, excess Na^+ suppresses K^+ uptake by roots (Jouyban 2012). Bartels and Sunkar (2007) also stated that Na^+ competes with K^+ for binding sites, and as a result, K^+ homeostasis is disrupted. Deficiency of K^+ causes disruption of plant metabolism, since K^+ is the most abundant cellular cation and plays an important role in maintaining cell turgor, membrane potential and enzyme activities.

1.1.2 Nutrient uptake imbalance. Salinity has a detrimental effect on nutrient uptake by plants as nutrient availability, transport and partitioning are affected by salinity (Niste et al. 2014). For example, NaCl loading results in nutrient deficiencies in plants because of the competition of Na^+ and Cl^- with essential nutrients such as K^+, $Ca^{2+,}$ and NO_3^- (Yokoi et al. 2002). Moreover, there is a correlation between increased NaCl concentration and decreased nutrient levels such as N, P, Ca, K, and Mg in species including fennel, peppermint and lemon (Bartels and Sunkar 2007).

Munns (2002, 2005) developed a concept of 'two-phase growth-response to salinity', based on plant response over time (**Figure 1-1**). In the first phase, plant growth reduces sharply (within several minutes) after NaCl stress due to osmotic changes. This is called the osmotic effect and initially reduces the plant's ability to absorb water. After the initial decrease in cell growth, a gradual recovery was observed until it reaches a steady state, which depends upon the salt concentration in soil zone (Munns 2002). Then, a second phase starts that is slower than the first and can be prolonged over days, weeks, or even months. During this phase, salt toxicity more strongly affects older leaves compared to younger ones. It also reduces total photosynthetic leaf area and accelerates senescence (Munns 2002).

Based on this two-phase concept, both salt-tolerant and salt-sensitive plants respond similarly to initial growth reduction because of the osmotic effect on roots. However, in the second phase, salt-sensitive plants respond differently from salt-tolerant plants. Salt-sensitive plants suffer from salt toxicity to a greater degree because of their inability to prevent salt accumulation in leaves during transpiration (Munns 2005).

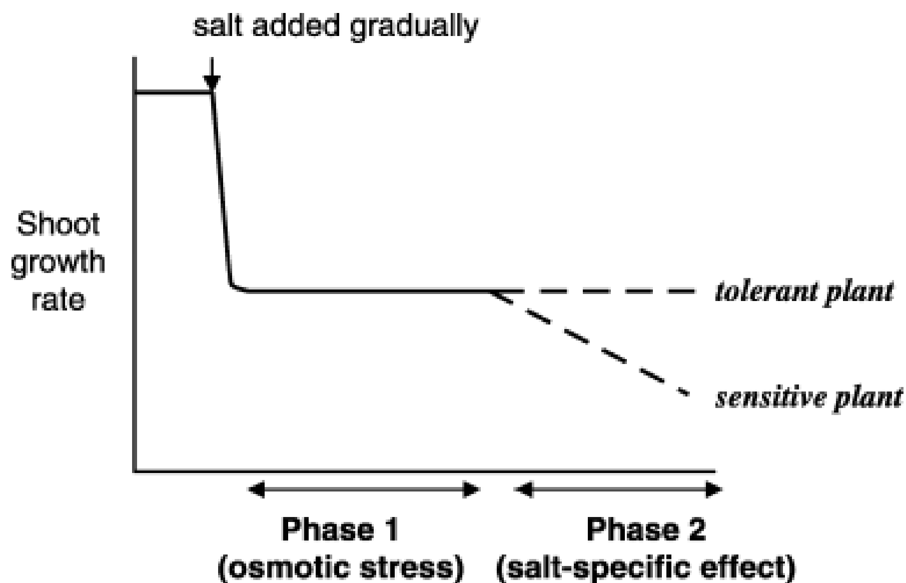


Figure 1-1. Schematic illustration of the 'two-phase growth response to salinity' (adapted from Munns 2005).

1.2 Plant Response to Drought Stress

Drought is a critical source of agricultural yield loss (Farooq et al. 2009; Jaleel et al. 2009). According to Jaleel et al. (2009) drought stress is a combination of reduced water content and diminished water potential, leading to turgor reduction, increased stomatal closure, and thus reduced cell growth and development. Photosynthesis and other metabolic processes in plants are inhibited by water deficiency that causes plant cell death (Jaleel et al. 2009). Farooq et al. (2009) also mentioned that drought stress suppresses plant growth by affecting various physiological and biochemical processes, such as photosynthesis, respiration, translocation, ion uptake, carbohydrate metabolism, and nutrient uptake. But the response of plants to drought stress differs at various organizational levels, and between plant species (Jaleel et al. 2009). It also depends on level of intensity and duration of stress (Jaleel et al. 2009).

Drought stress reduces seed germination and impairs seedling establishment (Harris et al. 2002; Kaya et al. 2006). Cell growth is severely hampered by drought, because lower turgor pressure leads to less water flow from xylem to the surrounding cells (Taiz and Zeiger 2006). Drought also impairs translocation of water and nutrients in roots and shoots of plants (Farooq et al. 2009). Nutrient uptake and nutrient unloading mechanisms are adversely affected by drought stress due to reduced amount of inorganic absorption (Garg 2003), but the effect also depends on the plant species and even genotypes within a species. Garg (2003) also reported that water stress causes an increase in N uptake and a decrease in P uptake, but there was no effect on K uptake.

Drought induces stomatal closure and limits CO_2 uptake, which is important for photosynthesis (Lawlor 2002). Farooq et al. (2009) stated that stomatal opening and closing is mostly affected by water deficiency compared to other components of plant water relations. In addition, alteration of leaf temperature plays a role in controlling leaf water status during drought stress. The stress caused by CO_2 limitation can result in photoinhibition and the destruction of photosynthetic pigment-protein complexes (Anjum et al. 2003).

1.2 Oxidative Stress Induced by Drought and Salt Stress

Generation of reactive oxygen species (ROS) is a secondary effect of abiotic stress on plants (Bartels et al. 2001; Apel and Hirt 2004). ROS include singlet oxygen ($^1O_2$), superoxide anion radicals (O_2^-), hydroxyl radicals (OH^-), and hydrogen peroxide (H_2O_2) (Apel and Hirt 2004). ROS are highly reactive and toxic to plant cells when they are generated in large amounts. They result in irreversible damage to proteins, lipids, carbohydrates, and DNA, and can even lead to cell death (Gill and Tuteja 2010). Generation of excessive amounts of ROS due to abiotic stress can play a major role in agricultural yield loss (Bartels and Sunkar 2007).

Chloroplasts, mitochondria, or peroxisomes are major sources of ROS in plant cells, because of their high rate of oxidizing metabolic activity or intense electron flow (**Figure 1-2**) (Gill and Tuteja 2010). ROS are produced in the chloroplast when singlet oxygen is generated from chlorophyll due to direct transfer of excitation energy and also during the Mehler reaction at photosystem I (Miller et al. 2010). Thus chloroplasts of plant cells are affected mostly by the accumulation of ROS when exposed to light during stress conditions. Moreover, concentrated

ROS suppresses repair damage to photosystem II and also inhibits protein production (Miller et al. 2010; Wilson et al. 2006).

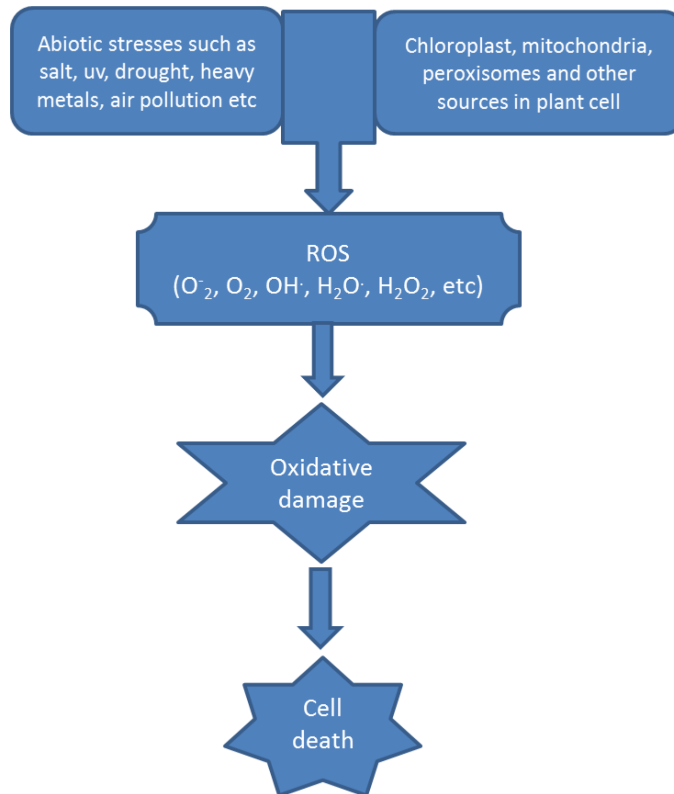


Figure 1-2. ROS generation due to abiotic stresses leads to ultimate cell death (adapted from Gill and Tutega 2010)

1.4 Mechanisms of NaCl and Drought Stress Tolerance

In response to abiotic stress, plants alter their metabolism, growth and development (Bartels and Sunkar 2007). To mitigate salt and drought stress, plants use two strategies: stress adaptation or stress avoidance. These mechanisms used vary on differences in stress perception, signal transduction, and appropriate gene expression programs, or metabolic pathways of stress tolerant plants. Zhu (2001) mentioned that stress-sensitive plants can become gradually adapted

to and acquire some degree of stress tolerance by acclimation and proper expression of genes responsible for adaptation.

1.4.1 Compatible solutes and osmotic adjustment. Generally, salt stress and drought induce some common physiological responses that are related to water consumption and ROS generation (Rodriguez et al. 2010). Despite salt or drought stress, plants must maintain internal water potential below that of the soil to maintain water uptake (Tester and Davenport 2003). One of the ways it can be achieved is to increase intra-cellular osmotica either by uptake of solutes from soil, or synthesis of intra-cellular metabolic solutes (Tuteja 2012).

Synthesis or accumulation of compatible solutes is a typical response for stress tolerance; common in all organisms, ranging from microbes to animals and plants (Sirraj and Sinclair 2002; Yokoi et al. 2002). Low molecular weight and highly soluble compatible compounds are non-toxic and remain unchanged in intra-cellular concentrations. Generally, they protect cells from stress by: adjustment of cellular water potential, detoxification of ROS, protection of membrane integrity, and stabilization of enzyme/protein structure and functions (Wani et al. 2013). Compatible solutes thus protect cellular components of plants from water stress and dehydration and are often termed osmoprotectants (Ahmed et al. 2014).

Though most of the compatible solutes are organic, some essential ions such as K^+, play similar roles (Yokoi et al. 2002). Examples of organic osmotic solutes are simple sugars (mainly fructose and glucose), sugar alcohols (glycerol and methylated inositols) and complex sugars (trehalose, raffinose and fructans) (Bohnert and Jensen 1996). Nuccio et al. (1999) mentioned some other compatible solutes that include quaternary amino acid derivatives (proline, glycine betaine, β-alanine betaine, proline betaine), tertiary amines (1,4,5,6-tetrahydro-2-mehyl-4-carboxyl pyrimidine), and sulfonium compounds (choline sulfate, dimethyl sulfonium propionate).

Compatible solutes can accumulate to high levels without affecting plant biochemistry (Bohnert and Jesen 1996), because they have little impact on pH of charge balance in the cytosol or organelles. Thus, they are also able to maintain enzyme activities under saline conditions. At high concentrations compatible solutes contribute to osmotic adjustment and act as

osmoprotectants (Yokoi et al. 2002). These compounds are found mainly in the cytosol and vacuole to adjust transportation of high concentration of salts inside and outside of cell (Yokoi et al. 2002). Compatible solutes also protect plants from toxic ions and dehydration (Yokoi et al. 2002; Zhu 2001). In addition, under salt stress, some compatible solutes protect plants from ROS toxicity by producing ROS scavenging products (Yokoi et al. 2002; Bohnert and Jensen 1996). Thus, osmoprotectants can help protect plants from salt and drought stress in multiple ways.

1.4.2 ROS defence mechanism. In general ROS are produced in low amounts in plants, but abiotic stresses disrupt plant metabolism and can result in significant increases in ROS concentration, leading to cellular damage (Apel and Hirt 2004). ROS can react with DNA, protein, and lipids, and in the absence of proactive mechanisms they damage cell structure and function (Evelin et al. 2009). Plant cells and organelles such as chloroplasts, mitochondria, and peroxisomes generate antioxidant machinery systems as a form of protection from toxic effects of ROS (Gill and Tuteja 2010). These antioxidant defense systems can be enzymatic or non-enzymatic (Bartels and Sunkar 2007). Generally non-enzymatic antioxidants include ascorbate (vitamin C), glutathione, tocopherol (vitamin E), flavonoids, alkaloids, and carotenoids, whereas enzymatic mechanisms consist of superoxide dismutase (SOD), peroxidases (POD), and catalase (CAT). Apel and Hirt (2004) discussed the elaborate classification of antioxidant systems. These systems either react with an active form of oxygen to keep them at a low level (i.e. superoxide dismutases, catalase and peroxidases), or regenerate oxidized antioxidants (glutathione, glutathione reductase, ascorbate, mono- and de-hydroascorbate). The first group of enzymes is involved in the detoxification of O_2^- and H_2O_2 ions, while the second system is responsible for the removal of these ions from different cellular compartments and organelles (Porcel et al. 2012). Because abiotic stress often results in increased levels of ROS, detoxification systems are a crucial component of stress acclimation.

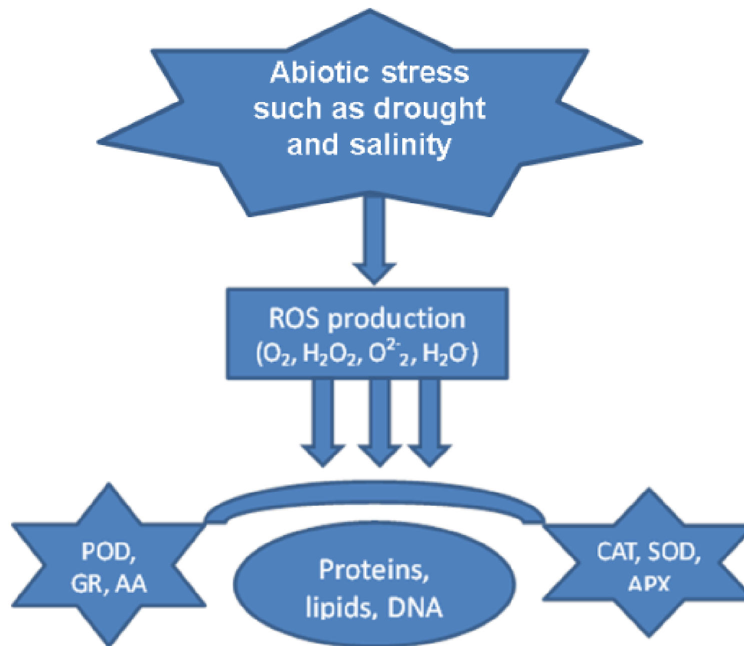


Figure 2-3. Antioxidant enzyme activity in ROS scavenging mechanism after abiotic stress (adapted from Farooq et al. 2009)

1.4.3 Specific mechanisms for salt and drought tolerance. During NaCl stress, Na^+ competes with K^+ for binding sites (Jouyban 2012). This competition may be mitigated by higher accumulation of $Ca^{2+,}$ since calcium helps to maintain potassium transport and potassium/sodium selectivity under NaCl stress (Zhu 2003). Mainly calcium affects an intercellular signaling pathway that regulates the expression and activity of potassium and sodium transporters (Zhu 2003). Plants also mitigate NaCl stress by ion homeostasis mechanisms (Hasegawa et al. 2000; Zhu 2003). Generally three mechanisms are employed to protect against accumulation of excess Na^+ in the symplast of plant cells. For example, firstly, Na^+ transporters (molecular identity is unknown) prevent entrance of Na^+ into plants (Zhu 2003; Maathuis et al. 2014); secondly, accelerate Na^+ compartmentalization in the vacuole (Zhu 2003; Maathuis et al. 2014); thirdly, Na^+ extrusion, when cystolic Na^+ can be transported back to the external medium or to the apoplast (Maathuis et al. 2014).

Drought stress mainly impairs water relations of plants at cellular, tissue and organ levels and results in damage to plants cell mainly due to dehydration (Beck et al. 2007). Plants may escape drought through short life cycle or early reproduction (Farooq et al. 2009). Other mechanisms are reducing water loss by transpiration, or improving water uptake through an extensive and prolific root system (Farooq et al. 2009). Limiting the number and area of leaves to reduce water consumption is one morphological adaptation to drought stress (Kavar et al. 2007). On the other hand, many physiological mechanisms are employed to mitigate drought stress, including (but not limited to) osmotic adjustment, accumulation of osmoprotectants and antioxidants, conservation of cell and tissue water, stabilization of cell membrane integrity, and production of plant growth regulators (Farooq et al. 2009).

1.5 Plant Symbionts

With regards to plants, Yang et al. (2013) defined symbionts as microorganisms that maintain relationships with plants without showing any pathogenic symptoms. They are able to promote plant growth and resistance to environmental constraints in return for carbohydrates fixed by the plant during photosynthesis. Fossil evidence indicates the relationship among plants and symbionts has been persistent throughout the evolutionary history of land plants (Harman 2011). Moreover, close association of plants and microorganisms is known from both aquatic to terrestrial environments (Yang et al. 2013). Plant symbionts were broadly classified into two groups: fungal symbionts; and bacterial symbionts (**Figure 1-4**).

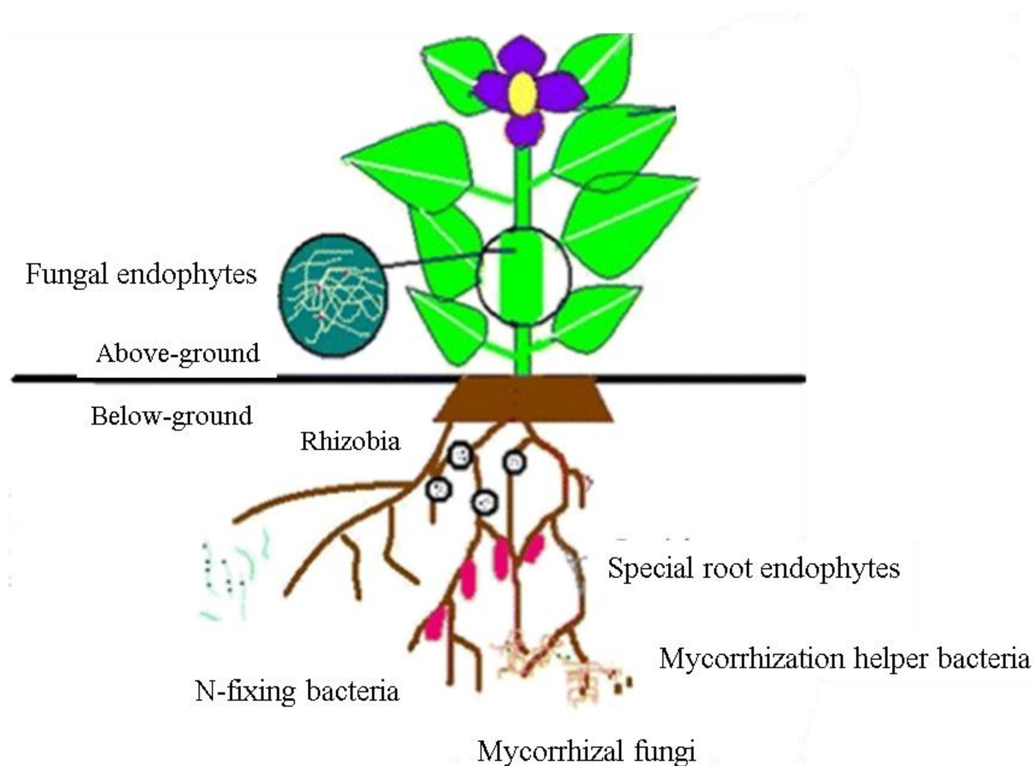


Figure 1-4.Association among selected plant symbionts, host plant, and other microbes (modified from Yang et al. 2012)

1.5.1 Fungal symbionts. Plant symbionts are common elements of the rhizosphere, particularly in non-cultivated soils, where they play a vital role in structure and function of plant communities (Petrini 1996; Rodriguez and Redman 1997). The fossil record indicates that fungal symbionts have been closely associated with plant communities for more than 400 million years, and fossil forms of these symbionts suggest that they are very much like current-day mycorrhizal and dark septate fungal endophytes (Rodriguez and Redman 2008).

Brundrett (2006) differentiated fungal symbionts into two functional groups based on plant colonization pattern, transmission, and ecological function. These two groups are: fungal endophytes; and mycorrhizal fungi. Fungal endophytes were defined as the fungi that grow within living plant tissues, but do not show any symptoms of their association to plants (Brundrett 2006). While these fungi can be pathogenic or beneficial, usually they colonize plant

tissues without showing pathogenic symptoms (Saikkoneen et al. 2008). Brundrett (2006) characterized mycorrhizal fungi based on the localized interface of specialized hyphae, the synchronized plant-fungus development, and benefits to plants for nutrient transfer (**Table 1-1**). Although endophyte association can be differentiated from mycorrhizal association by several criteria, they have similar characteristics too. For instance, both have similar pattern of nutrient exchange in host plants (Brundrett 2006).

Table 1-1. A comparison of functional characteristics of mycorrhizal fungi and fungal endophytes in plants (adapted from Brundett 2006)

Criteria	**Mycorrhizal fungi**	**Fungal endophytes**
Morphology	They form specialized hyphae in specialized plant organ	They form relatively unspecialized hyphae
Development	Synchronized	Not synchronized
Impact on fungus	Fungi are strongly dependent on plants for nutrients supply	Fungus moderately and weakly dependent on plants
Impact on plant	Strong or weak benefit	Weak harm or benefit
Nutrient transfer	Synchronized transfer; fungus serves as a strong sink	Passive transfer; fungus does not serve as a strong sink

1.5.2 Fungal endophytes. Systemic, septate fungal endophytes (hereafter, endophytes) grow and live entirely within host tissue and emerge only after host death (Rodriguez and Redman 2008). Endophytes comprise a phylogenetically diverse group of Dikarya. Most of them belong to the Ascomycota, while some of them belong to the Basidiomycota (Brundrett 2006; Rodriguez et al. 2009).

Fungal endophytes were classified by Rodriguez et al. (2009) into four classes according to host range, colonization pattern, mode of transmission, and types of conferred fitness benefits (**Table 1-2**). Class 1 endophytes are classified as clavicipitaceous (generally associated with grasses) while Classes 2, 3, and 4 are classified as non-clavicipitaceous (associated with non-vascular plants, ferns and allies, conifers, and angiosperms). Class 1 endophytes generally

colonize grasses and reside only in plant shoots causing systemic infections (in other words, systemic association) (Rodriguez et al. 2008). The other three groups of endophytes have comparatively broad ranges of hosts (Rodriguez et al. 2009). Class 2 endophytes colonize both above-ground (shoot and/or root) and below-ground tissues (only roots). In contrast, Class 3 endophytes colonize only above ground tissues, while Class 4 endophytes colonize only below ground tissues. Different classes of endophytes also maintain different patterns of infection (association) to the host. For example, Class 3 endophytes form localized infections (localized association), whereas Class 2 and Class 4 endophytes are able to form systemic infections in plants. Fungal endophytes can be transmitted either vertically from one generation to the next generation through seeds and vegetative propagules, or horizontally by their spores (Carroll 1988). According to Rodriguez et al. (2008), Class 1 endophytes are transmitted both horizontally and vertically, whereas Class 2, Class 3 and Class 4 endophytes are generally transmitted horizontally (**Table 1-2**).

Table 1-2. Characterization of functional classes of endophytes (adapted from Rodriguez et al. 2008)

Criteria	**Clavicipitaceous**	**Non-clavicipitaceous**		
	Class1	Class2	Class3	Class4
Host range	Narrow	Broad	Broad	Broad
Plants tissue (s) colonized	Shoot and rhizosphere	Shoot, root and rhizosphere	Shoot	Root
In plant colonization intensity	Extensive	Extensive	Limited	Extensive
In plant biodiversity intensity	Low	Low	High	Unknown
Mode of transmission	Vertical and horizontal	Horizontal	Horizontal	Horizontal
Fitness benefits	Non-habitat adapted	Non-habitat adapted and Habitat-Adapted	Non-habitat adapted	Non-habitat adapted

1.5.2.1 Functional role of fungal endophytes in plant growth and development. Many studies report that fungal endophytes may produce bioactive compounds or secondary metabolites with antifungal, antibacterial or antiherbivorous activities, and play a functional role in plant growth and development (**Figure 1-5**) (Zhao et al. 2011; Rai et al. 2014). Dighton (2003) mentioned that fungal endophytes improve the nutrient content of plants in addition to supplying secondary metabolites and growth regulators. These growth regulator compounds may contribute to both plant growth promotion and plant tolerance to environmental stresses (Rai et al. 2014). For example, *Trichoderma virens* synthesizes indole-type compounds such as indole-3-acetic acid, indole-3-acetaldehyde, and indole-3-ethanol that may promote plant growth by mimicking natural plant auxins (Contreras-Cornejo 2009).

During germination, fungal endophytes help to degrade cuticle cellulose of seeds which increases carbon availability for seedlings, leading to improved germination, vigor, and seedling establishment (Rai et al. 2014). Endophytes may contain certain metabolites to produce plant growth regulators and thus promote seed germination (Bhagbati and Joshi 2009).

Lekberg and Koide (2005) reported that improving water and nutrient uptake is one of the mechanisms by which fungal endophytes confer benefits to plants. Moreover, fungal endophytes contribute in solubilizing plant nutrients (such as phosphorus) that are unavailable to plants in certain soils and also in fixing atmospheric nitrogen (Pineda et al. 2010).

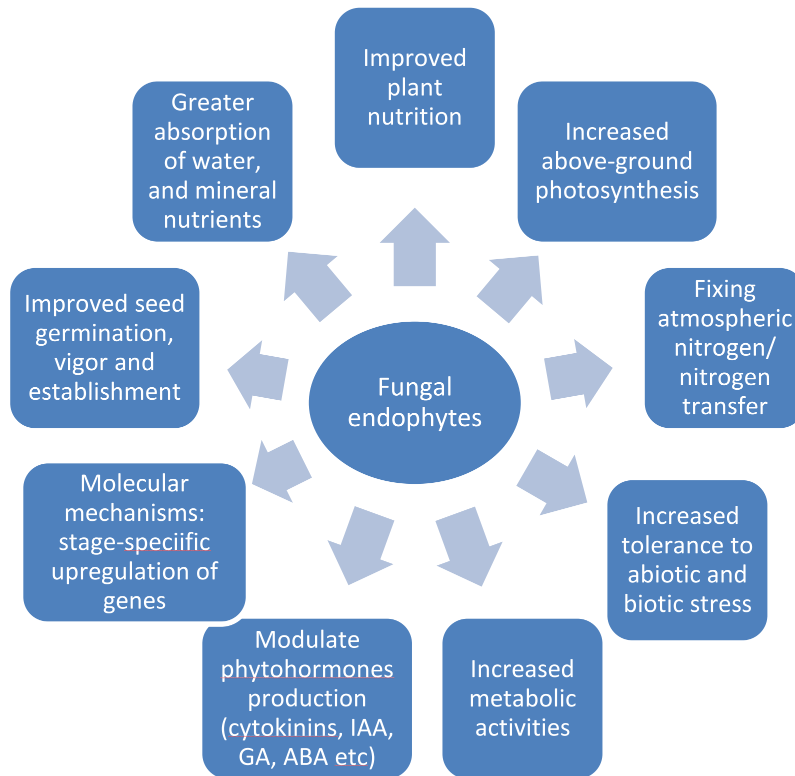


Figure 1-5. Functional role of fungal endophytes in plant growth promotion (modified from Rai et al. 2014)

1.5.2.2 Fungal endophyte colonization leads to increased plant stress tolerance. Many studies have reported that fungal endophytes may mitigate environmental stresses such as herbivory, drought, heat, salt, metals, and disease to host plants (Redman et al. 2002; Arnold et al. 2003; Marquez et al. 2007; Rodriguez et al. 2008). Redman and Rodriguez (2007) showed that systemic fungal endophytes confer habitat-specific stress tolerance to plants. For instance, when endophytes are isolated from grasses growing under particular environmental constraints (such as heat, lack of water, high salt concentrations), they confer the same functional stress adaptability to genetically diverse species such as rice, wheat, watermelon, and tomato (Rodriguez et al. 2012). Moreover, Rodriguez et al. (2008) studied the habitat-specific stress

tolerance conferred to plants by fungal endophytes isolated from varying environmentally stressful conditions (*Curvularia protuberata* from geothermal soils – high heat, *Fusarium culmorum* from coastal beach – high salinity). The authors defined this habitat-specific stress tolerance conferred by endophytes to plants as Habitat-Adapted (HA) symbiosis; however, the processes contributing to plant success are not fully understood (Rodriguez et al. 2009). Woodward et al. (2012) hypothesized that plant-fungal symbiosis triggers metabolic and gene expression changes that confer habitat-specific stress tolerance.

Rodriguez et al. (2008) reported that systemic endophytes confer habitat-specific stress tolerance to genetically divergent host plants. Endophyte-conferred tolerance is HA stress tolerance when these tolerances are the result of habitat-specific selective pressures (Rodriguez et al. 2009). Other benefits of colonization by fungal endophytes are called non-habitat adapted if they are conferred on any growth substrate (Rodriguez et al. 2008). The goal of my thesis is to determine if fungal endophytes isolated from plants growing in saline soil in Saskatchewan can provide the benefits of HA symbiosis to non-adapted tomato plants grown under drought or high salt conditions.

1.6 Objectives and Hypotheses

In order to address my goal of understanding if novel fungal endophyte strains can provide the benefits of HA-symbiosis, I have several objectives.

1.6.1 Objective and hypothesis of Chapter 2 entitled, 'A fungal endophyte strategy for mitigating the effect of salt and drought stress on plant growth'

1.6.1.1 Objective. Determination of growth performance by measuring biomass, photosynthetic efficiency, and ROS activity of endophyte-colonized and uncolonized plants under acute NaCl and intermittent drought stress.

1.6.1.2 Hypothesis. Endophyte-colonized tomato plants have better biomass, photosynthetic efficiency and less oxidative stress compared to uncolonized plants under intermittent drought and NaCl stress.

1.6.2 Objective and hypothesis of Chapter 3 entitled, 'Characterization of systemic fungal endophyte-induced abiotic stress-tolerance by alteration of proline accumulation and photosynthetic efficiency'

1.6.2.1 Objective. Determination of photosynthetic efficiency and proline accumulation in endophyte-colonized plants under acute and chronic NaCl stress and drought stress.

1.6.2.2 Hypothesis. Systemic endophytes improve maximal photosynthetic efficiency and alter proline accumulation in tomato plants under NaCl stress and drought stress.

1.6.3 Objective and hypothesis of Chapter 4 entitled, 'Protective effects conferred on tomato plants growing in saline or drought conditions by systemic fungal endophytes isolated from plants growing on saline soils in Saskatchewan'

1.6.3.1 Objectives. Detection and estimation of *in vitro* ROS generation and osmolyte concentration in endophyte-colonized plants under NaCl stress and drought stress.

1.6.3.2 Hypothesis. Systemic fungal endophytes modulate ROS generation, osmolyte concentration and photosynthetic efficiency in tomato plants under NaCl and drought stress.

1.7 References

Adiku SGK, Renger M, Wessolek G, Facklam M, Hecht-Bucholtz C (2001) Stimulation of the dry matter production and seed yield of common beans under varying soil water and salinity conditions. Agricultural Water Management 47: 55-68

Ahmad P, Wani MR, Azooz MM, Tran LP (2014) Improvement of Crops in the Era of Climatic Changes. Springer. Doi: 10.1007/978-1-4614-8830-9

Anjum F, Yaseen M, Rasul E, Wahid A, Anjum S (2003) Water stress in barley (*Hordeum vulgare* L.). II. Effect on chemical composition and chlorophyll contents. Pak. J. Agric. Sci 40: 45-49

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55: 373-399

Arnold AE, Mejía LC, Kyllo D, Rojas EI, Maynard Z, Robbins N, Herre EA (2003) Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences 100: 15649-15654

Atkinson NJ, Urwin PE (2012) The interaction of plant biotic and abiotic stresses: from genes to the field. J Exp Bot 63: 3523-3543

Azad K, Kaminskyj S (2015) A fungal endophyte strategy for mitigating the effect of salt and drought stress on plant growth. Symbiosis (special issue) 1-6

Babitha K, Ramu S, Pruthvi V, Mahesh P, Nataraja KN, Udayakumar M (2013) Co-expression of AtbHLH17 and AtWRKY28 confers resistance to abiotic stress in *Arabidopsis*. Transgenic Res 22: 327-341

Bartels D, Sunkar R (2007) Drought and salt tolerance in plants. Crit Rev Plant Sci 24: 23-58

Bartels PH, Montironi RM, Bostwick D, Marshall J, Thompson D, Bartels HG, Kelley D (2001) Karyometry of secretory cell nuclei in high-grade PIN lesions*. Prostate 48: 144-155

Beck EH, Fettig S, Knake C, Hartig K, Bhattarai T (2007) Specific and unspecific responses of plants to cold and drought stress. J Biosci 32: 501-510

Bhagobaty R, Joshi S (2009) Promotion of seed germination of Green gram and Chick pea by *Penicillium verruculosum* RS7PF, a root endophytic fungus of *Potentilla fulgens* L. Adv. Biotech 8: 7-15

Bohnert HJ, Jensen RG (1996) Strategies for engineering water-stress tolerance in plants. Trends Biotechnol 14: 89-97

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to environmental stresses. Plant Cell 7: 1099

Brundrett MC (2006) Understanding the roles of multifunctional mycorrhizal and endophytic fungi. In: Schulz BJE (ed.) Microbial root endophytes. Springer, pp 281-298. doi: 10.1007/3-540-33526-9

Carroll G (1988) Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. Ecology 69 (1): 2-9

Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann Bot 103: 551-560

Contreras-Cornejo HA, Macias-Rodriguez L, Cortes-Penagos C, Lopez-Bucio J (2009) *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in *Arabidopsis*. Plant Physiol 149: 1579-1592

Dighton J (2003) Fungi in ecosystem processes. CRC Press. New Jersey, USA

Dregne HE (1983) Desertification of arid lands. In: Physics of desertification. Springer, pp 4-34. dio: 10.1007/978-94-009-4388-9_2

Evelin H, Kapoor R, Giri B (2009) Arbuscular mycorrhizal fungi in alleviation of salt stress: a review. Annals of Botany 104: 1263-1280

Farooq M, Wahid A, Kobayashi N, Fujita D, Basra S (2009) Plant drought stress: effects, mechanisms and management. In: Sustainable Agriculture, Springer, pp 153-188

Garg B (2003) Nutrient uptake and management under drought: nutrient-moisture interaction. Curr.Agric 27: 1-8

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry 48: 909-930

Gupta B, Huang B (2014) Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. Int J Genomics 2014: 701596

Hamdia MA , Shaddad MAK (2010) Review of salt tolerance of crop plants. Journal of Stress Physiology and Biochemistry 6: 64-90

Harman GE (2011) Multifunctional fungal plant symbionts: new tools to enhance plant growth and productivity. New Phytol 189: 647-649

Harris D, Tripathi R, Joshi A (2002) On-farm seed priming to improve crop establishment and yield in dry direct-seeded rice. In: Pandey S (ed.) Direct seeding: Research Strategies and Opportunities, International Research Institute, Manila, Philippines, pp 231-240

Hasegawa PM, Bressan RA, Zhu J, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annual Review of Plant Biology 51: 463-499

Jaleel CA, Manivannan P, Wahid A, Farooq M, Al-Juburi HJ, Somasundaram R, Panneerselvam R (2009) Drought stress in plants: a review on morphological characteristics and pigments composition. Int.J.Agric.Biol 11: 100-105

Jouyban Z (2012) The Effects of Salt stress on plant growth. Technical Journal of Engineering and Applied Sciences 2: 7-10

Kavar T, Maras M, Kidrič M, Šuštar-Vozlič J, Meglič V (2008) Identification of genes involved in the response of leaves of *Phaseolus vulgaris* to drought stress. Mol Breed 21: 159-172

Kaya MD, Okçu G, Atak M, Çıkılı Y, Kolsarıcı Ö (2006) Seed treatments to overcome salt and drought stress during germination in sunflower (*Helianthus annuus* L.). Eur. J. Agron. 24: 291-295

Khan, MA. and Duke NC. (2001) Halophytes- A resources for the future. Wetland Ecology and Management 6: 455-456

Läuchli A, Grattan S (2007) Plant growth and development under salinity stress. In: Jenks MK (eds) Advances in molecular breeding toward drought and salt tolerant crops. Springer pp 1-32

Lawlor DW (2002) Limitation to photosynthesis in water-stressed leaves: stomata vs. metabolism and the role of ATP. Ann. Bot. 89 Spec No: 871-885

Lekberg Y, Koide R (2005) Is plant performance limited by abundance of arbuscular mycorrhizal fungi? A meta-analysis of studies published between 1988 and 2003. New Phytol 168: 189-204

Maathuis FJ, Ahmad I, Patishtan J (2014) Regulation of Na fluxes in plants. Frontiers in Plant science 5, doi: 10.3389/fpls.2014.00467

Mahajan S, Tuteja N (2005) Cold, salinity and drought stresses: an overview. Archives of Biochemistry and Biophysics 444: 139-158

Marquez LM, Redman RS, Rodriguez RJ, Roossinck MJ (2007) A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. Science 315: 513-515

Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ 33: 453-467

Munns R (2002) Comparative physiology of salt and water stress. Plant, Cell Environ 25: 239-250

Munns R (2005) Genes and salt tolerance: bringing them together. New Phytol 167: 645-663

Murata N, Takahashi S, Nishiyama Y, Allakhverdiev SI (2007) Photoinhibition of photosystem II under environmental stress. Biochimica et Biophysica Acta (BBA)-Bioenergetics 1767: 414-421

Niste M, Vidican R, Rotar I, Stoian V, Pop R, Miclea R (2014) Plant Nutrition Affected by Soil Salinity and Response of Rhizobium Regarding the Nutrients Accumulation. ProEnvironment 7: 71-75

Nuccio ML, Rhodest D, McNeil SD, Hanson AD (1999) Metabolic engineering of plants for osmotic stress resistance. Curr Opin Plant Biol 2: 128-134

Petrini O (1996) Ecological and physiological aspects of host-specificity in endophytic fungi. In: Endophytic Fungi in Grasses and Woody Plants. APS Press: St Paul, pp 87–100.

Pineda A, Zheng S, van Loon JJ, Pieterse CM, Dicke M (2010) Helping plants to deal with insects: the role of beneficial soil-borne microbes. Trends Plant Sci 15: 507-514

Porcel R, Aroca R, Ruiz-Lozano JM (2012) Salinity stress alleviation using arbuscular mycorrhizal fungi. A review. Agronomy for Sustainable Development 32: 181-200

Rai M, Rathod D, Agarkar G, Dar M, Brestic M, Pastore GM, Junior MRM (2014) Fungal growth promotor endophytes: a pragmatic approach towards sustainable food and agriculture. Symbiosis 62: 63-79

Ramoliya PJ, Patel HM, Pandey AN (2004) Effect of salinization of soil on growth and macro- and micro-nutrient accumulation in seedlings of *Salvadora persica* (Salvadoraceae). For Ecol Manage 202: 181-193

Redman R, Roossinck M, Maher S (2002) Field performance of cucurbit and tomato plants colonized with a nonpathogenic, mutualistic mutant (path-1) of *Colletotrichum magna* (Teleomorph : Glomerella magna; Jenkins & Winstead). Symbiosis 32: 55-70

Rodriguez R, Redman R (1997) Fungal life-styles and ecosystem dynamics: biological aspects of plant pathogens, plant endophytes and saprophytes. Adv Bot Res 24: 169-193

Rodriguez RJ, Woodward C, Redman RS (2012) Fungal influence on plant tolerance to stress. Biocomplexity of plant–fungal interactions. In: Oxford: Wiley-Blackwell, pp 155-163

Rodriguez RJ, Henson J, Van Volkenburgh E, Hoy M, Wright L, Beckwith F, Kim Y, Redman RS (2008) Stress tolerance in plants via habitat-adapted symbiosis. The ISME journal 2: 404-416

Rodriguez RJ, Woodward C, Redman RS (2010) Adaptation and Survival of Plants in High Stress Habitats via Fungal Endophyte Conferred Stress Tolerance. In: Seckbatch (eds) Symbioses and Stress. Springer 17: 461-476, DOI 10.1007/978-90-481-9449-0_23

Rodriguez RJ, White JF, Jr., Arnold AE, Redman RS (2009) Fungal endophytes: diversity and functional roles. New Phytol 182: 314-330

Rodriguez R, Redman R (2008) More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. J Exp Bot 59: 1109-1114

Saikkonen K, Wäli P, Helander M, Faeth SH (2004) Evolution of endophyte–plant symbioses. Trends Plant Sci 9: 275-280

Schwabe KA, Kan I, Knapp KC (2006) Drainwater management for salinity mitigation in irrigated agriculture. Am J Agric Econ 88: 133-149

Serraj R, Sinclair T (2002) Osmolyte accumulation: can it really help increase crop yield under drought conditions? Plant, Cell Environ 25: 333-341

Taiz L, Zeiger E (2006) Stress physiology. In: Plant Physiology (4th ed) Sinauer Associates, Inc., Sunderland, MA (2006), pp. 671–681

Tester M, Davenport R (2003) Na^+ tolerant and Na^+ transport in higher plants. Annals of Botany 91: 503-527

Tuteja N (2012) Improving Crop Resistance to Abiotic Stress: Vol. 1. John Wiley & Sons.

Wani SH, Singh NB, Haribhushan A, Mir JI (2013) Compatible solute engineering in plants for abiotic stress tolerance-role of glycine betaine. Curr Genomics 14: 157

Wilson KE, Ivanov AG, Öquist G, Grodzinski B, Sarhan F, Huner NP (2006) Energy balance, organellar redox status, and acclimation to environmental stress. Botany 84: 1355-1370

Woodward C, Hansen L, Beckwith F, Redman RS, Rodriguez RJ (2012) Symbiogenics: An Epigenetic Approach to Mitigating Impacts of Climate Change on Plants. HortScience 47: 699-703

Yang T, Chen Y, Wang X, Dai C (2013) Plant symbionts: keys to the phytosphere. Symbiosis 1-14

Yokoi S, Bressan RA, Hasegawa PM (2002) Salt stress tolerance of plants. JIRCAS working report 23: 25-33

Zhao J, Shan T, Mou Y, Zhou L (2011) Plant-derived bioactive compounds produced by endophytic fungi. Mini reviews in medicinal chemistry 11: 159-168

Zhu J (2003) Regulation of ion homeostasis under salt stress. Curr Opin Plant Biol 6: 441-445

Zhu J (2001) Plant salt tolerance. Trends Plant Sci 6: 66-71

CHAPTER 2

*****A version of this chapter has been published

A fungal endophyte strategy for mitigating the effect of salt and drought stress on plant growth

Kumkum Azad, Susan Kaminskyj

Symbiosis (special issue); DOI 10.1007/s13199-015-0370-y

Received: 15 August 2015 /Accepted: 15 December 2015

Statement of contribution:

Study conception and design: Kumkum Azad (primary contributor), Susan Kaminskyj

Acquisition of data: Kumkum Azad

Analysis and interpretation of data: Kumkum Azad

Drafting of manuscript: Kumkum Azad (primary contributor), Susan Kaminskyj

2.1 Introduction

Plants cope with environmental stress through physiological adaptation. Symbiotic fungi including systemic septate endophytes (hereafter, endophytes) are important for plant resilience in non-agricultural environments (Rodriguez et al. 2009). Abiotic factors that limit plant growth include drought, salinity, nutrient imbalance (toxicities and deficiencies), and temperature extremes (Hamdia and Saddad 2010). Worldwide, a fifth of cropland and a third of irrigated land are already affected by salinity (Shrivastava and Kumar 2015). Jamil et al. (2011) estimated that by 2050, half of all agricultural land could be desertified. Plants in natural and field situations regulate their metabolic pathways using mechanisms to detect and adapt to stress (Rodriguez et al. 2004; Gupta and Huang 2014). In this chapter, plant responses mediated by systemic fungal endophytes that confer tolerance to salinity and drought are examined.

Many plants have similar responses to soil dryness and salinity, at least during wilting (Bartels and Sunkar 2007). Loss of turgor induces stomatal closure leading to CO_2 limitation in leaf cells, and decreasing rates of photosynthesis. This in turn leads to increased reactive oxygen species (ROS) formation that damages cell structure/function, leading to even lower photosynthetic efficiency. Thus drought and salinity can play a major role in agricultural yield loss (Bartels and Sunkar 2007; Gill and Tuteja 2010).

Plants must keep their internal water potential below that of the soil to maintain water uptake and preserve cell turgor (Tester and Davenport 2003). Cell osmotic concentration can be increased by solute (especially K^+) uptake or by synthesis of osmotically active compounds called osmoprotectants or compatible solutes. These do not interfere with metabolism because they have little impact on pH and charge balance of the cytosol or luminal compartments (Yokoi et al. 2002; Farooq et al. 2009; Tuteja 2012). Some compatible solutes can act as ROS scavengers, protect membrane integrity (Porcel et al. 2012) and stabilize protein structure/function (Bohnert and Jensen 1996; Wani et al. 2013). Organic osmoprotectants include simple and complex sugars, sugar alcohols (e.g. mannitol) (Bohnert and Jensen 1996), as well as quaternary amino acid derivatives (e.g. proline and glycine betaine) and others (Nuccio et al. 1999). Notably, total osmoprotectant levels in halophytes range from 0.5-4.0 mol/L (Jouyban 2012).

Mechanistically, for plants to cope with *acute* salt stress (for example a storm surge on a low-lying marine coastline) ion homeostasis must be re-established and cellular damage must be repaired before growth can resume (Hamida and Saddad 2010; Maathuis et al. 2014). During NaCl stress, Na^+ competes with K^+ for protein binding sites. This can be mitigated by increased accumulation of Ca^{2+} (Jouyban 2012), likely via second-messenger regulation of K^+ and Na^+ transporters (Zhu 2003). For *chronic* salt stress, these responses must be commensurate with total salinity level and with ion composition.

Drought affects water relations at cellular, tissue and organ levels (Beck et al. 2007). Mitigation of drought stress includes escape and avoidance. Escape includes species-specific strategies: short life cycle/growth season, and early-season reproduction (Farooq et al. 2009). Avoidance includes reducing transpiration water loss by stomatal regulation and/or improving water uptake through an extensive or deep root system (Farooq et al. 2009). Shoot structure can also be altered during drought stress, limiting leaf number or area thus reducing transpiration (Kavar et al. 2008). However, agricultural crops do not necessarily have these features, and even if they had, overall productivity could be severely decreased. Thus, research into additional drought-tolerance strategies is needed.

Some fungal endophytes promote plant growth despite external environmental stresses (Rodriguez et al. 2009; Yang et al. 2013). These effects are generally associated with the biology of the fungal endophytes and thus, the effects can be transferred by fungal colonization to a diversity of plant species (Redman et al. 2001; Rodriguez et al. 2004; Marquez et al. 2007; Rodriguez et al. 2008; Redman et al. 2011) making them highly useful for agriculture and horticulture.

In this chapter of my thesis, growth performance of plants, colonized with systemic fungal endophytes (class 2: Rodriguez et al. 2009), are examined by measuring plant biomass, determining photosynthetic efficiency and ROS activity in response to acute NaCl stress and intermittent drought. I hypothesize that endophyte-colonized tomato plants have better biomass, photosynthetic efficiency and less oxidative stress compared to non-colonized plants under intermittent drought and NaCl stress.

2.2 Materials and Methods

2.2.1 Endophyte isolation, culture, identification, and storage. Plants were collected in Saskatchewan in the summer of 2012 and 2013 from the shores of saline lakes (Little Manitou Lake and Radisson Lake) and from the Mosaic Belle Plaine tailings management area (Appendix 1: Table 2-3). Roots were washed, surface-sterilized for 15 min in 0.6 % sodium hypochlorite, and then rinsed thoroughly with sterile water.

Root and shoot pieces were cut with sterile scissors and placed on 10 % potato dextrose agar (10 % PDA: 3.9 g PDA powder plus 15 g Bacto agar per litre of ultrapure water) supplemented after autoclaving with 50 µg/mL each of ampicillin, tetracycline, and streptomycin. Plates were incubated at room temperature. Fungal colonies grew from the plant pieces in 5-7 d. Systemic endophytes were isolated from root and shoot samples.

Dominant colony types were grown as pure cultures on 100 % PDA. Based on spore morphology and internal transcribed spacer (ITS) sequence analysis, strains 414 and 419 were identified as *Alternaria* spp (Woudernberg et al. 2013), whereas Hz613 was found to be a strain of *Trichoderma harzianum* (Samuels et al. 2015) (Appendix 1; supplemental Table 2-1 and Table 2-2). Strains were stored in screw-capped cryo-tubes in sterile water at 4 °C, in dry 250-400 mesh silica (Sigma) at -20 °C.

2.2.2 Plant growth condition. Surface-sterilized tomato (*Solanum lycopersicum* var. Rutgers) seeds were inoculated by shaking them for 30 min in dilute (10^3-10^4 spores/mL) suspensions of freshly-harvested spores of systemic endophytes, or the seeds were mock-inoculated in ultrapure water as a non-colonized control. We used *Fusarium culmorum* strain FcRed1, a previously-characterized saline tolerance conferring endophyte (Redman et al. 2011) as a positive control treatment, allowing the effectiveness of newly isolated SK strains to be compared.

Following inoculation, seeds were planted in double-decker Magenta boxes (MBs; Sigma; **Figure 2-1**). A hole was drilled in the bottom of the upper MB chamber in order to connect it by a wick to Hoagland's solution (Redman et al. 2011) for regular growth or an experimental treatment (**Figure 2-1a**). The lower chambers were calibrated to facilitate

measuring plant water use. The MBs were capped with translucent close-fitting plastic lids until the seedlings were about 6 cm tall (around 10th day of planting). Plants were grown at 22-23 ^{o}C and 23 % relative humidity with 12 h/d fluorescent light (350 μmol photons $m^{-2}s^{-1}$) in a growth room.

For NaCl-stress treatments, each MB contained 5 plants, and each treatment was performed in triplicate. NaCl stress induced by adding 300 - 500 mM NaCl in Hoagland's solution (250 mL) in the lower part of MB, applied to two-week-old plants for 20 d (**Figure 2-1c**). For drought-stress treatments, each MB had 6 plants and there were 6 MBs per treatment. For drought stress, the lower chamber was emptied (**Figure 2-1c**). Plants were rehydrated with reverse osmosis (RO) water for 2 d after each round of drought stress (**Figure 2-1d**), before harvesting for biomass measurements. Typical results for NaCl stress are shown in **Figure 2-1e**.

Biomass is a general measure of plant fitness. At the end of each experiment, plants were removed from the potting medium, and their roots were washed clean. Typically, plants were cut at the crown, so that root and shoot fresh and dry weight could be measured individually. Colonization of roots and shoots by the fungal endophyte was confirmed as for the original endophyte isolation (2.2.1).

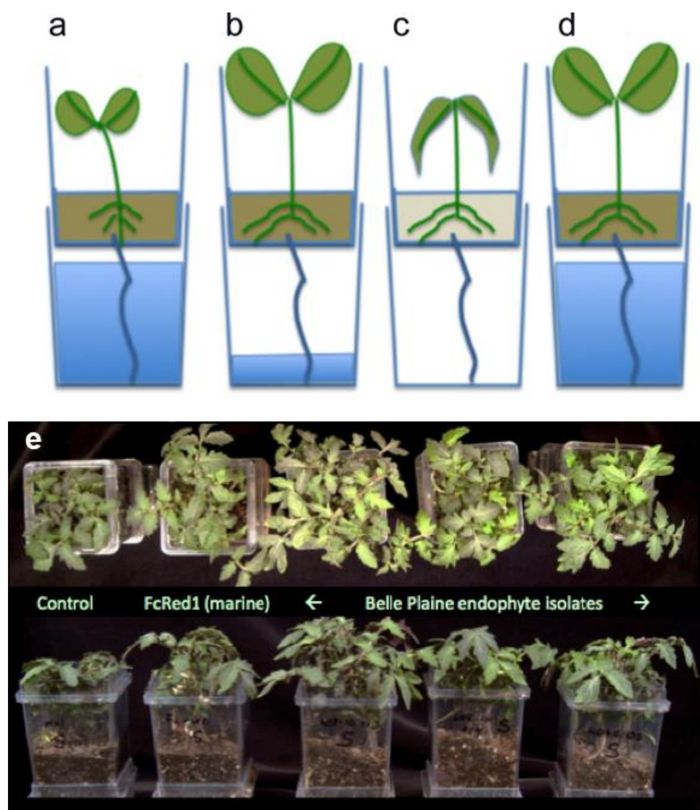


Figure 2-1. Experimental method (a-d) and (e) sample results for endophyte-mediated response to NaCl treatment. Seedling establishment (a), growth (b), imposed stress (here, drought; c), recovery (d). Typical response to 15 d NaCl stress (e).

2.2.3 Photosynthetic efficiency. The efficiency of Photosystem II (PSII) was measured with a portable chlorophyll fluorometer (PAM 2000, Heinz Walz Gmbh, Germany). This allows a non-invasive assessment of plant photosynthetic performance by measuring chlorophyll-a fluorescence (Zhou et al. 2015). Plants were dark adapted for 5 min. Then all of the reaction centres in PSII reaction centre were closed, and F_m (maximum fluorescence) was induced by using the PAM 200 halogen lamp to produce an 800 ms pulse of light (2500 micromols photons m^{-2} s^{-1}). The F_v (variable fluorescence) was calculated as the difference between F_m and F_o (minimal fluorescence). The ratio F_v/F_m [$(F_m-F_o)/F_m$] was used to estimate the quantum yield of PSII. Measurements were taken for the second-youngest leaf of plants from each replicates.

2.2.4 Localization of H_2O_2 accumulation. To localize and quantify H_2O_2, the second-youngest leaves were removed from plants and floated on 2 mg/mL aqueous 3,3-diaminobenzidine (DAB) (GoldBio, USA) for 4 h in the light (modified from Romero-Puertas et al. 2004). The H_2O_2 – DAB reaction produces a brown compound that is revealed after chlorophyll has been removed by boiling in 70 % ethanol for 20 min. Leaves were preserved in sterilized water and photographed for analysis.

2.2.5 Statistical analysis. The data obtained were analyzed by one-way analysis of variance (ANOVA) with SPSS version 22 (IBM). Duncan's multiple-range test was used to evaluate the significance of differences between treatments, when overall differences were found to be significant using ANOVA at $P<0.05$. For NaCl stress experiment, error bars represent ±SE of three replicates, while for drought stress experiment, error bar represent ±SE of six replicates. All experiments were conducted as a completely randomized block design. Control and strains of fungal endophytes were used as treatments for one-way ANOVA (data were separately analyzed by one-way ANOVA for each level of NaCl stress).

2.3 Results

2.3.1 Systemic fungal endophytes can mitigate the effect of NaCl stress. Seventeen day old endophyte-colonized and non-colonized plants (control plants) were treated with 300 mM or 500 mM NaCl for 20 d. There was no difference in fresh shoot biomass between endophyte-colonized and non-colonized plants (control plants) in the absence of stress (**Figure 2-2**). However, treatment of plants with 300 mM or 500 mM NaCl caused an approximately 6-7 times decrease in fresh shoot biomass, demonstrating a clear impact of NaCl stress on the plants. Endophyte colonization did not significantly alter fresh shoot biomass accumulation following either the 300 mM NaCl or 500 mM NaCl treatments (**Figure 2-2**). But, following the 300 mM treatment, endophyte colonization tended to result in a 30-50 % higher shoot biomass than non-colonized plants. Growth was even more severely reduced by 20 d in 500 mM NaCl (**Figure 2-2**), but again the differences between endophyte-colonized plants were not statistically significant ($P<0.05$).

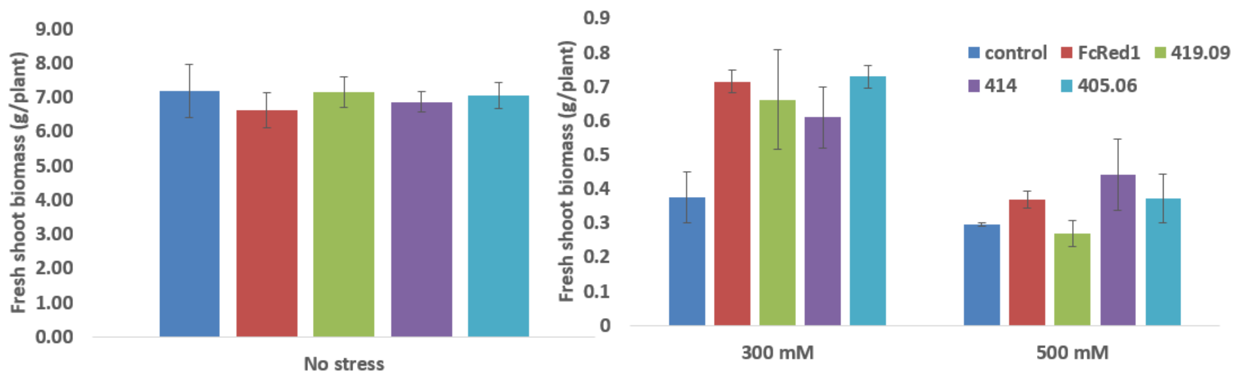


Figure 2-2. Effect of 300 mM, and 500 mM NaCl on fresh biomass of non-colonized (control), positive control (FcRed1), and plants colonized by endophytes collected in this study (419, 414, 405). There was no difference between endophyte-colonized and non-colonized plants in no NaCl stress condition. There was a strong trend for higher biomass in endophyte-colonized plants compared to the non-colonized grown in 300 mM NaCl, but not in 500 mM NaCl. Bars represent means ±SE of three replicates.

2.3.2 Systemic fungal endophytes can mitigate the effect of drought stress.

Endophyte-colonized and non-colonized tomato plants were treated with intermittent drought. Three-week-old plants were deprived of water for 10 d then an additional 7 d (each followed by 2 d recovery in RO water) before being harvested for biomass. There was no significant difference between non-colonized plants and plants colonized with FcRed1, the positive control endophyte. However, plants colonized with SK isolates Hz613, 419, and 414 all had significantly greater fresh root biomass and plants colonized with Hz613 and 414 also had greater fresh shoot biomass compared to non-colonized plants (**Figure 2-3**).

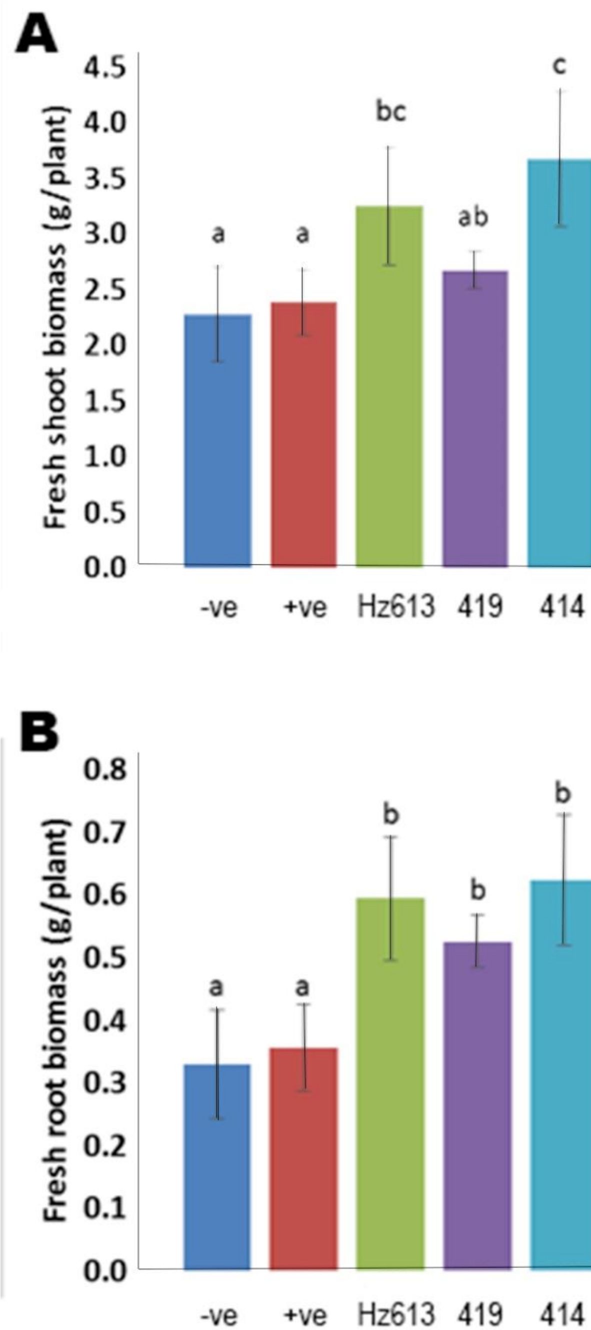


Figure 2-3. Effect of drought on A) shoot and B) root fresh biomass, comparing non-colonized or negative control plants with the positive-control FcRed1 and plants colonized by Saskatchewan saline endophytes (Hz613, 419, 414). Error bars represent ±SE of six replicates. ANOVA plus Duncan's multiple-range test was used to evaluate the significance of differences among treatments. Bars with different letters are significantly different at $P<0.05$.

2.3.3 Systemic fungal endophytes can support and maintain photosynthetic efficiency despite drought stress. Photosynthetic efficiency was assessed using the ratio of F_v/F_m (**Figure 2-4**). The first 10 d period of drought caused a decline in F_v/F_m by 10-30 % independent of endophyte colonization. However, after the second, 7 d drought stress event, plants colonized with endophyte strains 419 or 414 maintained their photosynthetic efficiency, to a greater degree than non-colonized (negative control) plants (**Figure 2-4**).

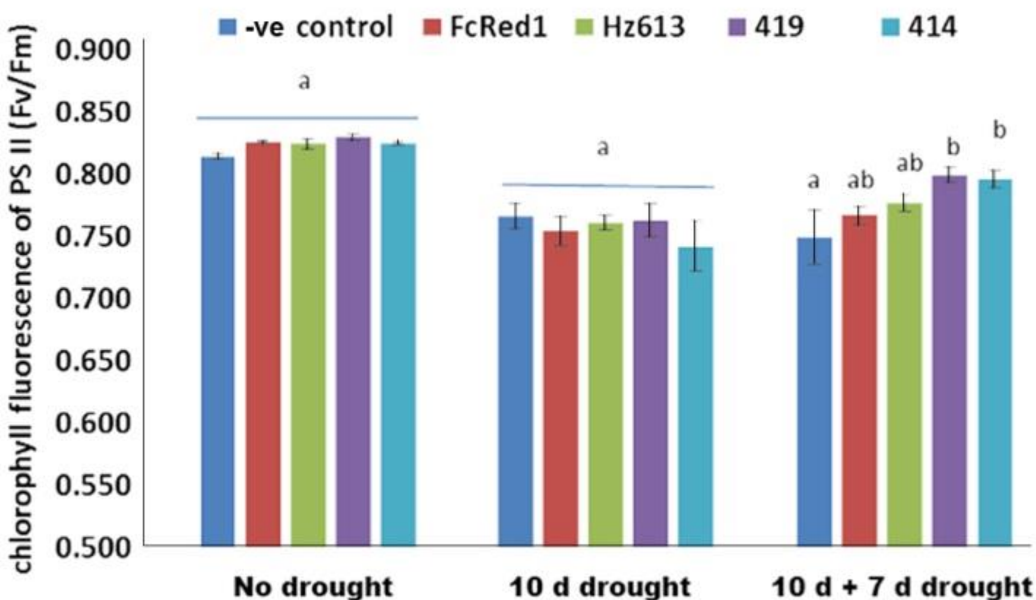


Figure 2-4. Effect of drought on photosynthetic efficiency of control (mock-inoculated, blue), FcRed1 (red) and Saskatchewan endophyte-colonized tomato plants (Hz613, 419, 414). Error bars represent ±SE. Three-week-old plants were exposed to 10 d then to 7 d drought, followed each time by 2 d RO water. After the second drought episode, endophyte-colonized plants had higher photosynthetic efficiency. Duncan’s multiple-range test was used to evaluate the significance of differences among treatments. Bars with different letters are significantly different ($P<0.05$).

2.3.4 Systemic fungal endophytes can reduce reactive oxygen species (ROS) accumulation associated with stress. H_2O_2 localization was used as a proxy to estimate accumulation of ROS in leaves of 300 mM NaCl treated tomato plants. Endophyte-colonized and non-colonized plants were tested by treating their second-youngest leaves with 2 mg/mL DAB. The H_2O_2-DAB reaction forms a brown pigment in the leaf tissue that was revealed after the leaves were decolorized. Following stress, endophyte-colonized plants had noticeably less brown pigmentation than control plants, suggesting lower accumulation of H_2O_2 (**Figure 2-5**).

Figure 2-5. Localization of H_2O_2 in tomato leaves grown in 300 mM NaCl for 20 d. The H_2O_2-DAB reaction produces a brown pigment that is visible after chlorophyll extraction. There was substantially less pigment in NaCl-treated plants colonized with endophyte strains collected from Saskatchewan saline locations than control or FcRed1-treated leaves.

2.4 Discussion

Higher biomass production by endophyte-colonized tomato plants following NaCl- or drought-stress is consistent with endophyte-mediated habitat-adaptive tolerance (Redman et al. 2001; Rodriguez et al. 2008; Redman et al. 2011; Yadav et al. 2012; Estrada et al. 2013). The results suggest that plants colonized by our Saskatchewan saline endophyte strains were metabolically more efficient than negative- or positive-control plants when challenged by salt or drought. Most interesting, endophyte-colonized plants responded relatively better after a second period of drought stress compared to the first, suggesting that habitat-adaptive tolerance is a dynamic and ongoing process. We are exploring the mechanisms responsible.

Photosynthetic efficiency assesses the energy yield of photosystem II for a standardized rate of illumination, which is related to the carbon input for plant metabolism (Woodward et al. 2012; Estrada et al. 2013). Endophyte colonization increased photosynthetic efficiency following drought-stress. Noticeably, endophyte-colonized plants recovered photosynthetic efficiency (Fm/Fv) to a greater extent than non-colonized plants following drought stress treatments. The mechanisms for these changes are under investigation. Fortunately, we can exploit these phenomena even before we fully understand them.

ROS generation is a common response to stress in eukaryotes and prokaryotes alike, particularly in chloroplasts and mitochondria. Leaves are the main photosynthetic organs for plants, and are a major site for oxidative respiration. Using DAB to localize and quantify H_2O_2 as a proxy for other ROS species, Endophyte-colonized plants had lower H_2O_2 levels in their leaves following NaCl-stress. Thus, our endophytes appear to reduce stress-induced ROS generation. Comparable results have been presented by Kotchoni et al. (2006), Rodriguez et al. (2008),

Redman et al. (2011), and Yadav et al. (2012). As above, the ultimate mechanisms for these changes are still under investigation.

Taken together, our study shows that the application of endophytes, isolated from plants growing in natural saline environments, can be a valuable addition for agricultural practices in arid and saline environments.

2.5 References

Bartels D, Sunkar R (2007) Drought and salt tolerance in plants. Crit Rev Plant Sci 24: 23-58

Beck EH, Fettig S, Knake C, Hartig K, Bhattarai T (2007) Specific and unspecific responses of plants to cold and drought stress. J Bioscience 32: 501-510

Bohnert HJ, Jensen RG (1996) Strategies for engineering water-stress tolerance in plants. Trends Biotechnol 14: 89-97

Estrada B, Aroca R, Barea JM, Ruiz-Lozano JM (2013) Native arbuscular mycorrhizal fungi isolated from a saline habitat improved maize antioxidant systems and plant tolerance to salinity. Plant Science 201: 42-51

Farooq M, Wahid A, Kobayashi N, Fujita D, Basra S (2009) Plant drought stress: effects, mechanisms and management. In: Sustainable Agriculture. Springer, pp 153-188

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Bioch 48: 909-930

Gupta B, Huang B (2014) Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. Int J Genomics 2014: 701596

Hamdia MA, Shaddad MAK (2010) Review of salt tolerance of crop plants. J Stress Physiol Bioch 6: 64-90

Jamil A, Riaz S, Ashraf M, Foolad M (2011) Gene expression profiling of plants under salt stress. Crit Rev Plant Sci 30: 435-458

Jouyban Z (2012) The effects of salt stress on plant growth. Tech J Eng Applied Sci 2: 7-10

Kavar T, Maras M, Kidrič M, Šuštar-Vozlič J, Meglič V (2008) Identification of genes involved in the response of leaves of *Phaseolus vulgaris* to drought stress. Mol Breeding 21: 159-172

Kotchoni SO, Kuhns C, Ditzer A, Kirch H, Bartels D (2006) Over-expression of different aldehyde dehydrogenase genes in *Arabidopsis thaliana* confers tolerance to abiotic stress and protects plants against lipid peroxidation and oxidative stress. Plant Cell Environ 29: 1033-1048

Maathuis FJ, Ahmad I, Patishtan J (2014) Regulation of Na^+ fluxes in plants. Front Plant Sci 5: 1-9

Marquez LM, Redman RS, Rodriguez RJ, Roossinck MJ (2007) A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. Science 315: 513-515

Nuccio ML, Rhodest D, McNeil SD, Hanson AD (1999) Metabolic engineering of plants for osmotic stress resistance. Curr Opin Plant Biol 2: 128-134

Porcel R, Aroca R, Ruiz-Lozano JM (2012) Salinity stress alleviation using arbuscular mycorrhizal fungi. A review. Agronomy Sust Devel 32: 181-200

Redman RS, Dunigan DD, Rodriguez RJ (2001) Fungal symbiosis from mutualism to parasitism: who controls the outcome, host or invader? New Phytol 151: 705-716

Redman RS, Kim YO, Woodward CJ, Greer C, Espino L, Doty SL, Rodriguez RJ (2011) Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. PLoS One 6: e14823

Rodriguez RJ, Redman RS, Henson JM (2004) The role of fungal symbioses in the adaptation of plants to high stress environments. Mitig Adapt Strateg Glob Change 9: 261-272

Rodriguez RJ, Henson J, Van Volkenburgh E, Hoy M, Wright L, Beckwith F, Kim Y, Redman RS (2008) Stress tolerance in plants via habitat-adapted symbiosis. The ISME Journal 2: 404-416

Rodriguez RJ, White JF Jr, Arnold AE, Redman RS (2009) Fungal endophytes: diversity and functional roles. New Phytologist 182: 314-330.

Romero-Puertas M, Rodríguez-Serrano M, Corpas F, Gomez Md, Del Rio L, Sandalio L (2004) Cadmium-induced subcellular accumulation of O_2^- and H_2O_2 in pea leaves. Plant Cell Environ 27: 1122-1134

Samuels GJ, Chaverri P, Farr DF, McCray EB (2015) *Trichoderma* online, Systematic Mycology and Microbiology Laboratory, ARS, USDA. Retrieved October 27, 2015, from /taxadescriptions/keys/TrichodermaIndex.cfm

Shrivastava P, Kumar R (2015) Soil salinity: A serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. Saudi J Biol Sci 22: 123-131

Tester M, Davenport R (2003) Na^+ tolerant and Na^+ transport in higher plants. Ann Bot-London 91: 503-527

Tuteja N (2012) Improving Crop Resistance to Abiotic Stress: Vol. 1. John Wiley & Sons

Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several Cryptococcus species. J Bacteriol 172: 4238-4246

Wani SH, Singh NB, Haribhushan A, Mir JI (2013) Compatible solute engineering in plants for abiotic stress tolerance - role of glycine betaine. Curr Genomics 14: 157-165

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications 18: 315-322

Woodward C, Hansen L, Beckwith F, Redman RS, Rodriguez RJ (2012) Symbiogenics: an epigenetic approach to mitigating impacts of climate change on plants. HortScience 47: 699-703

Woudenberg JHC, Groenewald JZ, Binder M, Crous PW (2013) *Alternaria* redefined. Studies in Mycology 75: 171–212

Yadav NS, Shulka PS, Jha A, Agarwal PK, Jha B (2012) The *SbSOS1* gene from the extreme halophyte *Salicornia brachiata* enhances Na^+ loading in xylem and confers salt tolerance in transgenic tobacco. BMC Plant Biology 12: 1-18

Yang T, Chen Y, Wang X, Dai C (2013) Plant symbionts: keys to the phytosphere. Symbiosis 59: 1-14

Yokoi S, Bressan RA, Hasegawa PM (2002) Salt stress tolerance of plants. JIRCAS Working Report 23: 25-33

Zhou R, Yu X, Kjær KH, Rosenqvist E, Ottosen C, Wu Z (2015) Screening and validation of tomato genotypes under heat stress using F v/F m to reveal the physiological mechanism of heat tolerance. Environ Exp Bot 118: 1-11. doi:10.1016/j.envexpbot.2015.05.006.

Zhu J (2003) Regulation of ion homeostasis under salt stress. Curr Opin Plant Biol 6: 441-445

CHAPTER 3

*A version of this chapter will be submitted for publication.

Characterization of systemic fungal endophyte-induced abiotic stress-tolerance by alteration of proline accumulation and photosynthetic efficiency

Kumkum Azad[1], Zakia Boubakir[1], Susan Kaminskyj[1], and Jim Basinger[2]
Department of Biology[1] and Department of Geological Sciences[2]

Statement of contribution:

Study conception and experimental design: Kumkum Azad (primary contributor), Susan Kaminskyj

Acquisition of data: Kumkum Azad

Analysis and interpretation of data: Kumkum Azad

Study conception, experimental design, and acquisition of data for proline assay: Zakia Boubakir, Kumkum Azad

Drafting of manuscript: Kumkum Azad (primary contributor), Susan Kaminskyj, Jim Basinger

3.1 Introduction

Systemic fungal endophytes (hereafter, 'endophytes') grow within plant roots, shoots, and leaves without showing disease symptoms (Brundrett 2006; Rodriguez et al. 2009; Yang et al. 2013; Azad and Kaminskyj 2015). Some systemic endophytes isolated from plants growing in harsh environments confer 'habitat-adapted' tolerance to those conditions (Rodriguez et al. 2009; Azad and Kaminskyj 2015). Thus, identifying HA-conferring endophytes that could convey to crop plants a broad tolerance to environmental stress would be a fast and effective way to improve agricultural performance. It would be much faster, for example than breeding for increased stress tolerance, because it would not require a balance between positive and negative plant traits.

Salinity and drought induce similar physiological and biochemical imbalances that are associated with reduced growth rate and eventually lower yield performance of plants (Bartels and Sunkar 2007). These effects include imbalanced osmolytes (also known as compatible solutes or osmoprotectants), lower photosynthetic efficiency, and overproduction of reactive oxygen species (ROS) (Bartels and Sunkar 2007). Accumulation of compatible solutes is a common mechanism of plants to mitigate salinity and drought stress without affecting normal biochemistry of plants (Bohnert et al. 1995; Yokoi et al. 2002). They contribute to the alleviation of environmental stress by maintaining cellular osmotic adjustment, protecting cell membrane turgidity, stabilizing protein or enzyme activities, and scavenging ROS (Hayat et al. 2012). In addition to K^+ accumulation (K is an essential inorganic nutrient), many compatible solutes are organic, including simple and complex sugars, alcohols, amino acids, and sulfonium compounds (Bohnert and Jensen 1996; Nuccio et al. 1999). Proline is a commonly found compatible solute in plants. Its accumulation can be induced by salinity, drought, low temperature, UV radiation, and heavy metal stresses (Hayat et al. 2012). Accumulation of proline in the cytosol, chloroplasts, and mitochondria helps to maintain cell turgor and osmotic adjustment (Szabados and Savouré 2010). Proline can also stabilize membranes and proteins, neutralize redox potential, and detoxify ROS (Ashraf and Foolad 2007; Hayat et al. 2012), but the actual physiological and biochemical mechanisms of proline are still unclear (Szabados and Savouré 2010).

Photosynthetic efficiency can be used an indicator of plant stress (Zhou et al. 2015). There are both direct and indirect effects of salinity and drought on photosynthesis (Chaves et al. 2009). Typically, direct effects are caused by decreasing stomatal conductance. The stomata close to reduce water loss from the plant, leading to decreased gas exchange. This leads to lower CO_2 levels inside the leaf, and increased rates of photorespiration (Chaves et al. 2009). As CO_2 levels decline there will be limited substrate available for the primary carbon fixation enzyme Rubisco. A lack of CO_2 thus results in the accumulation of NADPH and ATP within the chloroplast, disrupting the cellular redox-state and enzyme balance (Huner et al. 1998; Wilson et al. 2006). One consequence of the closed stomata is therefore an imbalance in the electron transport chain leading to acidification of the thylakoid lumen (Wilson et al. 2006). An indirect or secondary effect of drought or salt stress is the over reduction of the photosynthetic electron transport chain. This leads to increased rates of photoinhibition and the production of ROS (Chaves et al. 2009). Stress from drought or salinity is compounded by the fact plants continue to be exposed to light (Wilson et al. 2006).

Because drought and salt stress have been common events during evolutionary time, plants have developed many ways to protect themselves during stress: photorespiration releases CO_2 and uses NADPH and ATP; photoprotection decreases the functionality of photosynthesis by diminishing light energy input; and photoinhibition is the controlled degradation and rebuilding of PSII (Wilson et al. 2006). The process of photoprotection and photinhibition can be estimated at the plant-level using chlorophyll fluorescence techniques, which is termed as PSII efficiency (Fv/Fm). A decrease in Fv/Fm suggests a down regulation of photosynthesis due to a combination of photoprotection and photoinhibition and can be considered a measure of stress impact (Zhou et al. 2015).

Systemic fungal endophytes are known to produce secondary metabolites in host plants and can be a beneficial association for adapting environmental stress tolerance (Rai et al. 2014). For example, synthesis of plant growth regulators such as gibberellins, cytokinins, abscisic acid, and auxin by systemic fungal endophytes may contribute in growth and development of host plants in harsh environments (You et al. 2012). Moreover, endophytes may improve uptake of nutrients such as phosphorus and iron which are not accessible to plants in certain soils (Pineda et al. 2010). For instance, in low-iron environments, plants exploit siderophores (iron-chelating

agents) produced by microorganisms as an iron-source (Neilands 1995; Winkelmann 2002; Gangwar et al. 2012). Siderophores are low molecular mass (<1000 Da) compounds that assemble through well-defined pathway and comprise lateral chains and functional groups with high iron affinity (Neilands 1995). Microbial siderophores are grouped into: catecholates (produced by only bacteria), hydroxamates (produced by fungi and bacteria), and α–carboxylates (produced by Zygomycete fungi), based on the binding sites with iron (Winkelmann 2002; Baakza et al. 2004).

In the current study, fitness benefits that endophytes (isolated from salt tolerant plants) can confer on host plants are assessed. Following inoculation of tomato plants with a series of endophytes isolated from saline locations in Saskatchewan, I monitored biomass accumulation, proline accumulation, and photosynthetic efficiency in plants exposed to continuous and chronic NaCl or drought stress. In this chapter, I hypothesize that systemic endophytes improve maximal photosynthetic efficiency and alter proline accumulation in tomato plants under NaCl stress and drought stress.

3.2 Materials and Methods

3.2.1 Isolation, culture and identification of systemic fungal endophytes. Saline tolerant fungal endophytes were isolated from plants growing in saline lake shores (Little Manitou Lake and Radisson Lake) and from Mosaic Corp. potash tailings management areas at Belle Plaine, SK (Azad and Kaminskyj 2015). Surface-sterilized (soaked for 15 min in 0.6% sodium hypochlorite, then washed exhaustively with sterile water) root and shoot pieces were placed on 10 % potato dextrose agar (PDA: 3.6 g PDA powder, 15 g Bacto agar, 1 L ultrapure water) supplemented with ampicillin (50 µg mL^{-1}), tetracycline (50 µg mL^{-1}), and streptomycin (50 µg mL^{-1}) to allow fungal growth without bacterial contamination (Rodriguez et al. 2008; Azad and Kaminskyj 2015).

Several fungal colonies with different colors appeared in 5 to 7 days from plant parts and dominant colonies were selected for isolation to pure culture on 100% PDA medium (**Figure 3-1**). Fungal strains were identified by spore morphology and molecular identification techniques

(Azad and Kaminskyj 2015). Strains 419, 417, 414, and 405 were identified as *Alternaria* sp. and Hz613 was identified as *Trichoderma harzianum* (Azad and Kaminskyj 2015).

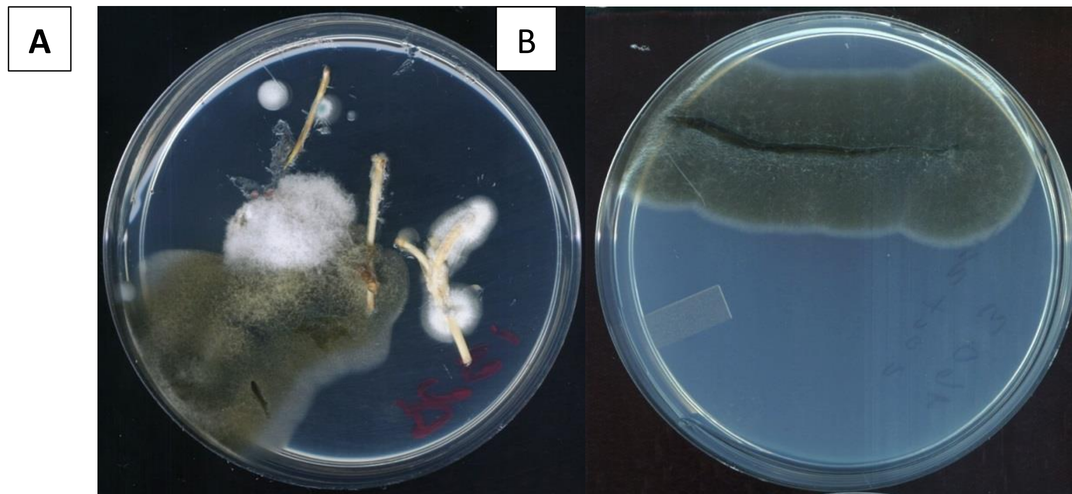


Figure 3-1. Primary and secondary endophyte cultures

A) Endophytes growing from roots and shoots of plants on 10% PDA (3.9 g PDA powder plus 15 g agar/L) supplemented with 50 µg mL^{-1} each of ampicillin, tetracycline, and streptomycin;

B) Pure culture of the dominant fungal strain on 10% PDA.

3.2.2 Plant growth conditions. Tomato (*Solanum lycopersicum* var. Rutgers) seeds were surface sterilized with 0.6 % sodium hypochloride for 15 min. Surface-sterilized seeds were inoculated with 10^3-10^4 spores/mL each of fungal strains (for endophyte-colonized plants) or mock inoculated with sterile water (for non-colonized plants) by gently shaking for 30 min (Azad and Kaminskyj 2015).

Inoculated-seeds were planted in sterile double-decker Magenta Boxes (MBs). The MBs were prepared in the following way: upper boxes were filled with equivalent amounts (~150g) of Sunshine Mix no. 3, sealed and autoclaved at 121°C for 15 min. A cotton rope between the boxes

acted as a wick for nutrient solutions (Azad and Kaminskyj 2015) (**Figure 3-2**). Prior to planting, the sterilized-soil in the MBs were flooded with 250 mL of 1X Hoagland's solution (Rodriguez et al. 2008). Inoculated seeds planted in the MBs were grown at 22 ^{o}C and 23 % relative humidity with 12-h fluorescent light (350 µmol photons $m^{-2}s^{-1}$) in a growth room (Azad and Kaminskyj 2015).

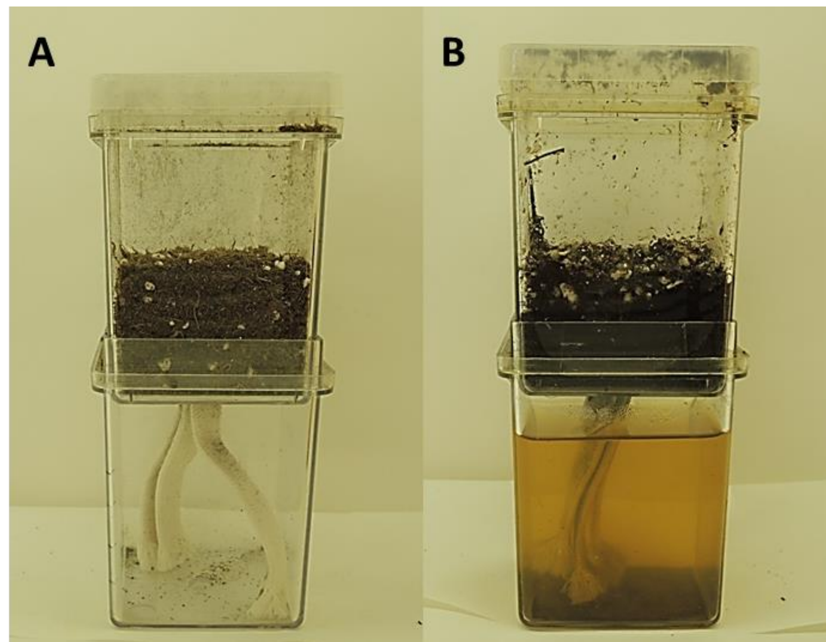


Figure 3-2. Magenta box preparation.

A) Magenta boxes (MBs) were prepared by drilling a 10 mm hole in the upper box. A knotted wick connects the upper and lower boxes. The upper box contains ~150 g Sunshine mix no. 3, which was capped before autoclaving at 121^{0}C for 15 min. Caps were kept in place until seedlings were about 12 d old.

B) Sterilized MBs were flooded through the top chamber with 250 mL of 1x Hoagland's solution, after which +/- inoculated seeds were planted. Stress was applied by altering the solution.

3.2.3 Drought and salinity stress treatments. Plants were exposed to two different patterns of NaCl stress: acute NaCl stress and chronic NaCl stress. In these experiments, each MB contained 5 plants and each treatment had three replicate MBs. For acute NaCl stress, 3 week old plants were watered with 300 mM NaCl in 1X Hoagland's solution (Appendix 2: Figure 3-13). After 10 d, plants were given reverse osmosis (RO) water for 2 d. For chronic NaCl stress, plants (3 week old) were exposed to three consecutive rounds of 10 d NaCl stress. In each round, plants were watered with 100 mM NaCl in 1X Hoagland's solution for 10 d, followed by 2 d of recovery with RO water. The same pattern was followed in another experiment during which plants were watered with 200 mM NaCl in 1X Hoagland's solution for 10 d, followed by 2 d recovery with RO water.

For drought stress, the lower chambers of the MBs were emptied and plants were allowed to grow for 10 d without water, followed by rehydration with RO water for 2 d. In this experiment, 5 plants were grown in each MB and three replicate MBs were used per treatment. In another drought stress experiment, plants were allowed to grow for 7 d without water, followed by rehydration with RO water for 2 d. In this experiment, 6 plants were grown in each MB and six replicate MBs were used per treatment.

3.2.4 Biomass measurement and assessment of endophyte colonization. Plants were removed from MBs and the roots were washed carefully (Azad and Kaminskyj 2015). Fresh and dry, shoot and root biomass was measured separately after each experiment. Three plants from each treatments were selected randomly to confirm colonization with systemic endophytes (Azad and Kaminskyj 2015). After the colonization test, endophytes were isolated as pure culture (**Figure 3-3**).

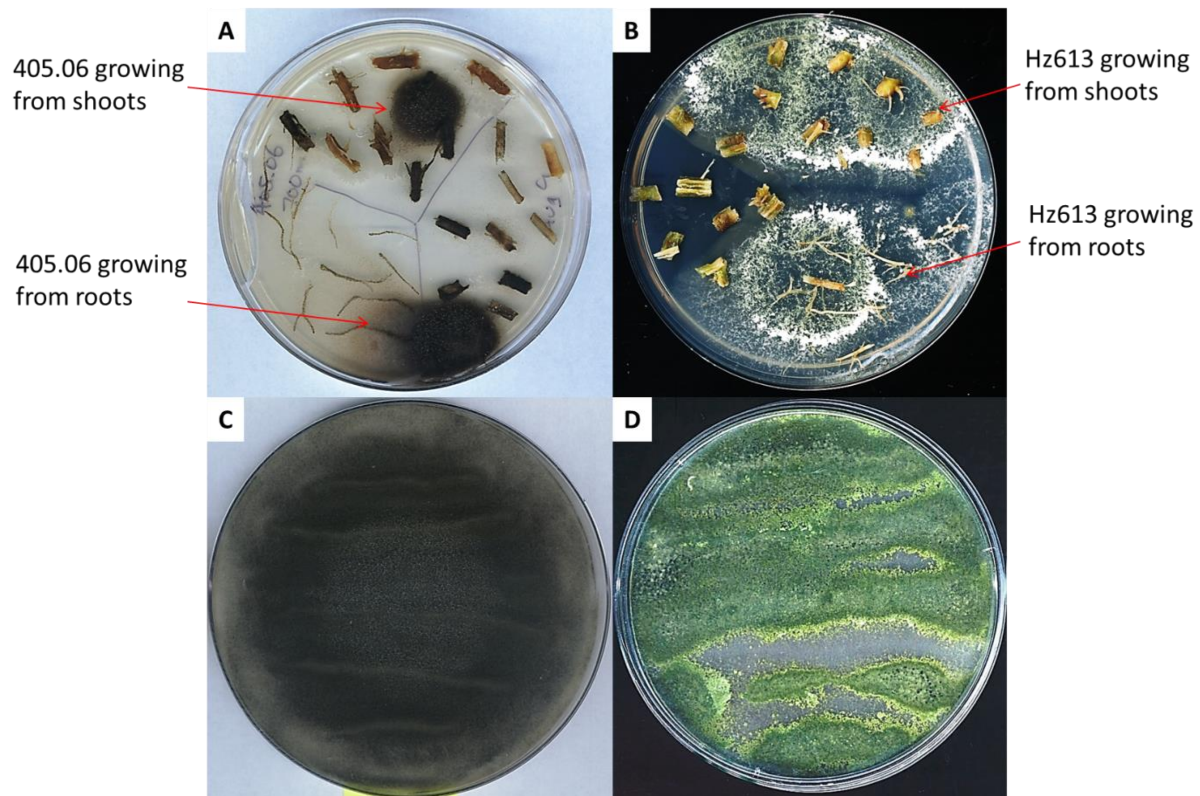


Figure 3-3. Endophyte-colonization test after abiotic stress experiment and reacquisition of endophytes

A) 405 strain growing from representing parts of shoot and roots of colonized plants

B) Hz613 strain growing from representing parts of shoots and roots of colonized plants

C) pure culture of 405 strain on 100% PDA

D) pure culture of Hz613 strain on 100% PDA

3.2.4.1 Root-endophytes colony visualization. Lactofuschin fluorescence (Kaminskyj 2008) was used to visualize endophyte hyphae in plant roots after abiotic stress experiments. Root samples were autoclaved for 20 min in 10 % KOH using wide glass vials topped with glass marbles to prevent evaporation. Following autoclaving, they were rinsed twice with 70 % ethanol to remove the KOH. After clearing KOH, the roots were stained overnight with

lactofuchsin (0.1 % acid fuchsin in 85 % lactic acid) at 60 °C. After staining, roots were rinsed twice at room temperature with de-staining solution DLAG (distilled water: 85 % lactic acid: glycerol = 1: 1: 1) and then de-stained in DLAG at 65 °C for 3 h. The de-stained roots were mounted in PVAG (4 g polyvinyl alcohol powder: 50 mL distilled water: 20 mL glycerol) with a cover slip, and endophyte colonies in roots were examined by confocal fluorescence illumination, using a FITC filter set and 534 nm excitation (confocal) were used to assess the presence of endophytes in plant roots (**Figure 3-4**).

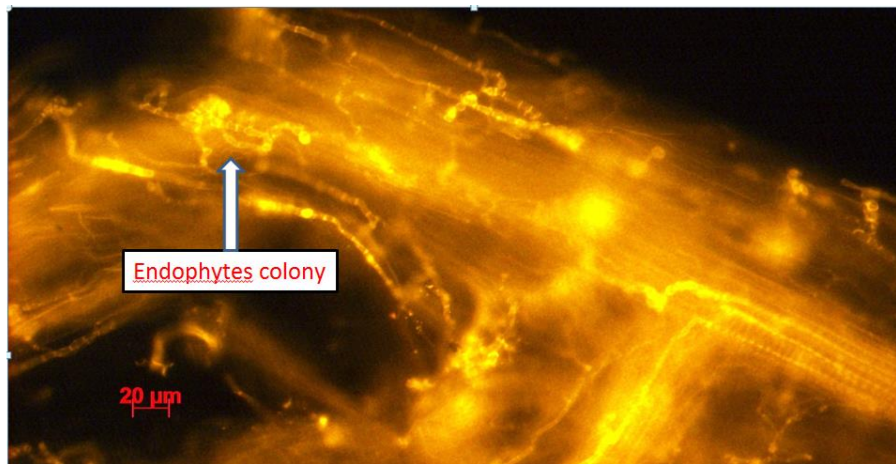


Figure 3-4. Visualization of endophyte colony in plant roots under epifluorescence microscopy using lactofuschin staining method (adapted from Kaminskyj, 2008)

3.2.5 Fluid use efficiency test. In order to assay fluid use efficiency of endophyte-colonized and non-colonized plants, fluid consumption of these plants were measured. Initially, 200 mL of 1X Hoagland's solution was placed in the lower chamber of MBs at the time of seed planting. Lower chambers of MBs were refilled with 1X Hoagland's solution once plants consumed it. After 21 d, fluid remaining in the lower chambers was measured and fluid usage was calculated as mL consumed in 21 days (modified from Redman et al. 2011).

3.2.6 Photosynthetic efficiency assay. Photosystem II PSII efficiency was measured with a chlorophyll fluorometer (Walz, Germany, model PAM 2000). Chlorophyll-*a* fluorescence was used to quantify plant photosynthetic efficiency (Zhou et al. 2015; Azad and Kaminskyj 2015). The quantum yield of PSII is the ratio between actual fluorescence yield (F_v) and the maximum fluorescence yield (F_m) in the dark-adapted state (Zhou et al. 2015; Azad and Kaminskyj 2015). Measurements were taken using the second-youngest leaf of six different plants from each treatment.

3.2.7 Proline accumulation assay. A method adapted from Bates (1973) was used to assay free-proline accumulation in plant fresh leaves. The second-youngest leaves (approximately 0.3 g) of endophyte-colonized plants and non-colonized control plants were homogenized in 6 mL of 3% aqueous sulfosalicylic acid. Homogenates of plant tissues were centrifuged at 4000 rpm for 10 min, then 0.5 mL of supernatant was mixed with previously prepared 0.5 mL of acid ninhydrin (1.25 g ninhydrin + 30 mL glacial acetic acid + 20 mL of 6 M phosphoric acid) and 0.5 mL of glacial acetic acid. This mixture was boiled at 100℃ for 1 h, and then the reaction was terminated in an ice bath. The reaction mixtures were added to 4 mL of toluene, followed by vortexing for 15-20 sec. These mixtures were left at room temperature for approximately 5 min to allow the aqueous phase, containing chromophore to separate from the toluene in the upper layer. The upper layer was then removed and its absorbance was determined at 520 nm with a UV-Vis spectrophotometer (Genesys 20). Pure toluene was used as a blank. The proline concentration was calculated by comparing the value against a standard curve derived from known concentrations of L-proline (Sigma-Aldrich, USA).

3.2.8 Siderophore and indole-type compound assay. To assay siderophore production, we used a method adapted from Alexander and Zuberer (1991) that detects all classes of siderophore (Preez-Midranda et al. 2007). Endophyte strains were grown on chrome azurol S (CAS) agar prepared by following Alexander and Zuberer (1991). Three different solutions were used to prepare this medium: the indicator solution, the buffer solution, and the nutrient solution. Firstly, the indicator solution was prepared by mixing 10 mL of 1 mM $FeCl_3.6H_2O$ (in10 mM HCl acid) with 50 mL of an aqueous solution of CAS (1.21 mg/mL). The purple solution of CAS was slowly added, with constant stirring, to a 40 mL solution of 1.82 mg/mL CTAB (Cetyl trimethylammonium bromide). The resulting dark blue solution was autoclaved and then cooled

to 50℃. Secondly, the buffer solution is a mixture of 30.24 g of PIPES (piperazine-N, N-bis [2-ethanesulfonic acid]) in 750 mL of salt solution containing KH_2PO_4 (0.3 g), NaCl (0.5 g), and NH_4Cl (1.0 g). The pH was adjusted to 6.8 with 50% KOH, and water was added to bring the volume to 800 mL. Agar powder (15g) was added to the buffer solution, followed by autoclaving, and then cooled to 50℃. Thirdly, the nutrient solution contains 1mL of a standard micronutrient solution (493 mg $MgSo_4.7H_2O$, 11 mg $CaCl_2$, 104 mg H_3bO_3, 1.17 mg $MnCl_2.4H_2O$, 1.2 mg $ZnSO_4.7H_2O$, 0.4 mg $CuSO_4.5H_2O$, 1.0 mg $Na_2MoO_4.2H_2O$) and 2.4 g of PDB in 70 mL of water. Then this solution was autoclaved and cooled to 50℃ and was added to the buffer solution along with 30 mL filter-sterilized 10% (w/v) casamino acids. Finally, the indicator solution was added with continuous stirring to avoid air bubbles in the media.

Microorganisms growing on CAS-agar compete for Fe with the indicator dye chromo-azurol S (CAS). Siderophores that remove iron from CAS medium are indicated by the change of blue CAS-agar to orange-red (Milagres et al. 1999). *Rhizopus.* sp (collected from the Biology Department, U of S) was used as a siderophore-producing positive control (Baakza et al. 2004). *Rhizopus* sp. and our endophyte strains were cultured on CAS-agar for 7-14 d. After one week, the samples were examined to determine if the blue CAS-agar medium turned pink or orange, indicating siderophore production.

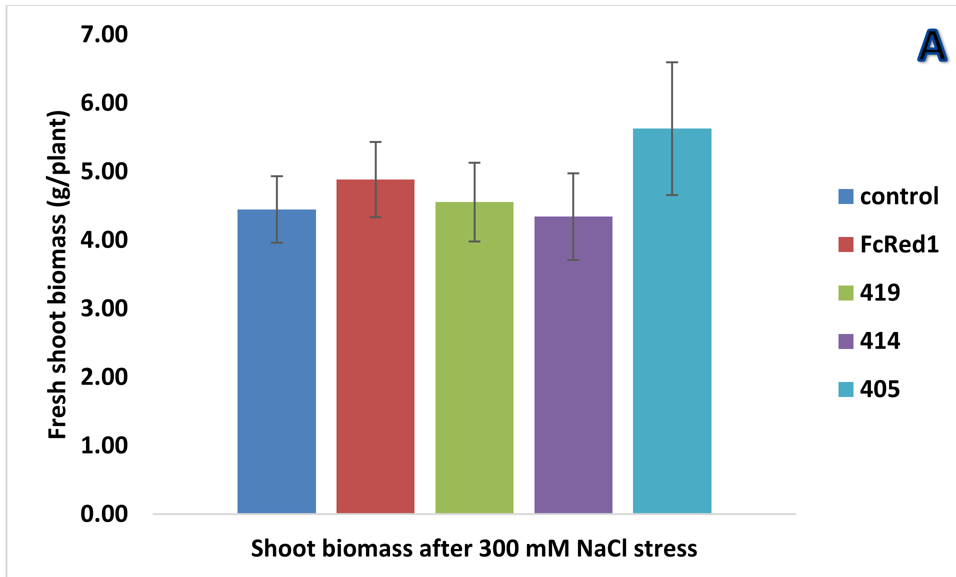
A modified spot-test method (Miller and Wright 1982) was used to evaluate the ability of endophytes to synthesize indole containing compounds. Endophytes were cultured on 1 g/L tryptophan in 10 % PDA (trp-PDA). Sterile filter paper disks were placed on the trp-PDA and inoculated. After one week, filter disks with endophyte culture were removed and placed into petri plates containing 20 mL of fresh Ehrlich reagent (modified from Srivastava and Shaw 1962). Ehrlich reagent was prepared by mixing 2 g of p-dimethylamino benzaldehyde with 50 mL of 100% ethanol, followed by adding 50 mL of concentrated HCl to this solution. Filter disks with endophyte mycelium were placed in this reagent for 5 min to monitor potential color change. Development of pink-red-purple indicated production of indole-type compounds.

3.2.9 Statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA) employing SPSS software (version 22, IBM). Duncan's multiple-range test was used to evaluate the significance of differences between treatments, when overall differences were

found to be significant using ANOVA at $P<0.05$. For both NaCl and drought stress experiments, error bars represent ±SE of three replicates. All experiments were conducted as a completely randomized block design. Control and strains of fungal endophytes were used as treatments for one-way ANOVA (data were separately analyzed by one-way ANOVA for each level of NaCl stress).

3.3 Results

3.3.1 Effects of acute salt stress on biomass. Growth performance of endophyte-colonized tomato plants in acute NaCl stress was assessed by comparing fresh biomass (both root and shoot) of endophyte-colonized plants and non-colonized control plants (Appendix 2: Figure 3-13). In the absence of NaCl stress, there were no significant differences in shoot and root biomass between endophyte-colonized and non-colonized plants (see Figure 2.2; Azad and Kaminskyj 2015). After 10 d of 300 mM NaCl stress, only the 405-inoculated plants exhibited a higher shoot biomass accumulation compared to control plants (**Figure 3-5A**). On the other hand, all of the endophyte-colonized plants had 40-50 % greater root biomass compared to non-colonized control plants (**Figure 3-5B**). However, the differences observed in both shoot biomass and root biomass between endophyte-colonized and non-colonized tomato plants was not statistically significant. (**Figure 3-5**).



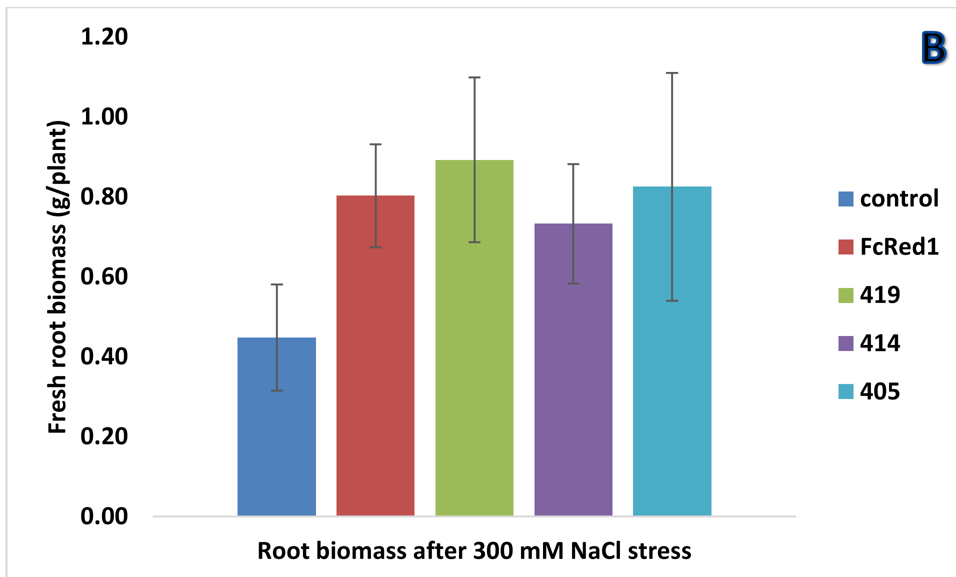
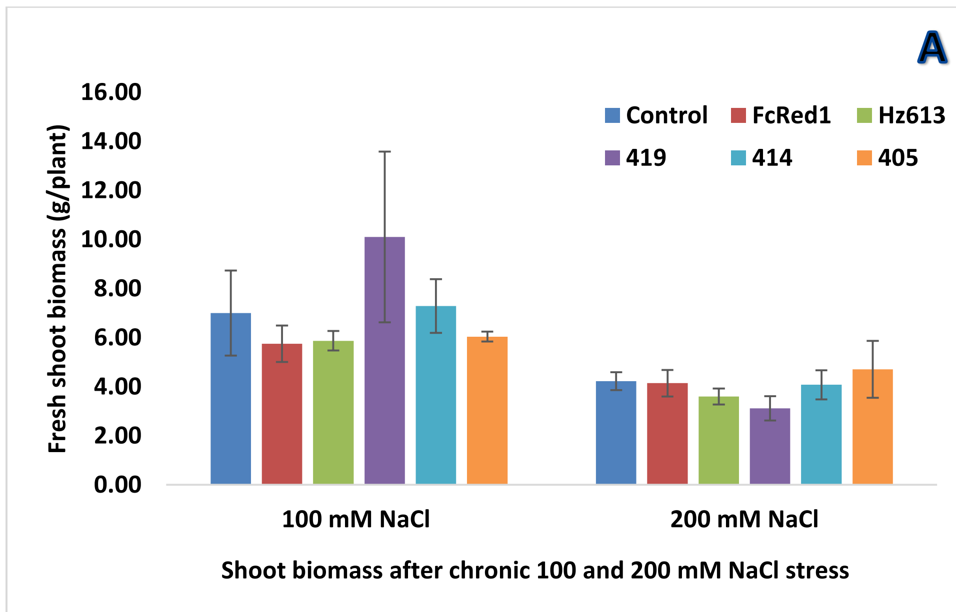


Figure 3-5. Effect of 300 mM NaCl (10 d) on shoot (A) and root (B) biomass accumulation in endophyte-colonized and non-colonized plants. Data represent the means ±SE of three replications. There was no significant difference (One-way ANOVA, $P<0.05$) in shoot or root observed in endophyte-colonized plants.

3.3.2 Effects of chronic salt stress on biomass. To assess long-term effects of endophyte colonization on salinity tolerance, plants were exposed to 30 d of 100 mM or 200 mM

chronic NaCl stress (three rounds of 10 d stress, followed by 2 d recovery in RO water after each round). Following 200 mM NaCl stress, plant biomass (both root and shoot) decreased by approximately 20% compared to plants exposed to the 100 mM NaCl stress (**Figure 3-6**). However, the differences in shoot biomass (**Figure 3-6A**) and root biomass (**Figure 3-6B**) observed between the endophyte-inoculated plants were not significant.



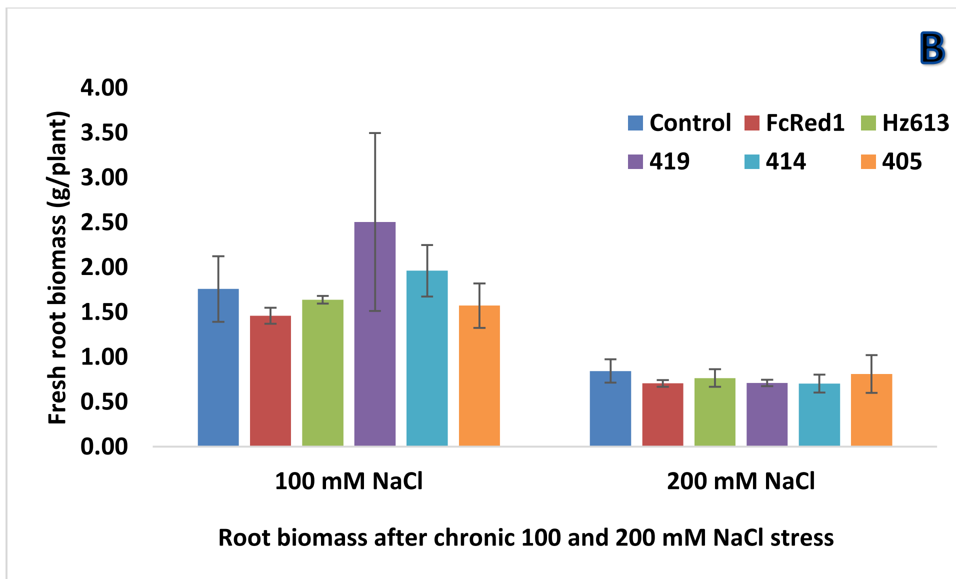


Figure 3-6. Effects of chronic NaCl stress (100 mM and 200 mM) on shoot (A) and root (B) biomass of endophyte-colonized and non-colonized plants. Data represent the means ±SE of three replications. There was no significant difference (One-way ANOVA, $P<0.05$) in shoot and root biomass of endophyte-colonized and non-colonized-plants. But root biomass decreased significantly (Duncan's multiple range test) upon 200 mM NaCl stress regardless of colonization.

3.3.3 Effects of chronic salt stress on photosynthetic efficiency. Photosynthetic efficiency was assessed using chlorophyll a fluorescence of dark-adapted second-youngest leaves of plants during chronic NaCl stress (section 3.2.6). There was no change in Fv/Fm of plants after the 1st round 10 d treatment with either 100 mM or 200 mM NaCl (**Figure 3-7A and Figure 3-7B**). However, after the 2nd round 10 d of 100 mM NaCl stress, some of the endophyte-colonized plants were better able to preserved a high level of Fv/Fm when compared to the non-colonized controls (**Figure 3-7A**). In particular, the FcRed1, Hz613, and 419 inoculated plants performed better than the control. Similarly, following the 2nd treatment with 200 mM NaCl, all of the endophyte-colonized plants, except 405, maintained their level of PSII function at a higher level than uninoculated control plants (**Figure 3-7B**). Following the 3rd round of 10 d NaCl stress, Fv/Fm of all plants sharply declined regardless of colonization (data not showing).

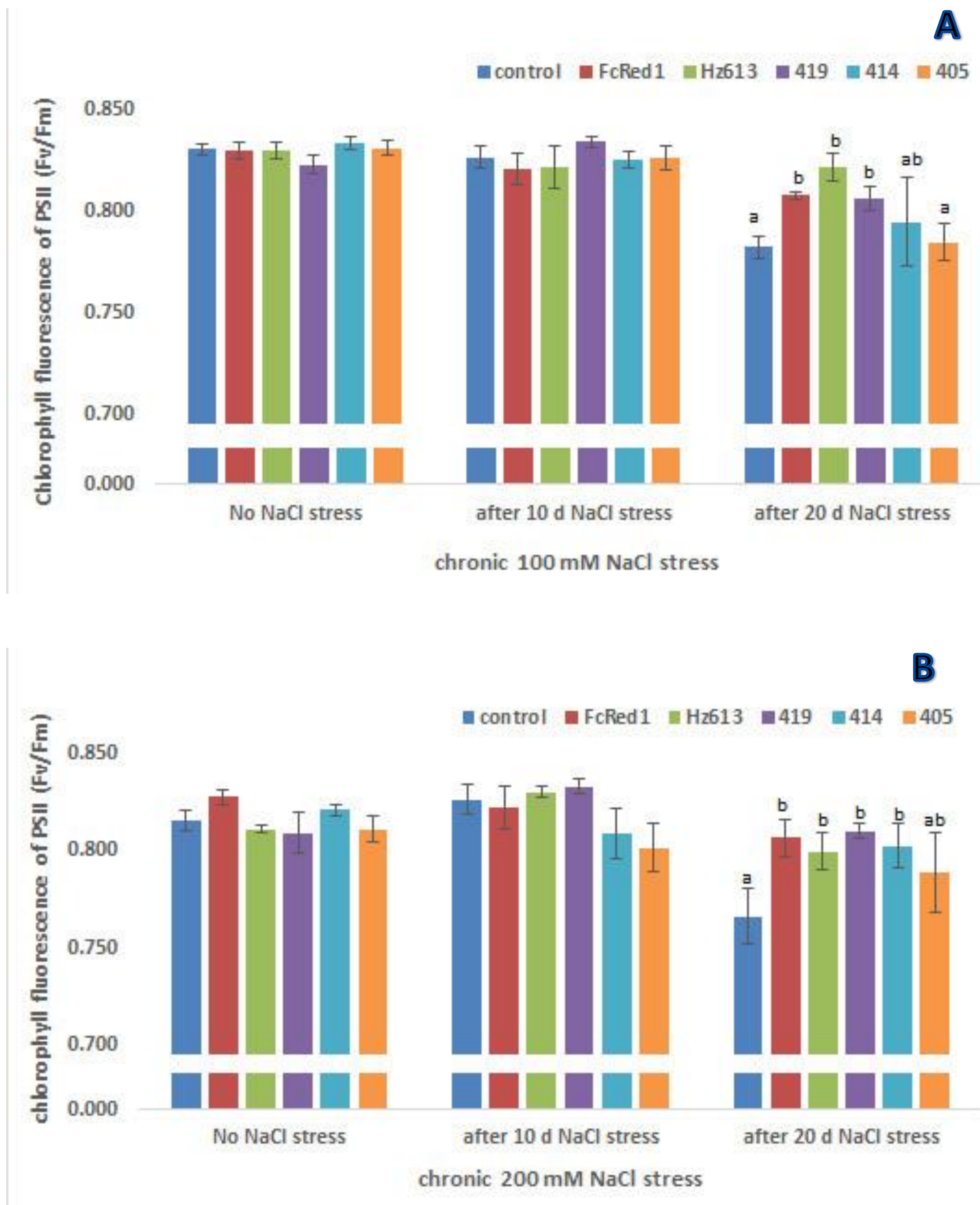


Figure 3-7. Effect of chronic salt stress using either 100 mM (A) or 200 mM (B) NaCl, on photochemical efficiency of PSII as measured by dark-adapted Fv/Fm. Data represent the means ±SE of three replications. Letters above the bars indicate significant difference. Three weeks old plants were exposed to two consecutive rounds of either 100 mM or 200 mM NaCl (10 d stress, then 2 d recovery in each round). After the second round of stress the endophyte-colonized plants had significantly higher photosynthetic efficiency (One-way ANOVA and Duncan's multiple range test, $P<0.05$) than non-colonized plants.

3.3.4 Effects of acute drought stress on biomass. Previously we found significantly higher biomass in endophyte-colonized plants compared to non-colonized plants during intermittent drought (three consecutive rounds of 10 d, 7 d, and 6 d) stress (see Figure 2-3; Azad and Kaminskyj 2015). In this chapter, we observed 10 d long periods of drought stress on endophyte-colonized and non-colonized plants. After 10 d period of drought stress, there was no difference between endophyte-colonized plants and non-colonized plants, apart from a trend toward increased fresh shoot (10-20 %) and fresh root biomass (10-30 %) in endophyte-colonized plants (**Figure 3-8A and Figure 3-8B**).

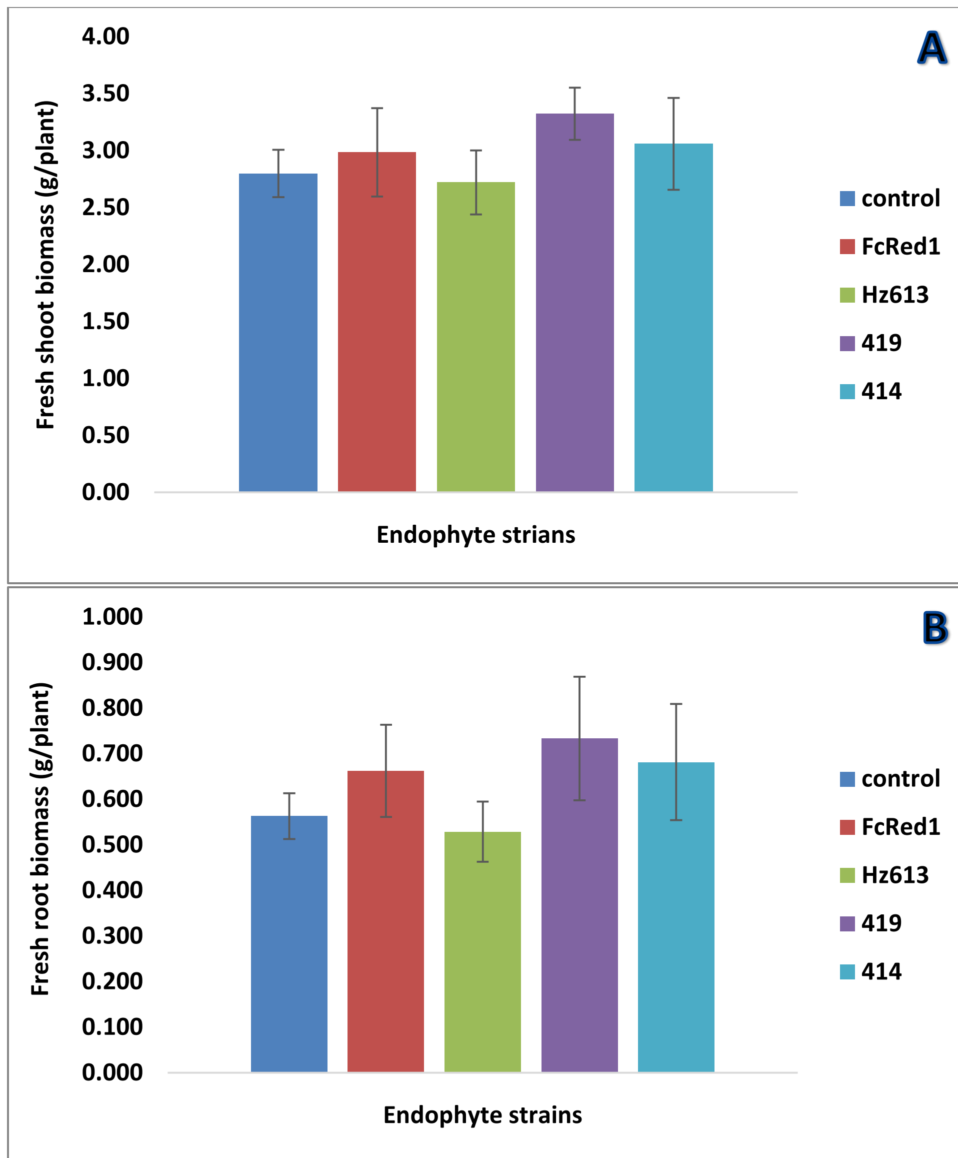


Figure 3-8. Effects of continuous drought stress (10 d) on shoot (A) and root (B) biomass of endophyte-colonized and non-colonized plants. Data represent the mean ± SE of three replications.

3.3.5 Fluid use efficiency. Fluid use efficiency in endophyte-colonized and non-colonized plants was assessed by measuring the volume of 1X Hoagland's solution consumed by plants (mL/g fresh weight and mL/g dry weight) over the course of 21 d. Total volume of fluid

used per plant was divided by the fresh and dry weight per plant. Endophyte-colonized plants tended to consume 10-20 % less fluid than non-colonized plants, although this difference was not statistically significant (**Figure 3-9**).

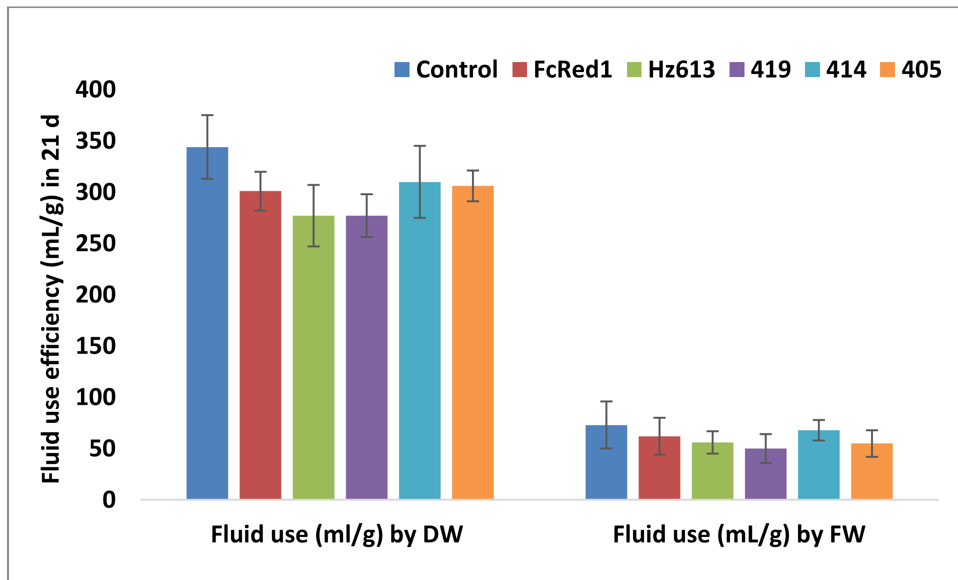


Figure 3-9. Effect of endophyte colonization on fluid use efficiency (by fresh weight = FW and by dry weight = DW). Data represent the means ±SE of four replications. There was no significant difference (One-way ANOVA, $P<0.05$) in fluid use efficiency of endophyte-colonized and non-colonized plants.

3.3.6 Proline accumulation. Free proline accumulation was assessed in three-week old endophyte-colonized and non-colonized plants in the absence of drought stress. There was no difference in proline accumulation regardless of endophyte colonization (**Figure 3-10A**). However, when plants were grown without water for 7 d, free proline accumulation increased more than 100-fold compared to plants in the absence of drought stress (**Figure 3-10B**). In addition, endophyte-colonized plants tended to have almost 25 % greater free proline

accumulation after 7 d drought compared to non-colonized plants, but this difference was not statistically significant (One-way ANOVA, P<0.05) (**Figure 3-10B**).

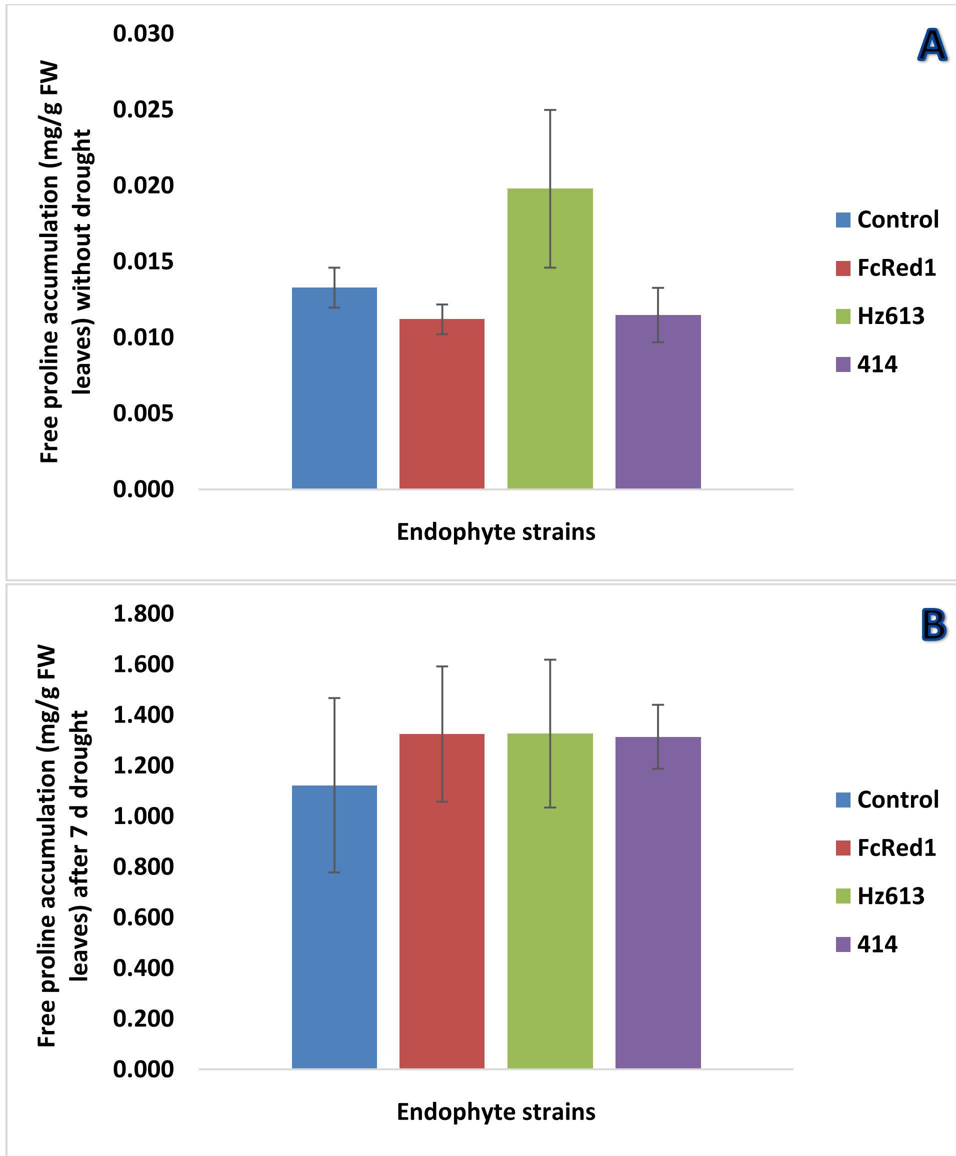


Figure 3-10. Accumulation of proline in well-watered plants (A) compared to plants exposed to 7 d of drought (B).

3.3.7 Siderophore and indole production by systemic fungal endophytes. Systemic fungal endophytes were cultured on CAS-agar for 10 d to assay siderophore production. The blue CAS- agar turned orange-pink-purple in the presence of siderophore production by endophytes (section 3.2.8). Only *Rhizopus* sp (a positive control species) produced a detectable level of siderophores, turning the blue agar dye into an orange product (**Table 3-1**; Appendix 2: supplemental Figure 3-11). Endophyte strains: FcRed1 and Hz613 grew moderately on CAS agar, but did not change its colour on CAS agar. In contrast, strains 419, 414, and 405 failed to grow on CAS agar (Appendix 2; supplemental Figure 3-11).

Endophytes grown on filter disks in tryptophan medium were tested with Ehrlich reagent (section 3.2.8). A colour change of filter disks from white to pink-red-purple indicated indole production. Strains FcRed1, 419, 417, 414, and 405 produced indoles, unlike Hz613 (**Table 3-2**; Appendix 2: supplemental Figure 3-12).

Table 3-1. Siderophore production by isolated systemic fungal endophytes

Endophytes	CAS reaction (blue CAS-agar turn into orange color)
Rhizopus sp.	+
FcRed1	-
Hz613	-
419	-
414	-
405	-

Table 3-2. Indole-type compounds production by isolated systemic fungal endophytes

Endophytes	Ehrlich reaction (filter paper turn into pink)
FcRed1	+
Hz613	-
419	+
414	+
405	+

3.4 Discussion

NaCl salinity and drought inhibit plant growth and development, triggering stress at the cellular and molecular levels. This is a significant problem in agriculture because almost 10 % of total agricultural land is salt-contaminated or drought affected (Bartels and Sunkar 2007). In our previous and current studies, tomato plants showed a trend of increased tolerance to NaCl and drought when colonized by systemic fungal endophytes isolated from saline habitats. NaCl and drought tolerance conferred by some systemic fungi including FcRed1 was previously reviewed by Rodriguez et al. (2008), Redman et al. (2011), and Woodward et al. (2012). Our goal in this chapter was to determine the level of effectiveness of our newly isolated endophytes obtained from saline sites in Saskatchewan.

3.4.1 Systemic fungal endophytes and increased biomass. Previously, we observed greater fresh shoot biomass in endophyte-colonized tomato seedlings than non-colonized plants upon 20 d NaCl stress followed by 2 d RO water rehydration (Chapter 2; Azad and Kaminskyj 2015). These results made us interested to observe the effects of repeated, short-term NaCl stress on growth performance of endophyte-colonized tomato plants.

Endophyte-colonized tomato plants had greater fresh root growth than non-colonized plants (approximately 2 fold) when plants were exposed to 300 mM NaCl stress, but there was no effect of endophyte colonization on fresh shoot biomass (**Figure 3-5**). However, increased fresh shoot biomass (approximately 30%) was observed in endophyte-colonized compared to non-colonized plants (Chapter 2; Azad and Kaminskyj 2015). Moreover, endophytes had no observable effect on fresh shoot and root biomass of plants after chronic 100 mM and 200 mM NaCl stress (**Figure 3-6**). These results suggested that our endophyte strains may act to protect plants or even promote plant growth at NaCl concentrations above 200 mM. This result was consistent with previous findings Rodriguez et al. (2008), Mei and Flinn (2010), and Redman et al. (2011), using other fungal endophytes.

3.4.2 Systemic fungal endophytes and increased photosynthetic efficiency. Photosynthetic efficiency (F_v/F_m) of endophyte-colonized and non-colonized plants was observed following chronic (periodic) 100 mM and 200 mM NaCl stress (three consecutive rounds of 10 d NaCl stress and 2 d RO water rehydration). In the absence of NaCl stress and

following 10 d of NaCl stress (both 100 mM and 200 mM) F_v/F_m of dark-adapted control plants and endophyte-colonized plants were in the same range; F_v/F_m was approximately 0.800 to 0.830 (**Figure 3-7A and 3-7B**). However, photosynthetic efficiency of these plants was decreased after 20 d of 100 mM and 200 mM NaCl (**Figure 3-7**), indicating that periodic NaCl stress has adverse impact on photochemistry of plants. At this level of stress significant effects of endophytes were found on colonized-plants compared to control plants (**Figure 3-7A and 3-7B**). A key point to consider following these experiments is the F_v/F_m measurements were the only ones that demonstrated a significant difference between the non-colonized controls and endophyte-colonized plants. It can be suggested that this simple chlorophyll fluorescence technique might be a very sensitive and robust method for identifying prospective endophytes that confer increased salinity and drought tolerance to colonized plants.

Our results correspond with the findings of Woodward et al. (2012). There was no difference in photochemical efficiency between symbiotic and nonsymbiotic plants in the absence of NaCl stress, while significant differences in photochemical efficiencies were observed in symbiotic plants under 300 mM NaCl stress (Woodward et al. 2012). This result suggested that colonized plants can better balance the light they absorb with their metabolic demands, when compared to non-colonized control plants (Woodward et al. 2012). On the other hand, our findings contradicted the observation of Meloni et al. (2003). There was no effect of 21 d of 200 mM NaCl stress on photosynthetic efficiency of cotton plants (Meloni et al. 2003). But salt stress may cause stomatal closure and results less carbon availability and carbon fixation in plants (Meloni et al. 2003). Therefore, our endophytes provided protection for photosynthetic efficiency of plants in response to periodic NaCl stress, which is more common in field condition with periodic rain. The protective role of endophytes was also observed upon intermittent drought stress in our previous study (Chapter 2; Azad and Kaminskyj 2015). One additional caveat to these findings is the amount of NaCl used in the experiments since endophyte-colonized plants showed better adaptability to photosynthetic efficiency in response to periodic 100 mM and 200 mM NaCl stress. Although these amounts of NaCl stress are unlikely under field condition, our experiment showed that plants can adapted with these amounts of periodic NaCl stress.

3.4.3 Systemic fungal endophytes and fluid use efficiency. Decreased fluid consumption correlates to better water use efficiency which can be an efficient mechanism for endophyte-conferred drought tolerance in host plants (Rodriguez et al. 2008). Better fluid use efficiency (approximately 20 %) was observed in endophyte-colonized plants compared to non-colonized plants (**Figure 3-4**). Our findings correlate with those of Rodriguez et al. (2008) and Redman et al. (2011). They showed colonization with systemic fungal endophytes correlated with a decrease in fluid consumption of up to 50% in host plants (Redman et al. 2011; Rodriguez et al. 2008).

3.4.4 Systemic fungal endophytes and proline accumulation under drought. Our previous studies suggested that systemic fungal endophytes isolated from saline habitats improve growth and photosynthetic efficiency in intermittent drought stress (Chapter 2; Azad and Kaminskyj 2015). We explored whether other drought-related factors would change in a comparable fashion for colonized plants.

Overproduction of proline during water deficiency is an indicator of drought tolerance (Parkhi et al. 2009). Proline helps plants to regulate nitrogen accumulation that leads to membrane stability under NaCl stress (Yadav et al. 2012). Higher proline levels also correlate with extreme drought events. It is stated that higher cellular proline levels help proved proline-induced stress tolerance in endophyte-colonized plants (Elbersen and West 1996). We observed almost 25% higher proline accumulation in colonized-plants than non-colonized plants (**Figure 3-10B**), consistent with the findings by Bayat et al. (2009). In a hydroponic culture of tall fescue, proline content was two-fold greater (627 µg/g leaf FW) in endophyte-colonized plants compared to endophyte-free plants (343 µg/g leaf FW) under extreme drought stress (Bayat et al. 2009). However, Elbersen and West (1996) observed endophyte-free fescue plants had more proline than endophyte-colonized plants under water stress.

Proline accumulation is correlated to osmotin or osmotin-like protein (Barthakur et al. 2001). Over-expression of osmotin in transgenic plants confers tolerance to biotic and abiotic stress (Barthakur et al. 2001; Parkhi et al. 2009; Patade et al. 2013). It was determined that osmotin protein contributes in osmotic adjustment under abiotic stress by elevating free-proline accumulation (Barthakur et al. 2001). Proline level also increased remarkably in osmotin over-

expressed transgenic tomato plants under cold stress (Patade et al. 2013). Osmotin-overexpressed tobacco seedlings (two and half weeks old) also contain higher proline level under short period (8 d) of drought stress (Parkhi et al. 2009). Osmotin-expressed tobacco plants accumulated higher proline compared to wild type in response to drought (5d) and NaCl stress (200 mM) (Barthakur et al. 2001). Therefore, our endophyte strains may trigger osmotin protein to elevate proline accumulation under drought leading to drought tolerance in host plants. This would reflect another mechanism that is enhanced in plants colonized by endophytes, and add to their decreased need for water under drought or salinity stress.

3.4.5 Siderophore and indole detection from isolated systemic fungal endophytes. We assessed the isolated endophytes' ability to synthesize siderophores and indoles. We observed some of our endophytes: 419, 414, and 405 failed to grow on CAS-agar. The failure of growing microorganisms on CAS-agar may be the toxic effect of CTAB (cetyle trimethyl ammonium bromide) on fungi and Gram-positive bacteria (Alexander and Zuberer 1991). Several researchers modified the preparation of CAS-agar medium for successful growth of different species of fungi and bacteria (Milagres et al. 1999; Perez-Miranda et al. 2007). For example, instead of PDB media/nutrient media, only 0.9% of agarose was used as a gelling agent in CAS-agar media and it was applied over agar medium of cultivated microorganisms and was observed the color change (in 15 min) due to Fe-affinity (Perez-Miranda et al. 2007). On the other hand, we observed moderate growth of FcRed1 and Hz613 on CAS-agar medium that was prepared in traditional method. But there was no indication of siderophore synthesis from these endophytes (**Table 3-1; Appendix 2:** supplemental Figure 3-11). Therefore, it is inconclusive from our experiment if our isolated fungal endophytes have strong Fe-affinity to their surrounding environments.

Phytohormones (e.g auxin, cytokinin) synthesized by microorganisms such as plant-associated bacteria (Costacurta and Vanderleyden, 1995) and fungi (Gruen, 1959) were reported previously by several researchers. We used Ehrlich reagent to examine the ability for indole production by systemic fungal endophytes. We cultured endophytes on tryptophan-rich PDA to investigate their ability for indole production. A positive Ehrlich reaction (**Table 3-2**) indicated our systemic fungal endophytes produce indoles, suggesting that endophyte-promoted plant growth in extreme environments may be partly due to indoles synthesized *in planta*. This

corresponds to the findings of Redman et al. (2011). Similar observations were made for *Penicillium funiculosum* LHLO6 (Khan et al. 2012), *Trichoderma virens* (Contreras-Cornejo et al. 2009), *Fusarium oxysporum* (Hasan 2002), and *Penicillium indica* (Sirrenberg et al. 2007).

The systemic fungal endophytes used in this study seem to confer some protection to the tomato plants used in our study. While growth promotion and increased biomass accumulation under the conditions here was not clearly significant, a trend was observed in endophyte-colonized plants. Interestingly, the endophyte colonized plants showed a lower impact on photosynthesis by drought and salinity stress, based on our F_v/F_m measurements. This may have resulted in altered biomass accumulation if longer-term studies were conducted. Our subsequent measurements into the mechanistic details of how the endophytes could be altering plant physiology to promote drought or salinity tolerance were inconclusive. However, increased proline content and the production to plant growth regulator precursors may be due in part of endophyte colonization. An exciting aspect of these results is the role of the endophytes in promoted increasing biomass and other physiological responses (photosynthetic efficiency and proline accumulation) in tomato plants. These endophytes would not have co-evolved with tomato plants, thus our results suggest that endophyte-conferred drought and salt tolerance can be supported in genetically diverse plants. This would make it much easier for agricultural specialists to acclimatize plants to harsh environments, compared to traditional breeding approaches. In future studies, we will examine specific modes of action of the endophytes in regulating plant physiological processes in response to extreme salt and drought stress.

3.5 References

Alexander D, Zuberer D (1991) Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. Biol Fertility Soils 12: 39-45

Ashraf M, Foolad M (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot 59: 206-216

Azad K, Kaminskyj S (2015) A fungal endophyte strategy for mitigating the effect of salt and drought stress on plant growth. Symbiosis 1-6

Baakza A, Vala A, Dave B, Dube H (2004) A comparative study of siderophore production by fungi from marine and terrestrial habitats. J Exp Mar Biol Ecol 311: 1-9

Bartels D, Sunkar R (2005) Drought and salt tolerance in plants. Crit Rev Plant Sci 24: 23-58

Barthakur S, Babu V, Bansa K (2001) Over-expression of osmotin induces proline accumulation and confers tolerance to osmotic stress in transgenic tobacco. Journal of Plant Biochemistry and Biotechnology 10: 31-37

Bates L, Waldren R, Teare I (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39: 205-207

Bayat F, Mirlohi A, Khodambashi M (2009) Effects of endophytic fungi on some drought tolerance mechanisms of tall fescue in a hydroponics culture. Russian Journal of Plant Physiology 56: 510-516

Bohnert HJ, Jensen RG (1996) Strategies for engineering water-stress tolerance in plants. Trends Biotechnol 14: 89-97

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to environmental stresses. Plant Cell 7: 1099

Brundrett MC (2006) Understanding the roles of multifunctional mycorrhizal and endophytic fungi. Microbial root endophytes. Springer, pp 281-298

Change B, Maehly A (1955) Assay of catalases and peroxidase. Meth Enzymol 2: 764-775

Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann Bot 103: 551-560

Chung K, Shilts T, Ertürk Ü, Timmer L, Ueng PP (2003) Indole derivatives produced by the fungus *Colletotrichum acutatum* causing lime anthracnose and postbloom fruit drop of citrus. FEMS Microbiol Lett 226: 23-30

Contreras-Cornejo HA, Macias-Rodriguez L, Cortes-Penagos C, Lopez-Bucio J (2009) *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. Plant Physiol 149: 1579-1592

Costacurta A, Vanderleyden J (1995) Synthesis of phytohormones by plant-associated bacteria. Crit Rev Microbiol 21: 1-18

Elbersen H, West C (1996) Growth and water relations of field-grown tall fescue as influenced by drought and endophyte. Grass Forage Sci 51: 333-342

Gangwar M, Rani S, Sharma N (2012) Diversity of endophytic actinomyetes from wheat and its potential as plant growth promoting and biocontrol agents. J Adv Lab Res Biol 3: 18-23

Gruen HE (1959) Auxins and fungi. Annual review of plant physiology 10: 405-440

Hasan H (2002) Gibberellin and auxin-indole production by plant root-fungi and their biosynthesis under salinity-calcium interaction. Acta Microbiol Immunol Hung 49: 105-118

Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A (2012) Role of proline under changing environments: a review. Plant signaling & behavior 7: 1456-1466

Huner NP, Öquist G, Sarhan F (1998) Energy balance and acclimation to light and cold. Trends Plant Sci 3: 224-230

Kaminskyj SG (2008) Effective and flexible methods for visualizing and quantifying endorhizal fungi. Mycorrhizae: Sustainable Agriculture and Forestry. Springer, pp 337-349

Mei C, Flinn BS (2010) The use of beneficial microbial endophytes for plant biomass and stress tolerance improvement. Recent patents on biotechnology 4: 81-95

Meloni DA, Oliva MA, Martinez CA, Cambraia J (2003) Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. Environ Exp Bot 49: 69-76

Milagres AM, Machuca A, Napoleão D (1999) Detection of siderophore production from several fungi and bacteria by a modification of chrome azurol S (CAS) agar plate assay. J Microbiol Methods 37: 1-6

Miller JM, Wright JW (1982) Spot indole test: evaluation of four reagents. J Clin Microbiol 15: 589-592

Neilands JB (1995) Siderophores: structure and function of microbial iron transport compounds. J Biol Chem 270: 26723-26726

Nuccio ML, Rhodest D, McNeil SD, Hanson AD (1999) Metabolic engineering of plants for osmotic stress resistance. Curr Opin Plant Biol 2: 128-134

Parkhi V, Kumar V, Sunilkumar G, Campbell LM, Singh NK, Rathore KS (2009) Expression of apoplastically secreted tobacco osmotin in cotton confers drought tolerance. Mol Breed 23: 625-639

Patade VY, Khatri D, Kumari M, Grover A, Mohan Gupta S, Ahmed Z (2013) Cold tolerance in Osmotin transgenic tomato (Solanum lycopersicum L.) is associated with modulation in transcript abundance of stress responsive genes. Springerplus 2: 117-1801-2-117.

Pérez-Miranda S, Cabirol N, George-Téllez R, Zamudio-Rivera L, Fernández F (2007) O-CAS, a fast and universal method for siderophore detection. J Microbiol Methods 70: 127-131

Pineda A, Zheng S, van Loon JJ, Pieterse CM, Dicke M (2010) Helping plants to deal with insects: the role of beneficial soil-borne microbes. Trends Plant Sci 15: 507-514

Rai M, Rathod D, Agarkar G, Dar M, Brestic M, Pastore GM, Junior MRM (2014) Fungal growth promotor endophytes: a pragmatic approach towards sustainable food and agriculture. Symbiosis 62: 63-79

Redman RS, Kim YO, Woodward CJ, Greer C, Espino L, Doty SL, Rodriguez RJ (2011) Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. PLOS one6: e14823

Rodriguez RJ, Woodward C, Redman RS (2012) Fungal influence on plant tolerance to stress. Biocomplexity of plant–fungal interactions.Oxford: Wiley-Blackwell 155-163

Rodriguez RJ, Henson J, Van Volkenburgh E, Hoy M, Wright L, Beckwith F, Kim Y, Redman RS (2008) Stress tolerance in plants via habitat-adapted symbiosis. The ISME journal 2: 404-416

Rodriguez RJ, White JF, Jr., Arnold AE, Redman RS (2009) Fungal endophytes: diversity and functional roles. New Phytol 182: 314-330

Sirrenberg A, Göbel C, Grond S, Czempinski N, Ratzinger A, Karlovsky P, Santos P, Feussner I, Pawlowski K (2007) *Piriformospora indica* affects plant growth by auxin production. Physiol Plantarum 131: 581-589

Srivastava BS, Shaw M (1962) the biosynthesis of indole acetic acid in Melampsora Lini (Pers.) Canadian Journal of Botany 40: 309-315

Szabados L, Savouré A (2010) Proline: a multifunctional amino acid. Trends Plant Sci 15: 89-97

Wilson KE, Ivanov AG, Öquist G, Grodzinski B, Sarhan F, Huner NP (2006) Energy balance, organellar redox status, and acclimation to environmental stress. Botany 84: 1355-1370

Winkelmann G (2002) Microbial siderophore-mediated transport. Biochem Soc Trans 30: 691-696

Woodward C, Hansen L, Beckwith F, Redman RS, Rodriguez RJ (2012) Symbiogenics: An Epigenetic Approach to Mitigating Impacts of Climate Change on Plants. HortScience 47: 699-703

Yadav NS, Shukla PS, Jha A, Agarwal PK, Jha B (2012) The SbSOS1 gene from the extreme halophyte Salicornia brachiata enhances Na+ loading in xylem and confers salt tolerance in transgenic tobacco. BMC Plant Biol 12:188-2229-12-188

Yang T, Chen Y, Wang X, Dai C (2013) Plant symbionts: keys to the phytosphere. Symbiosis 1-14

Yokoi S, Bressan RA, Hasegawa PM (2002) Salt stress tolerance of plants. JIRCAS working report 23: 25-33

You YH, Kang SM, Choo YS, Lee JM (2012) Fungal diversity and plant growth promotion of endophytic fungi from six halophytes in Suncheon Bay. Journal of microbiology and biotechnology 22: 1549-1556

Zhao J, Shan T, Mou Y, Zhou L (2011) Plant-derived bioactive compounds produced by endophytic fungi. Mini reviews in medicinal chemistry 11: 159-168

Zhou R, Yu X, Kjær KH, Rosenqvist E, Ottosen C, Wu Z (2015) Screening and validation of tomato genotypes under heat stress using F v/F m to reveal the physiological mechanism of heat tolerance. Environ Exp Bot 118: 1-11. doi:10.1016/j.envexpbot.2015.05.006.

CHAPTER 4

*A version of this chapter will be submitted for publication.

Protective effects conferred on tomato plants growing in saline or drought conditions by systemic fungal endophytes isolated from plants growing on saline soils in Saskatchewan.

Kumkum Azad[1], Susan Kaminskyj[1], and Jim Basinger[2]

Statement of contribution

Study conception and design: Kumkum Azad (primary contributor), Susan Kaminskyj

Acquisition of data: Kumkum Azad

Analysis and interpretation of data: Kumkum Azad

Drafting of manuscript: Kumkum Azad (primary contributor), Susan Kaminskyj, Jim Basinger

4.1 Introduction

Systemic fungal endophytes contribute to plant establishment in extreme environments, through a process called habitat-adapted symbiosis (Redman et al. 2001; Rodriguez et al. 2008). The protective effects of fungal endophytes that are involved in Habitat-Adapted (HA) symbiosis include the rapid activation of plant stress signaling pathways or the production of anti-stress components in host plants (Mei and Flinn 2010; Redman et al. 1999).

Plants adapt to environmental challenges by complex biochemical, physiological and genetic modification (Bartels and Sunkar 2007). Extreme salinity and drought impedes plant physiology by disrupting intercellular ion homeostasis and osmotic adjustment, followed by membrane dysfunction, and metabolic aberration (Bartels and Sunkar 2007; Farooq et al. 2009; Zhu 2003). The reduction in soil water content makes water acquisition by the plant more difficult. This leads to the closing of stomata in plant leaves to conserve water. As a result, the photosynthetic machinery becomes CO_2 limited and faces an accumulation of O_2 inside the leaf. As photosynthesis faces feedback limitation, photosystem II (PSII) undergoes increased photoinhibition, due to the over reduction of the electron transport chain, and the accumulation of reactive oxygen species (ROS). ROS are a common consequence of stressful environments (Bartels and Sunkar 2007). They can be both signals that regulate plant gene expression and damaging factors which degrade proteins, membranes, and even DNA (Miller 2010). Plants survive in saline soil by using different mechanisms to maintain water balance including: exclusion of extracellular ions, compartmentalization, cellular osmotic adjustment, and upregulation of antioxidant systems (Bartels and Sunkar 2007; Zhu 2003). The mechanism of drought tolerance involves many processes that lead to osmotic adjustment and /or changes in stomatal structure (Chaves et al. 2009). It is thought that fungal endophytes can help in the adjustment phase and protect the plant from some of the adverse effects of drought and salinity.

Reactive oxygen species (ROS) are highly reactive and toxic compounds that include both free- radicals (O_2^-, superoxide; OH^-, hydroxyl, HO_2^-, perhydroxy and RO^-, alkoxy) and non-radicals (singlet oxygen and H_2O_2, hydrogen peroxide) (Gill and Tuteja 2010). ROS are produced as byproducts of regular metabolic pathways such as photosynthesis and respiration in

mitochondria, chloroplasts, and peroxisomes (Apel and Hirt 2004; Gill and Tuteja 2010). Singlet oxygen is one of the main ROS produced by plants in times of environmental stress. It is caused by the interaction of light with chlorophyll molecules in the leaf. Because ROS are a natural part of plant metabolism, surfeit ROS is typically scavenged by the well-developed set of enzymatic and non-enzymatic antioxidant defense mechanisms in plants (Apel and Hirt 2004). Non-enzymatic antioxidants comprise ascorbate, glutathione, tocopherol, flavonoids, alkaloids, and carotenoids, while enzymatic antioxidants encompass superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, and catalases (Apel and Hirt 2004; Gill and Tuteja 2010). The balance between the production of ROS and antioxidant defense components is disrupted by biotic and abiotic stress and can lead to a sharp rise in intercellular ROS generation (Apel and Hirt 2004; Gill and Tuteja 2010). In consequence, the loss of balance in ROS production causes oxidative damage in plant cell which can lead to delayed growth, reduced photosynthetic efficiency, and in extreme cases, death of the cell or plant (Apel and Hirt 2004).

Osmolytes (also called compatible solutes) including proline, soluble sugar, and amino acids are important biological indicators of stress tolerance in plants (Ashraf and Foolad 2007). They accumulate in high amounts without influencing normal plant physiology and biochemistry (Bohnert and Jensen 1996). Osmolytes are mostly organic in nature, apart from essential ions like K^+ (Yokoi et al. 2002). Organic osmlolytes comprise simple sugars (fructose and glucose), sugar alcohols (glycerol, mannitol, and methylated inositols), complex sugars (trehalose, raffinose and fructans), quaternary amino acid derivatives (proline, glycine betaine, β-alanine betaine, proline betaine), tertiary amines (1,4,5,6-tetrahydro-2-mehyl-4-carboxyl pyrimidine), and sulfonium compounds (choline *o*-sulfate and dimethyl sulfonium propionate) (Nuccio et al. 1999; Bohnert and Jensen 1996). In addition to maintaining cellular osmotica, they protecting cell membrane turgidity, stabilize protein structure and enzyme activity (Hayat et al. 2012). Some osmolytes can also protect plants by acting as ROS-scavenging compounds (Bohnert and Jensen 1996; Hayat et al. 2012). As a result, they serve as osmoprotectants and ROS scavengers in the same time (Chapter 2; Porcel et al. 2012; Hayat et al. 2012; Azad and Kaminskyj 2015).

In this chapter, we observe the effects of systemic fungal endophytes in qualitative and quantitative ROS generation, osmolytes, and photosynthetic efficiency modulation in host plants in response to salt stress and drought stress. In this chapter, we hypothesize that systemic fungal

endophytes modulate ROS generation, osmolyte concentration and photosynthetic efficiency in tomato plants under NaCl and drought stress.

4.2 Materials and methods

4.2.1 Plant growth conditions. Tomato (*Solanum lycopersicum* var. Rutgers) seeds were inoculated with systemic fungal endophytes at spore concentration of 10^{-3}- 10^{-4} spore/mL (Chapter 2: section 2.2.2; Azad and Kaminskyj 2015). Prior to inoculation, seeds were surface sterilized with 0.6% sodium hypochlorite (NaOCl) for 15 min. Then, seeds were shaken in the fungal spore suspension for 30 min. Un-inoculated control seeds (for non-colonized plants) were shaken in sterile water to simulate the inoculation process (Chapter 2; Azad and Kaminskyj 2015).

Control and inoculated seeds were then planted in sterile, double-decker Magenta boxes (MBs) that contained Sunshine Mix no. 3 in the upper layer and Hoagland's solution in the bottom layer (Rodriguez et al. 2008). A top-knotted cotton rope was used to connect upper layer and bottom layer (Azad and Kaminskyj 2015). Plants were grown at 22°C and 23 % relative humidity with 12-h fluorescent light regime (350 μmol photons $m^{-2}s^{-1}$) in a growth room (Chapter 2: section 2.2.2; Azad and Kaminskyj 2015).

4.2.2 Salt and drought stress treatments and harvesting. Three week old endophyte-colonized and non-colonized plants were exposed to different levels of NaCl stress (400 mM, 500 mM, and 600 mM in separate experiments) for 15 d by adding NaCl in Hoagland's solution in the bottom layer of MBs (Chapter 2: section 2.2.2; Azad and Kaminskyj 2015). To induce drought stress, watering was terminated for 11 d by decanting off the 1X Hoagland's solutions in the lower layer of MBs and letting the soil dry out over time (Chapter 2: section 2.2.2; Azad and Kaminskyj 2015). After the stress treatments were terminated, plants were rewatered with reverse osmosis water (RO) for 48 hr (Appendix 3: Figure 4-14), and then harvested for biomass measurement and physiological assessment. Roots and shoots of the plants were cut separatly and their biomasses were determined. The fresh weight of roots and shoots were measured immediately after harvesting. For dry weight measurements, roots and shoots were dried at room temperature for 24 hr, followed by 55˚C oven temperature for 48 hr.

4.2.3 Physiological tests

4.2.3.1 Detection and estimation of in vivo superoxide (O_2^-) generation. *In vivo* detection of superoxide (O_2^-) in plants was accomplished by histochemical staining of leaves with nitro-blue tetrazolium (NBT). A modified method adapted from Yadav et al. (2012) and Ramel et al. (2006) was used to detect *in vivo* localization of O_2^- after treating leaves with NBT.

Typically, second-youngest leaves of endophyte-colonized and non-colonized plants were excised and were floated with 0.8 mM of NBT. Samples were kept in the dark at room temperature for 5 hr. Following incubation, leaves were boiled with 95% ethanol for 20 min in order to bleach chlorophyll. Subsequently, ethanol was removed and the leaves were preserved in sterile ultra-pure water. Blue stains, caused by formazan precipitates due to the reaction of NBT with O_2^-, were viewed and photographed.

The modified method of Ramel et al. (2009) was used to quantify the O_2^- content of leaves. NBT-stained leaves were homogenized in liquid nitrogen, followed by mixing in a 2 M KOH-DMSO solution (1:1.6, v/v). The leaf tissue homogenates were centrifuged for 10 min at 12000 rpm. The absorbance of formazan in the supernatants was measured immediately at 630 nm, using a UV-Vis spectrophotometer (Genesys, 60). The absorbance at 630 was compared with a standard curve obtained from known amounts of NBT in the KOH-DMSO mix. Six plants per treatment were used in this experiment.

4.2.3.2 Detection and estimation of in vivo hydrogen peroxide (H_2O_2) generation. *In vivo* detection of hydrogen peroxide (H_2O_2) in plants was accomplished by histochemical staining of leaves with 3, 3- diaminobenzidine (DAB). A modified method adapted from Yadav et al. (2012) and Ramel et al. (2006) was used to detect *in vivo* localization of H_2O_2 after treating leaves with DAB.

Typically, second-youngest leaves of endophyte-colonized and non-colonized plants were excised and were floated with 1 mg/mL of DAB. Samples were kept under light at room temperature for 6 hr. Brown spots on the leaves are due to formazan precipitations resulting from the reaction of DAB with H_2O_2. Following incubation, leaves were boiled with 95% ethanol for 20 min in order to remove the chlorophyll from the leaf. Subsequently, ethanol was removed and

the leaves were preserved in sterile ultra-pure water. The brown stains observed on leaves indicate DAB- H_2O_2 reaction.

The modified method of Ramel et al. (2009) was used to estimate the amount of H_2O_2 contents in leaves. DAB-stained leaves were homogenized in liquid nitrogen; followed by mixing in 0.2 M $HClO_4$. The leaf tissue homoginates were centrifuged for 10 min at 12000 rpm. The absorbance of the supernatants was measured at 450 nm with a UV-Vis spectrophotometer (Genesys, 60). The absorbance was then compared with a standard curve obtained from adding known amounts of H_2O_2 to a 0.2 M $HClO_4$-DAB solution. Six plants per treatment were used in this experiment.

4.2.3.3 Determination of total Osmolyte concentration. A modified method adapted from Rodriguez et al. (2008) was used in order to assay plant osmolyte concentrations. Before and after abiotic stress treatments (15 d NaCl stress and 11 d drought stress), osmolyte concentration of endophyte-colonized plants and non-colonized plants were assessed. Approximately 100 mg of lower stem tissues were ground in liquid nitrogen. Then 500 µL water was added and the sample centrifuged for 5 min at 6000 rpm. Resulting supernatant was used to measure osmolality (mOsm/kg) of plants, with a vapor pressure osmometer (Wescor 5500).

4.2.3.4 Photosynthetic efficiency assay. Photosystem II (PSII) efficiency was measured with a chlorophyll fluorometer (Walz, Germany, model PAM 2000) using the method described previously (Chapter 2: section 2.2.3; Azad and Kaminskyj 2015). Following 5 minutes of dark adaption, reaction centers in PSII were closed and F_m (maximum fluorescence) was induced by using the PAM 200 halogen lamp, an 800 ms pulse of light (2500 micromols photons m^{-2} s^{-1}) (Zhou et al. 2015). Then F_v (variable fluorescence) was calculated as the difference between F_m and F_o (minimal fluorescence). Finally F_v/F_m was calculated to estimate the quantum yield of PSII.

4.2.4 Statistical analysis. *P*-values were determined by one-way analysis of variance (ANOVA) and data were analysed by using SPSS software (version 22; IBM corp.). Duncan's multiple-range test was used to evaluate the significance of differences between treatments, when overall differences were found to be significant using ANOVA at $P<0.05$. For both NaCl

and drought stress experiments, error bars represent ±SE of six replicates. All experiments were conducted as a completely randomized block design. Control and strains of fungal endophytes were used as treatments for one-way ANOVA (data were separately analyzed by one-way ANOVA for each level of NaCl stress).

4.3 Results

4.3.1 Maintenance of growth during drought and salinity stress in endophyte-colonized plants. Growth of endophyte-colonized plants was assessed by comparing shoot and root biomass of endophyte-colonized plants to non-colonized plants after 15 d of NaCl stress (400 mM, 500 mM, and 600 mM) or 11 d of drought stress.

In the absence of NaCl stress, there was no significant difference in biomass of endophyte-colonized and non-colonized plants. After 15 d continuous NaCl stress (400 mM, 500 mM, and 600 mM), biomass of plants (both root and shoot) decreased significantly compared to no-stressed plants. However, there was no observable difference in root and shoot biomass between endophyte-colonized and non-colonized plants (**Figure 4-1**).

On the other hand, after 11 d continuous drought stress followed by 2 d of rehydration, plants colonized with endophyte 414 had significantly higher shoot biomass compared to non-colonized plants (**Figure 4-2**) (Appendix 3: Figure 4-14). But there was no significant difference (One-way ANOVA, $P<0.05$) in dry root biomass of endophyte-colonized and non-colonized plants.

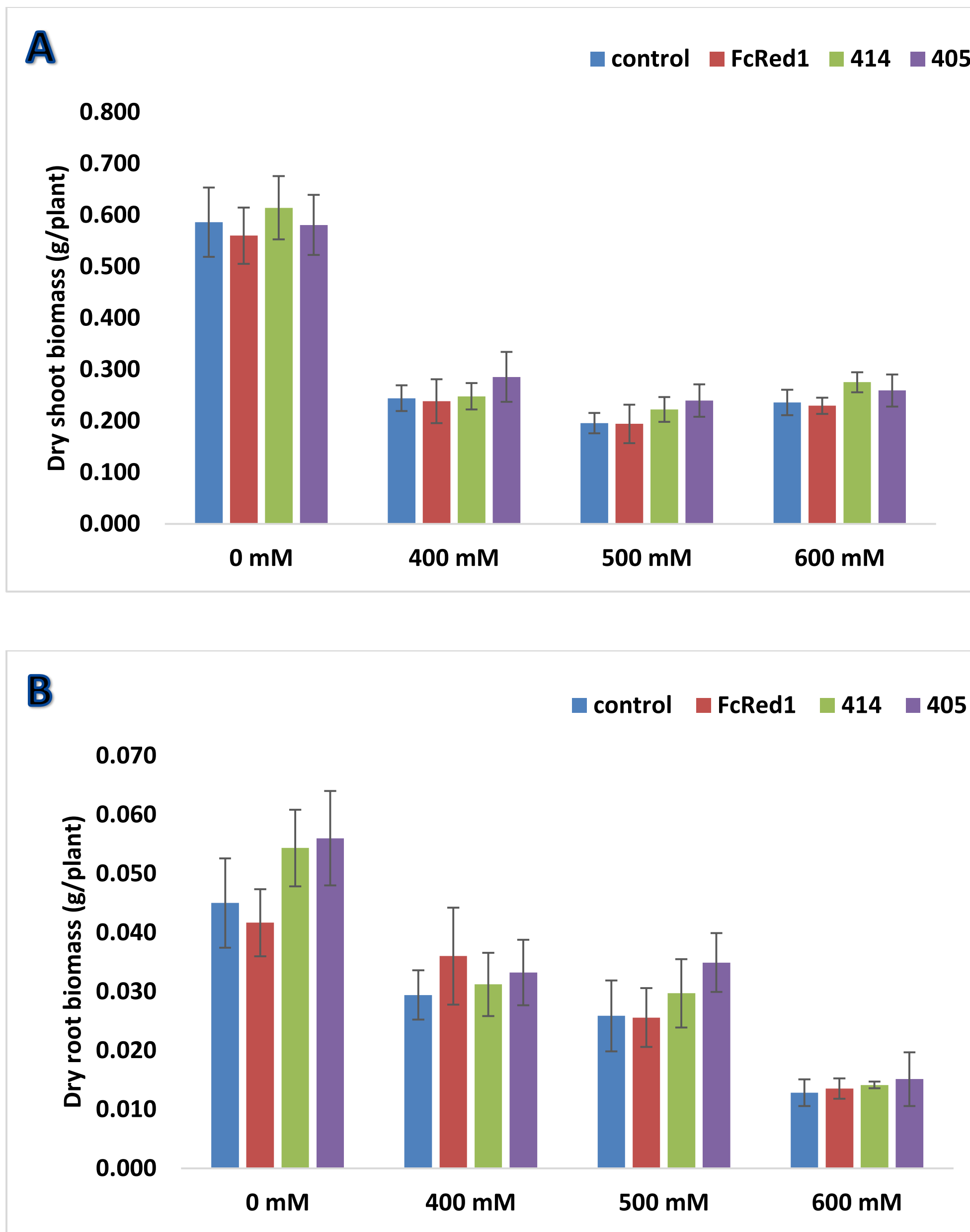


Figure 4-1. Effects 15 d period of 400 mM, 500 mM, and 600 mM NaCl on shoot (A) and root (B) biomass comparing endophyte-colonized and non-colonized plants. Data represent the means ±SE of six replications.

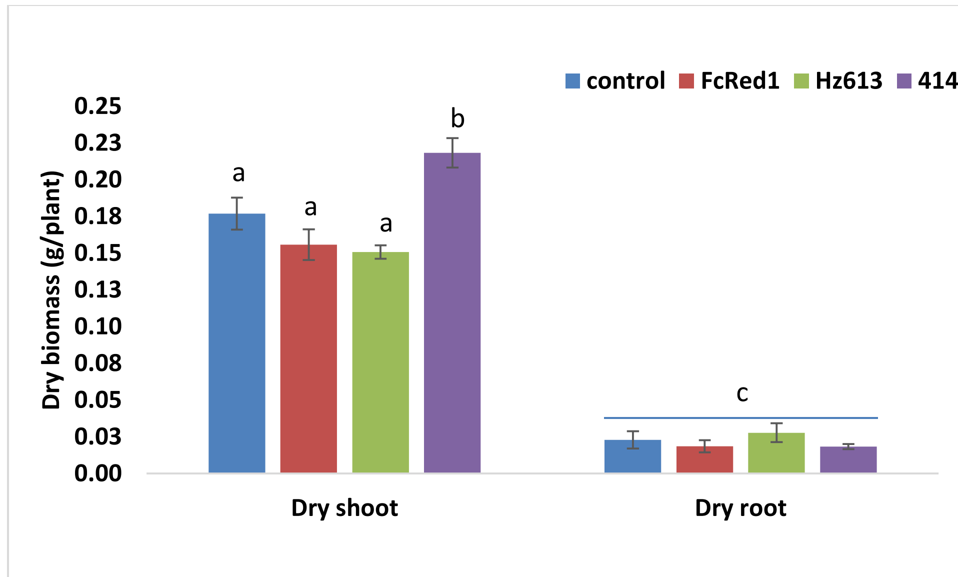
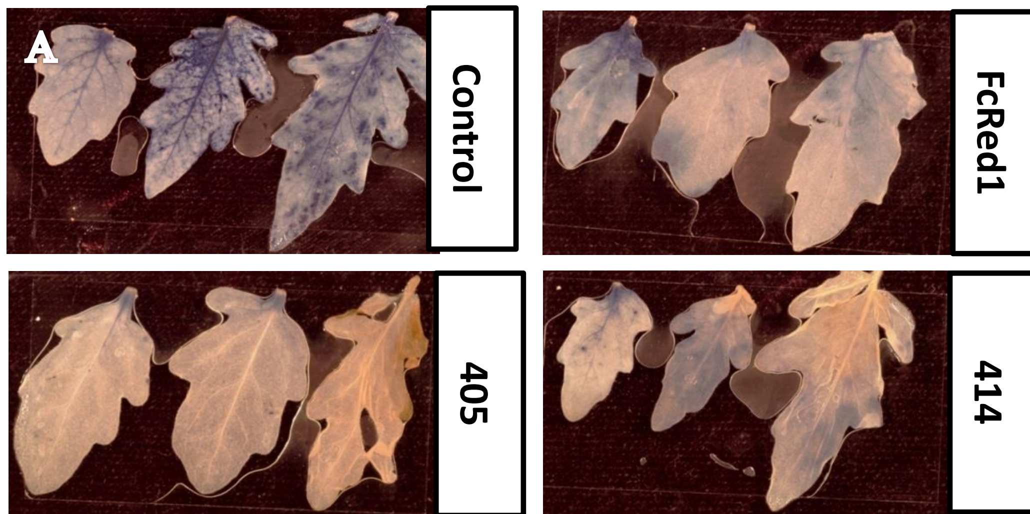


Figure 4-2. Effects of 11 d of drought on dry shoot and root biomass of endophyte-colonized and non-colonized plants. Data represent the means ±SE of six replications. Bars with the same letter are not significantly different (Duncan's multiple range test, $P<0.05$).

4.3.2 *In vivo* localization and estimation of superoxide (O_2^-) accumulation following drought and salinity stress. The qualitative and quantitative accumulation of O_2^- was analyzed in plants by treating second-youngest leaves of plants with NBT (section 4.2.3.1). *In vivo* accumulation of O_2^- in presence of NBT was indicated by the blue stain formation on plant leaves. Leaves of endophyte-colonized plants accumulated less blue stain and thus less O_2^- than non-colonized plants (**Figure 4-3A**). This was confirmed by a quantitative estimation of O_2^- content in plant leaves. At 600 mM NaCl stress, non-colonized plants had significantly higher accumulation of O_2^- contents compared to endophyte-colonized plants (**Figure 4-3B**). Similar pattern of O_2^- accumulation was observed upon the 11 d drought stress experiment. Non-colonized plants had approximately two times more accumulation of O_2^- contents compared to endophyte-colonized plants upon 11 d drought stress (**Figure 4-4**).



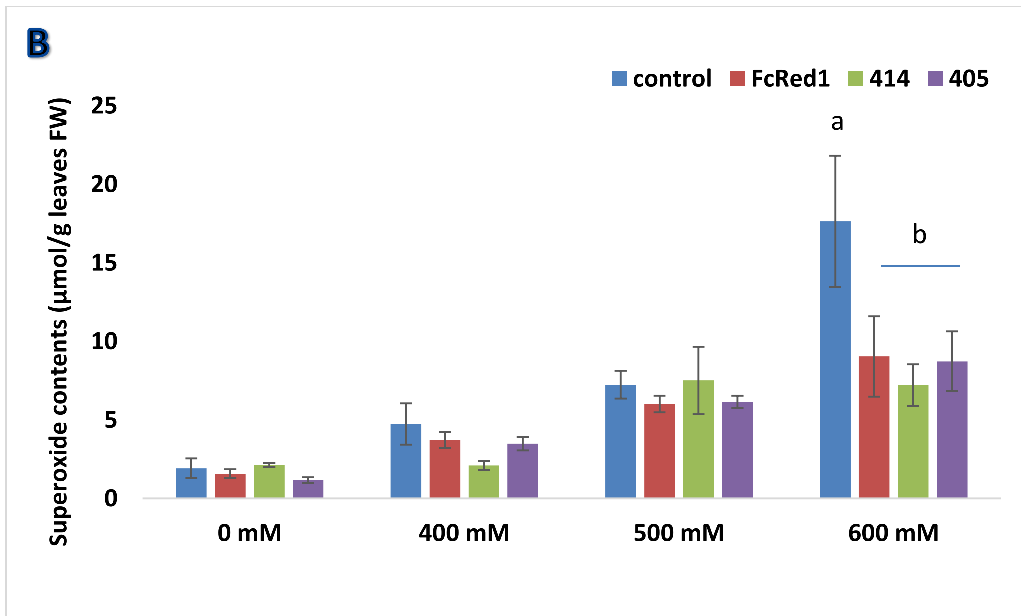
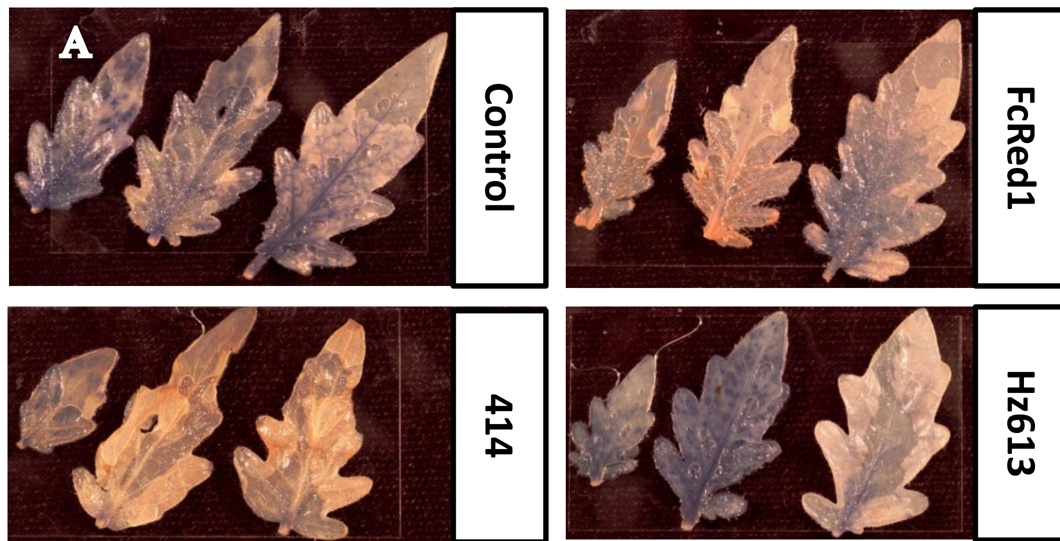


Figure 4-3. Qualitative (A) and quantitative (B) estimation of superoxide accumulation in leaves of endophyte-colonized and non-colonized plants after 15 d NaCl stress. Data represent the means ±SE of six replications. Bars with the same letter are not significantly different (Duncan's multiple range test, $P<0.05$).



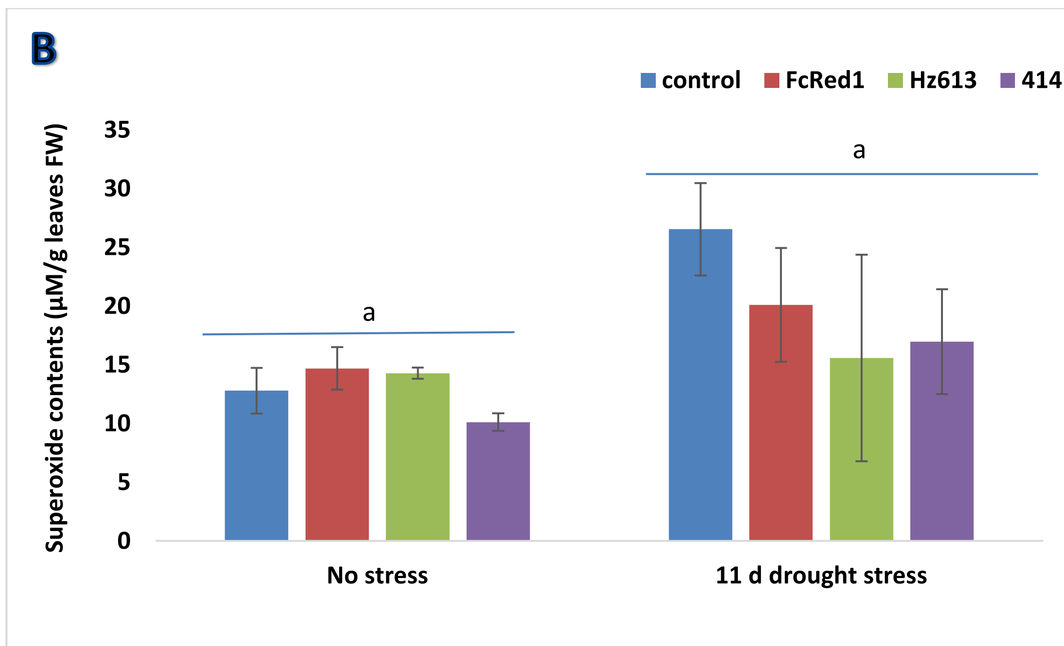
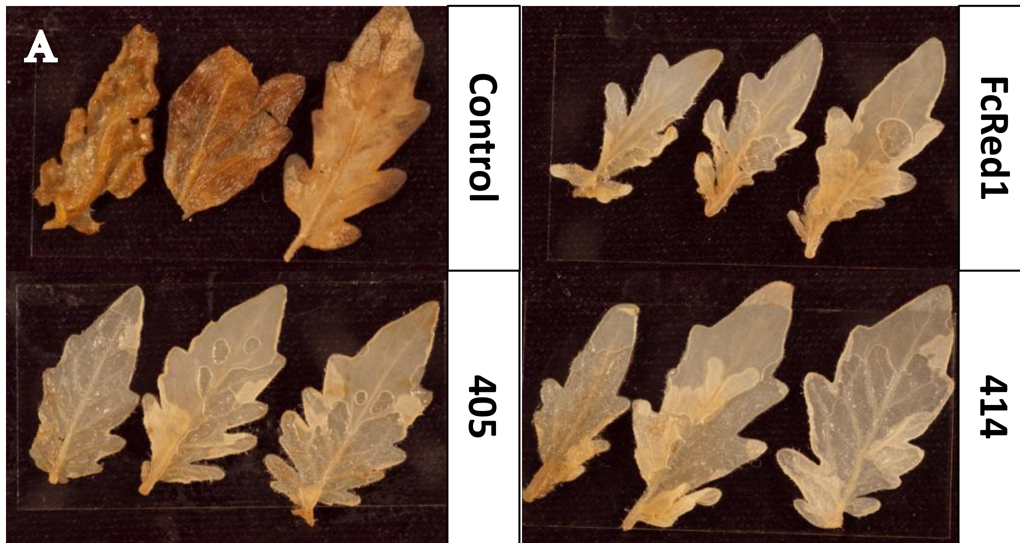


Figure 4-4. Qualitative (A) and quantitative (B) estimation of superoxide content using NBT reaction in tomato plants exposed to 11 d drought stress. Data represent the means ±SE of six replications. Bars with the same letter are not significantly different (Duncan's multiple range test, $P<0.05$).

4.3.3 *In vivo* localization and estimation of hydrogen peroxide (H_2O_2) accumulation following drought and salinity stress. The qualitative and quantitative accumulation of H_2O_2 was analyzed in plants by treating second-youngest leaves with DAB (section 4.2.3.2). *In vivo* accumulation of H_2O_2 content in the presence of DAB was indicated by brown stains formation on plant leaves, and it was observed that leaves of endophyte-colonized plants accumulated less H_2O_2 when compared to non-colonized plants in response to NaCl stress (**Figure 4-5A**). This result agreed to the quantitative estimation of H_2O_2 contents in plant leaves (**Figure 4-5B**). On the other hand, there was no difference in endophyte-colonized and non-colonized plants in response to DAB treatments after 11 d drought stress **(Figure 4-6**).



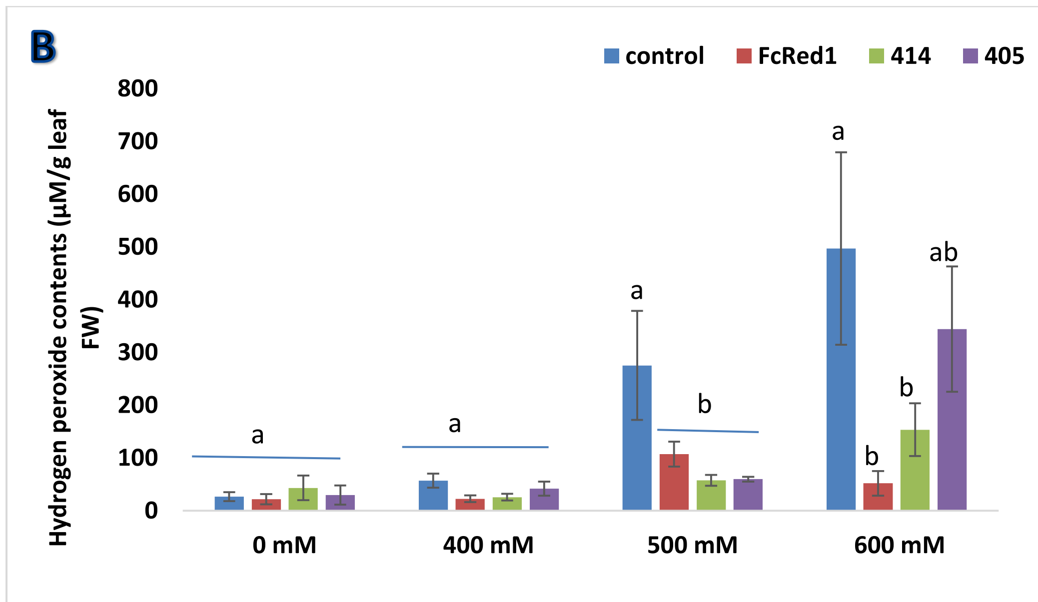
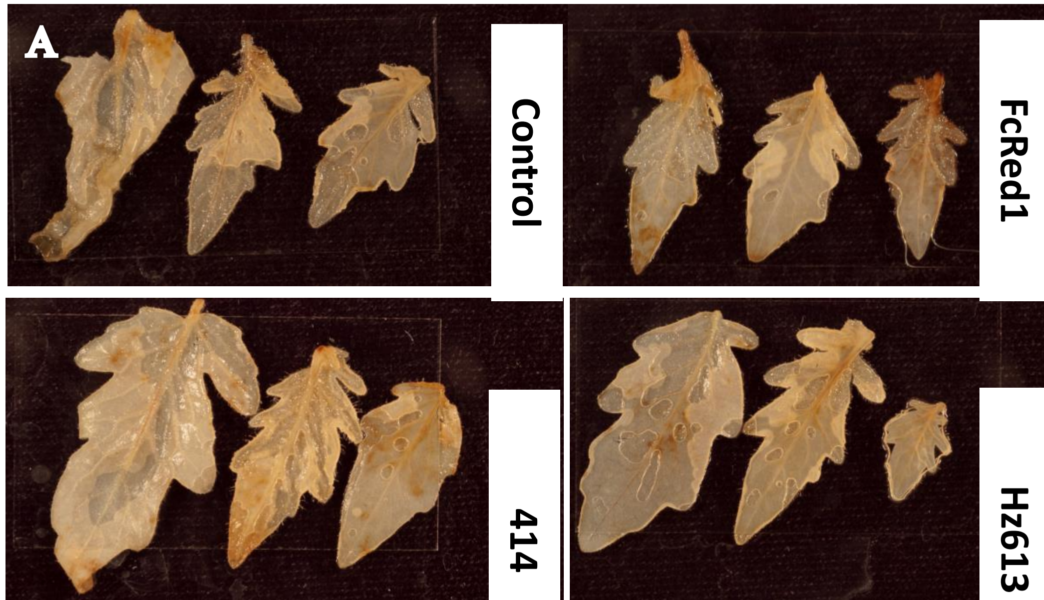


Figure 4-5. Qualitative (A) and quantitative (B) measure of H_2O_2 content in plants exposed for 15 d to NaCl: 0 mM, 400 mM, 500 mM, or 600 mM. Data represent the means ±SE of six replications. Bars with the same letter are not significantly different (Duncan's multiple range test, $P<0.05$).



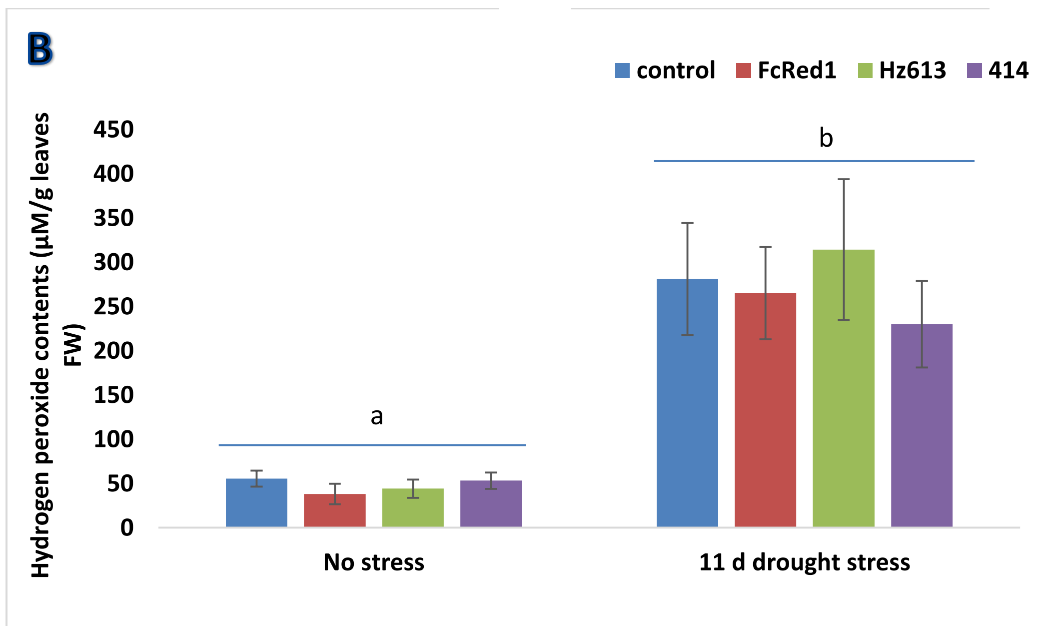


Figure 4-6. Qualitative (A) and quantitative (B) estimation of H_2O_2 content in tomatoes exposed to drought**.** Data represent the means ±SE of six replications. Bars with the same letter are not significantly different (Duncan's multiple range test, P<0.05).

4.3.4 Modulated osmolyte concentration following drought and salinity stress.

Osmolyte concentrations (amount of solutes per kg water) of plants were measured with a vapour pressure osmometer (Wescor 5500) after NaCl stress and drought stress (section 4.2.3.3). Upon exposure to 15 d NaCl stress (400 mM, 500 mM, and 600 mM) the osmolyte concentration of plants increased with the increase of NaCl concentration regardless of the presence of an endophyte. Osmolality of plants was increased almost two fold in plants exposed to 600 mM NaCl salinity compared to the no NaCl control condition (**Figure 4-7**). However, no significant difference was observed in endophyte-colonized and non-colonized plants (**Figure 4-7**). In contrast, osmolality of plants decreased after 11 d of drought stress and notably, drought stress reduced osmolyte concentration in endophyte-colonized plants to a greater degree when compared to non-colonized plants (**Figure 4-8**).

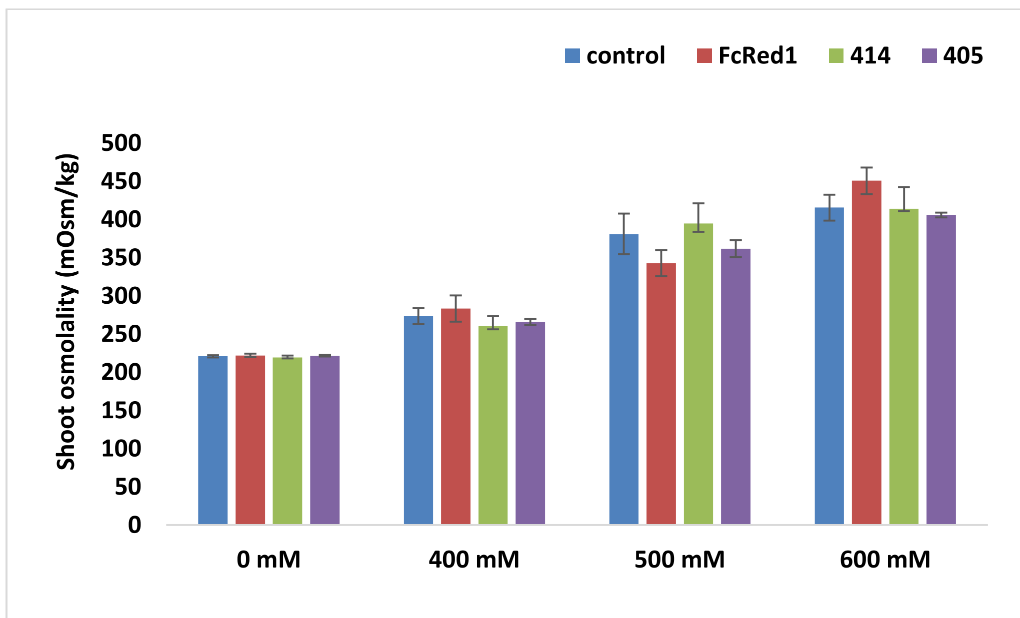


Figure 4-7. Effect of NaCl stress (400 mM, 500 mM, and 600 mM) on shoot osmolyte concentration. Increasing NaCl concentration correlated with higher osmolality of plants. One-way ANOVA showed no significant difference in osmolyte concentration between endophyte-colonized and non-colonized plants in response to NaCl ($P<0.05$). Data represent the means ±SE of six replications.

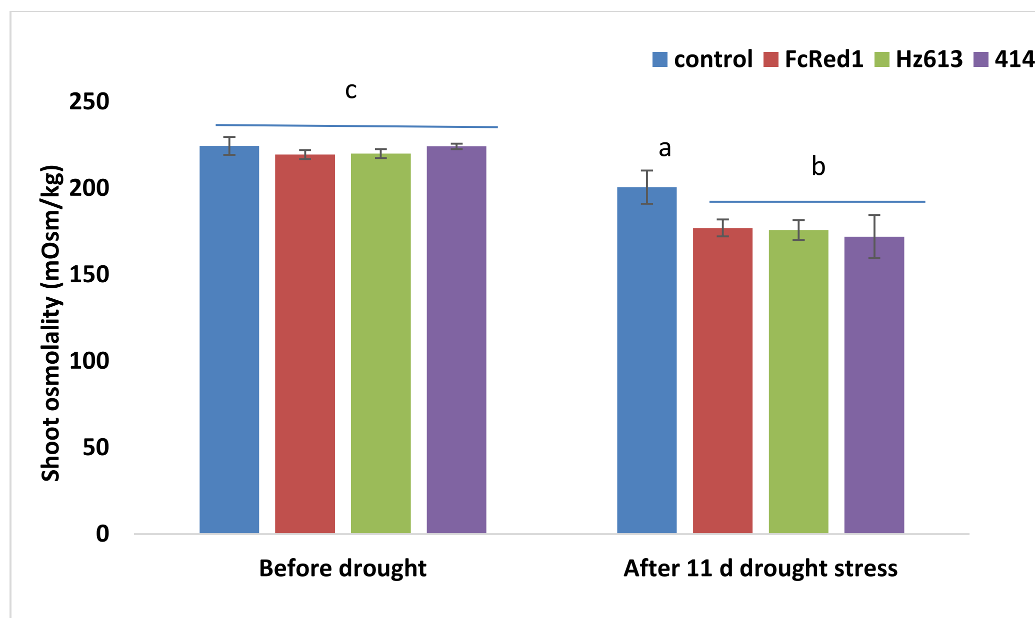


Figure 4-8. Effect of 11 d drought on shoot osmolyte concentration for non-colonized and endophyte-colonized tomatoes. After 11 d drought, osmolyte concentration was significantly decreased in the endophyte-colonized than the non-colonized plants. Data represent the means ±SE of six replications. Bars with the same letter are not significantly different (Duncan's multiple range test, $P<0.05$).

4.3.5 The impact of endophyte colonization on PSII photochemical efficiency following drought and salinity stress. Efficiency of PSII photochemistry as determined by chlorophyll fluorescence was measured before and after abiotic stress exposure (section 4.2.3.4). Continuous exposure to a 15 d period of NaCl stress resulted decreased photosynthetic efficiency of plants regardless of treatments. There was no observable difference in photosynthetic efficiency between endophyte-colonized plants and non-colonized plants upon exposure to 400 mM and 500 mM NaCl stress. However, significantly higher photosynthetic efficiency (approximately 30%) in endophyte-colonized plants was noticed in 600 mM NaCl stress (**Figure 4-9**).

On the other hand, after 11 d continuous drought stress and 2 d rehydration with RO water, photosynthetic efficiency of plants decreased almost 30% regardless of treatments, but

there was no significant difference (one-way ANOVA, P<0.05) between endophyte-colonized and non-colonized plants (**Figure 4-10**).

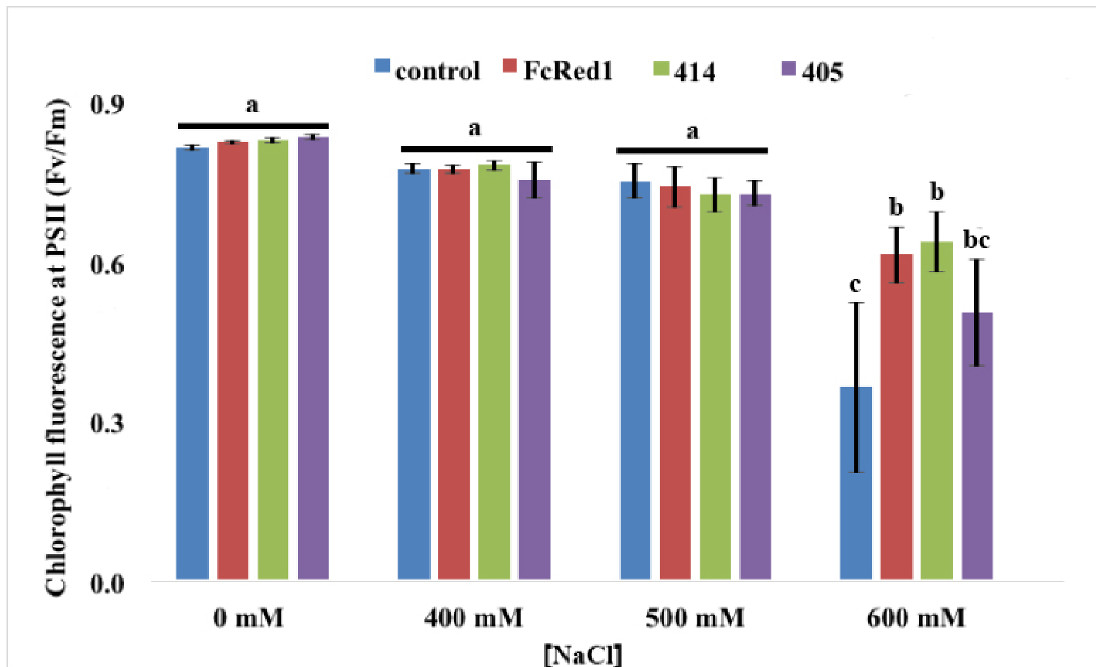


Figure 4-9. Effect of NaCl (400 mM, 500 mM, and 600 mM) on photosynthetic efficiency of endophyte-colonized and non-colonized tomato plants. Photosynthetic efficiency decreased significantly with NaCl treatment (ANOVA, P<0.05), but for 400 mM and 500 mM NaCl there was no difference between endophyte-colonized and non-colonized plants. At 600 mM NaCl, two of the endophyte strains significantly outperformed the non-colonized. Data represent the means ±SE of six replications. Bars with the same letter not significant different (Duncan's multiple range test, P<0.05).

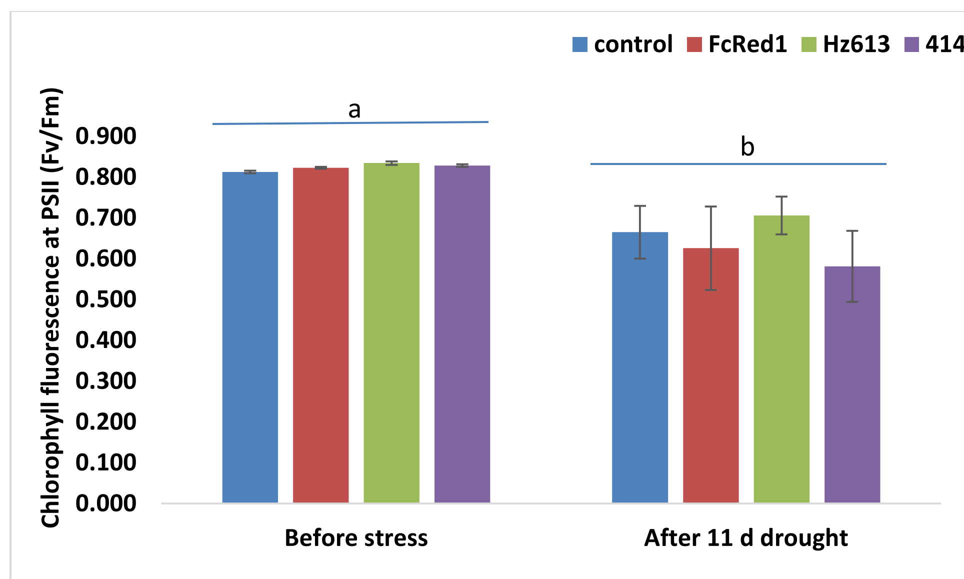


Figure 4-10. Effect of 11 d drought on photosynthetic efficiency of endophyte-colonized and non-colonized tomato plants. Photosynthetic efficiency decreased significantly (Duncan's multiple range test, $P<0.05$) after 11 d drought for both endophyte-colonized and non-colonized plants. But there was no significant difference between endophyte-colonized and non-colonized plants (One-way ANOVA, $P<0.05$). Data represent the means ±SE of six replications. Bars with the same letter not significantly different.

4.4 Discussion

4.4.1 Growth promotion. The association of systemic fungal endophytes with host plants improves growth performance of tomato plants in salinized and dry soil by enhancing NaCl and drought stress tolerance. Previously we observed systemic fungal endophytes induced increased shoot biomass in tomato plants (17 d old) exposed to a 20 d period of 300 mM NaCl stress (Chapter 2; Azad and Kaminskyj 2015). Further, increased root biomass was observed in endophyte-colonized plants (21 d old) compared to control plants upon 10 d period of 300 mM NaCl stress treatment (Chapter 3). However, there was no effect of systemic fungal endophytes on plant biomass when plants were exposed to chronic 100 mM and 200 mM NaCl stress (Chapter 3). These results are in agreement with reports in the literature ((Rodriguez et al. 2008;, Redman et al. 2011; Yadav et al. 2012; Estrada et al. 2013). I was interested to observe the effect

of endophytes on plant growth above 300 mM NaCl for longer periods. Our hypothesis was that when plants were severely stressed, the protective effects of the endophytes would be more apparent. Plant growth response to 400 mM and 500 mM were similar, with significantly declined biomass accumulation observed in both conditions (**Figure 4-1**). Above 300 mM NaCl stress may cause a disruption of plant enzyme activity due to ionic effects on protein structure and function (Yadav et al. 2012). On the other hand, root biomass of plants dropped sharply in 600 mM NaCl stress, even though shoot biomass was same like the biomass in 400 mM and 500 mM NaCl stress (**Figure 4-1**). This result indicated roots were more sensitive than shoots to extreme salt stress.

One of the endophytes we isolated from plants growing in saline sites in Saskatchewan (414) promoted significantly higher dry shoot biomass in colonized tomato plants (plants colonized with 414) compared to non-colonized plants, or plants colonized by the other endophytes, after 11 d drought stress and 2 d rehydration with RO water (**Figure 4-2**). This result corresponds to our previous study associated with endophyte-induced growth promotion after intermittent drought stress (Chapter 2; Azad and Kaminskyj 2015).

4.4.2 Reduced ROS/oxidative stress. Endophytes may alleviate oxidative stress in extreme NaCl and drought stress. Analysis of superoxide accumulation after 15 d NaCl stress and 11 d drought stress indicated endophyte colonization may reduce oxidative stress (**Figure 4-3** and **Figure 4-4**). Our results correspond to the findings of Redman et al. (2011) and Rodriguez et al. (2008). We also observed lower accumulation of H_2O_2 in endophyte-colonized plants exposed to 500 mM and 600 mM NaCl stress (**Figure 4-5**), which was in agreement with our previous work (Chapter 2; Azad and Kaminskyj 2015,). On the other hand, in our study H_2O_2 accumulation did not show difference in colonized-plants and non-colonized control plants upon the 11 d drought stress (**Figure 4-6**). These results suggest that endophyte colonization is better able to protect the plants exposed to salt stress than those under drought conditions. Perhaps, under this condition, endophytes were better able to help the plant to exclude NaCl.

Overproduction of ROS under abiotic stress is well-established by numerous reports (Kotchoni et al. 2006; Redman et al. 2011; Yadav et al. 2012). Endophytes may induce antioxidants machinery in endophyte-colonized plants and protect them from oxidative stress in

response to salt stress. Endophyte (*Piriformospora indica*) colonized barley plants showed increased activity of antioxidants such as ascorbate peroxidase upon NaCl stress (100-300 mM) (Baltruschat et al. 2008). Higher antioxidant activities (catalase (CAT), glutathione reductase (GR), glutathione S-transferase (GST) and superoxide dismutase (SOD) were observed in plants colonized with *Piriformospora indica*, which was also correlated with improved biomass and root length (Kumar et al. 2009). In addition, significantly higher ascorbate level was found in *P. indica*-colonized salt tolerant barley plants (Waller et al. 2005). Thus, while we cannot tell from our results whether the endophyte plants are experiencing reduced stress levels, or if they are better able to cope with the increased stress, the end result is an overall reduction in ROS accumulation. One can certainly use this as general evidence of greater stress tolerance.

4.4.3 Modulated osmolyte concentration. Abiotic stresses cause a change in plant-water relation resulting in accumulation of osmolytes or compatible solutes (Bohnert et al. 1995). NaCl stress changed the pattern of osmolyte concentration, but our systemic fungal endophytes did not influence plants shoot osmolality following 400, 500, or 600 mM NaCl stress (**Figure 4-7**). Our results correspond to the findings of Vera-Estrella et al. (2005). Cell sap osmolality in *Arabidopsis* was increased in parallel with NaCl salinity (Vera-Estrella et al. 2005). But our study was a disagreement with Redman at el. (2011). In the absence of NaCl stress, shoot osmolytes of colonized plants were higher, but in the presence of 300 mM NaCl stress osmolytes of colonized-plants increased 20% while osmolytes of non-colonized plants increased 50% (Redman et al. 2011).

In contrast, in the absence of drought stress there was no difference in osmolyte concentration of endophyte-colonized plants and non-colonized plants. However, systemic fungal endophytes significantly decreased osmolyte concentration in colonized-plants compared to non-colonized plants upon 11 d drought stress (**Figure 4-8**). This finding corresponds to a heat stress experiment of Rodriguez et al. (2008). After 12 d heat stress (50℃), non-colonized control plants had higher osmolyte concentration, while endophyte-colonized plants maintained lower osmolyte concentration (Rodriguez et al. 2008). However, our previous study showed endophytes increased free proline (most widely distributed osmolyte) accumulation under short period of drought (8 d) stress; 25% free proline accumulation was found in endophyte-colonized plants compared to non-colonized plants (Chapter 3). Perhaps proline is an especially powerful

osmotic protectant, which keeps the plants in better physiological condition without requiring a significant increase in the overall tissue osmolarity. Systemic fungal endophytes may responsible to increase matrix potential of host plants, leading to osmotic adjustment under stress (Rodriguez et al. 2008). Redman et al. (2011) reported osmolytes in colonized plants varies upon stress type, genotype of plants, or genotype of endophytes. Thus, again the endophytes obtained from saline sites in Saskatchewan had an impact on tomato plants during extreme stress conditions.

4.4.4 Modulated photosynthetic efficiency. Photosynthetic efficiency often used as a sensitive indicator of abiotic stress in plants (Zhou et al. 2015; Azad and Kaminskyj 2015). As physiological conditions impact photosynthesis due to energy imbalances, the first place the effects can be observed is often at the level of PSII function. Endophyte-colonized plants did not show beneficial effect following 400 and 500 mM NaCl stress. However, following 600 mM NaCl stress, endophyte-colonized plants maintained almost 30% higher photosynthetic efficiency compared to non-colonized plants, indicating beneficial effects of endophytes during extreme NaCl stress (**Figure 4-9**). This result is an agreement with Woodward et al. (2012). In the absence of NaCl stress, higher activity of PSII in endophyte-colonized plants were observed, indicating higher light absorbance by the chlorophyll pigments of colonized-plants (Woodward et al. 2012). However, we did not observe this effect.

PSII photochemical efficiency declined significantly after 11 d drought stress, but there was no difference between endophyte-colonized and non-colonized plants. This result is a disagreement with our previous results where endophyte-colonized plants had significantly increased photosynthetic efficiency during intermittent drought stress (Chapter 2; Azad and Kaminskyj 2015). Perhaps the results from this experiment reflected the plants' capacity to recover from drought stress regardless of the presence of the endophytes.

To sum up, **s**ystemic fungal endophytes isolated from plants growing in saline sites in Saskatchewan were able to confer tolerance to tomato plants in extreme salt and drought stress. We can suggest that this increased tolerance is due to the endophytes enhancing plant growth and modulating physiological and biochemical parameters. The endophytes also appeared to promote plant adaptability to the stress conditions by influencing osmolyte accumulation and protecting photosynthetic efficiency. The presence of systemic fungal endophytes led to the accumulation

of lower amounts of ROS which we suggest would lead to decreased oxidative damage in the plants. It is suggested endophyte-colonized plants may have better protection against oxidative damage by improving antioxidant machineries under salt and drought stress. Growth performance and physiological responses of endophyte-colonized host plants can be indicators of stress tolerance conferred by systemic fungal endophytes. Finally, our results suggest that fungal endophyte study is a good approach to take to develop systems that could be used to protect a broad range of crop species that are sensitive to salt and drought stress. If specific endophytes could be identified which convey broad tolerance of plants to abiotic stress, it would remove the need to breed stress tolerance cultivars. This could be a much faster, more efficient and more effective way to protect plants from stress brought on by climate change or salinization caused by over irrigation.

4.5 References

Aharon R, Shahak Y, Wininger S, Bendov R, Kapulnik Y, Galili G (2003) Overexpression of a plasma membrane aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions but not under drought or salt stress. Plant Cell 15: 439-447

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu.Rev.Plant Biol. 55: 373-399

Ashraf M, Foolad M (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot 59: 206-216

Azad K, Kaminskyj S (2015) A fungal endophyte strategy for mitigating the effect of salt and drought stress on plant growth. Symbiosis, pp 1-6

Baltruschat H, Fodor J, Harrach BD, Niemczyk E, Barna B, Gullner G, Janeczko A, Kogel K, Schäfer P, Schwarczinger I (2008) Salt tolerance of barley induced by the root endophyte Piriformospora indica is associated with a strong increase in antioxidants. New Phytol 180: 501-510

Bartels D, Sunkar R (2005) Drought and salt tolerance in plants. Crit Rev Plant Sci 24: 23-58

Bohnert HJ, Jensen RG (1996) Strategies for engineering water-stress tolerance in plants. Trends Biotechnol 14: 89-97

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to environmental stresses. Plant Cell 7: 1099

Brugnoli E, Lauteri M (1991) Effects of Salinity on Stomatal Conductance, Photosynthetic Capacity, and Carbon Isotope Discrimination of Salt-Tolerant (*Gossypium hirsutum* L.) and Salt-Sensitive (*Phaseolus vulgaris* L.) C(3) Non-Halophytes. Plant Physiol 95: 628-635

Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann Bot 103: 551-560

Estrada B, Aroca R, Barea JM, Ruiz-Lozano JM (2013) Native arbuscular mycorrhizal fungi isolated from a saline habitat improved maize antioxidant systems and plant tolerance to salinity. Plant Science 201: 42-51

Farooq M, Wahid A, Kobayashi N, Fujita D, Basra S (2009) Plant drought stress: effects, mechanisms and management. Sustainable Agriculture. Springer, pp 153-188

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry 48: 909-930

Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A (2012) Role of proline under changing environments: a review. Plant signaling & behavior 7: 1456-1466

Kotchoni SO, Kuhns C, Ditzer A, Kirch H, Bartels D (2006) Over-expression of different aldehyde dehydrogenase genes in *Arabidopsis thaliana* confers tolerance to abiotic stress and protects plants against lipid peroxidation and oxidative stress. Plant, Cell Environ 29: 1033-1048

Kumar M, Yadav V, Tuteja N, Johri AK (2009) Antioxidant enzyme activities in maize plants colonized with *Piriformospora indica*. Microbiology 155: 780-790

Mei C, Flinn BS (2010) The use of beneficial microbial endophytes for plant biomass and stress tolerance improvement. Recent patents on biotechnology 4: 81-95

Nuccio ML, Rhodest D, McNeil SD, Hanson AD (1999) Metabolic engineering of plants for osmotic stress resistance. Curr Opin Plant Biol 2: 128-134

Parkhi V, Kumar V, Sunilkumar G, Campbell LM, Singh NK, Rathore KS (2009) Expression of apoplastically secreted tobacco osmotin in cotton confers drought tolerance. Mol Breed 23: 625-639

Porcel R, Aroca R, Ruiz-Lozano JM (2012) Salinity stress alleviation using arbuscular mycorrhizal fungi. A review. Agronomy for Sustainable Development 32: 181-200

Ramel F, Sulmon C, Bogard M, Couee I, Gouesbet G (2009) Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced tolerance in *Arabidopsis thaliana* plantlets. BMC Plant Biol 9: 28-2229-9-28

Redman RS, Dunigan DD, Rodriguez RJ (2001) Fungal symbiosis from mutualism to parasitism: who controls the outcome, host or invader? New Phytol 151: 705-716

Redman RS, Kim YO, Woodward CJ, Greer C, Espino L, Doty SL, Rodriguez RJ (2011) Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. PLOS one6: e14823

Redman RS, Freeman S, Clifton DR, Morrel J, Brown G, Rodriguez RJ (1999) Biochemical analysis of plant protection afforded by a nonpathogenic endophytic mutant of *Colletotrichum magna*. Plant Physiol 119: 795-804

Rodriguez RJ, Henson J, Van Volkenburgh E, Hoy M, Wright L, Beckwith F, Kim Y, Redman RS (2008) Stress tolerance in plants via habitat-adapted symbiosis. The ISME journal 2: 404-416

Vera-Estrella R, Barkla BJ, Garcia-Ramirez L, Pantoja O (2005) Salt stress in *Thellungiella halophila* activates Na+ transport mechanisms required for salinity tolerance. Plant Physiol 139: 1507-1517

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Huckelhoven R, Neumann C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102: 13386-13391

Woodward C, Hansen L, Beckwith F, Redman RS, Rodriguez RJ (2012) Symbiogenics: An Epigenetic Approach to Mitigating Impacts of Climate Change on Plants. HortScience 47: 699-703

Yadav NS, Shukla PS, Jha A, Agarwal PK, Jha B (2012) The SbSOS1 gene from the extreme halophyte Salicornia brachiata enhances Na+ loading in xylem and confers salt tolerance in transgenic tobacco. BMC Plant Biol 12:188-2229-12-188

Yokoi S, Bressan RA, Hasegawa PM (2002) Salt stress tolerance of plants. JIRCAS working report 23: 25-33

Zhou R, Yu X, Kjær KH, Rosenqvist E, Ottosen C, Wu Z (2015) Screening and validation of tomato genotypes under heat stress using F v/F m to reveal the physiological mechanism of heat tolerance. Environ Exp Bot 118: 1-11. doi:10.1016/j.envexpbot.2015.05.006.

Zhu J (2003) Regulation of ion homeostasis under salt stress. Curr Opin Plant Biol 6: 441-445

CHAPTER 5: GENERAL DISCUSSION

Agriculture of the future has two major environmental challenges: *local* impact on land use due to human activities; and *general* effects of global climate change. Locally, growing human population and industrial intensity means the pressure to use land will inevitably increase. Urban sprawl (typically, cities are founded on prime agricultural land) and rural resource exploitation (including for example, mine tailings and brownfield sites) reduce the amount of agricultural lands. Meanwhile, irrigation in arid and semi-arid areas can cause soil salinization further decreasing the productivity of marginal crop land. Over 6% of total lands and approximately 30% of total irrigated lands are estimated to be salt-contaminated soils (Chaves et al. 2009). Generally, unpredictable weather patterns and increasing frequency and intensity of severe weather events (floods, cyclones, droughts) can render formerly arable lands unproductive (FAO 2011). To continue growing plants for food security of our growing population, on diminished soils, we need plants that are better able to grow under suboptimal conditions. Our data show that, consistent with Rodriguez et al. (2008), Mei and Flinn (2010), Yuan et al. (2010), Hamilton et al. (2012), Rai et al. (2014), and Yuan et al. (2010), symbiotic fungi isolated from plants growing in stress inducing conditions may be able to provide protection to a broad variety of plants. This would reduce the need for specific breeding programs and greatly accelerate the production of high-producing, stress tolerant crops.

5.1 Tomato Plants Experimentally Colonized with Saskatchewan Saline-tolerant Endophytes Grew Better than Control Plants on Saline and/or Dry conditions

Fresh and dry biomasses are general indicators of plant growth that address related aspects of performance; these are particularly informative when related to water-use efficiency. Endophyte-colonized and non-colonized plants were exposed to high concentrations of NaCl. In the absence of NaCl- or drought-stress, there was no difference in biomass of endophyte-colonized and non-colonized control plants (Chapter 2, 3, 4).

In chapter 2, seventeen day old plants were exposed to 300 mM NaCl stress for 20 d. We observed better fresh shoot biomass (approximately 30%) of endophyte colonized plants compared to non-colonized plants in response to 300-500 mM NaCl stress (Chapter 2; Azad and Kaminskyj 2015). In chapter 3, three week old plants were exposed to 300 mM NaCl stress for

10 d. We observed better fresh root biomass (approximately 20%) in colonized plants compared to non-colonized plants (Chapter 3). In another experiment, plants were exposed to chronic NaCl stress (100 and 200 mM in separate experiments). There was no observable difference in fresh biomass (both root and shoot) of colonized-plants and non-colonized plants in response to chronic NaCl stress (Chapter 3). In chapter 4, plants were exposed to 400, 500 and 600 mM NaCl stress for 15 d. There was no difference in biomass (both root and shoot) of colonized- and non-colonized plants (chapter 4). These results suggested that endophytes had effects on plants biomass in response to NaCl stress, while plants' age during stress and duration of stress exposure were determining factors.

Notably, saline endophytes enhanced plants biomass not only following NaCl stress conditions but also in response to drought. Endophyte-colonized plants had significantly higher fresh shoot biomass after periodic drought (Chapter 2; Azad and Kaminskyj 2015). Moreover, higher dry shoot biomass was observed in endophyte-colonized plants after 11 d continuous drought stress (Chapter 4). There was no noticeable effect of colonization on dry root biomass of plants after drought stress, which may be a negative consequence of dehydration on plants root system.

5.2 Plant Physiological Aspects Related to Colonization with Systemic Fungal Endophytes

Typical physiological responses of plants under stress include osmolyte adjustment, modulated photosynthetic efficiency, and ROS generation. Systemic fungal endophytes may promote NaCl and drought stress tolerance by altering stress related physiological aspects of plants (Mei and Flinn 2010; Woodward et al. 2012; Yuan et al. 2010).

Osmolytes increase is a common physiological response of plants during stress. Although increased NaCl stress caused increased osmolyte concentration in plants, endophyte colonization did not show any effect on osmolytes of plants in response to NaCl stress (Chapter 4). In contrast, endophytes may contribute in decreasing osmolyte concentration when plants were exposed to 11 d drought stress (Chapter 4). These results indicated modulated osmolyte concentration in plants due to endophyte colonization. However, osmolyte concentration in endophyte-colonized plants varies with the type of stress (Redman et al. 2011). Of course if the

plants were experiencing diminished stress because of endophyte colonization, one would expect lower levels of stress-induced osmolyte production.

Proline is an amino acid that acts as an osmolyte. Proline content noticeably increases in plants that experience water deficient condition (Farooq et al. 2009; Hayat et al. 2012; Verbruggen and Hermans 2008). Ideally, free proline accumulation is measured under drought stress and used as an indicator to assess the severity of stress (Bates et al. 1973; Bayat et al. 2009; Elbersen and West 1996; Parkhi et al. 2009). Greater accumulation (approximately 20 %) of free proline in endophyte-colonized plants after 7 d drought stress was studied (Chapter 3). Our result was an agreement with Bayat et al. (2009). But another study showed endophyte-promoted drought tolerance in plants, which is related to higher proline accumulation (Elbersen and West 1996).

Photosynthetic efficiency of plants was measured as photosystem II chlorophyll-*a* fluorescence. In the absence of stress, both endophyte-colonized and non-colonized plants maintained a similar range of photochemical efficiency (Chapter 2; Azad and Kaminskyj 2015). Endophyte colonization caused higher photosynthetic efficiency when plants were exposed to chronic or periodic NaCl stress (Chapter 3), suggesting decreased stress in these plants. Although there was no effect of endophytes after 10 d of 100 mM and 200 mM NaCl stress, endophyte-colonized plants recovered after a second 10 d NaCl stress (both 100 mM and 200 mM), indicating systemic fungal endophytes are able to maintain photochemical efficiency under these stress conditions (Chapter 3). On the other hand, continuous 15 d period of NaCl stress ($\geq$ 400 mM) cause sharp declination of photosynthetic efficiency, apart from a recovery of colonized-plants in 600 mM NaCl stress (Chapter 4). These results suggested that endophytes may help recovering photosynthetic efficiency of plants during chronic but comparatively lower amount of NaCl stress (100 and 200 mM). But continuous exposure to high amount of NaCl stress caused irreversible damage to photochemical efficiency of the plants. Our results were agreement with Woodward et al. (2012) and Meloni et al. (2003).

Photochemical efficiency of plants was adversely affected by 11 d drought stress regardless of endophyte colonization (Chapter 4). Drought reduced stomatal activities and less CO_2 uptake for photosynthesis (Lawer 2002). It also leads to disruption of photosynthetic

pigments and components and interrupts activities of photosynthetic apparatus (Anjum et al. 2003). However, photochemical efficiency was maintained at a higher level in endophyte-colonized plants after periodic or intermittent drought stress (Chapter 2; Azad and Kaminskyj 2015). Both salt and drought stress may cause down-regulation of some photosynthetic genes, which has detrimental effects on photosynthesis of plants (Wilson et al. 2006; Chaves et al. 2009). These stresses also lead to increased damage to the photosynthetic apparatus due to ROS production and photoinhibition. Nevertheless, NaCl stress had more adverse effects than drought on photosynthesis since NaCl causes combined effects of dehydration and osmotic stress (Chaves et al. 2009).

Localization of superoxide and H_2O_2 were assessed as proxies for stress-induced ROS accumulation in plants. Following acute and periodic NaCl stress, endophyte-colonized plants showed less accumulation of ROS in their leaves compared to control plants (Chapter 2; Chapter 4). Lower ROS accumulation in endophyte-colonized plants correlated with higher accumulation of osmolytes in plants (Hayat et al. 2012). However, there was no remarkable difference in ROS generation in endophyte-colonized plants and control plants after continuous 11 d drought stress (Chapter 4). Again, it is unclear from our data whether the plants are experiencing less stress due to the endophyte presence, and hence produce less ROS, or whether the endophyte helps in ROS scavenging. Regardless, the diminished ROS production suggests that the plants are able to stay healthier following the NaCl stress events if the endophyte is present.

Endophytes are known to promote plant growth under stress in part by regulating hormones such as indole acetic acid (IAA) (Contreras-Cornejo et al. 2009; Costacurta and Vanderleyden 1995; Hasan 2002; Khan et al. 2012; Redman et al. 2011; Sirrenberg et al. 2007). An experiment on plant indole content suggested endophytes have the potential to produce indole compounds (Chapter 3). Similar results were shown by Redman et al. (2011), Khan et al. (2012), Contreras-Cornejo et al. (2009), Sirrenberg et al. (2007), and Hasan (2002). IAA may contribute in alteration of root structure and facilitate root surface area for fungal colonization that can improve water and nutrients absorption capacities (Contreras-Cornejo et al. 2009; Costacurta and Vanderleyden 1995; Sirrenberg et al. 2007).

Thus, looking at my data as a whole, it appears that the endophytes we isolated can confer some added stress tolerance to the colonized tomato plants. While it may be premature to begin large-scale inoculation experiments, further study of how the endophytes are protecting the plants is warranted. In addition, it would be interesting to determine whether other plants can be protected by the endophytes isolated from saline environments in Saskatchewan, and what types of responses could be seen in field plot studies.

5.3 Future Studies

In my thesis research I investigated potential fungal endophytes that confer tolerance for plant growth on saline or dry environments. I explored the aspect of habitat-adapted symbiosis to enhance plants growth on salt contaminated soil and water deficient condition.

I demonstrated the effect of colonization of systemic fungal endophytes, which were isolated from saline habitats, on growth performance and physiological responses of plants. Current results are promising and indicate endophyte-promoted plants growth on saline and dry soil, although further research and field trials are needed to reach a precise conclusion. Based on current findings such as better biomass and modulated physiological responses of plants due to endophyte colonization, several future works can be suggested.

Indole production by systemic fungal endophytes suggested the possibility of indoles may be supplied by endophytes to host plants. However, further study is required to confirm this suggested symbiotic relationship between endophytes and the host plant. It is also important to quantify indoles that synthesized by endophytes.

Fluid consumption by plants showed a correlation between endophyte colonization and drought stress tolerance. We can also study stomatal conductance of plants since it has important role in water consumption of plants. This could also be linked to whole plant photosynthesis measurements, allowing a better measure of how the plants are functioning at the metabolic level when colonized by endophytes and exposed to drought stress.

Although accumulation of higher amount of osmolytes is a common acclimatization process to mitigate stress, the mechanism of osmolyte adjustment is still unclear. We noticed free

proline accumulation during drought stress, it also directs us to estimate the effects of proline accumulation to mitigate NaCl stress. It would also be interesting to know how much of the entire osmolite pool is composed of proline. Is it the major osmolite providing protection or is it a minor factor?

Numerous researchers (Baltruschat et al. 2008; Hamilton et al. 2012; Kumar et al. 2009; Waller et al. 2005) reported high antioxidant activities in endophyte-colonized plants. Our reports on ROS accumulation (Chapter 2 and Chapter 4) suggested lower oxidative stress on colonized-plants, indicating higher antioxidant activities. We need to determine which part of antioxidant machineries support oxidative stress in colonized-plants under stress.

Future studies could also focus on changes in gene expression due to plant-endophyte association. It is important to study functional genomics of plants coincidentally with physiological and biochemical approaches in order to investigate different mechanisms of plant metabolism under stress. This is because stress tolerance mechanisms in plants induce activation of a series of stress related genes and metabolites (Fatemeh et al. 2012). In addition, determination of endophyte-altered gene expression in colonized plants can be a future study since previous report showed endophyte-altered upregulation of specific plant genes (Woodward et al. 2012).

Finally we need to establish a field trial to test the effectiveness of our endophyte strains in field conditions where several stress parameters work together and impact plant growth and development. It will give us a better understanding of the efficiency of these strains to promote plant growth in natural conditions. We currently have an ongoing field trial project with *Trichoderma harzianum* in northern British Columbia. Expanding it, we could also explore the efficiency of these strains on agriculturally important crops including both dicot and monocot in order to observe interaction between different hosts and endophytes. A greater understanding of the interaction between these strains and other agriculturally important crop plants will facilitate effective application of these strains in agriculture.

5.4 References

Anjum F, Yaseen M, Rasul E, Wahid A, Anjum S (2003) Water stress in barley (*Hordeum vulgare* L.). II. Effect on chemical composition and chlorophyll contents. Pak.J.Agric.Sci 40: 45-49

Azad K, Kaminskyj S (2015) A fungal endophyte strategy for mitigating the effect of salt and drought stress on plant growth. Symbiosis, pp 1-6

Bates L, Waldren R, Teare I (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39: 205-207

Bayat F, Mirlohi A, Khodambashi M (2009) Effects of endophytic fungi on some drought tolerance mechanisms of tall fescue in a hydroponics culture. Russian Journal of Plant Physiology 56: 510-516

Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann Bot 103: 551-560

Contreras-Cornejo HA, Macias-Rodriguez L, Cortes-Penagos C, Lopez-Bucio J (2009) *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. Plant Physiol 149: 1579-1592

Costacurta A, Vanderleyden J (1995) Synthesis of phytohormones by plant-associated bacteria. Crit Rev Microbiol 21: 1-18

Elbersen H, West C (1996) Growth and water relations of field-grown tall fescue as influenced by drought and endophyte. Grass Forage Sci 51: 333-342

FAO-Adapt; Framework Programme on Climate Change Adaptation (2011) http://www.fao.org/docrep/014/i2316e/i2316e00.pdf

Farooq M, Wahid A, Kobayashi N, Fujita D, Basra S (2009) Plant drought stress: effects, mechanisms and management. Sustainable Agriculture. Springer, , pp 153-188

Hamilton CE, Gundel PE, Helander M, Saikkonen K (2012) Endophytic mediation of reactive oxygen species and antioxidant activity in plants: a review. Fungal Diversity 54: 1-10

Hasan H (2002) Gibberellin and auxin-indole production by plant root-fungi and their biosynthesis under salinity-calcium interaction. Acta Microbiol Immunol Hung 49: 105-118

Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A (2012) Role of proline under changing environments: a review. Plant signaling & behavior 7: 1456-1466

Khan AL, Hamayun M, Kim Y, Kang S, Lee I (2011) Ameliorative symbiosis of endophyte (*Penicillium funiculosum* LHL06) under salt stress elevated plant growth of Glycine max L. Plant Physiology and Biochemistry 49: 852-861

Lawlor DW (2002) Limitation to photosynthesis in water-stressed leaves: stomata vs. metabolism and the role of ATP. Ann Bot 89 Spec No: 871-885

Mei C, Flinn BS (2010) The use of beneficial microbial endophytes for plant biomass and stress tolerance improvement. Recent patents on biotechnology 4: 81-95

Meloni DA, Oliva MA, Martinez CA, Cambraia J (2003) Photosynthesis and activity of superoxide dismutase, peroxidase and glutathione reductase in cotton under salt stress. Environ Exp Bot 49: 69-76

Parkhi V, Kumar V, Sunilkumar G, Campbell LM, Singh NK, Rathore KS (2009) Expression of apoplastically secreted tobacco osmotin in cotton confers drought tolerance. Mol Breed 23: 625-639

Rai M, Rathod D, Agarkar G, Dar M, Brestic M, Pastore GM, Junior MRM (2014) Fungal growth promotor endophytes: a pragmatic approach towards sustainable food and agriculture. Symbiosis 62: 63-79

Redman RS, Kim YO, Woodward CJ, Greer C, Espino L, Doty SL, Rodriguez RJ (2011) Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. PLOS one6: e14823

Rodriguez RJ, Henson J, Van Volkenburgh E, Hoy M, Wright L, Beckwith F, Kim Y, Redman RS (2008) Stress tolerance in plants via habitat-adapted symbiosis. The ISME journal 2: 404-416

Sirrenberg A, Göbel C, Grond S, Czempinski N, Ratzinger A, Karlovsky P, Santos P, Feussner I, Pawlowski K (2007) *Piriformospora indica* affects plant growth by auxin production. Physiol Plantarum 131: 581-589

Verbruggen N, Hermans C (2008) Proline accumulation in plants: a review. Amino Acids 35: 753-759

Wilson KE, Ivanov AG, Öquist G, Grodzinski B, Sarhan F, Huner NP (2006) Energy balance, organellar redox status, and acclimation to environmental stress. Botany 84: 1355-1370

Woodward C, Hansen L, Beckwith F, Redman RS, Rodriguez RJ (2012) Symbiogenics: An Epigenetic Approach to Mitigating Impacts of Climate Change on Plants. HortScience 47: 699-703

Yuan Z, Zhang C, Lin F (2010) Role of diverse non-systemic fungal endophytes in plant performance and response to stress: progress and approaches. J Plant Growth Regul 29: 116-126

APPENDIX 1

Supplemental Information for Chapter 2

Table 2-1. Endophytes species identification

Endophytes	Species	Accession no	Primers
Hz613	*Trichoderma harzianum* P86-167	KJ439172.1	ITS4/ITS5
419.09	*Alternaria* sp	AY154681.1	ITS4/ITS5
	Alternaria sp. B13	GQ253348.1	NS1/NS4
	Alternaria malorum var. polymorpha	AY251081.2	LSU1Fd/LR5
417.03	*Alternaria* sp. IA20	AY154681.1	ITS4/ITS5
	Alternaria sp. B13	GQ253348.1	NS1/NS4
	Alternaria malorum var. polymorpha	AY251080.2	LSU1Fd/LR5
414	*Alternaria* sp. IA202	AY154681.1	ITS4/ITS5
	Alternaria sp. B13	GQ253348.1	NS1/NS4
	Alternaria malorum var. polymorpha	AY251080.2	LSU1Fd/LR5
406B.07	*Alternaria* sp. IA202	AY154681.1	ITS4/ITS5
	Alternaria sp. B13	GQ253348.1	NS1/NS4
	Alternaria malorum var. polymorpha	AY251080.2	LSU1Fd/LR5
405.06	*Alternaria* sp. IA202	AY154681.1	ITS4/ITS5
	Alternaria sp. F11A MAN-2013	KF703459.1	NS1/NS4
	Alternaria malorum var. polymorpha	AY251080.2	LSU1Fd/LR5
FcRed1	*Fusarium culmorun* (Collected from AST)		

Table 2-2. Oligonucleotides used in this study for PCR analysis

Primer name	Sequence (5´-3´)	Amplified region	Reference
ITS4 ITS5	TCCTCCGCTTATTGATATGC GGAAGTAAAAGTCGTAACAAGG	Internal transcribed spacer (ITS)	White et al. (1990); Woudenberg et al. (2013)
NS1 NS4	GTAGTCATATGCTTGTCTC CTTCCGTCAATTCCTTTAAG	Small sub-unit (SSU)	White et al. (1990); Woudenberge et al. (2013)
LSU1Fd LR5	GRATCAGGTAGGRATACCCG TCCTGAGGGAAACTTCG	Large sub-unit (LSU)	Vilgalys and Hester (1990); Woudenberg et al. (2013)

Table 2-3. Sites of plant collection

Endophytes*	**Soil conductivity (mOsm/cm)**	**Sites of collection**
FcRed1**	24.9	Marine beach
HZ613	12.8	Radisson Lake Shore (52°28´38.06´´N 107°23´35.95´´W)
419.09	16.6	Belle Plaine (50°25´46.39´´N 105°13´00.97´´W)
417.03	16.6	Belle Plaine (50°25´46.39´´N 105°13´00.97´´W)
414	63.2	Belle Plaine (50°25´46.39´´N 105°13´00.97´´W)
406B.07	10.8	Belle Plaine (50°25´46.39´´N 105°13´00.97´´W)
405.06	24.9	Little Manitou Lake shore (51°41´07.49´´N 105°23´50.69´´W)

*Strains of endophytes were named/ numbered according to sites/date of collection.
**FcRed1 was used as a positive control in this study. It is a marine beach endophyte collected from Adaptive Symbiotic technologies (AST) established by Rodriguez et al.

APPENDIX 2

Supplementary Information for Chapter 3

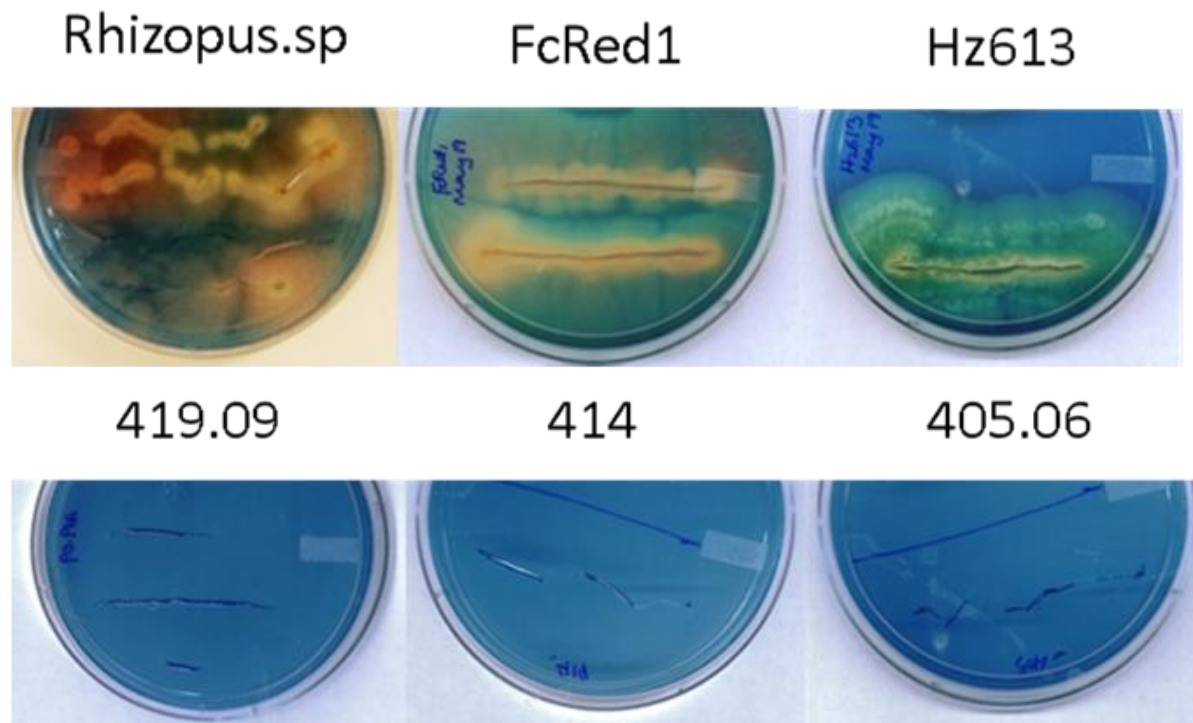


Figure 3-11. CAS-agar plates showing siderophores assay for endophytes: *Rhizopus* sp., FcRed1, Hz613, 419, 414, 405. Alteration of color in blue CAS-agar medium (blue agar changed into orange) suggested positive siderophores production by endophytes, while no color change of CAS-agar medium suggested there was no detectable amount of siderophores produced by endophytes.

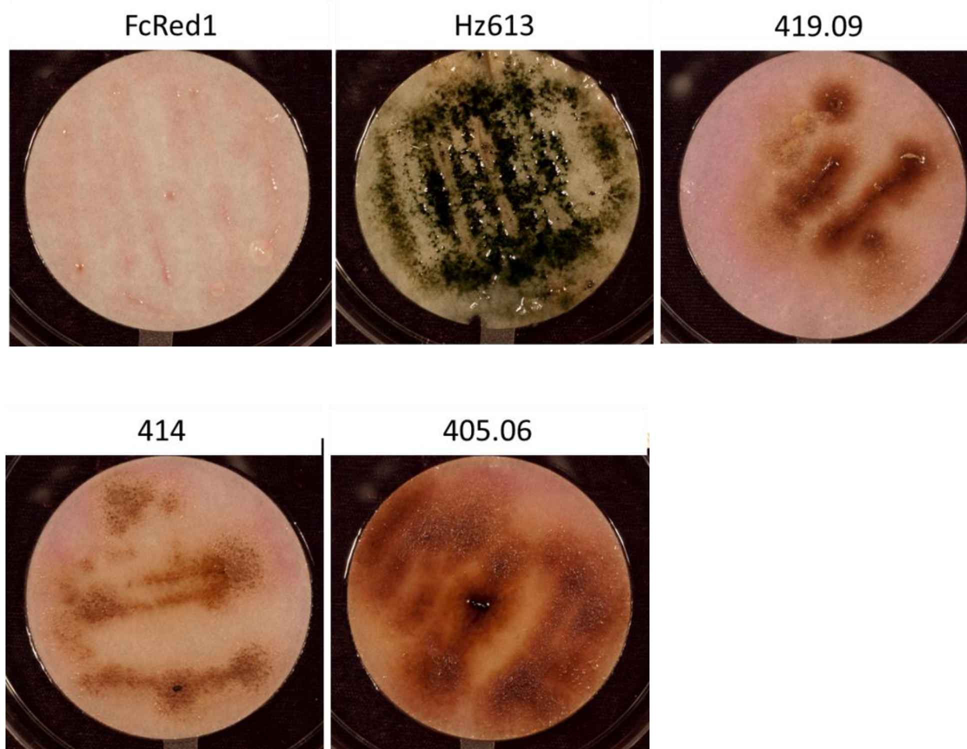


Figure 3-12. Detection of indole-type compounds using Ehrlich reagent. Endophytes were cultured on filter paper disks in tryptophan rich-PDA (10%) medium for 7 days. Filter paper disks were saturated with Ehrlich reagent for 5 min to detect indole-type compounds. Change of color in white filter disks indicated positive reaction with Ehrlich reagent, suggesting the endophytes produce indole-type compounds.

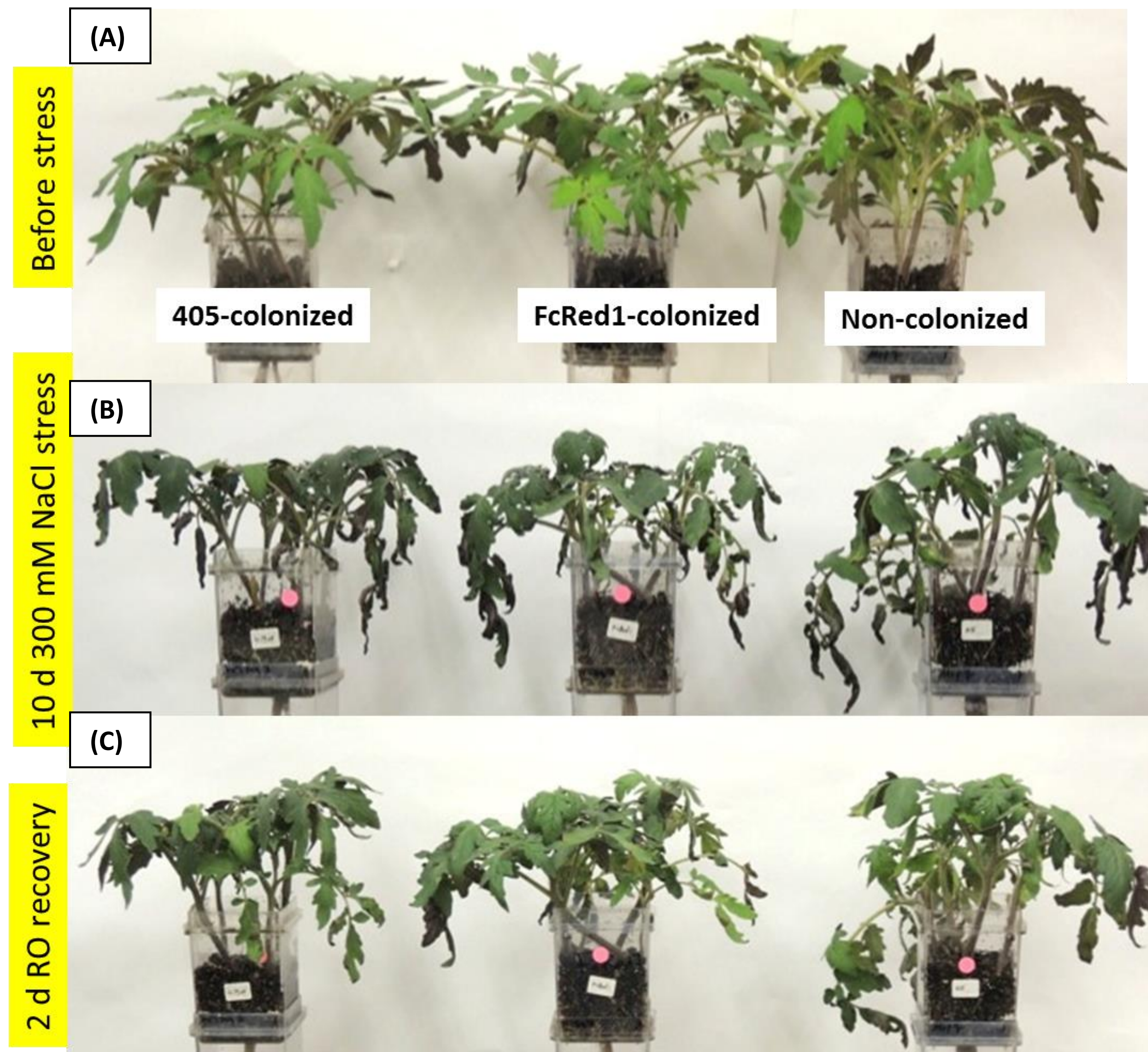


Figure 3-13. Three-week old plants treated with 300 mM NaCl for 10 d; (A) before stress, (B) after 10 d stress with 300 mM naCl, and (C) after rehydration with RO water for 2 d.

APPENDIX 3

Supplementary information for Chapter 4

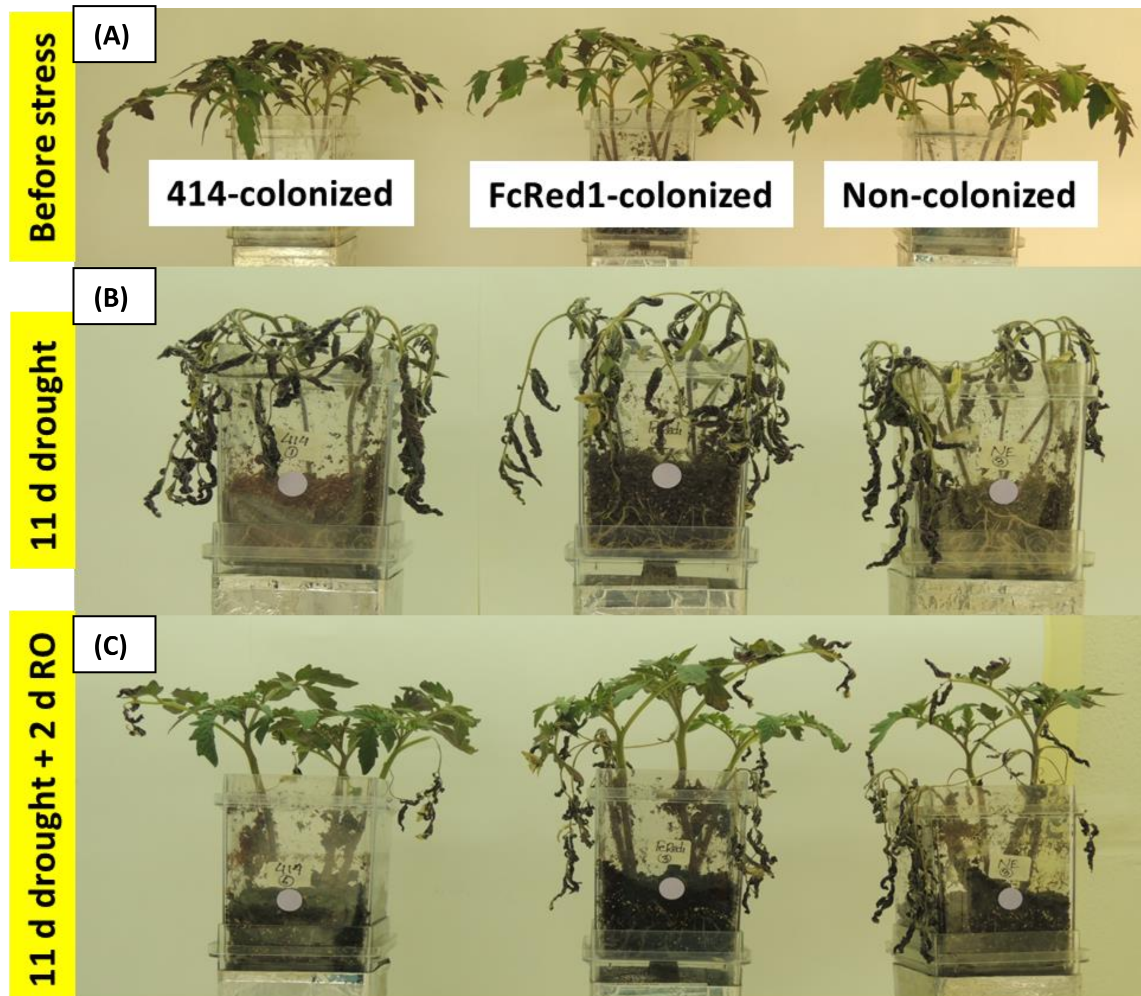


Figure 4-14. Three week old plants (A) treaded with 11 d continuous drought (B) and revived with RO water for 2 d (C).