# MOLECULAR REGULATION OF DROUGHT STRESS TOLERANCE IN SYMBIOTIC PULSES PRODUCED UNDER DROUGHT STRESS

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By

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# DEDICATION

To my dear parents, my husband and my lovely and beautiful daughter Sonal who provided guidance, support, encouragement and unconditional love throughout my way.

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# ABSTRACT

Peas and chickpeas are the most common varieties of pulses, the leguminous crops whose nutrient-reach grains are used to nourish the world's growing population. However, due to global climate change, abiotic stresses such as drought, high temperature and salinity are increasingly hindering crop health, yield and global food security. Increasing demands for food increases the importance and urgency of understanding how microbiomes may be exploited to increase crop yields and reduce losses caused by abiotic stress. In recent two decades, modern agricultural microbiology science is applying novel approaches to overcoming abiotic stresses. Yet, there are very few detailed studies highlighting the impacts of plant-associated endophytes on plant health and development when exposed to extreme drought.

In this study, a few selected fungal strains of *Penicillium* sp. SMCD 2206, and *Paraconiothyrium* sp. SMCD 2210, and bacterial strain of *Streptomyces* sp. SMCD 2215 endosymbionts were tested for their capacity to promote plant growth and reduce oxidative damage in tested plants grown under drought stress. A transfer of the stress tolerance from first ($F_1$) to second ($F_2$) generation was also tested. The study findings showed that under drought chickpea and pea $F_2$ seeds produced from $F_1$ inoculated (E+) with endophytes (SMCD 2206, SMCD 2210, and SMCD 2215) have high germination and better root and shoot growth compared to non-inoculated (E−) plants. Furthermore, the reactive oxygen species (ROS) level was assessed in chickpea and pea $F_2$ seeds and found that the fungal endophytes SMCD 2206, SMCD 2210, and SMCD 2215 reduced the oxidative damage under drought conditions in $F_2$ generation seeds produced from $F_1$ plants inoculated (E+) with these endophytes. The assessment of the impact of fungal endophytes on antioxidant gene expression found that endosymbionts downregulate antioxidant gene expression (proline, superoxide dismutase (SOD), manganese superoxide dismutase (MnSOD), dehydrin), indicating the significance of endophytes in stress tolerance. In addition, the quality of seeds in regard to protein content is also improved by fungal endophytes. Furthermore, the relationship between ROS level and seed germination was investigated, and found that an inverse relationship exists.

Overall, the endophytic symbionts SMCD 2206, SMCD 2210, and SMCD 2215 improve germination and plant growth, while reducing oxidative damage in second generation chickpea

and pea seeds under drought conditions. In addition, the endophytes pass on the stress tolerance to next generation; however, the mechanism of action remains obscure. In conclusion, endosymbionts have the potential to increase agricultural production under adverse environmental conditions. However, additional research at the molecular level is vital to understand the stress tolerance and inheritance mechanisms, and field/natural conditions are imperative to confirm the applicability of endophytes.

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| | |
|---|---|
| APX | Ascorbate Peroxidase |
| ASH | Ascorbic Acid |
| ABA | Abscisic Acid |
| ABRE /bZIP | ABA Response Element/ Basic Leucine Zipper Domain |
| AREB | ABA Responsive Element Binding Protein |
| ANOVA | Analysis of Variance |
| bp | Base Pairs |
| CAT | Catalase |
| CDC | Crop Development Centre |
| CDPK | Ca2+ Dependent Protein Kinases |
| DNA | Deoxyribonucleic Acid |
| DCFH-DA | 2', 7'-Dichlorofluorescein Diacetate |
| DREB/AP2 | Dehydrin Response Element Binding/Apetala 2 |
| ESTs | Expressed Sequence Tags |
| FAOSTAT | Food and Agriculture Organization of United Nations Statistic Division |
| FAO | Food and Agriculture Organization |
| GR | Glutathione Reductase |
| GSH | Glutathione |
| GOPX | Guaicol Peroxidase |
| GST | Glutathione -S- Peroxidase |
| HSD | Honest Significant Difference |
| $HO^{\circ}{}_{2}$ | Per Hydroxyl |
| $H_2O_2$ | Hydrogen Peroxide |
| IOP-PDA | Increased Osmotic Pressure-Potato Dextrose Agar |
| InsP | Inositol Polyphosphates |

| | |
|---|---|
| IYP | International Year of Pulses |
| LEA | Late Embryogenesis Abundant Proteins |
| LSD | Least Significant Difference |
| MDHAR | Monodehydroascorbate Reductase |
| MYC/MYB | Myelocytomatosis/Myeloblastosis |
| MnSOD | Manganese Superoxide Dismutase |
| NCBI | National Center for Biotechnology Information |
| Mt | Million Ton |
| M ha | Million Hectare |
| NTC | No Template Control |
| $O^{\circ}_2$ | Superoxide |
| $OH^{\circ}$ | Hydroxyl |
| $O_2$ | Singlet Oxygen |
| PCR | Polymerase Chain Reaction |
| PDA | Potato Dextrose Agar |
| PEG | Polyethylene Glycol |
| QPCR | Quantitative Real Time PCR |
| ROS | Reactive Oxygen Species |
| RNA | Ribonucleic Acid |
| SE | Standard Error |
| SMCD | Saskatchewan Microbial Collection and Database |
| SOD | Superoxide Dismutase |
| TF | Transcriptomic Factors |
| ul | Micro Litre ($10^{-6}$) |
| ZF-HD | Zinc Finger Homeodomain |

# CHAPTER 1.

# GENERAL INTRODUCTION

## 1.1 Introduction

As the result of climate change, pulses will be more frequently subjected to stress in semi-arid regions such as the Canadian prairies (Lahlali et al. 2014) during phenophases of pollen germination and flowering as well as seed formation and germination. Pulses (e.g., chickpea, pea, and lentil) are legumes producing grains in pods. In addition, they were domesticated approximately 11,000 years ago, and have spread almost worldwide (Zhoary and Hopf 2000). Depending on their adaptability to different ecological regions, these crops are further categorized into cool season (pea, chickpea, and lentil) (Cannon et al. 2009) and warm season (common bean and pigeon pea) (Zhu et al. 2005) crops. Pulses are also major contributors to the contemporary agriculture production industry due to their high nutritional and economic value. Specifically, food commodities created using pulses contain desirable carbohydrates, proteins, fibers, vitamins, and minerals (Mudryj et al. 2014) and have been recommended by Canadian government agencies to increase human health (Health Canada 2013) and global food security (Nyanga 2012). Furthermore, pulses help soil nitrogen improvement through N-root fixation, thereby maintaining sustainable soil quality and fertility for improvement of subsequent crop production (Burgess et al. 2012).

FAOSTAT (2012) reported that there are 77.5 million hectares of pulse production area in the world, and that these crops contribute 70.41 million tons of grains annually (about ~1 t of grain per hectare). North America, Canada, Middle East, and Asia are among the leading pulse producers worldwide (Roy et al. 2010). Canada has major role in pulse crop production as yearly pulse crops are grown on area exceeding than 2.3 million hectares (Pulse Canada) (http://www.pulsecanada.com/canadas-growing-regions). The major production of chickpeas,

peas and lentils in Canadian prairies come from Saskatchewan (Pulse Canada) (http://www.pulsecanada.com/canadas-growing-regions). For example, Statistics Canada (2011) reported the dominant role that Saskatchewan plays, as it accounts for 68.3 % of the total cultivated pea, 86.9 % of chickpea, and 96.0 % of lentil areas, respectively. However, in recent years there is noticeable increase in pulse demand as people are consuming healthy food choices (Faye 2010; USDA-ERS 2011).

Additionally, there is a continuous increase in world population, and by 2050 it will be necessary to increase current agriculture production by 60 % in order to meet food and nutritional security needs (Varshney et al. 2015). However, abiotic stresses limit the biomass production and yield wherever pulses are grown (Krishnamurthy 2011; Choudhary 2014). Since abiotic stresses usually impact cellular and biochemical processes, they subsequently disturb the normal functioning of plants (Koyro et al. 2012). Moreover, reduced water availability and drought are confounding and major stress-related factors for global pulse production (Ashraf et al. 2009; Jaleel et al. 2009). For instance, water scarcity alone reduced the production yield by 70 % (Boyer 1982), contributing to global food insecurity.

The combination of unstable climatic conditions and increased food demand resulting from the continuous increases in the world population makes it important to determine new ways to increase pulse crop production. Under continuously changing climatic conditions, it is particularly challenging to increase pulse production. Consequently, this calls for the innovative creation of new biotechnologies to mediate drought-related issues in pulse production that can augment the gap between demand and actual yield (FAOSTAT 2012; Varshney et al. 2013a; Bohra et al. 2014).

Recently, pulse-associated endosymbionts have received particular attention in the field of biotechnology. Vujanovic et al. (2012) reported that fungal endophytes confer stress resistance to pulses through alteration in gene expression patterns during seed germination. Specifically, the endosymbionts help plants combat the abiotic stress effects on growth and productivity by assisting the host genotype with adaptation to adverse conditions. In this case, there is a mutualistic relationship between plants and fungal endophytes, whereby endophytes support root growth and plant survival during drought, increase resistance to diseases and insects, and enhance nutrient uptake and yield (Schardl et al. 2004). For instance, *Piriformospora indica* endophytic fungus is root colonizer on many plant species that confers plant tolerance against

different biotic and abiotic stressors (Verma et al. 1998; Varma et al. 1999; Oelmuller et al. 2009). This kind of symbiosis or mutually beneficial plant-fungus relationship can enhance protect plants/hosts from drought stress (Nagabhyru et al. 2013). However, the fundamental mechanisms by which endophytes enhance plant drought tolerances remain under investigation. Although at first glance, these mechanisms seem complex and may consist of direct-trough contact and indirect-trough volatiles (Banerjee et al. 2014; Vujanovic et al. 2015), the effects of endophytes on plant metabolism, physiological, and physiochemical adaptations are evident (Bayat et al. 2009). By moderating the plant stress response via mycovitality (Hubbard et al. 2012), the endophytic fungi improves seed germination and agricultural traits *via* both genetic and epigenetic changes, which are also registered in $F_2$ plant offspring (Hubbard et al. 2014).

The aim of this study was to evaluate the effects of selected endophytes from the Saskatchewan Microbial Collection and Database (SMCD) on $F_2$ generation pulse seeds produced with and without endophytes under drought conditions. Mycovitality parameters such as seed vigor, reactive oxygen species status, and regulation of the expression of antioxidant and or resistance genes were assessed in both germinating seeds and germinants. Data were compared and analyzed in light of the molecular changes on the level of root and leaf tissue induced, as well as overall seed quality in terms of protein content. Further, benefits and potential challenges of using such plant-endophyte approach to ameliorate plant tolerance to drought are discussed. The results obtained from this study will assist in better understanding the effect of endophytes on pulse stress resistance against drought conditions.

## 1.2 Hypotheses

The use of endophytic symbionts is a potential method by which seed vigour and germination can be enhanced (Vujanovic et al. 2000) and plants can be protected from environmental stressors (Waller et al. 2005). Vujanovic and Vujanovic (2007) coined the term mycovitality to refer to this form of plant mycodependency. Typically, abiotic stress conditions lead to the production of reactive oxygen species (ROS), which are not only toxic to plant cells but also affect the expression of many genes including antioxidant genes (Bhattacharjee 2012). It is already known that fungal endophytes can increase plant tolerance against abiotic stress (e.g.,

heat and drought) in mature plants (Marquez et al. 2007; Rodriguez et al. 2008) via a process referred to as mycotrophy (Decklerc et al. 2005). Therefore, it was hypothesized that:

1. Endophytes improve seed germination in $F_2$ generation pulses produced under drought from $F_1$ plants inoculated with endophytes.
2. Downregulation of stress resistance genes in $F_2$ generation seeds and germinates exist and can be related to endophyte conferring drought adaptation to the plant/host. Symbiotic (E+, plant with endophyte) and non-symbiotic (E−, plant without endophyte) pulses express genes differently conferring drought resistance to the plant/host.
3. There will be more oxidative damage in non-symbiotic (E−) pulses compared to symbiotic (E+) pulses.
4. Symbiotically (E+) produced seeds will have better nutritional quality compared to non-symbiotic (E−) seeds.

### 1.3 Objectives

The objectives of this study were to research the effect of selected endophytes on $F_2$ chickpea and pea seeds produced under drought from $F_1$ plants inoculated with and without endophytes. Subsequently, *in-vitro* drought tests were employed with 5 % polyethylene glycol (PEG) (PDA amended with 5 % PEG) and increased osmotic pressure in PDA medium (IOP-PDA) that imitated osmotic stress and water availability conditions. This study had the following objectives -

1. To determine *in vitro* seed germination and morphological trait such as root and shoot length in $F_2$ generation seeds produced from $F_1$ generation plants inoculated with and without endophytes.
2. To study the expressions of various antioxidant genes that regulate drought resistance in symbiotic and non-symbiotic produced pulses by using first leaves samples and quantitative real time polymerase chain reaction (QPCR) method.
3. As indicators of oxidative stress, to measure reactive oxygen species in roots colonized by endophytes.
4. To measure the nutritional quality of inoculated and non innocculated seeds produced uuder drough stress.

In this thesis, the first chapter offers an introduction to the topic of interest. The second chapter then provides a literature review. Next, Chapter 3 discusses the role of fungal endophytes on drought stress tolerance in $F_2$ generation chickpeas. This is assessed in terms of seed germination, root and shoot length, reactive oxygen level in radicles, antioxidant gene expression, and seed quality and protein content under drought conditions. In this chapter, it is determined that different endophytes will affect various developmental processes in plants to different degrees. In Chapter 4, the results are provided and the role of fungal endophytes in improving drought stress tolerance to $F_2$ generation pea seeds produced under drought stress is discussed. Here, it is revealed that endophytes affect pea seed germination including roots, shoots length, as well as at a molecular level by changing antioxidant gene expression. Ultimately, it is concluded that endophytes affect seed quality and improve protein content.

# CHAPTER 2.

# LITERATURE SURVEY

## 2.1 Pulse importance

Pulses are leguminous annual crops which consist of seeds in a pod. Typically, the term pulse indicates crops which are mainly cultivated for dry seed. Pea, chickpea, lentil, kidney beans and some others are considered pulse crops. There are 670 to 750 genera and 18,000 to 19,000 species of legumes (Polhill et al. 1981).

The world's major producers of selected pulse crops are Canada, India, China, Myanmar and Brazil followed by other countries (FAO, 2006 - 2007). Pulse production in Canada is higher than 5.5 million ton per year (Parthiba 2015 - edited) http://www.thecanadianencyclopedia.ca/en/article/pulse-crops/. Nowadays Canada especially Saskatchewan has a major role in world total pulse production (Froehlich et al. 2015). Moreover, due to importance of pulses year 2016 is declared as 'International Year of Pulses (IYP)' by 68th UN General Assembly (A/RES/68/231, 2014).

### 2.1.1 Nutritional diet

Pulses are grown widely for food and animal feed. They are good source of protein, fiber and antioxidants (Rebello et al. 2014). Besides, pulse supplemented diets like chickpea are also a good source of calcium, magnesium, potassium, phosphorus, iron and zinc (Ibrikci et al. 2003). Legumes are major part of world agricultural production and fulfill the human dietary nitrogen requirement by 33 % (Vance et al. 2000). Legumes have diversified protein concentration range (18-40 %) depending on the species and cultivar of a species (Bliss 1990). Pulses are considered healthy diet as they are low in saturated fats and in addition also help in reducing cholesterol

level which decreases the chance of cardiovascular diseases and hypertension (Arnoldi et al. 2015).

### 2.1.2 Nitrogen fixation

Legumes have the ability to fix the atmospheric nitrogen in symbiotic association with *Rhizobium* bacteria resulting in improved crop-soil ecosystem, consequently, symbiosis is largely (80 %) responsible for biological nitrogen fixation (Zahran 2009). Major legumes crop fix roughly 40-60 (Mt) nitrogen annually, with addition of 3-5 (Mt) nitrogen to the natural ecosystem (Smil 1999; Drevon et al. 2015).Thus, pulse crops are grown alone or in combination with other crops on rotation basis to improve sustainability of soil quality and production. Moreover, continuous increase in cost of chemical fertilizers makes the symbiotic pulses as a good alternate for reducing the crops production cost. In addition, the use of chemical fertilizers increases the global warming and creates pollution to an alarming level which gives a state to think about ecologically favorable alternative including symbiotic legume crops which supplement the soil in an environment friendly manner (Bruijn 2015).

### 2.1.3 Other economic importance

Healthy animal production for meat, milk and dairy product requires grain crops and forage legumes (Wattiaux and Howard 2001; Boelt et al. 2015). Moreover, the interest for use of legume and legume flour in one or another form is increasing in many food products and baking industry including breads, extruded products and ready to eat snacks (Boye et al. 2010; Minarro et al. 2012).

Furthermore, legumes are symbiotic in nature and provide $N_2$ to plants, which decreases the dependency on fertilizers and reduces the cost for crop production as formulation of fertilizers by industries costs higher than US$ 100 billion (Bruijn 2015).

Moreover, extract from legumes has proved very useful ingredient for biochemical and pharmaceutical companies which synthesize insecticides and medicinal compounds (Morris Brad 2003). Legumes are also used in many biochemical manufacturing companies to prepare biodegradable plastics (Paetau et al. 1994), oils, dyes, inks and gums (Morris 1997). In addition,

they also provide raw material for food and chemical industries. However, they are mainly grown for human food and animal feed but they also support the raw material for paper industry, chemicals and medicine preparation; used as an ornamental plant; provide biological fences between different crops (Lewis et al. 2005).

## 2.2 Constraints in pulse production

Pulse crops are grown worldwide under different conditions but there are some biotic and abiotic factors such as pest, disease, salinity, chilling, freezing, heat and drought which limit the optimum production of yield (Suzuki et al. 2014). However, crop loss due to biotic stresses can be controlled by application of different types of chemicals but it is difficult to control the loss caused by abiotic stresses as climatic conditions are constantly changing. Also, abiotic stresses have advanced all over the world in one or another form and are primarily responsible for reducing the major crops yield by more than 50 % (Rodziewicz et al. 2014).

### 2.2.1 Abiotic stress - drought

Stress is a condition in which normal functioning of a plant system gets disturbed and many biochemical and physiological changes take place. Stresses produced by drought, freezing, heat, UV, heavy metals and salinity are termed abiotic stresses where drought, heat and salinity are dominant abiotic factors affecting plant growth and crop production worldwide (Rodziewicz et al. 2014). Furthermore, among abiotic stresses drought is very common and major problem worldwide (Johansen et al. 1994; Malhotra et al. 2004) which massively reduces the cool-season food legumes production (Saxena, 1993; Singh et al. 1994; Subbarao et al. 1995). Drought is a condition where limited water is available for plant use (Ganeshan et al. 2013). According to Kramer and Boyer, 1995 drought may be defined as a period where the water available for plant is less than the normal precipitation which reduces the plants growth and yield. So drought is primarily responsible for worldwide crop yield loss which may be higher than the total loss done by other stresses (Kramer 1983; Farooq et al. 2009).

Drought is mainly of two types; intermittent drought during vegetative phase and terminal drought during reproductive development which are responsible for the yield loss

(Erskine et al. 1994; Singh et al. 1997a; Serraj et al. 2004; Neumann et al. 2008). Furthermore, terminal droughts are more lethal than intermittent drought (Neumann et al. 2008). However, drought occurrence and intensity cannot be predicted as it is determined by many environmental factors including occurrence and distribution of rainfall, evaporative demands and moisture storing capacity of soils (Wery et al. 1994).

Furthermore, the severity of drought is continuously increasing and spreading all over the world, limiting the legumes production and creating difficulties to achieve the goal of feeding increasing population (Postel 2000; Toker and Mutlu 2011). In addition, the world's water supply is limiting and future food demand for rapidly increasing population will make the drought consequences more noticeable (Somerville and Briscoe 2001; Farooq et al. 2009).

### 2.2.2 Reactive oxygen species (ROS)

ROS are reactive oxygen molecule. However they are also produced in normal conditions during process of plant metabolism and work as a signaling molecule in different process (Sharma et al. 2012). In normal conditions there is balance between ROS production and scavenging by antioxidants but under stress conditions there is imbalance between ROS production and scavenging which leads to oxidative damage in different parts of plant cells (Sharma et al. 2012). Most of abiotic stresses cause the over production of reactive oxygen species (ROS) in different parts of plants like peroxisome, mitochondria and chloroplast leading to oxidative stress (Gill and Tuteja 2010; Sharma et al. 2012). ROS may be in form of free radicals as superoxide ($O_2^{\bullet -}$), per hydroxyl ($HO_{2^\circ}$), Hydroxyl ($^{\bullet}OH$) or as a non-radical form like hydrogen peroxide ($H_2O_2$) and singlet oxygen ($^1O_2$) (Gill and Tuteja 2010; Sharma et al. 2012). ROS commonly produced by absorption of energy or stepwise monovalent reduction as shown in (Sharma et al. 2012) Figure 2.1.

These are very reactive and toxic in nature, consequently, their overproduction leads to imbalance and causes damage to different components of cells lipids, protein and carbohydrate and DNA as mentioned in Figure 2.2 (Sharma et al. 2012). Thus, cell damage caused by ROS affects plants overall growth and productivity.

## 2.3 Plant - abiotic stress protection mechanism

Every living organism has some kind of protective system to save them from adverse conditions. Similarly, plants possess general defense mechanisms in addition of particular pathways of resistance against abiotic stressors. In addition, plants have developed various schemes to face the environmental stresses by drought adaptation, drought avoidance or tolerance depending on signal originated by plants at molecular and physiochemical level (Bartels and Sunkar 2005). However, plants respond to all stresses by adjusting metabolic, physiological processes and molecular gene expression in different tissues (Nakashima et al. 2009). Furthermore, under abiotic stresses, changes at molecular level lead to stress tolerance *via.* stress perception, signal transduction to different cells, change in gene expression and finally metabolic changes (Agarwal et al. 2006).

There are many common tolerance factors produced in adaptive response of a plant to various abiotic stresses as shown in Figure 2.3. Also, environmental stress-inducible genes can be mainly divided into two groups depending on protein products; (1) functional proteins such as late embryogenesis abundant (LEA), betaine, proline and other osmoregulators which provide plant cells resistance to environmental stresses while (2) regulatory proteins consist of transcription factors which regulate gene expression responsible for stress tolerance (Beck et al. 2007).

### 2.3.1 Antioxidant enzymes

The protective antioxidant systems of plants are composed of enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants are SOD (superoxide dismutase), APX (ascorbate peroxidase), GR (glutathione reductase), CAT (catalase), MDHAR (monodehydroascorbate reductase), GPX (glutathione peroxidase), DHAR (dehydroascorbate reductase), GOPX (Guaicol peroxidase), GST (glutathione -S- peroxidase) while non-enzymatic antioxidants are ASH (ascorbic acid), GSH (glutathione), alkaloid, phenolic compounds and proline (Gill and Tuteja 2010).

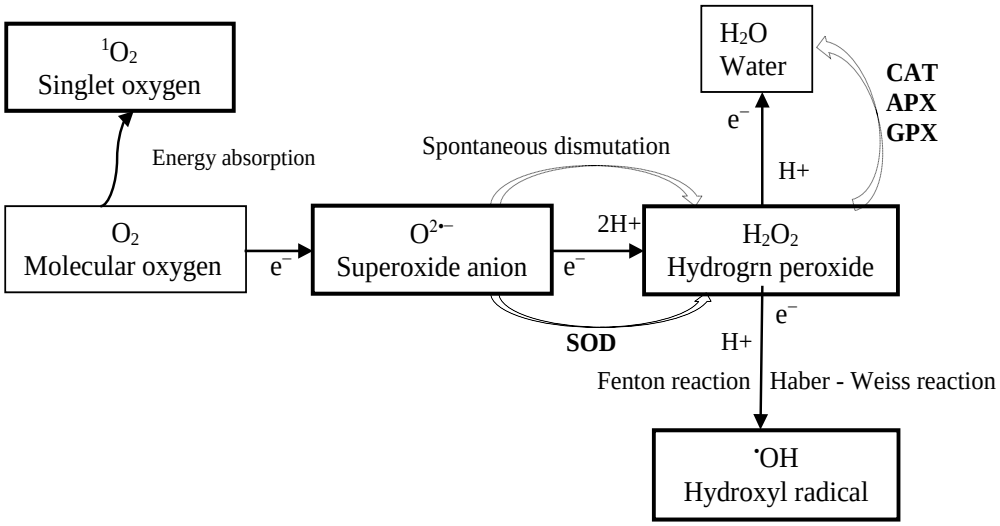



**Figure 2.1** Schematic representation of generation of reactive oxygen species (ROS) in plants - activation of $O_2$ occurs may occur by stepwise monovalent reduction of $O_2$ which leads to formation of $O_2^{\bullet-}$, $H_2O_2$, and $^{\bullet}OH$, whereas energy transfer to $O_2$ leads to formation of $^1O_2$. $O^{2\bullet-}$is easily dismutated to $H_2O_2$ either non-enzymatically or by superoxide dismutase (SOD) catalyzed reaction to $H_2O_2$ and finally converted to water by antioxidants. (Sharma et al. 2012).

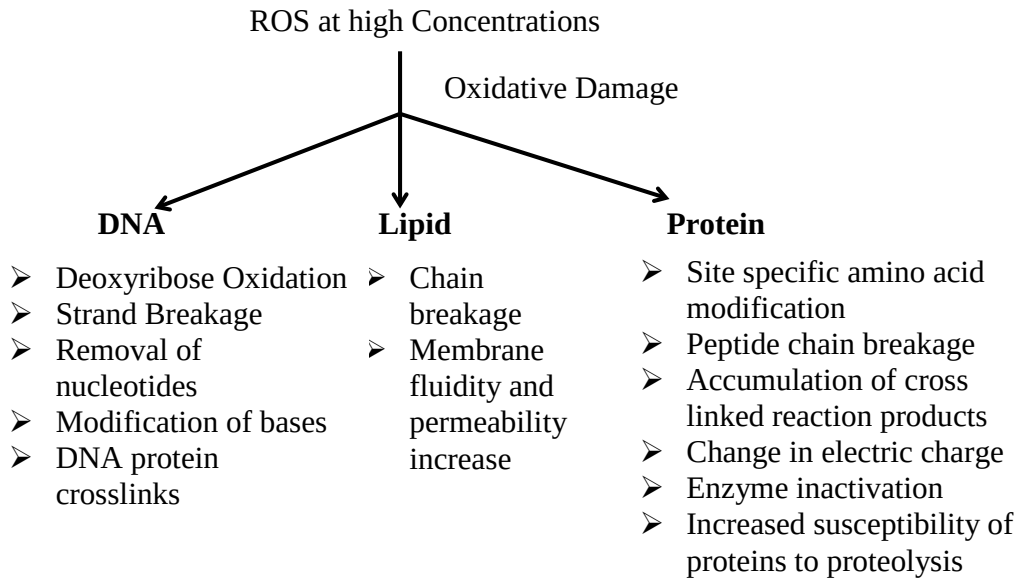



**Figure 2.2** Reactive oxygen species (ROS) induced oxidative damage to lipids, proteins, and DNA. (adapted from Sharma et al. 2012).

However, severity of oxidative damage depends on level of ROS and extent of lipid peroxidation in a cell which is controlled by activity of enzymatic antioxidants like SOD, CAT, peroxidases and amount of non- enzymatic antioxidants anthocyanins, ascorbic acid, carotenoids including others (Apel and Hirt 2004). Thus, for protection of plants, antioxidant enzymes scavenge the free radical or convert more reactive species into less reactive species (Gill and Tuteja 2010).

Upregulation of enzymes and antioxidants has been noticed under abiotic stresses in different plant varieties (Sharma et al. 2012). Also, high salinity produces water stress which is a form of drought (Romero et al. 2001) and upregulation of SOD activity under salt stress has been proved in different plants like *Lycopersicon esculentum* (Gapinska et al. 2008), *Cicer arietinum* (Kukreja et al. 2005) and mulberry (Harinasut et al. 2003). Upregulation of different antioxidants under drought also has been confirmed such as SOD in three cultivars of *Phaseolus vulgaris* (Zlatev et al. 2006), *Oryza sativa* (Sharma and Dubey 2005 a) and ascorbate peroxidase (APX) in *P. vulgaris* (Zlatev et al. 2006), *Picea asperata* (Yang et al. 2008).

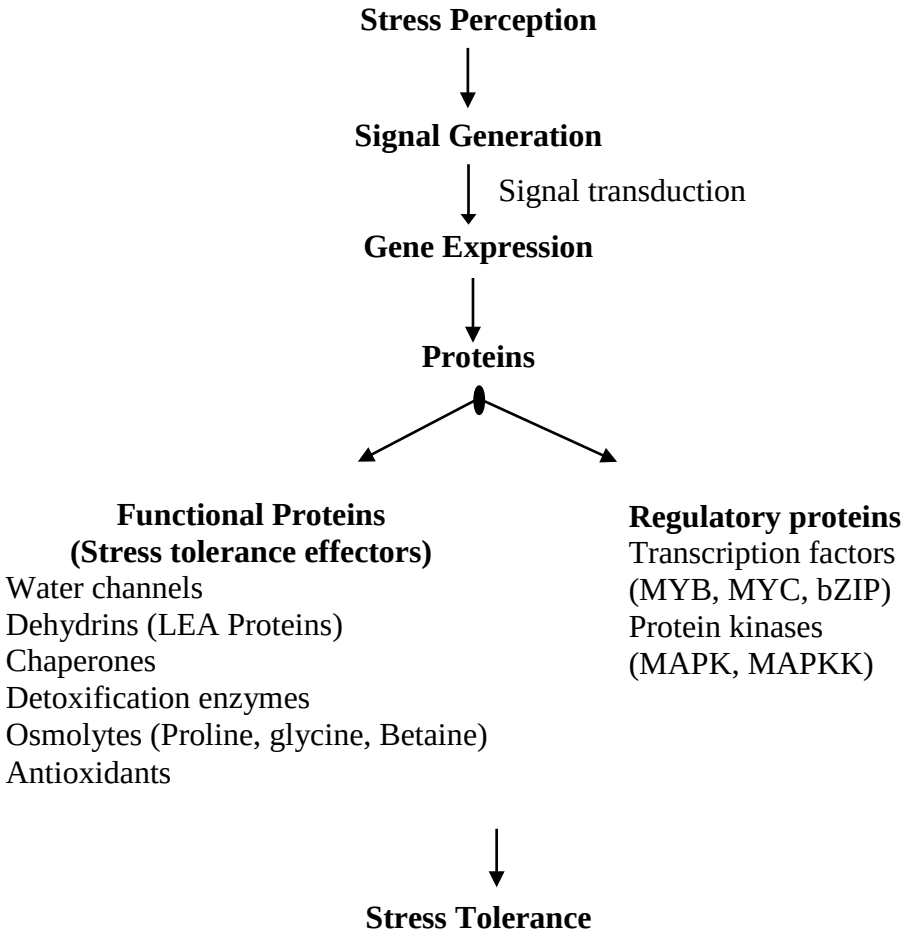



**Figure 2.3** Stress tolerance adaptive responses produced by plants in response drought stress. (adapted from Beck et al. 2007).

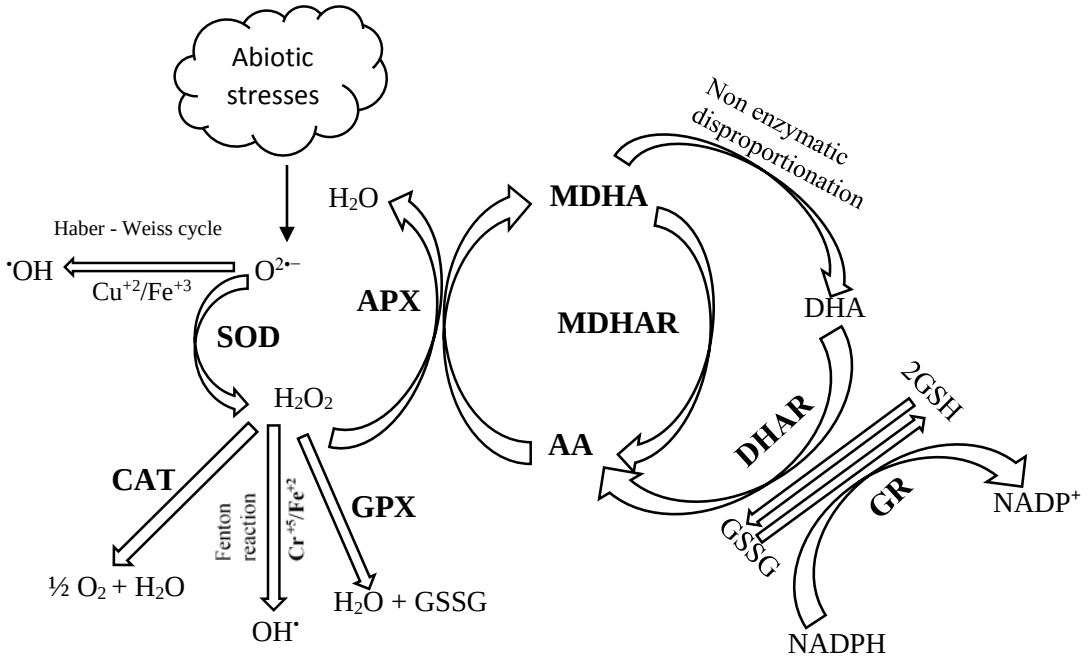



**Figure 2.4** ROS and antioxidant defense mechanism (Gill and Tuteja 2010).

In addition, under salt stress conditions upregulation of CAT (catalase) activity have been confirmed in *C. arietinum* leaves (Eyidogan and Oz 2005) and roots (Kukreja et al. 2005). Furthermore, proline is an osmolyte which is accumulated under stresses like, drought and high temperature and helps in stress tolerance (Yancy et al. 1982; Sairam et al. 2002). Generally, antioxidant gene upregulation is an indicator of plant better defense system to protect them from ROS by scavenging or reducing ROS production (Ara et al. 2013).

### 2.3.2 Signal transduction pathway and transcriptomic regulation of gene expression under drought stress

Drought stress signal leads to increase in $Ca^{2+}$ influx (Sanders et al. 1999; Knight 2000) and production of secondary messengers like ROS, as well as inositol polyphosphates (InsP) and abscisic acid (ABA) which are regulatory molecules (Xiong et al. 2002). Then secondary messengers lead to activation of phosphorylation cascades by involving $Ca^{2+}$ dependent protein kinases (CDPK) and mitogen activated protein kinases (MAPK) (Xiong et al. 2002).

Furthermore, the phosphorylation cascades influence the transcription factors activities which are responsible for overall gene expression and metabolic changes responsible for stress tolerance (Xiong et al. 2002; Ni et al. 2009) (Figure 2.5). In addition, these signal mechanisms are also accomplished with the help of other important molecules like adaptors, scaffolds, transferases and ubiquitination enzymes (Xiong and Zhu 2001).

Plants coding sequences consist of 7 % transcriptomic factors (TF) (Udvardi et al. 2007). Transcriptomic factors are proteins which bind to cis-elements of gene responsible for stress tolerance and result in upregulation and downregulation of related genes (Agarwal and Jha 2010). So transcriptomic factors play a major role in signal transduction pathways for stress tolerance (Nakashima et al. 2014). In addition to stress signal controlling factors, type and amount of stress also decides the expression of genes and amount of osmolytes produced (Pinheiro and Chaves 2011).

Drought induces ABA synthesis and accumulation (Mahajan and Tuteja 2005; Swamy and Smith 1999; Zhang et al. 2008b) which activate the signaling pathways responsible for stress tolerance (Tuteja 2007). Overall, Abscisic acid (ABA) is the major plant hormone involved in drought stress tolerance by regulating gene functions (Cutler et al. 2010; Kim et al. 2010).

The stress signals are mainly transmitted by ABA dependent and ABA independent pathways which decide the expression of antioxidant genes as well as accumulation of osmolytes in cells (Figure 2.6 adapted from Daniela et al. 2012; Loredana et al. 2011; Lata et al. 2011). Furthermore, the stress perception is controlled by transcription factors and stress tolerance can be enhanced by modifying these factors (Umezawa et al. 2006). In drought stress ABA-dependent pathways mainly include MYC/MYB, AREB/ABFs (bZIP) and ABA-independent pathways consist of DREB2 transcription factors (Daniela et al. 2012) while ZF-HD/NAC is controlled by both pathways (Agarwal and Jha 2010). Furthermore, after phosphorylation, AREB/ABFs binds to ABRE, MYC/MYB to MYCR/MYBR element while DREB2 and CBF4/DREB1D transcription factor binds to DRE element and activate the transcription factors responsible for stress responsive gene expression (Daniela et al. 2012). Most of DREB act without ABA but CBF4/DREB1D regulates gene expression in an ABA-dependent manner (Haake et al. 2002). Overall, transcriptomic factors bind to their respective cis elements and alter gene expression for different metabolites, proteins and antioxidants responsible for drought stress tolerance (Singh and Laxmi 2015). In addition, transcription activators and repressors also have

been modified by genetic engineering for drought tolerance in plants (Umezawa et al. 2006). In conclusion, some signaling pathways are specific while others are interrelated to each other enhancing the complexity of stress tolerance mechanism (Daniela et al. 2012).

## 2.4 Role of fungal endophytes

Fungal endophytes or endosymbionts are the eukaryotic microorganisms which live asymptomatically inside healthy plant tissues (Hyde and Soytong 2008). According to fossil records, plant and endophytic fungi have close relation as old as 400 million years indicating the role of endophytes in plant evolution and possibly endophytes put selective pressure on plants (Krings et al. 2007). Furthermore, some fungal endophytes are mutualistic in behavior as both the plant and microorganism get benefit from each other (Redman et al. 2001).

Moreover, endophytes also help plant in nutrition uptake (Read 1999), growth enhancement (Varma et al. 1999) and provide tolerance to disease (Redman et al. 2002b) drought and metals (Read 1999). Furthermore, in many studies it has been proven that symbiotic fungi also confer drought tolerance to plants (Clay and Schardl 2002). Overall, fungal and plant symbiosis process can provide stress tolerance to plants from different stresses by rapidly activating the host response system (Redman et al. 1999) or by synthesizing the anti-stress biochemicals by fungus (Bacon and Hill 1996; Schulz 2002) or by combination of both processes. Role of fungal endophytes in drought stress tolerance and inheritance by epigenetic changes also has been proved in study of wheat (Hubbard et al. 2014). But until now it is not much known whether endophytes confer the stress tolerance to following generation of pulses.

According to Carroll et al. (1974) endophytic fungi can be divided into: Class I endophyte which are constitutive mutualists and Class II endophyte which are inducible mutualists. It is considered that many of Class I clavicipitaceous endophytes are systemic and vertically transmitted by seeds (Lamabam et al. 2011) while Class II endophytes are horizontally transmitted from plants to plants and can inhabit the majority of the plants (Rodriguez et al. 2008). Furthermore, in many studies it has been found that both class I and class II endophytes play a role in abiotic stress tolerance as shown in table 2.1.

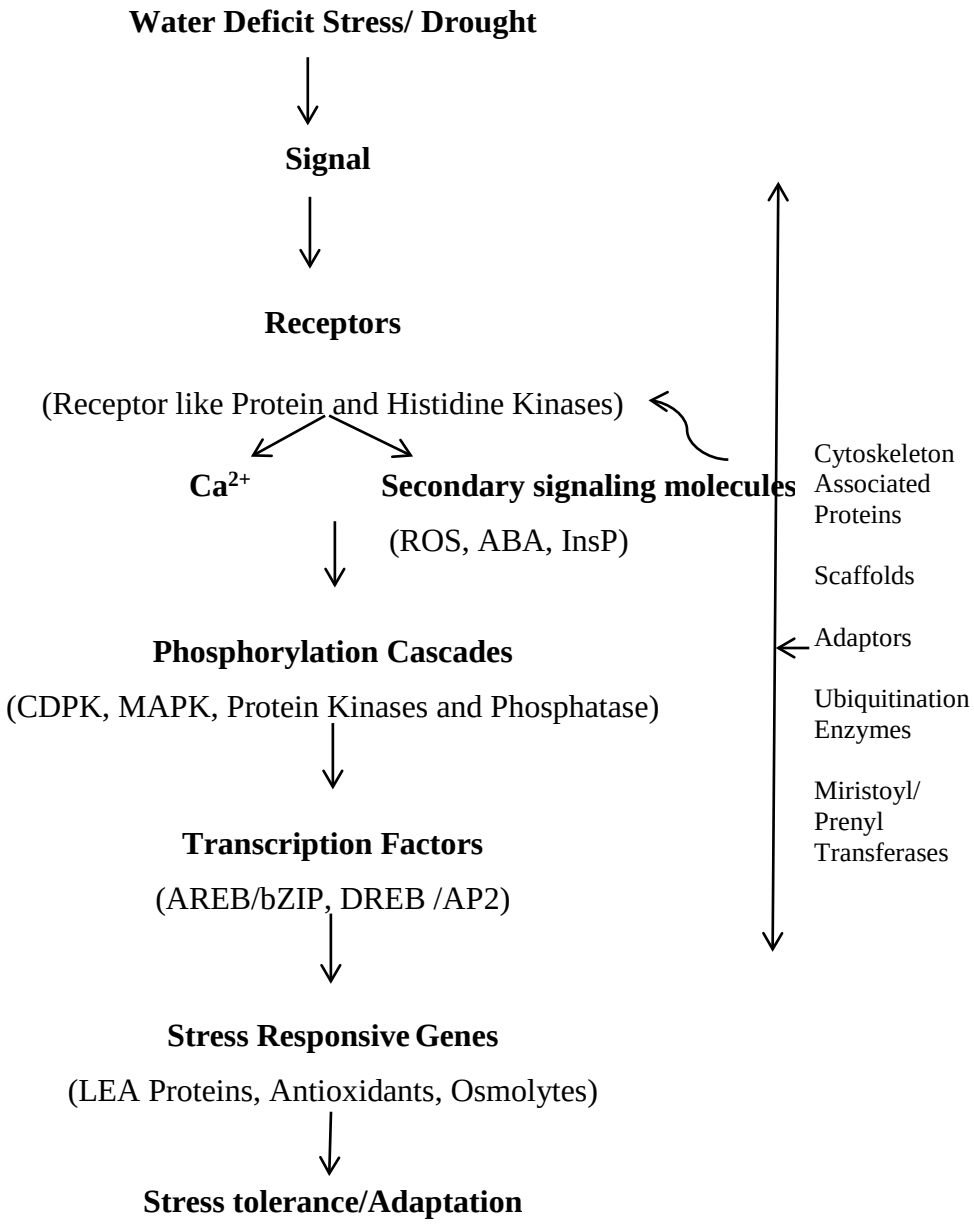



**Figure 2.5** Drought stress tolerance signal transduction pathway (adapted from Xiong et al. 2002). ROS- reactive oxygen species, ABA- abscisic acid, InsP - inositol polyphosphates, ABRE /bZIP - ABA response element/ basic leucine zipper domain, DREB/AP2 –dehydrin response element binding/apetala 2, LEA- late embryogenesis abundant proteins.

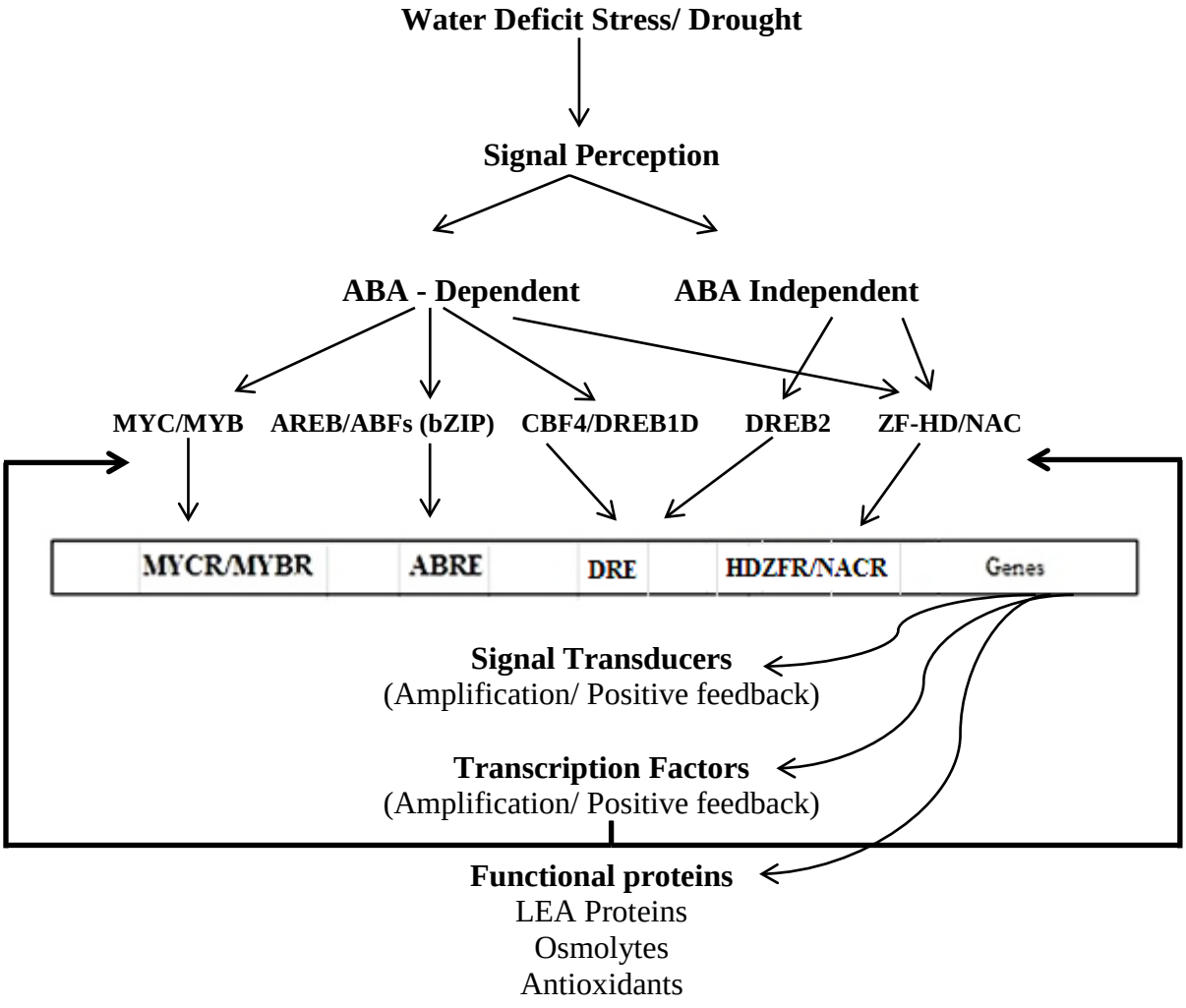



**Figure 2.6** Transcriptional regulation of stress-responsive genes in response to water deficit-stress/drought (adapted from Daniela et al. 2012; Loredana et al. 2011; Lata et al. 2011). ABA- abscisic acid, AREB- ABA responsive element binding protein, ABRE /bZIP - ABA response element/ basic leucine zipper domain, DREB - dehydrin response element binding, LEA- late embryogenesis abundant proteins, MYC/MYB- Myelocytomatosis/Myeloblastosis, ZF-HD - Zinc finger homeodomain.

## 2.5 Stress tolerance inheritance possibly related to epigenetics

Plants are sessile organisms and therefore face high amount of abiotic and biotic stresses in comparison of animals which are mobile (Hirayama and Shinozaki 2010; Petrov et al. 2015). Furthermore, crops are important for food and feed for humans and animals but constantly changing environmental conditions are heavily impacting their production. So it is a necessity to develop crop varieties which are stress tolerant. However, plant breeding (Sanghera et al. 2011) and genetic engineering (Kissoudis et al. 2014) methods for stress tolerance are very challenging. In addition, genetic engineering of transcription factors only provides partial stress tolerance and produces negative effects which impact plant growth and production under combination of other stresses (Kissoudis et al. 2014).

Epigenetic changes provide a novel method for plants stress tolerance and inheritance to subsequent generation without disturbing DNA sequences (Wang et al. 2011; Hubbard et al. 2014). Moreover, these heritable changes are produced by process of DNA methylation and post-translational modifications like histone modification without any change in DNA sequence of organism (Dhar et al. 2014). Furthermore, phenotypic variation produced by epigenetic changes also can be inherited to next generations (Johannes et al. 2009; Boyko and Kovalchuk 2010). In addition, epigenetic variations also regulate the gene expression responsible for stress tolerance (Chinnusamy and Zhu 2009). Wang et al. (2011) study showed drought tolerance in rice by process of DNA methylation.

## 2.6 Tissue specific gene expression in plants

Genetic based evidence as homeotic genes proves the tissue specificity of gene expression in plants (Somerville 1989; Edwards and Coruzzi 1990). Furthermore, in recent work (Wang et al. 2012) expression of total 88 unigenes (all assembled sequences having the same annotation were clustered into a unigene) was detected in drought-stressed root, however only 18 unigenes were found to be exclusively for root, and remaining were also expressed in leaf. Although, expression of total 52 unigenes was detected in drought-stressed leaf, yet only13 unigenes were particularly for leaf, and the rest were also expressed in root and only 9 unigenes were found to be expressed commonly in root and leaf stressed tissues. The unigene database is advanced by NCBI (National Center for Biotechnology Information) which provides a unigene

number to each sequence present in it. The unigene assembles ESTs (Expressed Sequence Tags) and other sequences of mRNA, as well as coding sequences defined for genomic DNA, into subgroups of similar sequences (Boguski and Schuler 1995; Wolfsberg and Landsman 2001). However, in pea (*Pisum sativum*) genes have been found which are highly expressed in roots but not any of them was strictly specific to the roots (Evans et al. 1988).

Overall, this suggests that genes may express specifically in tissues but in some studies such as Evans et al. (1988) there was not any cDNA very specific to root. However, epigenetic modifications responsible for stress tolerance are reported to be tissue specific (Wang et al. 2011).

## 2.7 Quantitative QPCR

There has always been an interest in studying the different pathways available in plant and animal systems to understand basic mechanism of growth and development including metabolic changes, disease resistance and stress tolerance. But it is not as simple due to complexity of pathways because of interlinking of different kind of genes. However, with the availability of molecular techniques it has become feasible to measure gene expression which depends upon mRNA quantity (Bustin 2002). The QPCR (Quantitative Real time PCR) has emerged as a powerful tool to measure the gene expression due to its accuracy, simplicity, speed and quantitative results from small amount of samples (Valasek and Repa 2005). In this technique data analysis can be done by absolute or relative quantification methods depending upon need of experiment (Schmittgen and Livak 2008). Relative quantification provides the results for changes in gene expression in form of fold changes while absolute quantification measures the copy number of gene (Schmittgen and Livak 2008). There are many studies which explain the power of QPCR in gene expression analysis. Pea antioxidant gene (CAT, APX, MnSOD, FeSOD, CuZn- SOD and GR) expression was assessed by QPCR (Panda and Matsumoto 2010). Moreover, the expression of cytokinin oxidase/dehydrogenase (CKX) gene was studied in differentially stressed leaves and roots of pea plant by real-time QPCR analysis (Irina et al. 2005).

**Table 2.1** - A few examples of fungal endophytes (class I and class II) which provide help in abiotic stress tolerance (adapted from Lamabam et al. 2011).

| Fungal Endophytes/Species | Abiotic stress | Host Plant | References |
|---|---|---|---|
| *Phialophora* sp. | Drought | *F. pratensis* | Malinowski et al. 1997 |
| *N. lolii* | Drought | Perennial ryegrass | Latch et al. 1985; Ravel et al. 1997 |
| *N. coenophialum* | Drought/ water stress | Tall fescue | Belesky et al. 1989; De Battista et al. 1990. |
| *Acremonium* sp. | Drought | Tall fescue | White et al. 1992 |
| *N. uncinatum* | Water stress | Meadow fescue | Malinowski et al 1995 |
| *Neotyphodium* sp. | Drought | *F. pratensis*<br>Perennial ryegrass<br>*F. arizonica* | Malinowski et al 1997<br>Baker et al 1997<br>Morse et al 2002 |
| *C. magna* (path-1*)* | Drought | *L. esculentum*<br>*C. annum* | Redman et al. 2001 |
| *C. magna* (L2.5) | Drought | *L. esculentum*<br>*C. annum* | Redman et al. 2001 |
| *C. musae* (927) | Drought | *L. esculentum*<br>*C. annuum* | Redman, et al. 2001 |
| *C. orbiculare* (683) | Drought | *L. esculentum* | Redman et al. 2001 |
| *C. gloeosporioides* (95-41A) | Drought | *L. esculentum* | Redman et al. 2001 |
| *Colletotrichum* sp. | Drought | *L. esculentum* | Rodriguez et al. 2004 a |
| *F. Culmorum* (Fc18) | Drought | *L. moillis*<br>*O. sativa*<br>*L. esculentum* | Rodriguez et al. 2008 |
| *F. culmorum* (FcRed1) | Drought | *L. moillis*<br>*O. sativa*<br>*L. esculentum*<br>*D. lanuginosum* | Rodriguez et al. 2008 |
| *C. magna* | Drought | *T. aestivum*<br>*L. esculentum* cv. Big Beef and Seattle's best<br>*C. annuum* cv. Calif. wonder<br>Watermelon | Rodriguez and Redman 2008 |

**Table 2.1 *cont*...**

| | | | |
|---|---|---|---|
| *C. gloeosprioides* | Drought | *L. esculentum* cv. Big Beef<br>*C. annuum* cv. Calif. Wonder | Rodriguez and Redman 2008 |
| *C. orbiculare* | Drought | *L. esculentum* cv. Big Beef | Rodriguez and Redman 2008 |
| *Fusarium* sp.<br>*Alternaria* sp. | Heat/ Drought | *L. esculentum* | Rodriguez and Redman 2008 |
| *C. musae* | Drought | *C. annuum* cv. Calif. *Wonder* | Rodriguez and Redman 2008 |
| *P. indica* | Drought | *Arabidopsis* sp. | Sherameti et al. 2008a |
| *T. hamatum* (DIS 219b) | Drought | *T. cacao* | Bae et al. 2009 |
| *C. protuberate* (CpMH206) | Drought | *D. lanuginosum*<br>*L. esculentum* | Rodriguez et. al. 2008 |
| *C. protuberate* (Cp4666D) | Drought | *D. lanuginosum*<br>*L. esculentum* | Rodriguez et. al. 2008 |
| *Cuvularia* sp. | Heat/ Drought | *L. esculentum* | Rodriguez and Redman 2008 |
| *P. indica* | Drought | *B. campestris* ssp. Chinensis | Sun et al. 2010 |
| *P. glomerata* LWL2<br>*Penicillium* sp. LWL3 | Drought/ Salinity | *O. sativa* | Waqas et al. 2012 |
| SMCD 2206, 2210, and 2215 Endophytes | Heat/ Drought | *Triticum* ssp. | Hubbard et al. 2012 |
| *P. indica* | Drought | *H. vulgare* | Ghabooli et al. 2013 |

In addition, transgene expression in Bt Maize was studied by QPCR (Trtikova et al. 2015). The verification of SuperSAGE (Serial analysis of gene expression) results of selected gene of *Cicer arietinum* salt stressed roots and nodule was performed by QPCR assays (Molina et al. 2011). Furthermore, Gao et al. (2008) performed QPCR gene expression on selected gene from constructed library to test the reliability of library. Together, these studies explain the reliability of QPCR technique in detection of gene expression.

# CHAPTER 3.

# MICROBIAL ENDOSYMBIONTS: DROUGHT STRESS TOLERANCE IN 2nd GENERATION ($F_2$) CHICKPEA (*CICER ARIETINUM*) SEEDS

## 3.1 Abstract

Chickpea is an important leguminous crop grown worldwide due to its nutritional and economic value. However, abiotic stress, primarily caused by drought, has limited chickpea production. This study highlights the endosymbiotic plant growth promotion as well as alleviation of abiotic stress in germinating chickpea seeds and seedlings under drought stress conditions. Seed produced by $F_1$ endosymbiotic plants under drought stress in controlled environment was used to conduct this 2nd generation ($F_2$) study. Endosymbionts improve seed germination and enhance root and shoot growth in 2nd generation seeds without endophytes when exposed to drought. Expression levels of antioxidant genes: proline, SOD-superoxide dismutase and dehydrin were downregulated, which characterizes enhanced oxidative stress tolerance and reduced reactive oxygen species (ROS) in host cells. The endosymbiont beneficial effect was adapted into an increased nutrient quality of 2nd generation seed. This study indicates the potential of the tested endosymbionts to moderate drought stress in 2nd generation plant by triggering epigenetic changes inherited across chickpea generations which correlated with enhanced resilience and improved agricultural traits in this globally important crop.

## 3.2 Introduction

Chickpea (*Cicer arietinum*) is an annual legume crop of the subfamily Faboideae (family Leguminosae) primarily cultivated for their high-protein seeds produced in pods. It is a representative of the West Asian Neolithic diploid (2x = 16) crop (Jain et al. 2013) grown on

~13.5 million ha throughout the world agro-regions: Asia-89.2 %, Oceania-4.2 %, Africa-3.6 %, Americas-2.4 %, and Europe 0.5 %, respectively (FAOSTAT 2014). In Canada, the chickpea seeded area of 77 k ha produced 177 kt, 2013-2014 (Agriculture and Agri-Food Canada, Canada: Outlook for Principal Field Crops, 2015-10-22) and up to 99 % was grown in the Saskatchewan prairie in 2014 (Saskatchewan Pulse Growers. The industry, Accessed online, Feb25/2016. http://saskpulse.com/about/the-industry).

Chickpeas are associated with a nutritious diet, as their seeds contain 20–30 % crude proteins, 40 % carbohydrates, and important minerals (Roorkiwal et al. 2014). Since the seed proteins contain essential amino acids, they are considered to be a good source for dietary protein (Jukanti et al. 2012). In addition to providing food with high nutritional value, chickpeas can help lower individual's cholesterol level (Crujeiras et al. 2007). The beneficial properties of chickpeas also include assisting in the prevention of obesity, diabetes, hypertension, and cancer (Jukanti et al. 2012). Furthermore, they play a major role in reducing agriculture production costs as chickpeas contribute to nitrogen fixation. Specifically, they have the capacity to fix up to 140 kg nitrogen ha-1 and obtain 80 % of the nitrogen requirement from symbiotic nitrogen fixation (Saraf et al. 1998). Additionally, it has been reported that chickpeas change the soil microbiome community, which influences the growth of successive wheat crops (Ellouze et al. 2013). Therefore, chickpeas are a biological means to increase soil fertility when used in crop rotation, thereby decreasing dependency on chemical fertilizers. Moreover, they are a source of raw material for the processing industry (USA dry pea and lentil council, Accessed online Dec 2015).

Climatic effects, particularly drought and heat, present major challenges to the global production of chickpeas (Millan et al. 2006). Drought is a continuously increasing abiotic stress factor (Vurukonda et al. 2016) that limits chickpea growth and development globally. Indeed, it is a major cause of chickpea yield loss (40–45 %) across the globe (Ahmad et al. 2005). An FAO (2012) report indicates that worldwide chickpea production under optimal conditions is 11.6 million t from a 12.3 million ha area where the average yield is less than ~1 t/ha (Roorkiwal et al. 2014). However, the potential yield of chickpeas under optimal conditions is 6 t/ha (Singh 1987). Consequently, this provides evidence of a large gap between current and optimal chickpea production.

Drought is a condition where limited water is available for plant use, and it affects seed germination (Kaya et al. 2006) and ultimately plant growth, and productivity. Under drought

conditions, reactive oxygen species (ROS) such as singlet oxygen, hydrogen peroxide, hydroxyl radicals, and superoxide's are produced, which impact the proper plant functioning (Sharma and Dubey 2005b; Sharma et al. 2012). Under normal conditions, the ROS level is kept in balance by plant antioxidant scavenging system (Sharma et al. 2012). However, drought conditions lead to an overproduction of ROS in plant cells, which disturbs the equilibrium between ROS production and antioxidant scavenging and results in apoptosis and cell death/damage (Gill and Tuteja 2010; Das and Roychoudhury 2014 ).

The antioxidant defense system of plants consists of enzymatic and non-enzymatic antioxidants (Gill and Tuteja 2010). Proline is one non-enzymatic antioxidant commonly synthesized in plants in response to stress conditions (Gill and Tuteja 2010). It can provide osmotic adjustment and be effective ROS scavenger (Kaur and Asthir 2015). Subsequently, increased proline accumulation in response to stress conditions might be due to increases in synthesis or decreases in proline degradation (Gill and Tuteja 2010). Superoxide dismutase (SOD) is an enzymatic antioxidant that is up regulated in response to stresses in order to protect plants from oxidative damage (Gill and Tuteja 2010). Additionally, dehydrins are hydrophilic proteins that appear to be common products in response to drought stress (Campbell and Close 1997). Therefore, under stress conditions, there is increase in activity of antioxidants to protect plants from oxidative damage (Sharma and Dubey 2005a).

Cultivated chickpea has a narrow genetic base making it difficult for breeders to produce new elite cultivars with durable resistance to major biotic and abiotic stresses (Mantri et al. 2007). Endophytic fungi and bacteria that live asymptomatically within plant tissues show tangible promise as symbiotically related to plants (Behie and Bidochka 2014). Moreover, plant roots colonized by endosymbionts result in improved plant growth, high seed yield, and stress tolerance (Sherameti et al. 2008). Their affiliation with plants also impacts several plant processes, including gene expression. For example, the endophytic fungus *Piriformospora indica* alters drought-related gene expression in leaves under stress conditions (Sherameti et al. 2008). As a result, the impact of endophytes on plants at a molecular level can be better understood by antioxidant gene expression.

Under stress conditions, the beneficial role of fungal endophytes in drought tolerance inheritance to the $F_2$ generation was recently shown in wheat seed (Hubbard et al. 2013). Specifically, it was suggested that some epigenetic changes at the molecular level might be the

reason for stress tolerance inheritance (Hubbard et al. 2014). Epigenetic changes are heritable changes at the molecular level that result in differences in functionality and plant properties without changing DNA sequences (Dhar et al. 2014). Overall, the relationship between plants and endophytes is complex and depends on many factors including the type and intensity of stress as well as the genotype of the crop and microbial endophytes (Berg and Smalla 2009; Ellouze et al. 2013.). While it has been proven that drought stress tolerance can be transferred to the $F_2$ generation in wheat (Hubbard et al. 2013), this has not been studied in pulses. In this context, it was hypothesized that endophytes will improve seed germination and plant morphological traits including root and shoot length. In addition, it is expected that fungal endophytes will reduce oxidative damage in plants. Thus, the germination assay and the level of oxidative damage in plant cells may provide an indication of the impact of endophytes on the plant system. Moreover, studying antioxidant gene expression will verify affiliation between endophytes and the plant. Moreover, the aim of this is to shed light on role of endophytes in conferring stress tolerance to $F_2$ generation chickpea seeds under drought conditions in an attempt to increase resistances in pulses by use of endophytes.

As a whole, climate changes and the continuously increasing population that are primarily responsible for worldwide food insecurity further increase the importance of studying chickpea crops. Utilizing endophytes may be a better strategy for improving crop production under stress conditions.

## 3.3 Hypotheses

1. The hypothesis was that $F_2$ seeds produced from chickpea $F_1$ plant inoculated with endophytes will have improved germination and morphological traits in comparison to non-inoculated plants.
2. Second hypothesis was that there will be some downregulation of stress resistance genes in seeds and germinates if the related endophyte confers some drought adaptation to the plant. In addition, it was expected that symbiotic (with endophyte) and non-symbiotic (without endophyte) produced chickpea seeds will differently express genes conferring drought resistance.

3. There will be more oxidative damage in non-symbiotic chickpea seeds in comparison of symbiotic chickpea.
4. Stress tolerant seeds will have higher nutritional quality.

## 3.4 Objectives

The aim of this study was to assess the effects of endophytes on $F_2$ generation chickpea seeds produced under drought from $F_1$ seeds with and without endophytes. The *in vitro* drought conditions imposed by increased osmotic pressure in PDA medium (IOP-PDA) and 5 % PEG (PDA amended with 5 % PEG). The following aspects were analyzed under drought stress.

1. To determine the impact of endophytes on $F_2$ generation chickpea seeds *in vitro* germination and on morphological traits such as root and shoot length.
2. To assess the role of endophytes in modulating antioxidant gene expression under drought conditions.
3. To find out whether the endophytes can scavenge ROS in chickpeas when exposed to: (1) an increased osmotic pressure (IOP) in a PDA growth medium, and/or (2) decreased water availability in a PEG medium by measuring reactive oxygen species.
4. To find out the nutritional quality of chickpea in terms of protein content.

## 3.5 Materials and methods

### 3.5.1 Media stress conditions

The current study created *in vitro* drought stress using polyethylene glycol (PEG). PEG was used to produce water deficit effect on *in vitro* plant growth representing drought stress condition (Lagerwerff et al. 1961; Veslues et al. 1998).

Potato Dextrose agar (PDA) (EMD, Germany) with 2 % agar and PDA modified with 5 % polyethylene glycol 8000 (PEG, Amersco, Solon, Ohio, USA) were prepared. Generally, the PDA medium provides standard moisture conditions, whereas the PEG medium provides drought conditions for *in vitro* germination experiments. In accordance with manufacturer recommendations, the measurement of the firmness of both media (PDA with 2 % agar and PDA amended with 5 % PEG) was checked by texture analyzer (Stable Microsystems Ltd. Surrey,

GU7 1YL, UK) (Figure 3.1) measuring the penetration force experienced by root tips during germination on these media. Additionally, the resistance of growth media was measured using a penetrometer according to manufacturer recommendations (Humboldt MFG. CO. Illinois, USA). The penetrometer was used to push a disc of 3 cm in diameter on both media with same force (Figure 3.2), and the reading was recorded. Higher penetrometer resistance is an indicator of mechanical impedance (any soil or medium which is too hard for root penetration) that reduces root growth (Bengough et al. 2011).


**Figure 3.1** Texture analyzer (Stable Microsystems Ltd.).

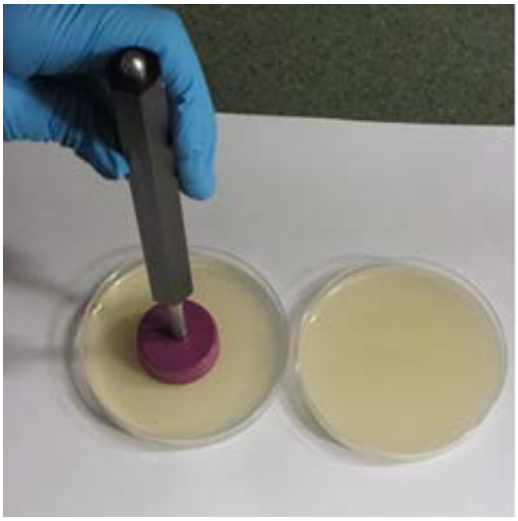


**Figure 3.2** Media penetration resistance measurement by penetrometer.

### 3.5.2 Chickpea seeds, endosymbionts and sterilization protocol

This study used $F_2$ generation chickpea CDC Vanguard (*desi*) seeds produced from $F_1$ non-inoculated (E−) and inoculated (E+) with filamentous, spore forming endosymbionts: fungal endosymbiotic *Penicillium* sp. SMCD 2206 and *Paraconiothyrium* sp. SMCD 2210 as well as bacterial symbiotic *Streptomyces* sp. SMCD 2215 plants subjected to drought stress. $F_2$ generation chickpea (CDC Vanguard) seeds produced in normal conditions from $F_1$ non-inoculated (E−) plants were used as a control. These seeds were obtained from greenhouse experiments performed earlier (Figure 3.3).

Seeds were surface sterilized by washing them with 95 % alcohol for 3 min followed by distilled water for 1 min, 6 % sodium hypochlorite (LAVO 6, Montreal, Quebec) for 1 min, and then three times with distilled water each for 1 min. After sterilization, seeds were placed on filter paper for quick drying and then inoculated on medium for germination. All tested $F_2$ seeds grown on PDA with 2 % agar (increased osmotic pressure - potato dextrose agar IOP-PDA medium) and PDA with 5 % PEG were free from microbes as produced under greenhouse sterile conditions (Vujanovic, personal communication). The first leaves were collected (Gao et al. 2008; Peng et al. 2009) and stored at -80ºC for use in further experiments (Peng et al. 2009).

### 3.5.3 Germination rate

Seeds sterilized as per the sterilization protocol (seed approved as free from endophytes) were germinated (3 seed/plate) in triplicate *in vitro* conditions under increased osmotic pressure (potato dextrose agar IOP-PDA medium) and under drought conditions (PDA amended with 5 % PEG medium) in petri plates and then sealed with parafilm (Bemis, Neenah, WI, USA). Then, they were incubated in the dark at room temperature (23 ± 1°C). Germination was determined by the emergence of radicals from seeds. Samples were observed on daily basis and the germination rate was determined by germination percentage (Vujanovic et al. 2015).

### 3.5.4 Plant morphology

On the $7^{th}$ day of germination, root length was measured using a ruler on three randomly selected replicates.

On the 9th day of germination, shoot length was measured using a ruler on three randomly selected replicates. The first leaves from each sample were collected and stored at -80ºC for use in further experiments.

### 3.5.5 ROS (reactive oxygen species) detection by DCFH-DA (2', 7'-dichlorofluorescein diacetate) method

Detection and measurement of ROS in 6 day old chickpea (CDC Vanguard) roots was analyzed using the DCFH-DA method (Figure 3.4). ROS detection of radicals performed by epifluorescence is based on the formation of a fluorescent compound from 2', 7'-dichlorofluorescein diacetate (DCFH-DA) when the acetate group is cleaved and non-fluorescent 2', 7'-dichlorofluorescein (DCFH) is oxidized to the fluorescent DCF product in a peroxidase-dependent reaction (Cathcart et al. 1983; Paul 2010). The cellular endogenous esterases activity is considered sufficient for this reaction (Rodríguez and Taleisnik 2012).

A Nikon C2 Confocal laser scanning microscope (Nikon Canada Inc.) was used to study ROS in 6 day old roots. About 0.5 cm of the root length was gently excised from all samples and quickly washed three times with distilled waterto remove any ROS produced as the result of cut stress. Then radicals/roots tips were placed into a solution of 50 µM DCFHDA (Sigma, Germany) and 20 mM phosphate buffer pH 6.0 and incubated in dark for 30 min. Next, these roots were quickly washed three times with 20 mM potassium phosphate ($KH_2PO_4$) (EMD chemicals Inc. Darmstadt, Germany) buffer (pH 6.0) and fluorescence intensity was measured by confocal microscopy which depends on green colour brightness excitation wavelength of ~ 488 nm and emission wavelength of ~ 525 nm (Figure 3.5). All samples were treated equally and processed quickly under the microscope to avoid photo bleaching.

For all samples, Region of Interest (ROI) and exposure time were set so that they were the same, and fluorescence was measured using a 10× lens. The fluorescence was measured in triplicate in the form of fluorescence intensity. The green colour intensity (as mentioned in the fluorescence scale - Figure 3.10 a) is the fluorescence produced due to the amount of ROS present (Figure 3.5).

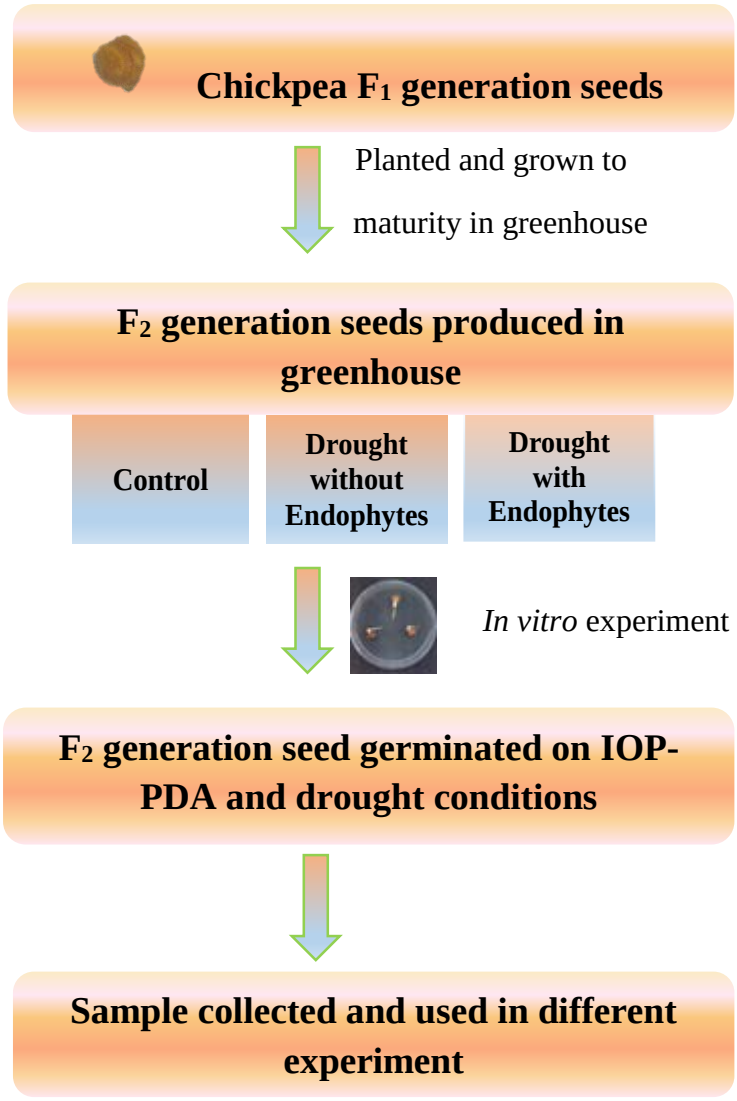



**Figure 3.3** Methodology for chickpea (CDC Vanguard) seed obtained for this study.

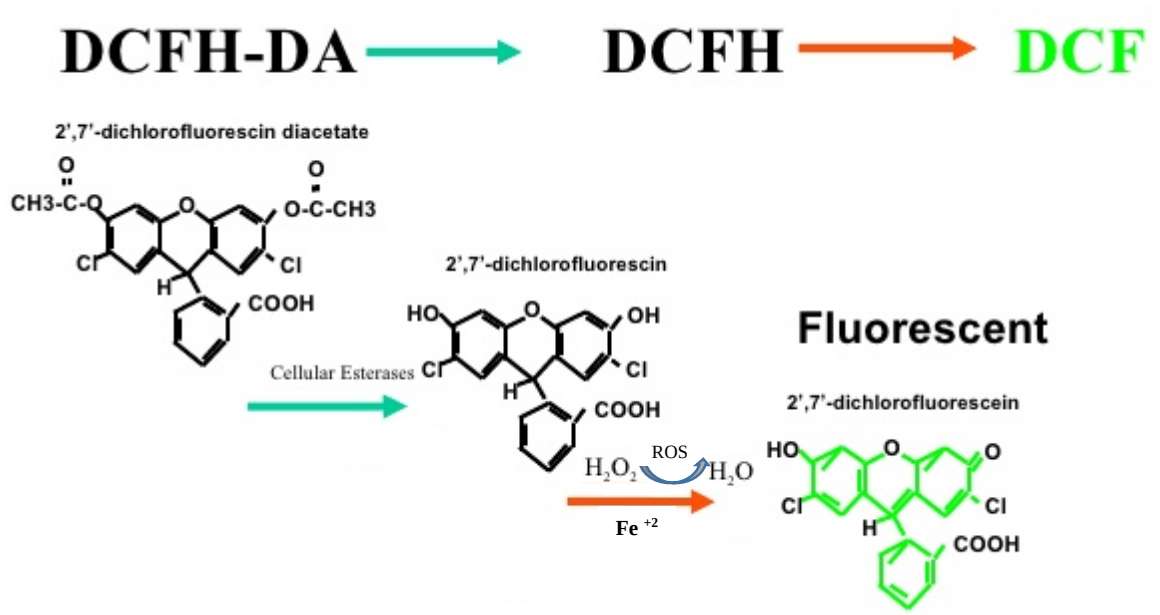



**Figure 3.4** Reactive oxygen species (ROS) detection by 2', 7'-dichlorofluorescein diacetate (DCFH-DA) method (adapted from Robinson 2006).

### 3.5.6 RNA extraction and cDNA synthesis

The Aurum$^{TM}$ Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA) was used to extract total RNA from the first leaves of the chickpea following the manufacturer's recommendations. RNA quality and concentration was measured by using nanodrop 2000c (Thermo Scientific, Wilmington, DE, USA). Additionally, following the manufacturer recommendations, the iscript cDNA synthesis kit (Bio-Rad Lab-oratories, Hercules, CA) was used for cDNA synthesis from extracted RNA by in the PCR machine (Eppendorf Mastercycler, EP gradient S, Germany). The cDNA synthesis was completed immediately following RNA extraction and stored at -80ºC.

### 3.5.7 Gene expression by QPCR

Primers for different antioxidant genes (proline, superoxide dismutase (SOD), dehydrin) as shown in Table 3.1 were used. The primers were selected on the basis of their specificity to the selected genes and performance in previous studies. The expression of antioxidant genes was measured in the first leaves using the real time PCR (QPCR) method and relative quantification.

The chickpea actin gene was used as an internal control to normalize gene expression. In addition, $F_2$ chickpea control sample produced under normal conditions was used as calibrator/untreated control. The formula $2^{-\Delta\Delta CT}$ = [(CT gene of interest - CT internal control) sample A - (CT – gene of interest - CT internal control) sample B] (Schmittgen and Livak 2008) was used to calculate the gene expression to determine fold changes. This form of the equation may be used to compare gene expression in two different samples (sample A and sample B). Specifically, since each sample is related to an internal control gene, data may be interpreted as "the expression of the gene of interest relative to the internal control in the treated sample compared with the untreated control" (Schmittgen and Livak 2008).

The MJ-Mini™ Personal Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) was used for qPCR, in accordance with the manufacturer's instructions. The PCR conditions used were 95˚C for 30 sec, 40 cycles of 95˚C for 15 sec, and 60˚C for 30 sec. In addition, melt curve analysis of 65˚C - 95˚C was used. The one reaction of 20 µl volume consisted of iTaq™ universal SYBR® Green supermix 10 µl, forward primer 1 µl (10 µM), reverse primer 1 µl (10 µM), template 1 µl (100 ng/ul), and nuclease free water (Qiagen) (7 µl). Each sample was run in triplicate with the internal control, negative control, and no template control (NTC). To check the specificity, accuracy, and consistency of the selected gene, melt curve analysis was conducted.

### 3.5.8 Seed quality

Seed quality analysis was conducted using Intertek Sunwest (Research Drive, Saskatoon, Saskatchewan, Canada) from randomly pooled 0.5 g samples. The seed protein content (% dry basis) was measured using the AOAC (2001.11) method and calculated according to the Ptn=N x 6.25 conversion factor.

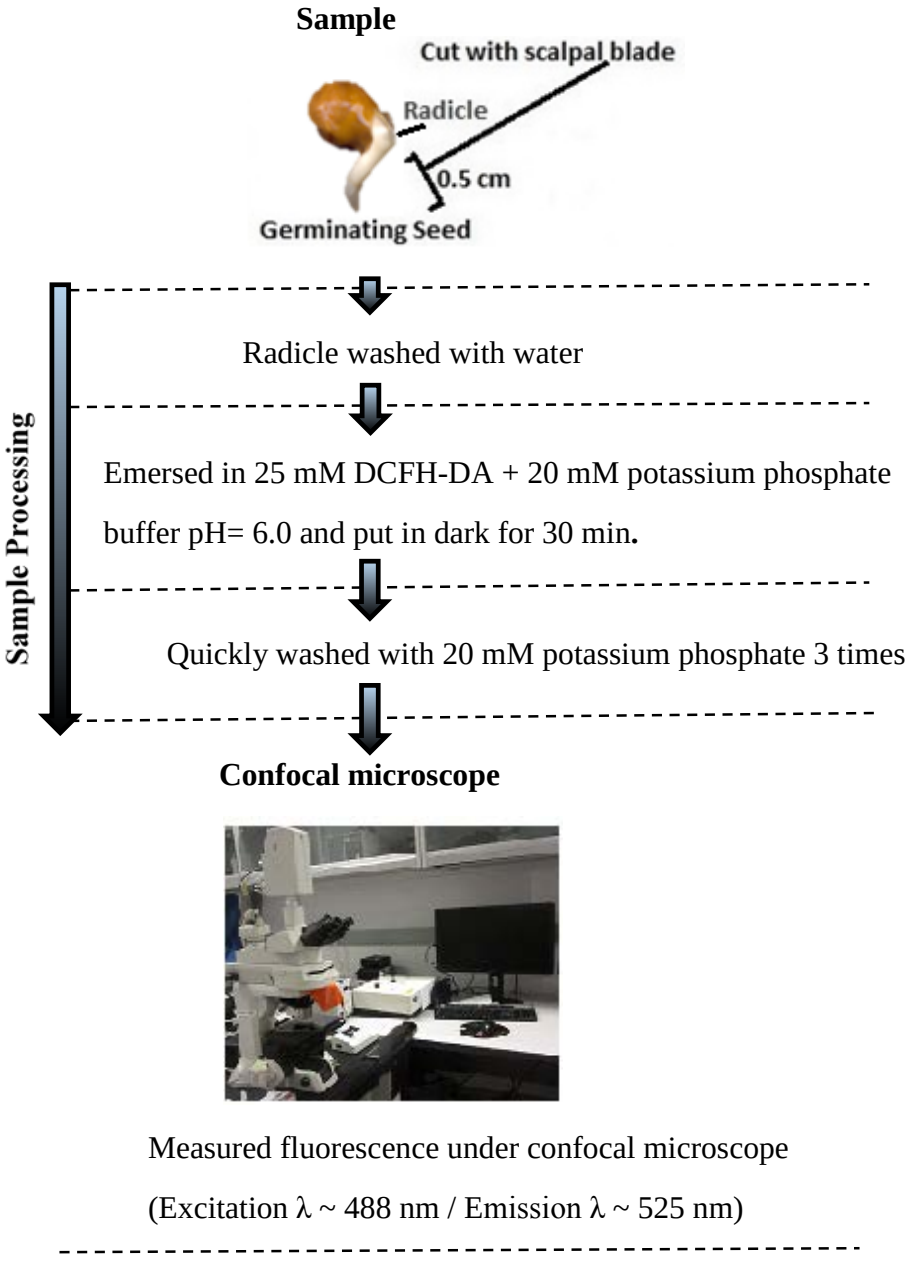



**Figure 3.5** Procedure of sampling and ROS detection in 6th day old root by DCFH-DA method.

**Table 3.1** List of primers specific to chickpea genes.

| Gene | Function | Primer sequence | Length of amplicon (bp) | References |
|---|---|---|---|---|
| Actin | Internal Control | F-5'catcaccctcggcatttc3'<br>R-5'cagccttaaccattccagtc 3' | 123bp | Peng et al., 2009. |
| Dehydrin | Antioxidant gene | F- 5' tggtggcactggagatg 3'<br>R- 3' aactacctgggttgtggg 5' | 195bp* | Gao et al., 2008.<br>*Bhattarai, T., Fettig, S., 2005. |
| Superoxide Dismutase (SOD) | Antioxidant gene | F5'gatcngacctaaacaagcaagtg3'<br>R-5'aaatgctggcggcagagtag 3' | 162 bp | Coram, T. E., Pang, E.C.K., 2006. |
| Proline | Antioxidant gene | F-5'aacgggacttcccgaagatgt 3'<br>F 5'gagaaaagagcaaagcccatg3' | 123 bp | Dopico B., Labrador E., June 2002. Submitted to the NCBI database. |

### 3.5.9 Statistical analysis

All experiments were performed in triplicate and the means and standard errors were calculated for seed germination, root length, shoot length, and ROS levels detection. Protein content was statistically analyzed using a one way analysis of variance (ANOVA) technique followed by *post hoc* Tukey honest significant difference (HSD) and least significant difference (LSD) test at $P \leq 0.05$ in SPSS (IBM SPSS statistic 22).

## 3.6 Results

### 3.6.1 Medium characteristics

The texture analyzer indicated that PDA medium is approximately 4.5 times more firm than the PDA medium amended with 5 % PEG (Figure 3.6). Consequently, PEG produces the effect of dehydration by reducing the availability of water. However, the PDA medium produced an increased osmotic pressure (IOP) in the medium due to its firmer nature than the PDA amended with 5 % PEG, which is semisolid in texture.

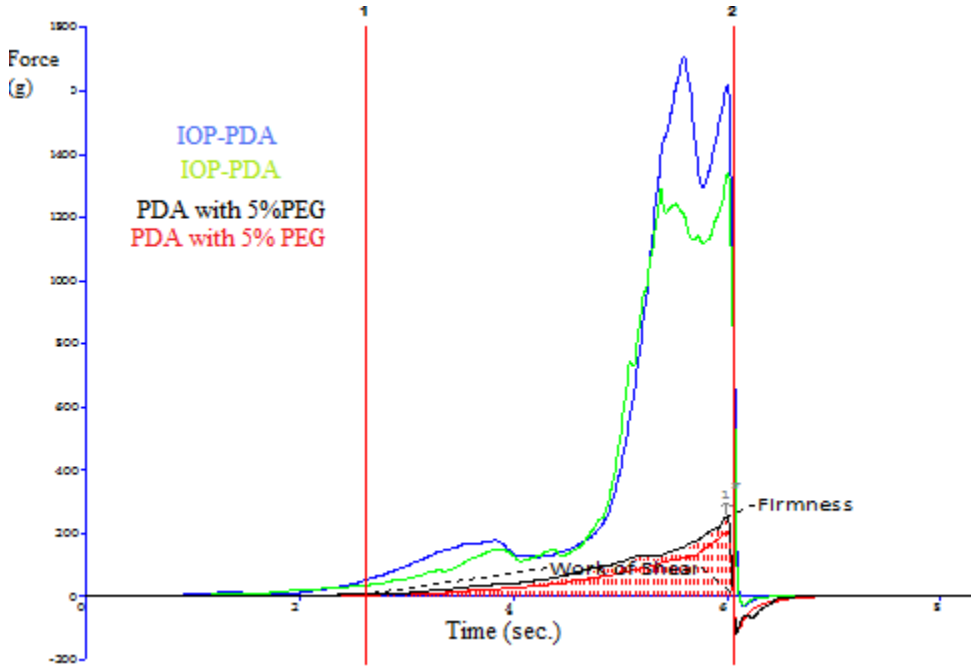



**Figure 3.6** Graph generated from the measurement of media firmness following application of texture analyzer (Stable Microsystems Ltd.).

The measurement conducted using penetrometers (Humboldt MFG.CO Illinois, USA) also revealed that PDA medium had 0.75 kg/cm$^2$ penetrometer resistance. This indicated that it had higher mechanical impedance and root penetration resistance in comparison to PDA amended with 5 % PEG, which resulted in less than 0.2 kg/cm$^2$ resistance (Figure 3.2).

### 3.6.2 Germination rate

Percent germination results showed that $F_2$ generation chickpea seeds - produced under drought stress by inoculation of $F_1$ chickpea plants with fungal endophytic SMCD 2206, SMCD 2210, and bacterial endophytic SMCD 2215 strains attained increased *in vitro* germination when the IOP-PDA medium was used compared to seeds - produced from non-inoculated $F_1$ chickpea plant under the same conditions. However, germination remained lower than the control seed sample. $F_2$ generation chickpea seed followed a similar germination pattern under drought stress conditions (PDA amended with 5 % PEG), and it was found that E+ (endophyte inoculated) $F_2$ generation seed showed higher germination compared to E− (non-inoculated) seeds.

Thus, $F_2$ generation chickpea seeds from E− ($F_1$) plants demonstrated low germination; whereas seeds from E+ ($F_1$) plants had 20 % higher germination under drought stress conditions (Figure 3.7).

### 3.6.3 Root length

It was found that $F_2$ generation chickpea seeds produced using inoculation of endophyte SMCD 2206 had increased root length compared to non-inoculated and control seed samples on the IOP-PDA medium. Additionally, there was increased germination in $F_2$ seeds from $F_1$ plants inoculated with SMCD 2206, SMCD 2210, and SMCD 2215 compared to $F_2$ generation chickpea seed from E− ($F_2$) plants under drought conditions. However, there was overall higher root length under PEG-drought compared to the IOP-PDA increased osmotic pressure and penetrometer resistance conditions (Figure 3.8).

### 3.6.4 Shoot length

The $F_2$ generation seeds produced under drought stress conditions from $F_1$ plants inoculated with fungal (SMCD 2206, SMCD 2210) and bacterial (SMCD 2215) endophytes had higher shoot length than non-inoculated (E−) sample, but lower length than control seeds under drought conditions (Figure 3.9). In addition, seeds with endophyte SMCD 2206 treatment showed higher shoot length compared to the other endophytes under drought conditions. Furthermore, $F_2$ seeds produced using inoculation of endophytes (E+) SMCD 2210 and SMCD 2215 resulted in higher shoot lengths on the IOP-PDA medium than the seeds produced with no inoculation (E−).

### 3.6.5 ROS detection by fluorescence

Results indicated that ROS production was increased in drought conditions as measured by green colour intensity which depends on the amount of ROS formation or production.
In addition, it was confirmed that endophyte treatment helps in reducing ROS production and accumulation in chickpea (CDC Vanguard) cells. In E+ treatments, fluorescence intensity values were low compared to E− treatments.

$F_2$ generation chickpea seed produced under drought conditions from $F_1$ plants inoculation with E+ (SMCD 2206, SMCD 2210, and SMCD 2215) produced less ROS compared to E− seeds exposed to IOP-PDA medium. Moreover, SMCD 2215 treatment in $F_1$ plants produced $F_2$ generation seeds that demonstrated less ROS production than controls using the IOP-PDA medium (Figure 3.10 b).

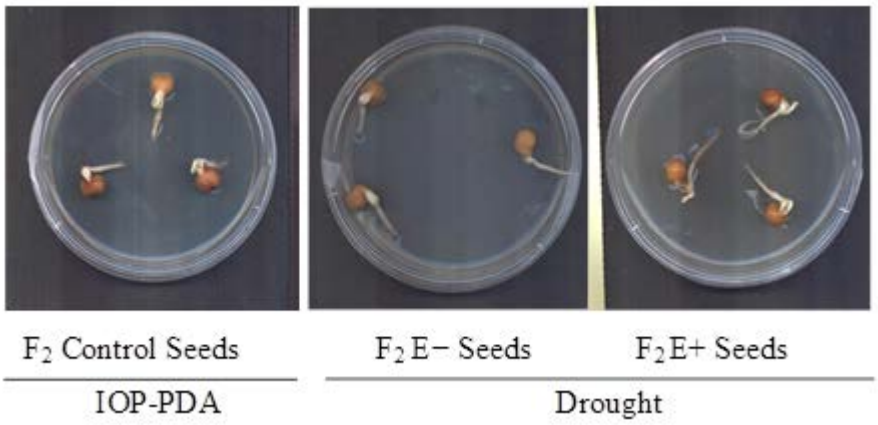
Additionally, ROS production under drought conditions was lower in seeds with endophyte treatment (E+) compared to those with no endophyte (E−) treatment and controls. Here, it was found that endophyte treatment helps in reduction of ROS production and accumulation in chickpea (CDC Vanguard) plant cells. However, the overall ROS production was high for the IOP-PDA medium, indicating high plant stress under an increased osmotic pressure.

### 3.6.6 Comparative analysis of germination rate and fluorescence intensity

Germination percentage on IOP-PDA media decreased ~ 40 % in comparison of drought conditions while fluorescence intensity was increased ~ 250 on IOP-PDA in contrast of drought conditions.

Fluorescence intensity was the measure of ROS production as shown in Figure 3.10. So on IOP-PDA medium there was high ROS production in comparison of drought conditions. It was observed that when there was high germination percentage there was low ROS production. In contrast, if germination percentage was low there was high ROS production. So overall there was an inverse relation between germination percentage and ROS presence (Figure 3.14).

(a)




(b)

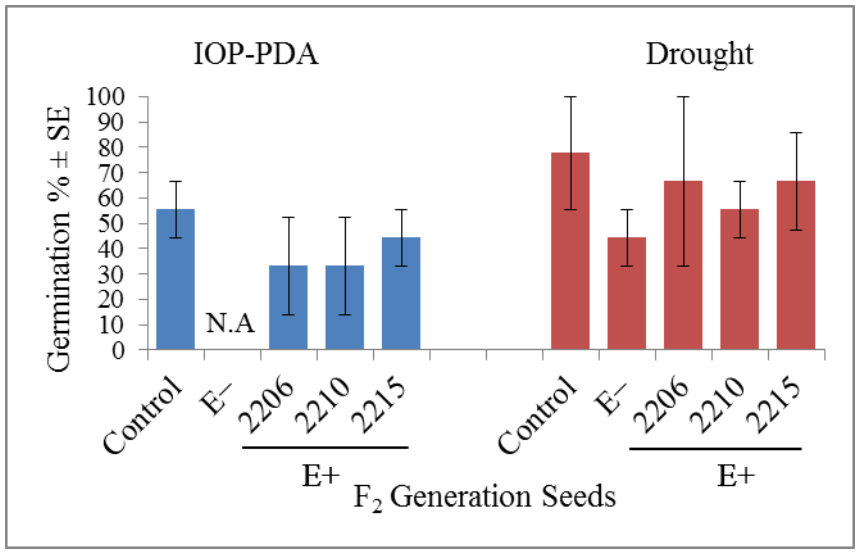



**Figure 3.7** (a*) In vitro* chickpea (CDC Vanguard $F_2$ generation) seed germination after 3 days on IOP-PDA (Increased Osmotic Pressure - PDA) and drought condition medium. (b) Germination rate values are means (n=9) and bars represent standard error (±SE). $F_2$ control seeds were produced from $F_1$ seeds by applying normal conditions, F2 (E−) seed were produced from $F_1$ seeds by applying drought stress without endophytes, $F_2$ (E+) seeds were produced from $F_1$ seeds by applying drought stress with endophytes. Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments.

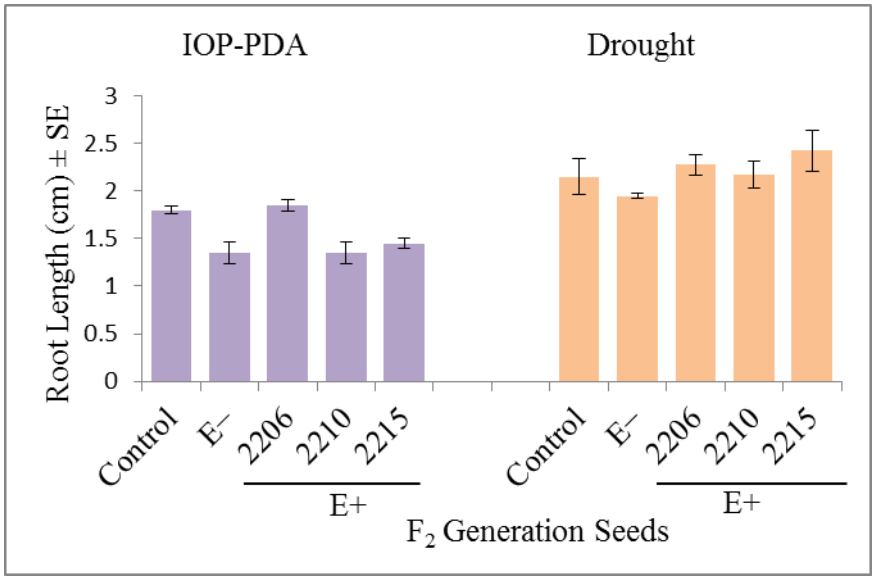



**Figure 3.8** Chickpea (CDC Vanguard $F_2$ generation) root length observed on the 7$^{th}$ day after germination for seeds using the IOP-PDA (Increased Osmotic Pressure - PDA) and drought condition medium. Mean values for root length (n=9) are provided, and the bars represent standard error (±SE). The $F_2$ seeds produced from $F_1$ seeds as $F_2$ control seeds under normal conditions and $F_2$ (E−) seeds under drought conditions without endophytes, while $F_2$ (E+) seeds under drought conditions with endophytes. Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments.

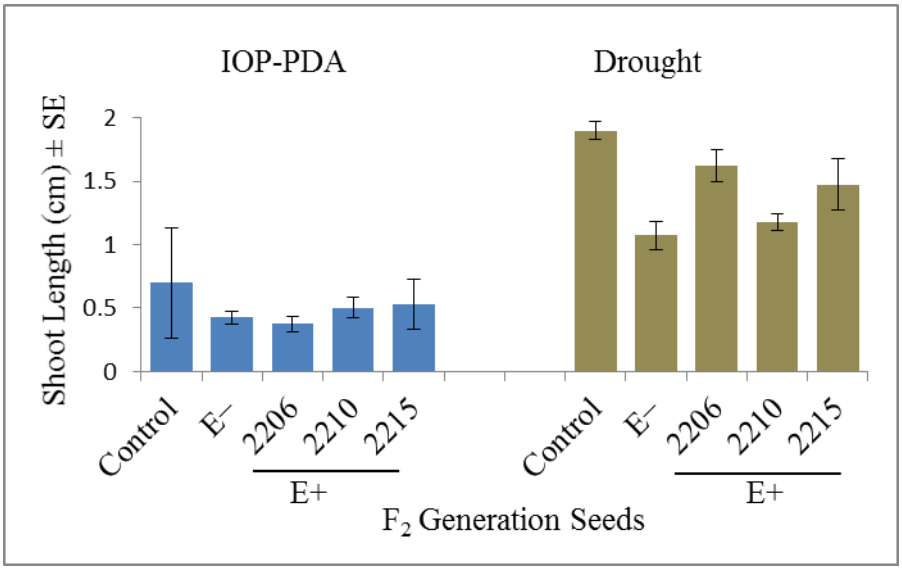
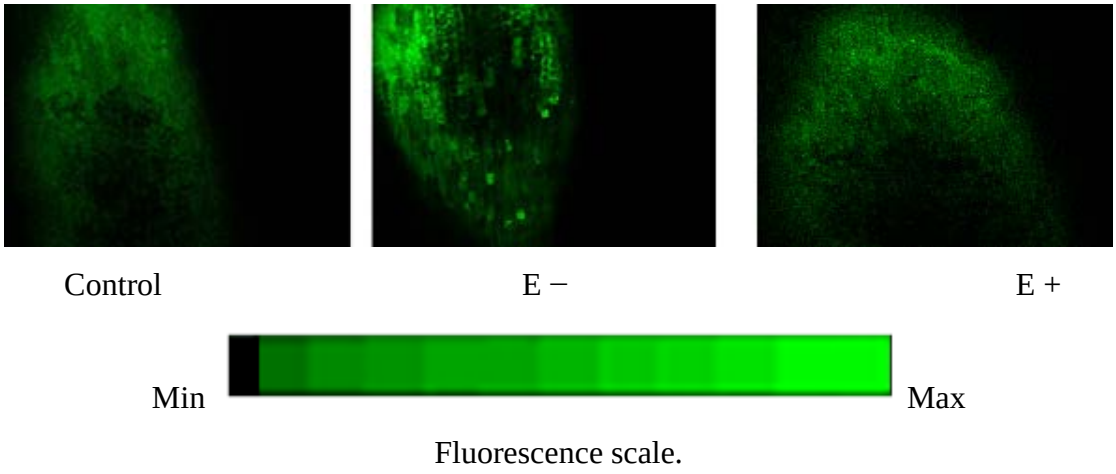
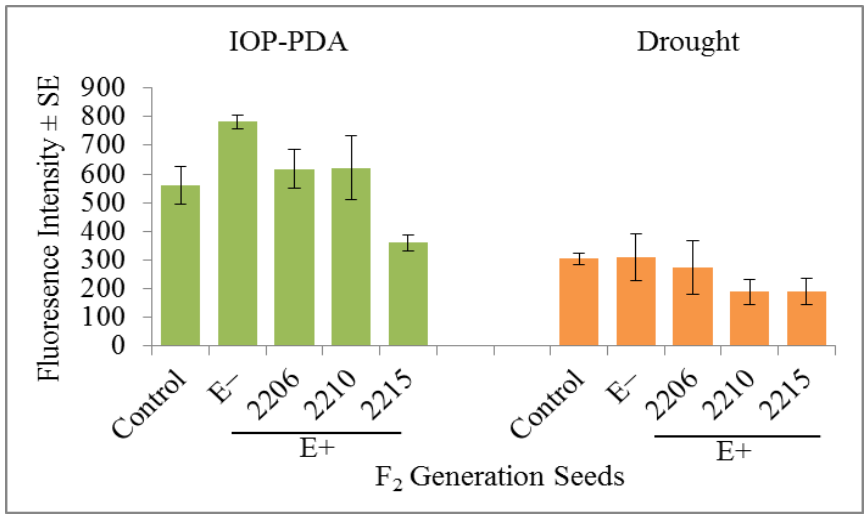



**Figure 3.9** Chickpea (CDC Vanguard $F_2$ generation) shoot length on 9$^{th}$ day after germination using IOP-PDA (Increased Osmotic Pressure - PDA) and drought condition medium. Mean values (n=9) are presented for shoot length values and the bars represent standard error (± SE). $F_2$ control seeds, $F_2$ (E−) seeds, and $F_2$ (E+) seed produced under normal conditions, drought stress without endophytes, and drought stress with endophytes, respectively from $F_1$ generation seeds. Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments.




(a)




(b)

**Figure 3.10** (a) Fluorescence scale - presented for visualization of different colour intensities (b) Detection of oxidative damage by reactive oxygen species production in 6 day old chickpea (CDC Vanguard $F_2$ generation) roots under drought conditions and on the IOP-PDA (Increased Osmotic Pressure - PDA) medium. Values for fluorescence intensity presented as means (n=9) and the bars represent standard error (±SE). $F_2$ control seeds under normal conditions, $F_2$ (E−) seed under drought conditions, and $F_2$ (E+) seeds under drought conditions with endophytes produced from $F_1$ seeds. Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments.

### 3.6.7 Gene expression by QPCR

#### 3.6.7.1 Proline synthesizing gene expression

The result showed that endophytes confer stress tolerance to the next generation and reduce proline synthesizing gene expression in $F_2$ generation chickpea (CDC Vanguard) seeds. $F_2$ generation chickpea seeds produced under drought conditions from $F_1$ plants inoculated with E+ (SMCD 2206, SMCD 2210, and SMCD 2215) down regulated proline synthesizing gene expression compared to E− seeds exposed to the IOP-PDA medium. The endophyte SMCD 2206 produced a 4-fold downregulation of proline synthesizing gene expression compared to $F_2$ generation seeds that received no endophyte treatment using the IOP-PDA medium. Furthermore, while under drought conditions, the proline synthesizing gene down regulated in $F_2$ generation seeds of plants inoculated with fungal endosymbiotic SMCD 2206 and SMCD 2210 strains but was up regulated in seeds treated with bacterial endosymbiotic SMCD 2215 strain (Figure 3.11). Overall, the downregulation of the proline gene in endophyte treated $F_2$ generation seeds indicate that both fungal and bacterial endophytes helped reduce oxidative damage to host under stress conditions.

#### 3.6.7.2 Superoxide dismutase (SOD) gene expression

Superoxide dismutase **(**SOD) gene expression was down regulated in $F_2$ generation chickpea seed produced under drought conditions from $F_1$ plants treated with endophytes (SMCD 2206, SMCD 2210, and SMCD 2215), while it was up regulated in seeds with no endophytic treatment on IOP-PDA medium. However, the mechanism of action is not yet clear. Moreover, SMCD 2206 and SMCD 2210 showed more downregulation than SMCD 2215 on the IOP-PDA medium. Overall, there was downregulation of the SOD gene in $F_2$ generation seeds produced under drought conditions from $F_1$ plants inoculated with SMCD 2206, SMCD 2210. In contrast, this became up regulated following SMCD 2215 treatment under drought conditions (Figure 3.12)

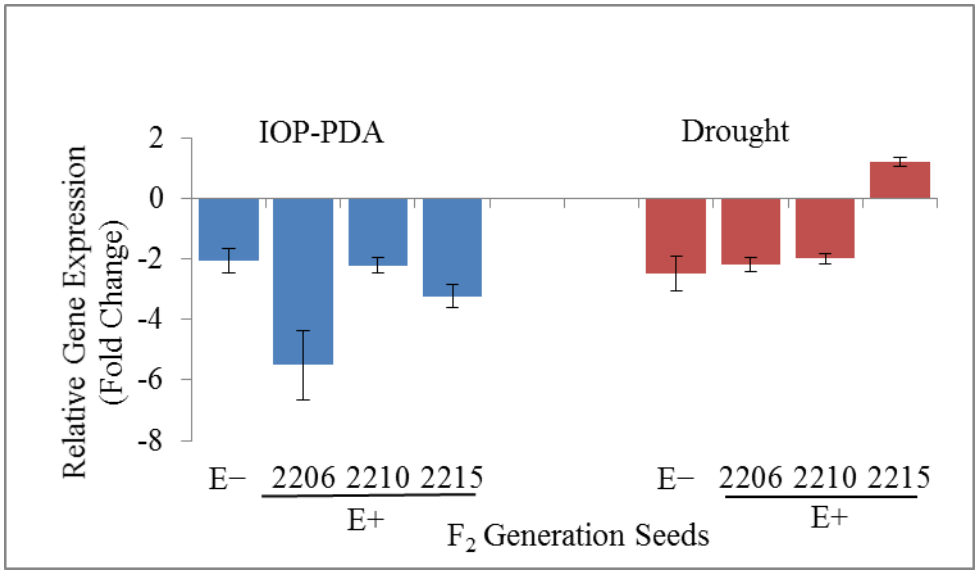



**Figure 3.11** Relative gene expression of Proline in chickpea (CDC Vanguard $F_2$ generation) first leaves on IOP-PDA (Increased Osmotic Pressure - PDA) and drought condition. F2 control seeds produced from $F_1$ seeds by applying normal conditions, $F_2$ (E−) seeds produced from $F_1$ seeds by applying drought stress conditions without endophytes, and $F_2$ (E+) seeds produced from $F_1$ seeds by applying drought stress with endophytes. Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments. The chickpea actin gene was used as an internal control to normalize gene expression. In addition, $F_2$ chickpea control sample produced under normal conditions was used as calibrator/untreated control. The formula $2^{-\Delta\Delta CT}$ = [(CT gene of interest - CT internal control) sample A - (CT – gene of interest - CT internal control) sample B] (Schmittgen and Livak 2008) was used to calculate the gene expression to determine fold changes. Means of three replicates were used to obtain the relative gene expression.

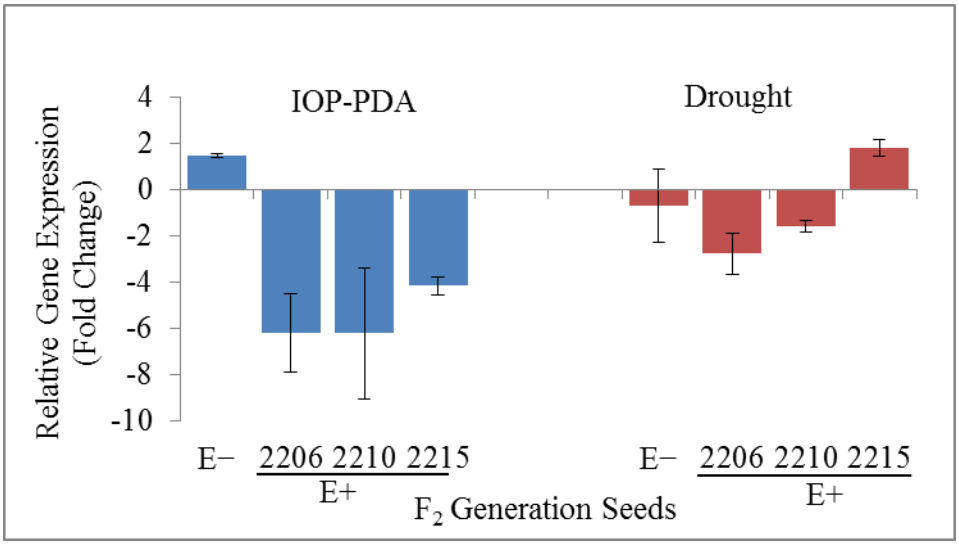



**Figure 3.12** Superoxide dismutase (SOD) relative gene expressions in the first leaves of chickpeas (CDC Vanguard $F_2$ generation) under IOP-PDA and drought stress conditions. $F_2$ control seeds under normal conditions, $F_2$ (E−) seed under drought conditions. $F_2$ (E+) seeds with endophytes under drought conditions were produced from $F_1$ seeds. Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments. The chickpea actin gene was used as an internal control to normalize gene expression. In addition, $F_2$ chickpea control sample produced under normal conditions was used as calibrator/untreated control. The formula $2^{-\Delta\Delta CT}$ = [(CT gene of interest - CT internal control) sample A - (CT – gene of interest - CT internal control) sample B] (Schmittgen and Livak 2008) was used to calculate the gene expression to determine fold changes. Means of three replicates were used to obtain the relative gene expression.

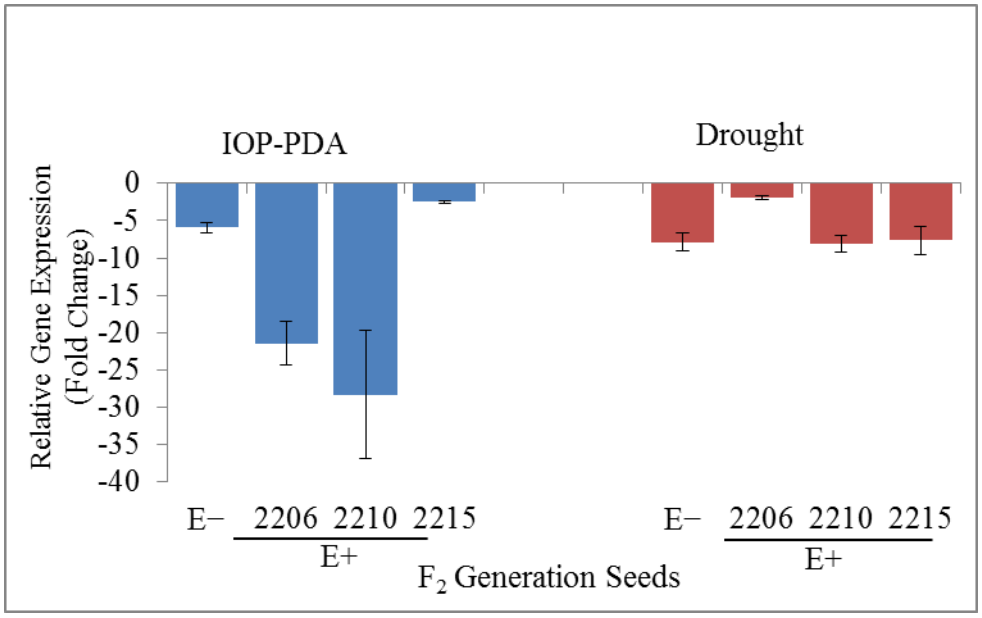



**Figure 3.13** Dehydrin relative gene expressions in the first leaves of chickpeas (CDC Vanguard $F_2$ generation) on IOP-PDA and drought stress conditions. $F_2$ control seeds, $F_2$ (E−) seeds, and $F_2$ (E+) seeds were produced under normal conditions, drought stress conditions without endophytes, and drought stress with endophytes, respectively, from $F_1$ generation seeds. Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments. The chickpea actin gene was used as an internal control to normalize gene expression. In addition, $F_2$ chickpea control sample produced under normal conditions was used as calibrator/untreated control. The formula $2^{-\Delta\Delta CT}$ = [(CT gene of interest - CT internal control) sample A - (CT – gene of interest - CT internal control) sample B] (Schmittgen and Livak 2008) was used to calculate the gene expression to determine fold changes. Means of three replicates were used to obtain the relative gene expression.

#### 3.6.7.3 Dehydrin gene expression

$F_2$ generation chickpea seeds produced under drought conditions from $F_1$ plants inoculated with E+ (SMCD 2206, SMCD 2210) resulted in an approximately 20-fold downregulation of dehydrin gene expression compared to E− seeds using the IOP-PDA medium (Figure 3.13).

Under drought conditions, the dehydrin gene was down regulated in $F_2$ generation seeds from plants inoculated with SMCD 2215. However, as previously mentioned, SOD and proline were up regulated following SMCD 2215 treatment.

### 3.6.8 Protein content

The effects of endophytes on seed quality in regard to protein content in $F_2$ generation chickpeas (CDC Vanguard). Here, It was found that chickpea seeds produced under drought conditions from seeds that had been treated with endophytes (SMCD 2206, SMCD 2210, and SMCD 2215) had higher 1.0 - 2.2 % protein content than controls E (−) and drought (E−) (Table 3.2).

## 3.7 Discussion

The present study investigated the role of selected fungal and bacterial endophytes on improved stress tolerance in 2$^{nd}$ generation chickpea seeds under drought stress. Important seed germination and plant growth parameters were assessed and compared with a shift in expression of antioxidant genes as well as ROS-toxic molecules accumulation in plant tissue; the two major indicators of the plant physiological health and resilience against abiotic stress (Sharma et al. 2012; Das and Roychoudhury 2014). Drought is a widespread problem and a major constraint in pulse production (Toker and Mutlu 2011). Plants may be affected by drought at any stage of the life cycle; however, certain stages including germination and seedling growth are critical **(**Pessarakli 1999). Specifically, the germination stage provides the basis for healthy plant development. For this study, a chickpea was exposed to decreased water availability (PEG-PDA) and increased mechanical impedance and root penetration resistance created *in vitro* using IOP-PDA medium to imitate drought conditions. Generally, crop cultivated under field conditions,

root elongation in drying soil is also limited by a combination of mechanical impedance and water stress (Bengough et al. 2011).

**Table 3.2** Seed protein analysis of $F_2$ generation chickpeas (Cultivar: CDC Vanguard; Generation: $F_2$ control (E−) seeds produced from $F_1$ seeds by applying normal conditions without endophytes, $F_2$ drought (E−) seeds produced from $F_1$ seeds by applying drought stress without endophytes, and $F_2$ (E+) produced from $F_1$ seeds by applying drought stress with endophytes. Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments. Different letters indicated significant differences in the ANOVA ($P \leq 0.05$), followed by the *post hoc* Tukey HSD tests).

| Variety/Treatement | Protein (%) dry basis |
|---|---|
| CDC Vanguard/Control E ─ (F2) | 9.74[a] |
| CDC Vanguard/Drought E ─ (F2) | 10.3[a] |
| CDC Vanguard/ E+ SMCD2206 (F2) | 12.5[c] |
| CDC Vanguard/ E+ SMCD2210 (F2) | 11.3[b] |
| CDC Vanguard/ E+ SMCD 2215 (F2) | 10.4[a] |

[a]Values with same letter are not significantly different from each other ($P \leq 0.05$).

### 3.7.1 Germination rate and root and shoot length

Endophytes tested in this study have the capacity to enhance wheat tolerance against heat and drought stress in both $F_1$ plants and $F_2$ generation seeds via mycovitality (Hubbard et al. 2013). Under drought stress conditions chickpea (CDC Vanguard) seed germination was enhanced by fungal SMCD 2206, SMCD 2210, and bacterial SMCD 2215 endophytes. Moreover, the results are consistent with other studies (Mastouri et al. 2010; Hubbard et al. 2012) that revealed that specific endophytes can enhance seed germination, root length, and shoot length in stress conditions such as drought. Moreover, seed germination is a vital process

that is necessary for plant growth and development. Thus, increasing seed germination using endophytes will be beneficial for increasing overall agricultural production.

Drought stress reduces root length in chickpeas (CDC Vanguard). The importance of root growth for maintaining crop yield is becoming recognized and of increasing interest to plant breeders (Gewin 2010). This is the case because plant development depends on healthy and longer root systems. Endophytes SMCD 2206, SMCD 2210, and SMCD 2215 assisted in the development of improved root system under drought conditions. The relationship between endophytes and plants produces structural changes such as increased root growth and longer root hairs (Malinowski et al. 2000), as well as some changes at physiochemical and molecular levels.

In addition, longer shoot is an indication of superior plant growth and biomass production. Our results confirm the role of endophytes in improved shoot length development of chickpea under stress conditions. Endophyte treated plants produced 75 % (first harvest), 113 % (second harvest), and 18 % (third harvest) higher shoot biomass than endophyte-free control plants (Ghimire et al. 2009). Plant shoot and root lengths are considered highly sensitive plant response parameters for exposure to any type of stress and are commonly used for measuring plant stress tolerance (Bayoumi et al. 2008). The present study shows that selected endophytes increase root and shoot under drought conditions are in agreement with the results on other crops (Mattos et al. 2008; Khan et al. 2011c).

### 3.7.2 Endophyte reduction of oxidative damage

Under stress conditions, ROS production ($H_2O_2$, $^{\cdot}OH$) leads to oxidative damage, which impact plant growth and in extreme condition leads to plant death (Gill and Tuteja 2010; Sharma et al. 2012; Das and Roychoudhury 2014). However, endophytes produce antioxidants that are known to reduce ROS (Ruma et al. 2013). In the present study ROS was measured in $F_2$ generation E+ and E− chickpea seeds. This analysis demonstrated that selected E+ helps reduce ROS level. Furthermore, it suggests that endophytes reduce oxidative damage in plant cells and antioxidant enzymes may not require upregulation for plant protection. However, the mechanism of action remains unclear. The results are in agreement with and earlier study by Shukla et al. (2015), that determined that endophyte *Trichoderma harzianum* treated wheat plants produced less (13–27 %) $H_2O_2$ compared to untreated (60 %) wheat plants under drought stress.

### 3.7.3 Comparative analysis of germination rate and fluorescence intensity

Macelo and Queila (2013) confirmed the role of ROS in seed dormancy and germination. Here, ROS level and germination percentage comparative analysis was performed. Typically, high concentration of ROS prevent radical emergence; thus, this role of ROS would act at the interface between signaling and deleterious effects (Bailly et al. 2008). As shown in Figure 3.14, there is an inverse relation between germination percentage and ROS presence. The results obtained are consistent with previous studies that report that a high concentration of ROS prevents radical emergence and decreases germination percentage. In this case, the ROS is highly reactive toward biomolecules and when ROS accumulates at a level higher than the threshold, it shifts from a signaling to deleterious role (Bailly et al. 2008).

### 3.7.4 Expression of antioxidant genes

In this study, the role of endophytes in improved seed germination and ROS scavenging seemed also related with orchestrated expression of antioxidant genes in chickpea. Consequently, the impact of endophytes on plants at a molecular level can be better understood by studying antioxidant gene expression. The antioxidant machinery is necessary and has a central role in plant protection from different environmental stresses including salinity, drought, and heat (Gill and Tuteja 2010; Das and Roychoudhury 2014). Many studies have found that gene expression for antioxidant enzymes is up regulated to protect plants from cellular injury caused by the ROS response to stress (Sharma et al. 2012; Kukreja et al. 2005). Proline, SOD, and dehydrin gene expression was assessed in $F_2$ generation chickpea seeds produced by drought conditions and inoculation with and without selected endophytes. It was found that endophytes play a role in reducing oxidative damage. Specifically, endophyte treatment down regulates the proline, SOD, and dehydrin gene expression in $F_2$ generation chickpea seeds. Furthermore, the fungal SMCD 2206, SMCD 2010 and bacterial SMCD 2215 endophytes had more considerable effect on the downregulation of proline and SOD on IOP-PDA medium.

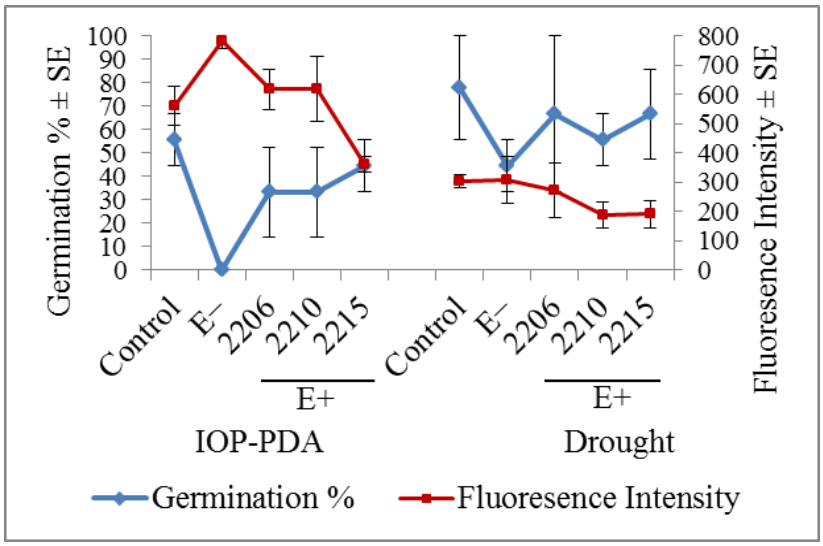



**Figure 3.14** Comparative analysis of germination rate (3rd day) and fluorescence intensity in chickpeas (CDC Vanguard $F_2$ generation) on IOP-PDA (Increased Osmotic Pressure - PDA) and under drought conditions. Endophyte treatments (E+) include SMCD 2206, SMCD 2210, and SMCD 2215. $F_2$ control seeds produced from $F_1$ through application of normal conditions. In addition, $F_2$ (E−) and $F_2$ (E+) seed produced from $F_1$ by applying drought stress with and without colonization, respectively. Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments.

However, the bacterial 2215 endophyte treatment did not show much noticeable downregulation of dehydrin on IOP-PDA medium, but fungal SMCD 2206, SMCD 2010 endophytes showed substantial downregulation. Furthermore, under drought conditons there was downregulation in treatments of SMCD 2206, SMCD 2210 and SMCD 2215 but not substantial except that SMCD 2206 noticeably downregulated SOD. In addition, under drought conditions bacterial SMCD 2215 strain showed considerable effect on regulation of dehydrin antioxidant gene expression by upregulating the proline and SOD while dowregulating dehydrin. It is known that dehydrin gene expression has been observed to be drought-regulated in both drought-tolerant and drought-susceptible cultivars (Wood and Goldsbrough 1997). Because dehydrin is

downregulated in the bacterial SMCD 2215 condition, the simultaneous upregulation of SOD and proline in SMCD 2215 treatment shows that this bacterial endophyte may help by differently regulating gene expression at a molecular level compared to fungal endophytes. Generally, antioxidant gene expression is up regulated under stress conditions to protect the plant (Kukreja et al. 2005). The overall, comparison of IOP-PDA and drought conditions showed that there was more considerable downregulation on IOP-PDA than drought conditions. However, E+ plants demonstrated downregulation of antioxidant gene expression under stress condition, signifying the importance of endophytes in plant stress management. Therefore, it seems that endophytes lead plants to experience less stress and do not require them to up regulate antioxidant gene expression for protection. These results are in agreement with Tripathi et al. (2013) study, where they inferred that induced chickpea stress was primarily characterized by the induction of abiotic stress responsive genes. Furthermore, the role of fungal species for improvement in plant growth was characterized by downregulation and unaffected expression of the majority of the 19 genes that were studied by Tripathi et al. (2013).

### 3.7.5 Seed nutritional quality

The seed protein of grain legumes is highly affected by the environment. For example, Burstin et al. (2011) and Gueguen and Barbot (1988) found that protein contents in legumes may vary within a variety depending on the environment. Furthermore, all environmental factors that impact nitrogen nutrition may also influence seed protein content through their effect on nitrogen availability (Biarnès et al. 2000). Thus, chickpea seed protein quality is highly determined by the genotype and environmental conditions (Frimpong et al. 2009). As a result, drought is a major factor that impacts the nutritional quality of chickpea seeds. In the current study, the protein content was evaluated in $F_2$ generation chickpea seeds produced under drought stress in both the presence and absence of selected endophytes. The results reported that the SMCD 2206, SMCD 2010, and SMCD 2215 endophyte treatments increased the chickpea seed protein content >1 % compared to controls. Consequently, endophytes benefit not only plant growth and productivity, but also increase the nutritional quality of the seed with important dietary properties.

Interestingly, this study depicted that stress tolerance provided by endophytic fungi can be transferred to the $F_2$ generation as the results of molecular level changes. Although still in its

infancy, the possible facilitator of these changes may be related to epigenetics. In some previous studies, the role of epigenetic changes in abiotic stress responses and tolerance has been reported (Sahu et al. 2013; Yuan et al. 2013). For instance, Hubbard et al. (2014) found that colonization of drought stressed wheat seedlings using selected fungal endophytes corresponded with altered DNA methylation that matched unstressed plants. In the present study, the presence of improved germination, and root and shoot length in $F_2$ generation plants confirms the inheritance of stress tolerance that likely result from epigenetic changes.

## 3.8 Conclusion

The endophytes selected in this study promote higher seed germination, root length, and shoot length in $F_2$ generation chickpeas under drought stress by reducing oxidative damage. Moreover, the presence of endophytes reduced oxidative damage and downregulate antioxidant genes expression. Thus, it is expected that there is an interaction between microbial and plant cells that result in some epigenetic changes at the molecular level. These can then be inherited to the next generation. Consequently, the symbiotic relationship between endophytes and plants in regard to their drought stress tolerance can be transmitted to the next generation. However, there is further need to study the epigenetic mechanism of inheritance at a molecular level, which could be included in future field studies and experiments for chickpea breeding programmes in drought-prone environments.

## 3.9 Connection to the next study

In the present study the role of fungal endophytes (SMCD 2206, SMCD 2210, and SMCD 2215) was investigated in regard to drought stress tolerance on second generation chickpea (*Cicer arietinum*) seeds. Here, it was found that selected endophytes improved seed germination as well as root and shoot lengths in second generation chickpeas under drought conditions. Additionally, they reduced oxidative damage and demonstrated downregulation of antioxidant genes in endophyte treated plants. Moreover, endophytes improved seed protein content. However, different crops may behave differently, even with the same microbes (Gundel et al. 2012; Qawasmeh et al. 2012). Chickpeas are drought resistant, whereas peas are considered

drought sensitive (Toker and Yadav 2010) .While the pea crops are relatively more sensitive to drought (Wilson et al. 1985; Pszcz´ et al. 2003), it would be interesting to examine the effects of the same endophytes on pea crops. Overall, results from this and next study will provide useful information that is applicable and may contribute to increased production of different crops.

# CHAPTER 4.

# ROLE OF MICROBIAL ENDOPHYTES IN DROUGHT STRESS TOLERANCE TO 2nd GENERATION ($F_2$) GENERATION PEA (*PISUM SATIVUM*) SEEDS

## 4. 1 Abstract

As the global economy grows, so too will the demand for high quality grain of pea due to its tremendous dietary values. Pea is a primary legume crop and has an important role to ensure food quality, global food security as well as economic development. However, abiotic stress conditions like drought, salinity, and heat have compromised its production. For instance, drought has the most detrimental effects on yield loss and grain quality throughout water limited agro-regions. It mainly affects plant defense system by stimulating overexpression of good genes-often with uncertain consequences on reactive oxygen species (ROS) accumulation in plant tissues/organs. The aim of this study was to use the agricultural biologicals-based on beneficial endophytes as a strategy to mitigate pea production problems under such adverse conditions. In this study, positive impact of microbial endophytes was highlighted on improved plant growth: germination, root length, and shoot length. In addition, it was observed that the modulation effect of endophytes in lowering level of ROS in leaves that correlated with down-regulated expression of proline, superoxide dismutase (SOD), and manganese superoxide dismutase (MnSOD) antioxidants. Moreover, the protein content was increased in 2nd generation seeds produced from $F_1$ plants colonized by endophytes. It was also found that some fungal endophytes help improve pea growth, root and shoot length, by decreasing the oxidative damage caused by ROS in pea under drought stress. Symbiotic microbe-pea interaction also resulted in improved seed protein content. Although, it was confirmed that fungal endophytes confer

drought tolerance to $F_2$ generation peas; the mechanism of stress tolerance inheritance remains unclear. A possible reason for stress tolerance inheritance might be the variance in genetic and molecular system via some epigenetic mechanism that still needs to be discovered.

## 4.2 Introduction

Pea is a diploid (2n = 14 chromosomes) and especially self-pollinated crop which comes among the earliest grain legumes of the old world (Zohary and Hopf 2000). It is an annual, cool seasoned leguminous crop consisting of seeds in a pod that varies in color (yellow and green). Pea is grown in different parts of world and mainly consists of two types: garden peas (wrinkled seeds) and field peas (smooth surface seeds). Peas originated somewhere in Ethiopia or the Mediterranean basin and Central Asia (Vavilov 1951). In addition, peas are produced worldwide including India, U.S. and Russia. However Canada, France and China are leading pea producer (USA Dry Pea and Lentil council, Accessed online Dec 2015). In Canada, Saskatchewan accounts for two-thirds of the dry pea area, Alberta for nearly one-third, and the remainder are seeded in Manitoba and British Columbia ("Canada: Outlook For Principal Field Crops, October 22, 2015 - Agriculture And Agri-Food Canada (AAFC)"). According to Statistics Canada and industry consultations, the dry pea seeded area in Canada was 1.34 M ha in 2013–2014 and production was 3.96 Mt ("Canada: Outlook For Principal Field Crops, October 22, 2015 - Agriculture And Agri-Food Canada (AAFC)")

Pea is mainly grown for human food and animal feed. It provides nutritional food as it contains high amount of protein and has high resistant starch content (Angioloni and Collar 2013). Moreover, it has comparatively low amount of anti-nutritional factors (Wang, Hatcher, and Gawalko 2008). Additionally, it provides raw material for many food companies. Since it helps in nitrogen fixation, it is also grown in rotation with cereal crops to conserve soil fertility (Crews and Peoples 2004).

Pea crops are particularly susceptible to drought stress (Wilson et al. 1985), which has many negative effects on pea growth and development, including stoppage of nitrogen fixation and reduction of the total biomass (Cousin 1997). Water scarcity results in drought conditions for plant growth and development. The continuous changes in climatic conditions are an indicator of more prevalent and high intensity drought periods in the future (IPCC 2014). The major part of

agricultural production is affected by drought conditions, which limit optimum production (Farooq et al. 2009) and increase the difference from required demand. Drought affects the overall development of plants at molecular, proteomic, and physiological level, resulting in reduction of the quantity and quality of crops produced (Alqudah et al. 2011). Under drought condition, there is an increase in level of ROS (reactive oxygen species) that leads to oxidative damage to different parts of the plant (Gill and Tuteja 2010; Das and Roychoudhury 2014). However, plants have antioxidant defense system (Gill and Tuteja 2010).

Plants antioxidant system consist of multiple enzymatic (SOD, MnSOD) and non-enzymatic (proline, dehydrin) defense molecules involved in cell stress tolerance by scavenging ROS (Gill and Tuteja 2010). Proline assesed in this study belongs to pyrroline-5-carboxylate reductase protein which have catalytic activity and helps in L-proline biosynthesis pathway (data not shown). In addition, dehydrin belongs to group 2 LEA (late embryogenesis abundant) proteins and there accumulation provide stress tolerance (Hanin et al. 2011). However, under extreme environmental conditions there is imbalance in ROS production and antioxidants scavenging which leads to oxidative damage (Gill and Tuteja 2010). Environmental changes that lead to conditions such as drought are unavoidable, but using fungal endophytes can reduce the severity of damage caused by drought stress. Previously, many studies (Rodriguez and Redman 2008; Hubbard et al. 2012; Waqas et al. 2012; Ghabooli et al. 2013) have been conducted on the beneficial role of endophytes in relation to plant development and stress tolerance.

Endophytes are the microorganisms living inside the parts of plants that show no disease symptoms (Clay and Schardl 2002; Schulz and Boyle 2005) and they are known plant companions in adverse environmental conditions (Redman et al. 2002; Read 1999). Their relation with plants improves plant growth and stress tolerance including modification of the microbial community of soil for consecutive crops (Singh et al. 2011; Ellouze et al. 2013; Iqbal et al. 2013). However, there are different theories for endophyte-induced changes in plant activity. For instance, these changes may be caused by phytohormone secretion (Waqas et al. 2014), improved nutrition acquisition (Singh et al. 2013), or by changing the gene expression of different biochemical pathways (Sherameti et al. 2008). Furthermore, endophytes have capacity to transmit horizontally or vertically, and their transmission provides better health to the host compared to non-colonized hosts (Clay and Schardl 2002). The extent of symbiosis depends on plants and microbes genotypes (Gundel et al. 2012; Qawasmeh et al. 2012). In addition, recent

studies using wheat have proven the role of fungal endophytes in drought tolerance (Hubbard et al. 2013) and inheritance to $F_2$ generation seeds through the possible mechanism of epigenetic changes (Hubbard et al. 2014). Epigenetic changes involve heritable changes in organism that do not cause any change in DNA sequences (Dhar et al. 2014). These consist of modifications such as DNA methylation, which promotes changes in gene expression engaged for stress tolerance, and these modifications can be inherited to next generation (Angers et al. 2010).

Until now, no research has been conducted on the impact of endophytes on pea stress tolerance and inheritance to the next generation. The application of any methods that can increase pea crop production will be beneficial to meet the increasing demand of the growing population. The role of endophytes can show us a path for better plant growth and development under stress conditions.

## 4.3 Hypothesis

1. It was hypothesized that $F_2$ seeds produced from pea $F_1$ plant treated with endophytes will have enhanced germination and root, shoot length than no endophyte treatments.
2. Secondly, it was hypothesized that there will be downregulation of antioxidant genes if endophytes confer stress tolerance to second generation pea seeds. Moreover, it was expected that different enzymatic and non-enzymatic antioxidant genes will be expressed differently in different endophytic treatments depending on the endophyte's compatibility with the plant.
3. The ROS are produced under stress conditions; therefore, it was also expected that if endophytes help the stress tolerance of plants then the seed produced with endophytes will have less ROS production compared to seed produced without endophytes.
4. It was hypothesized that, if endophytes help in stress tolerance, then the nutritional quality of endophyte inoculated seeds will be better compared to seeds that are not inoculated.

## 4.4 Objectives

The objectives were to study the effect of selected endophytes on $F_2$ pea seeds produced from $F_1$ plants inoculated and uninoculated with endophytes under drought. Accordingly, 5 % polyethylene glycol (PEG) (PDA amended with 5 % PEG) and increased osmotic pressure in PDA medium (IOP-PDA) were used to create *in-vitro* drought conditions. The following objectives were determined.

1 To assess seed germination capacity, root length, and shoot length in second generation pea seeds produced from $F_1$ plants with and without endophytes under drought stress.

2 Determine the effect of endophytes on antioxidant genes expression in second generation pea seeds under drought stress.

3 To study the effect of endophytes on oxidative stress in second generation pea seeds under drought stress by measuring reactive oxygen species.

4. To see the effect of endophytes on second generation pea seeds nutritional quality (protein content) under drought stress.

## 4.5 Materials and Methods

### 4.5.1 Media preparation

Potato Dextrose agar (PDA) (EMD, Germany) with 2 % agar and PDA modified with 5 % polyethylene glycol 8000 (PEG, Amersco, Solon, Ohio, USA) were used. Typically, for *in vitro* studies the PDA medium provides standard moisture conditions, whereas the PEG medium provides drought conditions. The measurement of the firmness of both media for checking the penetration force exerted by media on root tips during germination (PDA with 2 % agar and PDA amended with 5% PEG) was studied by texture analyzer (Stable Microsystems Ltd. Surrey, GU7 1YL, UK) following manufacturer instructions. Moreover, the resistance of growth media was also measured by using a penetrometer according to manufacturer recommendations (Humboldt MFG.CO Illinois, USA). The penetrometer was used to push a disc of 3 cm in diameter on both media with same force, and the reading was recorded. Bengough et al. (2011)

study showed that mechanical impedance reduces the root growth. Overall, the higher penetrometer resistance is a signal of mechanical impedance.

### 4.5.2 Pea seeds, endosymbionts and sterilization protocol

First generation pea (CDC Golden) plants colonized by SMCD 2206 (fungal endosymbiotic *Penicillium* sp.), SMCD 2210 (fungal endosymbiotic *Paraconiothyrium* sp.), and SMCD 2215 (bacterial symbiotic *Streptomyces* sp.) endophytes and non-colonized plants under drought stress were used to produce $F_2$ generation seeds. $F_2$ generation seeds produced under normal conditions without colonization were used as a control. The $F_2$ generation seeds used in this study were obtained from a greenhouse experiment that was previously conducted (Figure 4.1). Seed surface sterilization was done by 95 % alcohol for 3 min, distilled water for 1 min, 6 % *sodium hypochlorite* (LAVO 6, Montreal, Quebec) for 1 min and finally washed with distilled water 3 times (each 1 min). Seed free form microbes (as grown in greenhouse sterile conditions and surface sterilized - Vujanovic, personal communication) were grown on PDA with 2 % agar (increased osmotic pressure - potato dextrose agar IOP-PDA medium) and PDA with 5 % PEG. The first leaves were obtained (Gao et al. 2008; Mustafa et al 2009; Peng et al. 2009) and saved at -80°C (Munoz et al 1998, Peng et al. 2009).

### 4.5.3 Germination rate

Sterilized seeds (free from endophytes) were germinated on increased osmotic pressure (potato dextrose agar IOP-PDA medium) and 5 % PEG medium in petri-plates at room temperature (23˚C) in dark. A seed was considered germinated by emergence of radical and germination rate was expressed by germination percentage (Vujanovic et al. 2015).

### 4.5.4 Plant Morphology

The root length and shoot length were measured on the $6^{th}$ day of germination using a ruler on three randomly selected replicates. The first leaves were collected and stored at - 80˚C until further application in experiments.

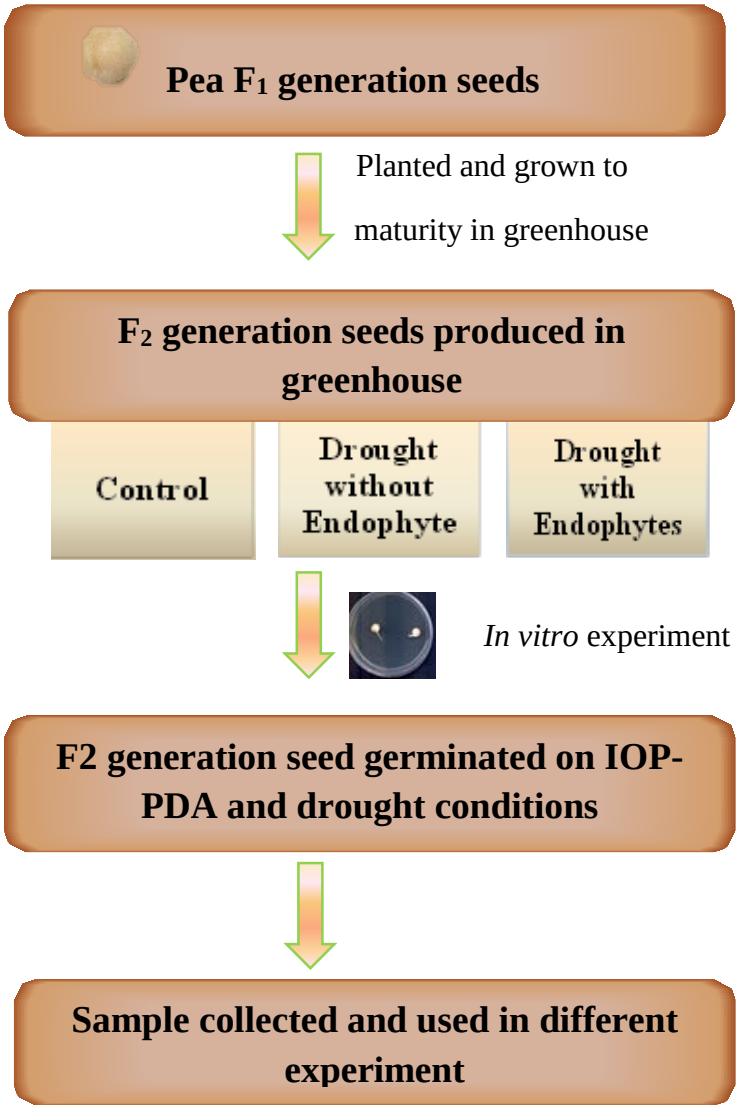



**Figure 4.1** Procedure used to obtain pea (CDC Golden) seeds/samples for this study.

### 4.5.5 ROS (reactive oxygen species) detection by DCFH-DA method

ROS detection by 2', 7'-dichlorofluorescein diacetate (DCFH-DA) depend on epifluorescence, in this the acetate group is cleaved and non-fluorescent 2', 7'-dichlorofluorescein (DCFH) is oxidized to the fluorescent DCF product in a peroxidase-dependent reaction (Cathcart et al. 1983). ROS was studied in 6 day old roots of pea by Nikon C2 Confocal laser scanning microscope (Nikon Canada Inc.). The root of length 0.5 cm was gently excised and quickly washed with water (three times) to remove any ROS produced due to cut stress. In next step radicals/roots tips were placed into a solution of 50 µM DCFHDA (Sigma, Germany) and 20 mM phosphate buffer pH 6.0 and incubated in dark for 30 min. Further, 20 mM potassium phosphate ($KH_2PO_4$) (EMD chemicals Inc. Darmstadt, Germany) buffer (pH 6.0) was used for root quick washes (three times) and fluorescence intensity was measured under the microscope (10× lens) by excitation wavelength of ~ 488 nm and emission wavelength of ~ 525 nm. Samples were treated uniformly (Region of Interest (ROI) and exposure time were set) and processed rapidly under the microscope to avoid photo bleaching. Fluorescence intensity was measured in triplicates.

### 4.5.6 RNA Extraction and cDNA synthesis

RNA was extracted from first leaves of pea by using Aurum$^{TM}$ Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA). In addition RNA concentration and quality was checked by nanodrop 2000c (Thermo Scientific, Wilmington, DE, USA). Furthermore, cDNA synthesis was performed by using the iscript cDNA synthesis kit (Bio-Rad Lab-oratories, Hercules, CA) and PCR machine (Eppendorf Mastercycler, EP gradient S, Germany). Instantly, after RNA extraction cDNA synthesis was performed and stored at -80ºC.

### 4.5.7 Gene expression by QPCR

The pea actin gene was used as an internal control and the relative gene expression of proline, superoxide dismutase (SOD), and manganese superoxide dismutase (MnSOD) antioxidants were studied in the first leaves using primers (Table 4.1) by real time PCR (QPCR) method. The primers selection was primarily based on their specifivity to selected genes. The pea

actin gene was used as an internal control to normalize gene expression. Moreover, $F_2$ pea control sample produced under normal conditions was used as calibrator/untreated control. Gene expression in terms of relative quantification (fold changes) is calculated by the formula $2^{-\Delta\Delta CT}$ = [(CT gene of interest - CT internal control) sample A - (CT – gene of interest - CT internal control) sample B] (Schmittgen and Livak 2008). Gene expression of two different samples can be calculated by the formula $2^{-\Delta\Delta CT}$, where each sample is correlated to an internal control gene, and data may be understood as "the expression of the gene of interest relative to the internal control in the treated sample compared with the untreated control" (Schmittgen and Livak 2008).

Following manufacturer instructions MJ-Mini™ Personal Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) was used at cyclic conditions of 95˚C for 30 sec, 40 cycles of 95˚C for 15 sec, and 60˚C for 30 sec for real time PCR. Furthermore, melt curve analysis was done at 65˚C - 95˚C. The iTaq™ universal SYBR® Green super mix was used and one reaction volume was 20 µl. All reactions were run in triplicates with internal control, NTC (no template control) and negative control. Melt curve analysis was performed to check the specificity and accuracy of gene expression.

### 4.5.8 Seed quality

Seed quality was measured by Intertek Sunwest (Research drive, Saskatoon, Saskatchewan, Canada) from 0.5 g randomly pooled samples. The AOAC (2001.11) method was applied to determine protein (% dry basis) content. Protein content was computed using the Ptn = N x 6.25 conversion factor.

### 4.5.9 Statistical analysis

In the current study, all experiments were executed in triplicate. Mean and standard error were determined for various experiments including seed germination, root length, shoot length, and ROS level. The one way analysis of variance (ANOVA) technique followed by *post hoc* Tukey honest significant difference (HSD) and least significant difference (LSD) tests were applied to determine the statistical significance of protein content at $P \leq 0.05$ using SPSS (IBM SPSS statistic 22).

**Table 4.1** Pea genes specific primer outline

| Gene | Function | Primer sequence | Length of amplicon (bp) | References |
|---|---|---|---|---|
| Actin | Internal Control | F5'gttccacaatgttccctggt 3'<br>R5'attctgcctttgcaatccac 3' | 192 bp | Mustafa et al., 2009. |
| Proline | Antioxidant | F-5'ctttgagatgagtagtagttgcgga3'<br>R-5'ccatgtctagtggccaaattg 3' | 188 bp | Williamson, C.L., Slocum, R.D., 1992. |
| Superoxide Dismutase (SOD) | Antioxidant | F-5' cttgtggtattattgggttgcaagg 3'<br>R-5'caagtgcagtcatatagccattgag 3' | 176 bp | Nakamura et al., 2003. |
| Manganese Superoxide Dismutase (MnSOD) | Antioxidant | F-5'gcagaaaaaccctatcctccgtgct 3'<br>R-5'gctccaaagctccgtagtcg 3' | 138 bp | Wong Vega et al., 1991. |

## 4.6 Results

### 4.6.1 Medium characteristic

The result of texture analyzer showed that PDA medium is ~ 4.5 times harder to penetrate than PDA amended with 5 % PEG. The penetrometers (Humboldt MFG.CO Illinois, USA) also indicated that PDA medium had 0.75 kg/cm$^2$ penetrometer resistance in comparison of 0.2 kg/cm$^2$ resistance of PDA amended with 5 % PEG. Overall, PEG produces dehydration effect by reducing availability of water while PDA creates increased osmotic pressure (IOP).

### 4.6.2 Germination rate

$F_1$ pea plants grown under drought conditions in conjunction with colonization by endophytes (SMCD 2206, SMCD 2210, and SMCD 2215) produced $F_2$ generation seeds that were found to improve germination on the IOP-PDA medium in comparison to non-colonized seeds produced under drought conditions. Here, it is proven that fungal endophytes enhance germination of $F_2$ generation pea seeds under stress conditions. The seed germination of plants

colonized by SMCD 2206 and SMCD 2215 endophytes was even higher than the control plants on the IOP-PDA medium (Figure 4.2). However, under drought conditions (PDA amended with 5 % PEG) colonized plants showed better germination than non-colonized plants, but this remained lower than the control plants.

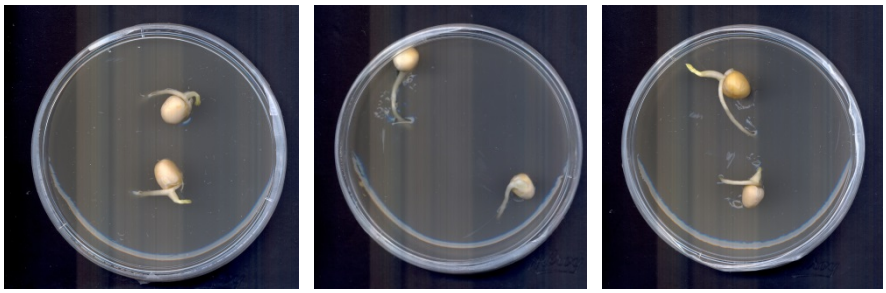
### 4.6.3 Root length

Drought stress reduces root growth, which disturbs plant growth and productivity. Thus, the role of endophytes on root growth was assessed here. It was found that $F_2$ generation pea seeds produced by inoculation of SMCD 2206 and SMCD 2215 endophytes had increased root length in comparison of non-inoculated seed (E−) on the IOP-PDA medium. Moreover, under drought conditions there was better germination in $F_2$ generation seeds of plants inoculated with SMCD 2206, SMCD 2210, and SMCD 2215 compared to $F_2$ generation pea seeds from E− plants (Figure 4.3).

### 4.6.4 Shoot length

Endophytes helped in improved shoot length and growth. The $F_2$ generation seed obtained under drought stress from $F_1$ plant colonized with endophytes (SMCD 2206 and SMCD 2210) got higher shoot length than non-inoculated (E−) plants on IOP-PDA medium. $F_2$ generation seeds produced by inoculation of endophyte (E+) SMCD 2206 had higher shoot length even than control seeds on IOP-PDA medium (Figure 4.4). However under drought conditions the $F_2$ generation seed produced under drought stress from $F_1$ plant inoculated with endophytes SMCD 2215 got higher shoot length than non-inoculated (E−) sample but lower than controls.

(a)




(b)

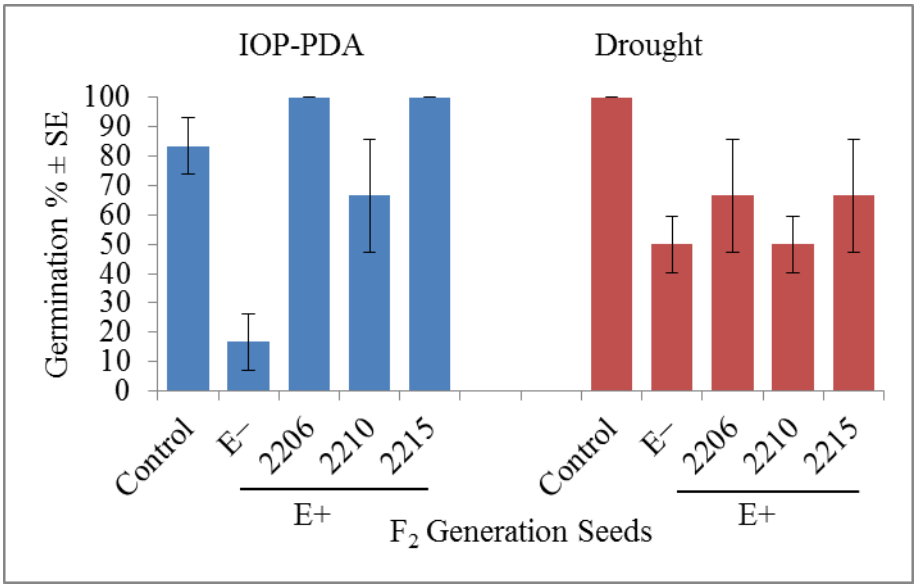



**Figure 4.2** (a) Pea (CDC Golden) $F_2$ generation *in vitro* seed germination after 4 days on IOP-PDA (Increased Osmotic Pressure) and drought condition medium. $F_2$ generation pea $F_2$ (E−) seeds produced from $F_1$ without colonization and $F_2$ (E+) seeds with endophytes under drought conditions where $F_2$ Control seed produced from $F_1$ by applying normal conditions. (b) The mean (n=6) germination percentage is presented and standard error (±SE) is illustrated by bars. Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments.

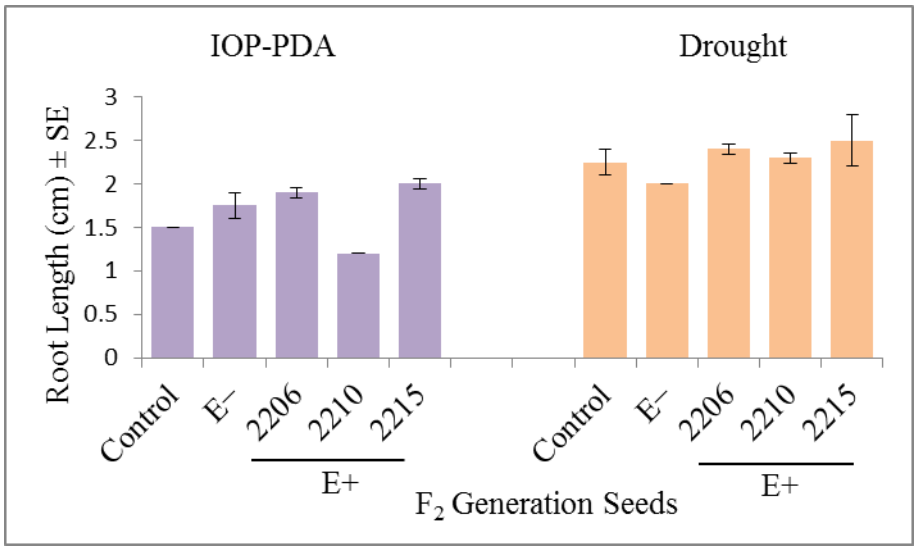



**Figure 4.3** Pea (CDC Golden $F_2$ generation) root length was measured at 6$^{th}$ day of germination on IOP-PDA (Increased Osmotic Pressure) and the drought condition medium. $F_2$ Control seeds originated from $F_1$ by applying normal conditions, $F_2$ (E−) seed without endophytes and $F_2$ (E+) seeds with endophytes originated from $F_1$ by applying drought stress. The mean (n=6) root length values are presented, and bars represent standard error (±SE). Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments.

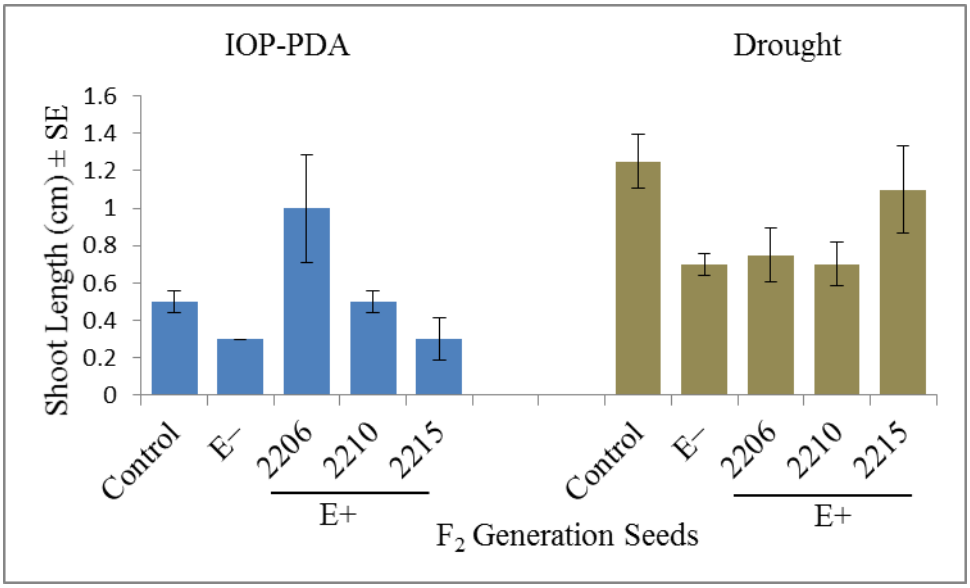
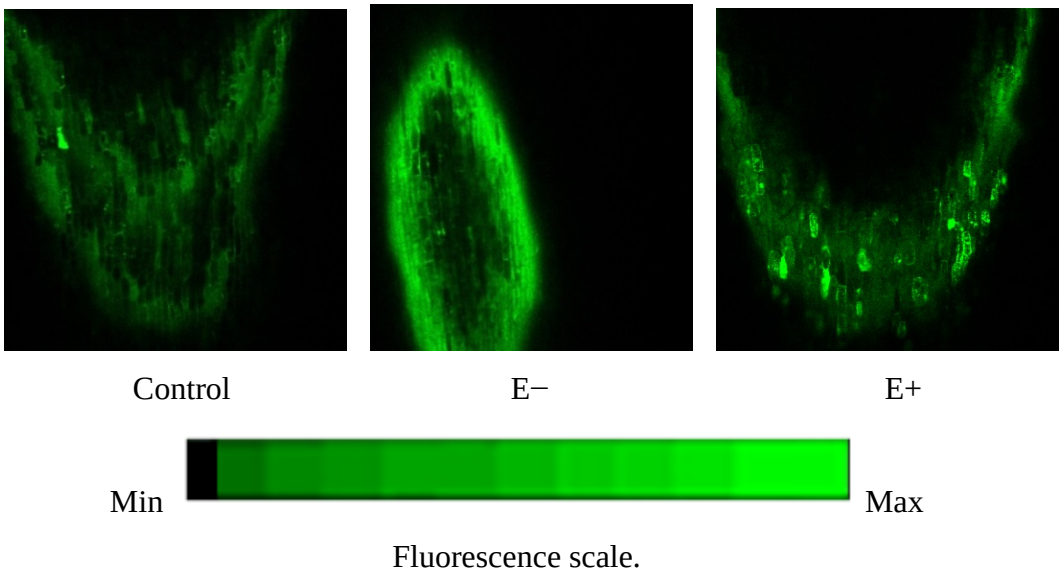
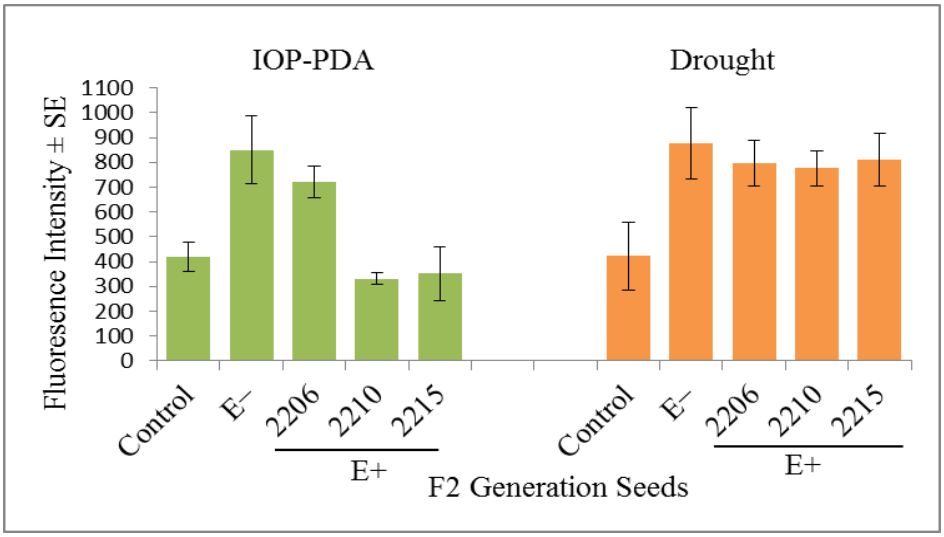



**Figure 4.4** Pea (CDC Golden $F_2$ generation) shoot length was recorded at 6$^{th}$ day of germination on IOP-PDA (Increased Osmotic Pressure) and the drought condition medium. $F_2$ Control seeds produced from $F_1$ by applying normal conditions, $F_2$ (E−) seed produced from $F_1$ by applying drought stress without an endophyte, and $F_2$ (E+) seeds produced from $_{F1}$ by applying drought stress with endophytes. Shoot length values are in means (n=6) and bars represent standard error (±SE). Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments.




(a)




(b)

**Figure 4.5** (a) Fluorescence scale- presented for visualization of different colour intensities (b) Oxidative damage was detected in pea (CDC Golden $F_2$ generation) 6$^{th}$ day old roots on IOP-PDA (Increased Osmotic Pressure) and drought conditions by measuring reactive oxygen species production. $F_2$ Control seeds produced from $F_1$ by applying optimal conditions while non-colonized $F_2$ (E−) seed produced under drought conditions. In addition, colonized $F_2$ (E+) seeds produced from $F_1$ by applying drought stress. Mean (n=6) fluorescence intensity values are presented, and bars

represent standard error (±SE). Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments.

### 4.6.5 ROS detection by DCFH-DA method

The effect of selected endophytes on ROS production was studied. It was observed that under drought stress conditions there is increase in ROS production that is directly proportional to the amount of green fluorescence (Figure 4.5).

$F_1$ pea plants under drought conditions inoculated with endophytes (SMCD 2206, SMCD 2210, and SMCD 2215) produced $F_2$ generation seeds with less ROS compared to plants without inoculation (E−). Moreover, plants inoculated with SMCD 2210 and SMCD 2215 endophytes produced less ROS than controls on the IOP-PDA medium. While ROS production under drought conditions was lower in endophyte treatments (E+) (SMCD 2206, SMCD 2210, and SMCD 2215) in comparison to no endophyte (E−) treatment, it was higher than control seeds.

### 4.6.6 Comparative analysis of germination rate and fluorescence intensity

Germination percentage on IOP-PDA medium was ~ 35 % lower than the drought conditions. Furthermore, fluorescence intensity under drought conditions was comparatively higher (~ 440) than the IOP-PDA medium. Overall, there was an inverse relationship between germination percentage and fluorescence intensity on both medium (Figure 4.9).

### 4.6.7 Gene expression by QPCR

#### 4.6.7.1 Proline synthesizing gene expression

Proline synthesizing gene expression decreased in colonized pea (CDC Golden) $F_2$ generation seed indicating the role of endophytes in stress tolerance inheritance. $F_2$ generation seed produced under drought conditions from $F_1$ generation by inoculation with endophytes (SMCD 2206, SMCD 2210, and SMCD 2215) were down regulated in terms of proline gene

expression compared to non-inoculated seeds on the IOP-PDA medium. Proline gene expression was downregulated 100-fold in $F_2$ generation seeds produced from parents treated with endophyte SMCD 2210 compared to no endophyte treatment on IOP-PDA medium. Under drought conditions, only seeds of $F_2$ generation SMCD 2206 showed 10-fold downregulation (Figure 4.6).

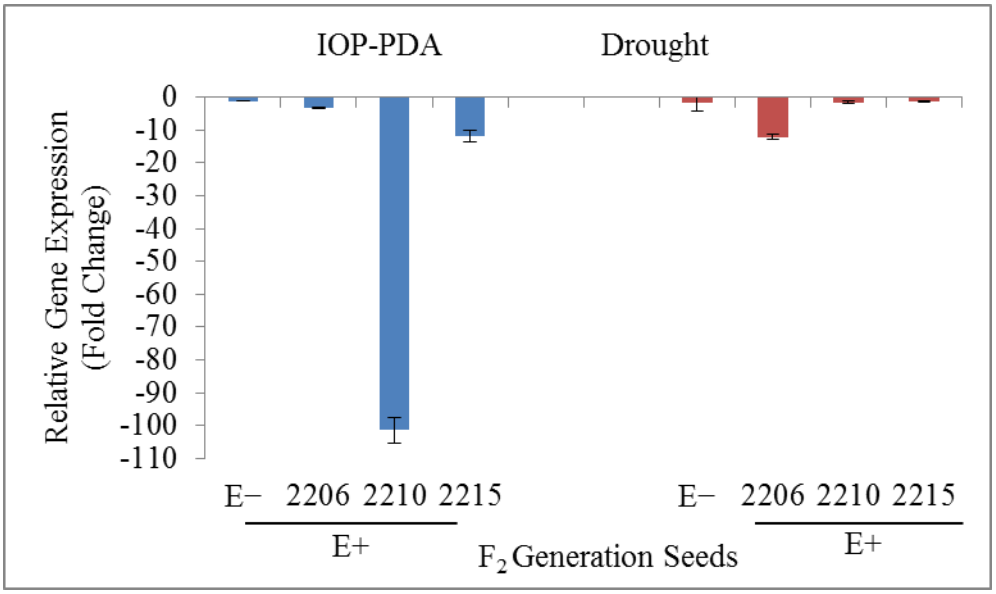



**Figure 4.6** Relative gene expression of proline in pea (CDC Golden $F_2$ generation) first leaves on IOP-PDA (Increased Osmotic Pressure) and drought condition. $F_2$ (E−) without endophytes and $F_2$ (E+) seed with endophytes produced from $F_1$ by applying drought stress while $F_2$ control seeds produced from $F_1$ by applying normal conditions. Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments. The pea actin gene was used as an internal control to normalize gene expression. Moreover, $F_2$ pea control sample produced under normal conditions was used as calibrator/untreated control. The formula $2^{-\Delta\Delta CT}$ = [(CT gene of interest - CT internal control) sample A - (CT – gene of interest - CT internal control) sample B] (Schmittgen and Livak 2008) was used to calculate the gene expression to determine fold changes. Means of three replicates were used to obtain the relative gene expression.

#### 4.6.7.2 Superoxide dismutase (SOD) gene expression

Fungal endophytes downregulate the SOD gene expression, indicating that presence of endophytes reduces stress conditions for plants. The SOD gene expression downregulated 50-fold in $F_2$ generation pea seeds produced under drought conditions from $F_1$ plant colonization with SMCD 2210 and 10-fold with SMCD 2215 in comparison to E− seeds on the IOP-PDA medium. However, under drought conditions, there was no considerable downregulation of SOD gene in $F_2$ generation seeds of plants inoculated with SMCD 2206 and SMCD 2210 and indication of SOD upregulation when treated with SMCD 2215 (Figure 4.7).

#### 4.6.7.3 Manganese superoxide dismutase (MnSOD) gene expression

It was found that plant antioxidant gene expression was altered in the company of fungal endophytes. $F_2$ generation pea seeds produced under drought conditions from $F_1$ plant inoculated with E+ (SMCD 2210 and SMCD 2215) down regulated MnSOD gene expression in comparison of E− seeds on the IOP-PDA medium while SMCD 2206 up regulated MnSOD gene expression (Figure 4.8). Under drought conditions, there was downregulation of genes in $F_2$ generation pea seeds produced under drought conditions from $F_1$ plants inoculated with E+ (SMCD 2206). However, there was no considerable gene downregulation when inoculated with SMCD 2210 and SMCD 2215 in comparison to E (−).

### 4.6.8 Protein content

The protein content in $F_2$ generation pea (CDC Golden) seeds produced under drought conditions from $F_1$ plants inoculated with E+ (SMCD 2206, SMCD 2210, and SMCD 2215), no inoculation E− (controls and drought), and found that endophytes treatment (E+) considerably increased the protein content compared to no endophyte treatment ( E−) under drought conditions (Table 4.2).

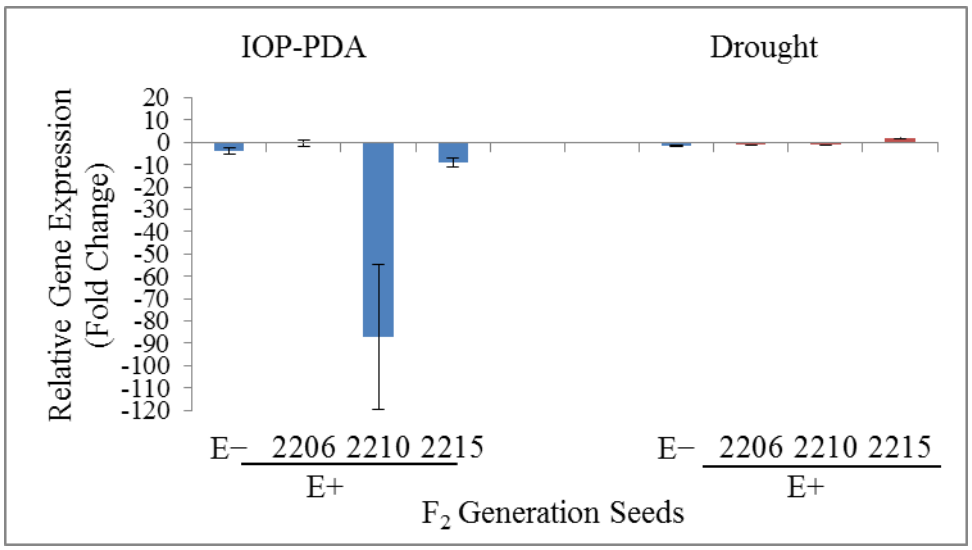



**Figure 4.7** SOD relative gene expression in pea (CDC Golden $F_2$ generation) first leaves on IOP-PDA (Increased Osmotic Pressure) and drought stress conditions. $F_2$ Control seeds produced from $F_1$ by applying normal conditions, $F_2$ (E−) seed produced from $F_1$ by applying drought stress without an endophyte, and $F_2$ (E+) seeds produced from $F_1$ by applying drought stress with endophytes. Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments. The pea actin gene was used as an internal control to normalize gene expression. Moreover, $F_2$ pea control sample produced under normal conditions was used as calibrator/untreated control. The formula $2^{-\Delta\Delta CT}$ = [(CT gene of interest - CT internal control) sample A - (CT – gene of interest - CT internal control) sample B] (Schmittgen and Livak 2008) was used to calculate the gene expression to determine fold changes. Means of three replicates were used to obtain the relative gene expression.

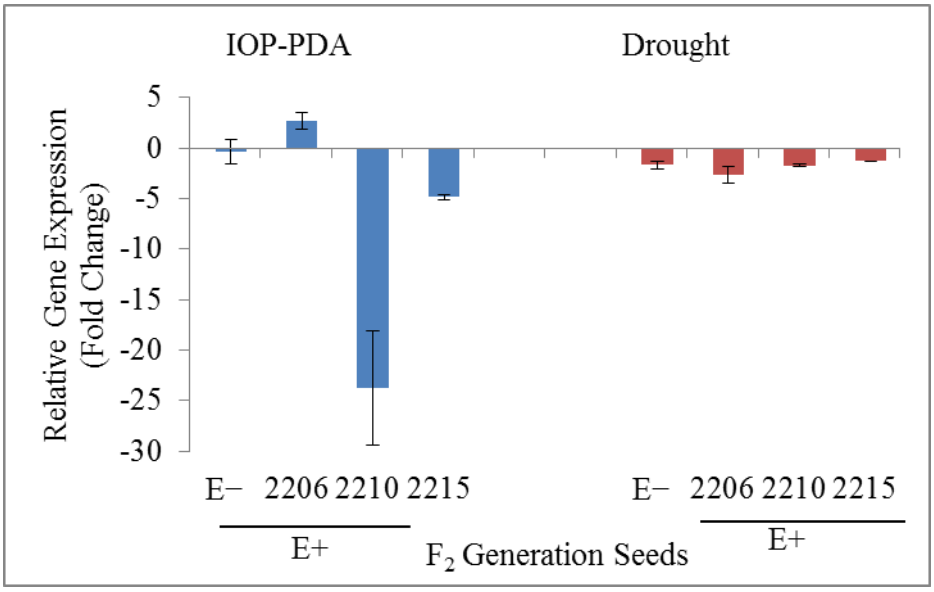



**Figure 4.8** Manganese superoxide dismutase (MnSOD) relative gene expression was determined in pea (CDC Golden) first leaves on IOP-PDA (Increased Osmotic Pressure) and drought stress conditions. $F_2$ Control seeds produced from $F_1$ by applying normal conditions, $F_2$ (E−) and $F_2$ (E+) seed produced from $F_1$ by applying drought stress without an endophyte and drought stress with endophytes, respectively. Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments. The pea actin gene was used as an internal control to normalize gene expression. Moreover, $F_2$ pea control sample produced under normal conditions was used as calibrator/untreated control. The formula $2^{-\Delta\Delta CT}$ = [(CT gene of interest - CT internal control) sample A - (CT – gene of interest - CT internal control) sample B] (Schmittgen and Livak 2008) was used to calculate the gene expression to determine fold changes. Means of three replicates were used to obtain the relative gene expression.

**Table 4.2** Seed protein analysis of second generation peas (Cultivar: CDC Golden; Generation: $F_2$ control (E−) seeds produced from $F_1$ by applying normal conditions without endophytes, drought $F_2$ (E−) seed without endophytes and $F_2$ (E+) seeds with endophytes produced from $F_1$ by applying drought stress. Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments. Different letters indicate significant differences at ($P \leq 0.05$ ANOVA, followed by *post hoc* Tukey HSD test).

| Variety | Protein (%) dry basis |
|---|---|
| CDC Golden/ Control E − (F2) | $24.0^{e}$ |
| CDC Golden/Drought E − (F2) | $16.7^{a}$ |
| CDC Golden/Drought E+ SMCD 2206 (F2) | $17.7^{b}$ |
| CDC Golden/Drought E+ SMCD 2210 (F2) | $18.6^{c}$ |
| CDC Golden/Drought E+ SMCD 2215 (F2) | $19.8^{d}$ |

[a]Values with same letter are not significantly different from each other ($P \leq 0.05$).

## 4.7 Discussion

In present research the effect of endosymbionts on 2nd generation pea seeds drought tolerance and inheritance to next generation was investigated. Seed germination and growths indicators like root and shoot length were studied. In addition change in antioxidant genes expression and effect on ROS production pattern were also documented. Antioxidant genes and ROS are important for studying the abiotic stress tolerance (Gill and Tuteja 2010; Sharma et al. 2012; Das and Roychoudhury 2014). Drought is a major problem and highly impact the pulse production (Khan et al. 2010; Toker and Mutlu 2011) especially pea crop (Wilson et al. 1985). To study this *in vitro* experiment was conducted on IOP-PDA and PDA amended with 5 % PEG medium which impose the drought condition by increasing root penetration resistance and reducing the availability of water, respectively.

### 4.7.1 Germination rate, root and shoot length

Drought sensitivity negatively impacts pea production, especially in earlier growth stages (Wilson et al. 1985, Heath and Dawkins; Lal 1985). Germination is an initial and important process for healthy plant development (Almansouri et al. 2001). Drought adversely affects the germination of pea seeds (Gamze et al. 2005). Thus, it is very challenging to meet the food requirements of the expanding population, and this is even more difficult in adverse climatic conditions (Somerville and Briscoe 2001). Consequently, any method that can help improve pea germination and overall productivity will be useful. In this context, fungal endophytes present a possibility to be explored in relation to plants under changing environmental conditions. We found that some selected fungal endophytes help in better germination and pass this trait to the next generation; however, the mechanism of action is not yet clear. $F_2$ generation pea seeds produced under drought conditions from $F_1$ plants inoculated with E+ have better germination compared to E−. Results from this study are in agreement with Hubbard et al. (2012) study on wheat where it has proven that fungal endophytes improve seed germination by improving plant stress tolerance under stress conditions. Endophytes degrade cellulose in the seed coat and improve the carbon acquisition within the seed, which improves seed germination and vigor (Jerry 1994).

Improved root length was observed in $F_2$ generation pea seeds produced from $F_1$ generation by inoculation compared to endophyte free plants. Plant productivity can be maintained under drought conditions by better root systems, including increased root length (Comas et al. 2013). Moreover, endophytes also increased shoot length in $F_2$ generation pea seeds produced under drought stress from $F_1$ generation by colonization. Endophytes are known as producers of various growth hormones incuding auxin and gibberellin (Khan et al. 2008; Hamayun et al. 2009). The improved growth parameters of a crop result in higher biomass production and increase overall crop productivity. As a result, increased root and shoot development in the presence of fungal endophytes confirms the potential of endophytes to improve plant growth and development in changing climatic conditions

### 4.7.2 Endophytes help in reduction of oxidative damage

Plants response to stress conditions by generation of ROS is an early event among other changes such as activation of transcription factors and changes in gene expression (Apel and Hirt 2004; Jajic et al. 2015). Moreover accumulation of ROS in high concentration causes oxidative damage to different parts of plants, which affects plant growth and, in extreme cases, causes plant death (Sharma et al. 2012). However, fungal endophytes can reduce the negative effects of ROS and can transfer the stress tolerance to the next generation. The E+ treatments in $F_1$ pea plants reduced ROS production in $F_2$ generation pea plants under drought conditions compared to E− plants. Our results are in agreement with the study of Rodriguez et al. (2008) that suggests that endophyte-inoculated plants have less oxidative damage because endophytes may help in scavenging ROS, activate plant scavenging system more efficiently, or stop the production of ROS under abiotic stress. Similarly, *T. harzianum* treated wheat plants produce less ROS compared to untreated plants (Shukla et al. 2015). Moreover, the findings are also in agreement with our study on chickpeas, as well as with other existing studies (Shukla et al. 2015).

### 4.7.3 Comparative analysis of germination rate and fluorescence intensity

Under stress conditions there is less seed germination and, even if seed is germinated, plants are unhealthy and overall productivity is lower than optimum (Muscolo et al. 2014). It has been found that the amount of ROS is critical for seed germination and that ROS production imbalance impacts the seed germination process (Bailly et al. 2008). Consequently, measurement of ROS levels and its relationship with germination is important for desired agricultural production. Overall, when there is high concentration of ROS, there is less germination percentage. In other words, there is an inverse relationship between ROS level and germination percentage (Figure 4.9).

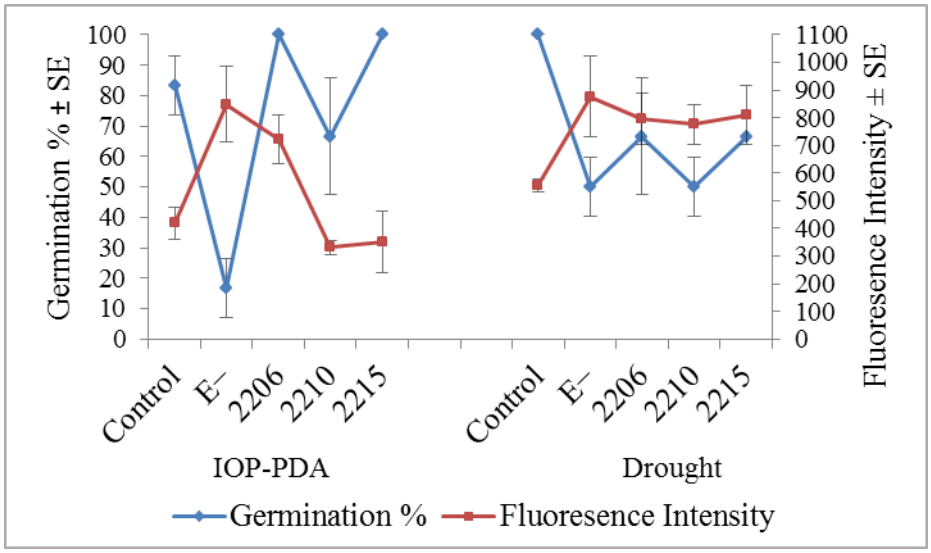



**Figure 4.9** Germination rate (4th day) and fluorescence intensity were determined in peas (CDC Golden $F_2$ generation) on IOP-PDA (Increased Osmotic Pressure) and under drought conditions. $F_2$ generation control seeds from $F_1$ under normal conditions, $F_2$ (E−) seed from $F_1$ under drought stress without an endophyte, and $F_2$ (E+) seeds from $F_1$ under drought stress with endophytes. Endophytes treatments (E+) were SMCD 2206, SMCD 2210, and SMCD 2215. Fungal endosymbiotic *Penicillium* sp. SMCD 2206, *Paraconiothyrium* sp. SMCD 2210 and bacterial symbiotic *Streptomyces* sp. SMCD 2215 were used for endosymbiotic treatments.

### 4.7.4 Expression of antioxidant genes

Antioxidants are ROS scavengers (Gill and Tuteja 2010). Under stress conditions, the antioxidant gene expression of plants is upregulated to protect plant from oxidative damage and cell injuries and provide stress tolerance (Kukreja et al. 2005; Harb et al. 2015). In addition, it has been proven that there is higher accumulation of proline osmolyte under stress conditions in order to protect plants (Ahmad et al. 2008 b; Hayat et al. 2012). Our results show that under drought stress conditions manganese superoxide dismutase (MnSOD) antioxidant gene is down regulated in $F_2$ generation pea seeds produced under drought conditions from $F_1$ plant inoculated with E + in comparison to E− but only SMCD 2206 treatments showed considerable downregulation. In addition, only SMCD 2206 treatment showed noticeable downregulation of

proline under drought conditions. However, majority of E+ and E− treatments did not change much proline and SOD expression under drought conditions but indicating the tendency toward downregulation except that SMCD 2215 is indicating towards upregulation. Moreover, SMCD 2210 and SMCD 2215 endophytes had more substantial effects on the downregulation of proline, SOD, and MnSOD antioxidant genes on the IOP-PDA medium while SMCD 2206 upregulated MnSOD. The results are in agreement with study of Elbersen and West (1996), observed that under drought conditions fescuse plants treated with endophytes had lower proline concentration and felt less stressed than those without infection. Moreover, it has been shown that plants company with endophytes can save them from stress and result in less activation of antioxidant machinery (Khan et al. 2012 a, 2012 b). Additionally, glutathione and lipid peroxidation activity have been found to be reduced in plants associated with fungal endophytes (Khan et al. 2012 c). Generally, endophytes help plants in stress tolerance by a mutualistic relation (Redman et al. 2001). These results suggest that endophytes in plants reduced stress so that the antioxidant genes do not need to be up regulated and are, instead, down regulated. Studying the effects of different endophytes on plant antioxidant gene expression will be helpful in understanding the complete mechanism of stress tolerance.

### 4.7.5 Seed nutritional quality

Pea provides a nutritional diet and health benefits as it contain high amount of protein (Barac et al. 2010). However, seed protein quality and quantity is affected and varied by growing conditions and genotypes (Martínez -Villaluenga et al. 2008; Wang et al. 2010). The effect of endophytes on protein content in $F_2$ generation pea seeds was measured and found that endophytes have the capacity to increase protein content. But, the pea protein content may be different for different varieties as protein content, composition, and properties are strongly influenced by genotype (Barac et al. 2010). Moreover, they inherit this property to the next generation. However, it is still necessary to study the detailed mechanism of this action.

As we know, seed protein is important from both nutrition and economic points of view, but continuously changing environmental conditions create uncertainty in pea seed production and quality. Consequently, to secure nutritional demand we need to find a way to maintain

consistency in production and quality. The use of endophytes can provide a potential solution to increase the quality and quantity of different crops.

## 4.8 Conclusion

It was found that selected endophytes improved pea seed germination and plant growth including root and shoot length under drought stress. Endophytes help in reduction of oxidative damage because there is less ROS production in colonized plants compared to non-colonized plants. For stress tolerance, antioxidant gene expression was measured and found that they are downregulated under drought conditions in plants influenced by endophytes. Typically, under stress, antioxidant gene expression becomes upregulated to protect plants; however, here it is proven that symbiotic endophytes alleviate the stress impact on the plant. Additionally, they pass this stress tolerance to $F_2$ generation pea seeds *via* the possible mechanism of epigenetic modification. More studies are required to further investigate these changes at proteomic and molecular levels.

# CHAPTER 5.

# GENERAL DISCUSSION

Abiotic stress such as drought and continuously increasing population enhance the problem of food security. To achieve the goal of sufficient food for everyone, we need to increase the agricultural production. Pulses such as chickpea and pea are high in protein and provide nutritious diet but their production is largely impacted by prevalent drought conditions (Kudapa et al. 2013; Osman Hany Samir 2015). Pea is more drought sensitive than chickpea (Toker and Yadav 2010) as pea nitrogen content is highly affected by stress conditions which results in reduced grain production (Cousin 1997; Neugschwandtner et al. 2015). In addition, chickpea can use more soil moisture than pea which might be due to pea inability to root deeper (Angadi et al. 2003) under drought stress. Plant microbiome such as beneficial bacterial and fungal endophytes may be a potential solution for increasing agriculture production in adverse environmental conditions. Hubbard et al. (2013) found that microbial endophytes can provide stress tolerance to wheat under heat and drought. However, until now there is not much knowledge on stress tolerance in chickpea and peas by endophytes, as well whether the stress tolerance provided by microbes can be inherited to the next generation. The effect of fungal (SMCD 2206, SMCD 2210) and bacterial (SMCD 2215) endophytes was studied on second generation chickpea and pea crops produced under drought conditions. It was hypothesized that the endophytes SMCD 2206, SMCD 2210 and SMCD 2215 can pass stress tolerance to the second generation chickpeas and peas under drought conditions and will improve seed germination resulting in overall increase of biomass. Moreover, it was also expected that there will be downregulation of antioxidant gene and less oxidative damage as there will be less ROS content in samples treated with endophytes. Furthermore, the endophyte can improve the nutrition quality in terms of protein content.

Germination is an essential process for plant development; however it is negatively impacted by drought conditions and decreases the germination percentage (Almansouri et al. 2001; Gamze et al. 2005; Yucel et al. 2010). The endophyte SMCD 2206, SMCD 2210 and SMCD 2215 treatments increase the *in vitro* seed germination in second generation chickpea and pea produced under drought condition including improvement in morphological traits such as root and shoot length. The findings of increased germination, root and shoot length by endophytes are in agreement with studies of Mastouri et al. (2010) and Hubbard et al. (2012). It is considered that degradation of cellulose present in seed coat and improvement in carbon acquisition by endophyte helps in improvement of germination process (Jerry 1994). In addition, growth hormones are also secreted by endophytes which increase the overall growth of plants (Khan et al. 2008; Hamayun et al. 2009). Moreover, it was also noted that there is an inverse relationship between germination percentage and ROS level. It is supported by Bailly et al. (2008) study that reported ROS level is critical for seed germination and high amount of ROS affect seed germination process.

In addition, endophytes help in reduction of ROS species in second generation chickpea and pea produced under drought conditions; however the mechanism of action is not clear yet. Generally, antioxidant genes upregulation occurs under stress conditions for plant protection from stresses (Kukreja et al. 2005; Harb et al. 2015). In the present study endophyte treatments result in less ROS production and, so possibly the antioxidant machinery do not need to upregulate. Consequently, it was found that the downregulation of different antioxidant genes in second generation chickpea and pea produced under drought condition. Proline, SOD, MnSOD and dehydrin antioxidant genes were mostly downregulated in different endophytic treatments up to various extents in both chickpea and pea crops. Khan et al. (2012 a), (2012 b) studies also reported that endophytes inoculation downregulates the antioxidant gene expression. Specifically, SMCD 2206 inoculated sample downregulated Proline, SOD, and MnSOD genes in chickpea on IOP-PDA medium but not in pea. However, SMCD 2210 and SMCD 2215 inoculation downregulated Proline, SOD, and MnSOD genes in second generation chickpea as well in pea on IOP-PDA medium. Pea is considered more sensitive to drought in comparison of chickpea (Toker and Yadav 2010). Thus, the same endophyte may provide varying degrees of stress tolerance depending on the genotype of crop (Saikkonen et al. 1999; Faeth et al. 2006).

Abiotic stresses such as drought reduce the protein content (Lecoeur and Guilioni 2010). Decrease in protein content is also an indicator of oxidative damage in plants (Moran et al. 1994). In our study, endophytes (SMCD 2206, SMCD 2210 and SMCD 2215) improved the protein content in second generation chickpea and pea crops under drought conditions. So endophytes do not only improve the protein content but also reduce the oxidative damage in plants by reducing ROS level.

The morphological data such as germination, root and shoot length as well as the molecular level changes studied by gene expression prove the beneficial role of endophytes in stress tolerance and inheritance to second generation chickpea and pea produced under drought conditions. However, further studies are needed on the endophytes under different stress conditions with different crops. Testing single or various combinations of endophytes might be a better strategy for stress tolerance. In addition, studying broad range of antioxidant genes at molecular level will increase the knowledge about stress tolerance inheritance by endophytes. Epigenetic modification such as DNA methylation is considered related with stress tolerance inheritance mechanism (Wang et al. 2010; Hubbard et al. 2014). Thus, by linking the strings of gene expression with proteomic and biochemical test results will provide a better picture of stress tolerance and inheritance by endophytes. To understand the role of different endophytes in epigenetic modifications, molecular and biochemical studies are also warranted. Finally, the applicability of endophytes for stress tolerance to increase the agriculture production can be confirmed by field studies.

# CHAPTER 6.

# GENERAL CONCLUSION

In conclusion, this study was driven by the idea that fungal endophytes help plants in adverse environmental conditions by reducing oxidative damage. Microbial endophytes not only increase plant growth and biomass but also help with stress tolerance (Chuansheng and Barry 2010). In addition, it has been found that endophytes can provide drought stress tolerance either by decreasing the use of water or by increasing the water use capability (Rodriguez et al. 2008).

In both legume crops studied here (chickpea and pea), potential for the use of endophytes in stress tolerance and improvement in seed quality is proven by improved germination efficacy and seed protein content. The results presented in this thesis are in agreement with previous studies (Varma et al. 1999; Clay and Schardl 2002; Ghabooli et al. 2013) that demonstrated that endophytes help facilitate stress tolerance and promote better growth and yield. However, the same endophyte may have different effects on different crops depending on the crop genotype (Gundel et al. 2012; Qawasmeh et al. 2012).

Mycovitality increased seed germination percentage in E+ (SMCD 2206, SMCD 2210, and SMCD 2215) compared to E− plants under both IOP and drought stress conditions in second generation peas and chickpeas, indicating that endophytes have the ability to inherit the stress tolerance capability to the next generation. Under drought conditions, there is an increase in ROS production in E− plants experiencing cell apoptosis while E+ plants have less ROS production, further proving the role of endophytes in stress tolerance. Our results show that ROS level, as measured by fluorescence intensity, is inversely related to germination efficacy. This signifies that if there is high ROS then there is less germination and vice versa. In conclusion, the germination process is affected by drought conditions as there is increased production of ROS, which limits germination and growth processes.

Antioxidant genes get upregulated in E− plants under stress conditions whereas E+ plants show downregulation of these genes, indicating the role of endophytes in stress tolerance. However, the mechanism of this action is still largely unknown.

It was observed that endophytes play a major role in plant stress tolerance by various changes at molecular and proteomic levels. Endophytes reduce ROS level so that antioxidant systems do not need to be upregulated. In contrast, antioxidant systems become downregulated. In conclusion, endophytes increase germination and overall root and shoot lengths by reducing oxidative damage and improve legume quality in terms of nutritional protein.

The confirmation of results may be warranted by large scale experiments at greenhouse and field levels. Further understanding the mechanism of action at molecular and proteomic levels will provide new insight and facts about the relationship between plants and endophytes. Therefore, the use of endophytes to increase agricultural production in unstable climatic conditions will be an inexpensive and environmental friendly potential solution to feeding the increasing world population. In future studies, omics can be used to develop a better understanding on the effect of plant-endophyte interaction for an improved stress tolerance in pulses.

# CHAPTER 7.

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