Amelioration of Plant Drought Stress by Plant Growth-Promoting Bacteria

by

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Author's declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Plant growth-promoting bacteria (PGPB) that contain the enzyme (1-aminocyclopropane-1-carboxylate) ACC deaminase protect a variety of plants against damage from various biotic and abiotic stresses. Here, the ability of bacterial strains *Pseudomonas* sp. UW4 (a rhizospheric PGPB) and *Pseudomonas fluorescens* YsS6 (an endophytic PGPB) and their respective (1-aminocyclopropane-1-carboxylate) ACC deaminase minus mutants, to ameliorate the effects of drought stress on canola (*Brassica campestris*) seedlings was assessed.

Initially, biochemical assays were used to estimate the levels of a number of the plant growth-promoting activities encoded within each of the four above-mentioned strains. These activities include: ACC deaminase activity, indole-3-acetic acid (IAA) production, siderophore synthesis and the trehalose concentration produced. Subsequently, the effect of the four bacterial strains on canola seedlings grown in the greenhouse in potting soil and subjected to different levels of drought stress (0, 6, 8, and 10 days) was measured. In all experiments, measurements were taken of shoot fresh weight, shoot dry weight, shoot protein concentration, leaf chlorophyll concentration, and shoot trehalose concentration. The results are discussed in terms of a previously developed model of PGPB functioning in the facilitation of plant growth.

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Dedication

To my parents Ali and Hayat

Who chose to complete their higher education through their children;

You who deserve to put their names on this work

To my husband Sami, and my children Mohammad, Fares, and Maryam

Who believe that my success is their success

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1. Introduction

Obtaining a sufficient amount of quality food to feed all of the people in the world, both now and in the future, is a serious global concern (Bresson *et al.,* 2014; Glick 2013). By 2050, more than 50% of the world's arable lands are expected to have serious plant growth problems, largely because of issues associated with drought and salinity. Moreover, there is a possibility of decreasing production of global food in the future because of global warming (Naveed *et al.,* 2014). In developed countries, for example, the annual crop yield increase is currently less than 1% while the increase in demand in those countries is around 3% annually (Naiman *et al.,* 2009). This problem is a consequence of several phenomena, including global warming, that can frequently lead to drought and severe water stress (Bresson *et al.,* 2014). Inhibition of plant growth is one of the results of these negative stresses in the natural environment (Kim *et al.,* 2012).

To address the above-mentioned issue, increasing crop water-use efficiency may occur either by better crop management or through the development of drought resistant/tolerant plants (Chen *et al.,* 2013a). To reach higher levels of productivity of these crops without any harmful effects to the environment, the soil quality and agricultural practices should be improved (García de Salamone *et al.,* 2012). In addition, researchers have to find strategies and technologies to increase crop yield and at the same time decrease the use of potentially harmful chemical fertilizers and pesticides (García de Salamone *et al.,* 2012). Currently, one of the biotechnological strategies that is being applied to induce environmental stress tolerance of plants is the judicious application of strains of plant growth-promoting bacteria (PGPB) (García de Salamone *et al.,* 2012).

1.1. Plant growth-promoting bacteria (PGPB)

Plant roots are surrounded by a thin layer of soil known as the rhizosphere, a region that is important for root activity and metabolism (Heidari & Glopayegani, 2012). In one gram of rhizosphere soil, there may be up to 10^8-10^{12} microorganisms, including about 10^3 different types of microorganisms (Naiman *et al.,* 2009). The rhizosphere is considered to be a nutrient-rich microhabitat. Bacteria are the most abundant type of microorganism found in the soil; commonly the concentration of bacteria that is found around the roots is much greater (by around 10- to 1000-fold) than the bacterial density in the rest of the soil (Glick, 1995). The presence of high levels of substances that bacteria can use as nutrient sources, exuded from plant roots, is the primary reason for the high concentration of bacteria around the roots. These exuded substances include amino acids, sugars, and organic acids (Glick, 2013). Of the many bacteria that are normally found in the soil, some have a largely beneficial interaction with plants (Bresson *et al.,* 2014), which is determined by the health of the plants and the fertility of the soil (Heidari & Glopayegani, 2012). These interactions typically occur when bacteria colonize the rhizosphere of many plant species, and promote plant growth by a variety of different mechanisms. In addition to rhizospheric PGPB, other beneficial plant-bacterial interactions include symbiotic relationships with the plant and endophytic bacteria that often are found near, on, or within the plant roots (Glick, 1995; Penrose & Glick, 2003; Gururani *et al.,* 2013). Many strains of *Pseudomonas* spp. are considered to be PGPB based on their genetic makeup and metabolic capabilities; however, not all strains of this genus act on plants as plant growth-promoting bacteria (Glick, 2013). Moreover, depending upon the bacterium, the plant, soil conditions and a variety of environmental

factors, the interactions between bacteria and plant roots may be beneficial, harmful, or inconsequential for the plant (Glick*,* 1995). Thus, for example, PGPB may not be useful to plants when the plants are grown in soil where the conditions are optimal (Glick, 2013).

1.1.1. Types of PGPB-plant interactions

There are multiple complex interactions among plants, bacteria, and soil (Gamalero & Glick, 2012). The bacteria that interact with plants may be divided into three common types. The first type is isolated from the area adjacent to the root surface called the rhizosphere. Secondly, endophytic bacteria can be isolated from internal plant tissues. Thirdly, some bacteria, including for example some strains of *Azospirillum*, can be isolated from either internal plant tissues as an endophyte or from external plant tissues as generally bound to plant roots (Dodd *et al.,* 2010). Plant susceptibility to biotic and abiotic stress is important in the stimulation of plant growth by PGPB (Bresson *et al.,* 2014). Rhizosphere bacteria also play a significant role in plant resource capture (Dodd *et al.,* 2010).

Some PGPB, such as strains of *Pseudomonas* spp., have abilities to produce organic acids or phosphatases and thereby facilitate the solubilization of inorganic or organic phosphorus from the soil. In addition, some have the ability to produce various phytohormones including auxins and cytokinins (Naiman *et al.,* 2009). Some *Pseudomonas* spp. strains are characterized as biocontrol PGPB, and control pathogenic microorganisms through the production of some molecules such as antibiotics,

siderophores, hydrogen cyanide, fungal cell wall-degrading enzymes, as well as through the induction of systemic resistance (Naiman *et al.,* 2009).

The relation between plants and endophytic (plant growth-promoting bacteria) PGPB has benefits for both partners because the bacteria are able to colonize a plant's interior and build a beneficial relationship by producing some important components for the plants (Rashid *et al.,* 2012). According to Rashid *et al.,* (2012) and Jiang *et al.,* (2012), some bacterial endophytes express the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, that converts ACC to ammonia and α-ketobutyrate for bacterial use. This type of conversion leads to a decrease of ethylene levels in the host plants. As a consequence, the bacteria promote plant growth (Rashid *et al.,* 2012). Under environmental stress, PGPB may utilize the production of ACC deaminase to promote plant growth by decreasing ethylene levels that are commonly produced by plants under stress (Jiang *et al.,* 2012), and the levels of plant hormones can be altered by rhizobacteria via decreasing of ethylene levels that occurs by the action of the bacterial enzyme ACC deaminase (Jiang *et al.,* 2012). For example, PGPB induce plant growth by cleaving ACC that leads to a decrease in inhibitory ethylene levels thereby stimulating plant growth (Belimov *et al.,* 2013). Endophytic bacteria can be isolated from internal plant tissues. Plants and endophytic bacteria both benefit from their relationship, while the bacteria promote the plant growth by decreasing the ethylene levels via the expression of ACC deaminase, the bacteria have an ability to colonize the plant's tissues and obtain nutrients and proliferate (Rashid *et al.,* 2012).

In fact, it has been suggested that in nature, the large majority of plants contain bacterial endophytes (Sturz *et al.,* 2000). Tomato, potato, wheat, sweet corn, cotton, and carrot plants are some examples of agricultural plants from which endophytes have been isolated. In most plants, these microorganisms are abundant in roots, but endophytes may also be found in leaves, seeds, and fruits (Krishnan *et al.,* 2012). Moreover, from a single plant, it is often possible to isolate several different species of endophytes (Ali *et al.,* 2012). Although bacterial endophytes utilize the same growth-promoting mechanisms as rhizospheric bacteria, as they can colonize inside the plant tissues they are less subject to environmental pressure and thus may be more effective under different environmental conditions (Ali, 2013). As far as researchers have been able to tell, endophytic bacteria that are found inside of a plant do not cause any obvious disadvantages for the plant. On the other hand, many endophytes have some features such as producing different secondary metabolites and protecting the plants against disease so that they are typically considered to be beneficial to their plant hosts (Krishnan *et al.,* 2012). Both the rhizospheric bacteria and endophytes are affected by the plant genotype; for example, the interaction of both endophytes and rhizospheric bacteria is altered when comparing how they respond to transgenic versus non-transgenic canola strains (Krishnan *et al.,* 2012).

1.1.2. Mechanisms that are used by PGPB

PGPB commonly colonize the rhizosphere and promote plant growth via several different mechanisms (Jiang *et al.,* 2012). PGPB have two main ways direct and indirect that used to promote plant growth: (Glick, 1995; Bhusan *et al.,* 2013). While the facilitation of environmental nutrient uptake or the provision of plants with bacterially

synthesized compounds are considered as direct plant growth promotion, the prevention of the harmful effects of various phyotpathogenic organisms is considered to be indirect plant growth promotion (Penrose & Glick, 2003; Gamalero & Glick, 2012; Glick, 1995; Glick, 2004). The various means of indirect plant growth promotion are often conceptualized as biological means of preventing the plants from being inhibited by the many disease-causing pathogenic agents that inhabit the soil, which means that in this case the PGPB are being employed as biocontrol agents in place of chemical agents which are often hazardous to animals, humans, and natural ecosystems (Penrose & Glick, 2003).

1.1.2.1. Direct mechanisms of plant growth promotion

To directly facilitate the proliferation of plants there are several mechanisms that PGPB may use including: 1) fixing atmospheric nitrogen and supplying it to the plants (Glick, 1995; Penrose & Glick, 2003; Glick, 2004), 2) synthesizing enzymes such as ACC deaminase that can modulate plant growth (Penrose & Glick, 2003), 3) synthesizing different metabolic chemical compounds (Dodd *et al.,* 2010) or phytohormones such as auxins and cytokinins (Penrose & Glick, 2003) which promote plant growth at different stages of growth (Glick, 2004), 4) solubilizing minerals such as phosphorus, 5) and biofertilization, i.e. promoting plant growth via improving plant nutrient uptake P, N, Fe (Gamalero & Glick, 2012), such as providing iron that is sequestered from the soil (Glick, 2004) via synthesizing siderophores (Penrose & Glick, 2003) because otherwise the amount of iron that is available for microbial growth would be low. After the plants take up and transport the iron-siderophore complex, the plants use a mechanism that can release the iron from the complex (Glick, 1995).

1.1.2.2. Indirect mechanisms of plant growth promotion

The prevention of the harmful effects of phytopathogenic organisms is considered as indirect plant growth promotion (Penrose & Glick, 2003; Gamalero & Glick, 2012; Glick, 1995; Glick, 2004). Indirect promotion prevents the plants from becoming diseased; which means that the PGPB are used as biocontrol agents. As a result, any PGPB should have some specific features to consider it as a biocontrol agent (Penrose & Glick, 2003). Those features may include any one or more of the following: 1) Antibiotic synthesis (Dodd *et al.,* 2010). 2) Providing plants with soluble iron that is obtained by secretion of iron-binding siderophores and thereby depriving phytopathogens of iron (Kloepper *et al.,* 1980. 3) The PGPB may produce some metabolites and antifungal compounds (Penrose & Glick, 2003) such as pathogenesis-related (PR) proteins or similar enzymes (Glick, 2015). 4) Production of volatile organic compounds that may be toxic to the pathogens. Some examples of these compounds are acetone, ethyl propionate, and phenethyl alcohol (Glick, 2015), and producing hydrogen cyanide (HCN) to help the plants that are infected by pathogenic fungi such as *Thielaviopsis basicola* that causes black root rot of tobacco (Glick, 2015). 5) Production of some enzymes that can disrupt the fungal cells such as chitinase, β-1,3-glucanase, proteinase, or lipase (Chernin *et al.,*1995). 6) Production of the enzyme ACC deaminase to lower the pathogen-induced production of stress ethylene in plants (Penrose & Glick, 2003). 7) Competitively excluding other rhizosphere organisms (Dodd *et al.,* 2010). 8) PGPB can induce plant resistance to pathogens via several signals from bacterial outer membrane protein polysaccharide such as *O*-antigenic side chain, siderophores, and salicylic

acid. This type of resistance is known as induced systemic resistance (ISR) (Bakker *at al.,* 2007).

A particular bacterium may use more than one mechanism to promote plant growth during the plant's lifecycle. For example, a PGPB could lower the plant's ethylene concentration thereby decreasing the ethylene inhibition effect on seedling root length, which is considered as a direct mechanism. In contrast, if the seedlings have consumed the nutrients that were contained within the seed, the same PGPB may subsequently provide the plant with nutrient components such as nitrogen, iron, and phosphorus from the soil (Penrose & Glick*,* 2003). To limit the damage to plants that occurs from phytopathogens, using PGPB as biological agents prevents the proliferation or functioning of fungal pathogens, at the same time the PGPB, if it contains ACC deaminase, may promote plant growth by decreasing the ethylene levels that result from fungal infection. In contrast, many chemical agents are hazardous to humans and animals, and these chemicals can accumulate in natural ecosystems (Glick*,* 2004). To increase the effect of PGPBs as biological agents, the bacterial *Pseudomonas* sp. UW4 ACC deaminase gene was isolated and then transformed into several known biocontrol bacterial strains that did not contain ACC deaminase (Glick*,* 2004). The root and shoot fresh weights of the plants that were treated with the ACC deaminase transformed strains were greater than the fresh weights of the plants that were treated with wild-type biocontrol strains (Glick*,* 2004).

1.2. Drought and water stress

In the natural environment, there are many factors that can affect plant growth, either positively or negatively. For example, temperature, flooding, drought, and toxic metals are some of the negative stresses that may inhibit plant growth (Kim *et al.,* 2012). Among different biotic and abiotic environmental stresses, drought is defined as an important environmental problem whose effects impact both plants and people (Glick, 2013). In addition, there is a concern that the world's water supply for future generations may become problematic as a consequence of widespread water scarcity. Drought and water stress result from, among other things, global climate change and improper irrigation practices in many areas of the world. These practices often completely disregard resource conservation and sustainability principles (Fereres *et al.,* 2007). In addition, there are other negative effects that are caused by plant drought including inhibition of photosynthesis leading to damage to chloroplasts, chlorophyll destruction, lipid peroxidation and protein loss (Heidari & Glopayegani*,* 2012). Inhibition of plant growth is the major effect of drought on plants and is caused as a response to drought by producing of some chemical signals in the plant such as abscisic acid (ABA) or ACC, which is subsequently converted to ethylene to decrease plant growth directly (Belimov *et al.,* 2009). Increasing plant growth and crop production are the targets of many scientific studies that have been undertaken as a result of decreasing water levels and environmental nutrients (Chen *et al.,* 2013B).

1.3. Physiological changes to plants as a consequence of water stress

"Since a plant cannot physically remove itself from its environment the way an animal can, a plant responds to various environmental stresses by modifying its metabolism

and physiology" (Kim *et al.,* 2012). Water stress and drought commonly cause significant levels of plant mortality and decreases of plant biomass. However, there are some plants species that are better able to survive and recover following drought when the stress conditions are removed. This occurs because of the nature of some of the physiological and metabolic changes that result from plant dehydration (Bresson *et al.,* 2014). Drought is a form of abiotic stress that impacts the water relations of a plant at both cellular and whole plant levels. Drought stress leads to a wide range of both specific and nonspecific damages to the plant. To increase the drought tolerance of the plants that are grown in drying and semi-drying soil, plants may be inoculated with native beneficial microorganisms (Heidari & Glopayegani*,* 2012). In the absence of beneficial microorganisms, drought stress may destroy the electron transport system in the plant leading to the formation of potentially toxic activated oxygen compounds such as H_2O_2, $O_2^{\cdot}$, and $OH^{\cdot}$ (Heidari & Glopayegani*,* 2012).

1.3.1. ACC

Drought or water stress leads to the inhibition of plant growth by stimulating the plant to send some chemical compounds such as ABA, which maintains the water status, and decreasing plant growth directly by stimulating the production of some plant signals such as ACC via its conversion to ethylene (Belimov *et al.,* 2009). The ethylene levels will not be elevated to the inhibition point, for the roots of the PGPB that contain ACC deaminase are bound to the roots or seed coats of developing plants (Glick*,* 2004). These bacteria facilitate the formation of longer roots within a few days after the seeds are planted (Penrose & Glick, 2003). ACC deaminase-containing bacteria stimulate root growth by decreasing the root ACC concentration and therefore decreasing root ethylene production, whole plant ethylene

production, and the xylem ACC concentration for the plants that grow in drying soil (Jiang *et al.,* 2012).

The bacterial synthesis of the enzyme ACC synthase, in addition to being regulated by plant regulatory and developmental factors, may also be induced by the high levels of indole-3-acetic acid (IAA) that are produced by the bacteria and taken up by the plant, thereby augmenting the plant's endogenous IAA pools. Thus, high levels of IAA can promote an increase in the synthesis of ACC. Some of the ACC that is produced by the plant is exuded by various plant tissues, taken up by the bacteria that contain ACC deaminase and then cleaved by bacterial ACC deaminase enzyme to produce ammonia and α-ketobutyrate (Penrose & Glick, 2001). The net result is that PGPB reduce the levels of ACC in the environment external to the plants by taking up and cleaving the ACC. As a consequence, the plant may exude more ACC to avoid a differential level between external and internal ACC (Glick *et al.,* 1998). Thus, the ethylene level in the plant would be reduced because of reducing of ACC levels (Penrose & Glick, 2001).

As reviewed in Gamalero *et al.* (2012) and Nascimento *et al.* (2014), a number of different research groups have demonstrated that many different soil microorganisms have ACC deaminase activity, including fungi such as *Penicillum citrinum* and bacteria such as *Pseudomonas* spp. In addition, it has been demonstrated for a wide range of environmental stresses that the bacteria that produce ACC deaminase can facilitate plant growth because of their ability to lower the ethylene content in plants (Gamalero & Glick, 2012). Also, it has been found that plants that are treated with ACC deaminase-containing bacteria can resist the

stress ethylene that results from a range of environmental stresses, both biotic and abiotic, and have longer roots (Ma *et al.,* 2003).

To promote plant growth and enhance root elongation, the bacteria should have ACC deaminase activity because, when they are tested in a laboratory setting, the mutant strains that lack this activity have little effect on plant growth especially during stressful conditions (Mayak *et al.,* 2004). Even though all ACC deaminase-containing bacteria can promote plant growth, when selecting strains to ameliorate the effects of drought, the bacterial strains that are isolated from dry soil are more effective than strains that are isolated from areas where water is more readily available (Fig. 1) (Mayak *et al.,* 2004; Timmusk *et al.,* 2011).

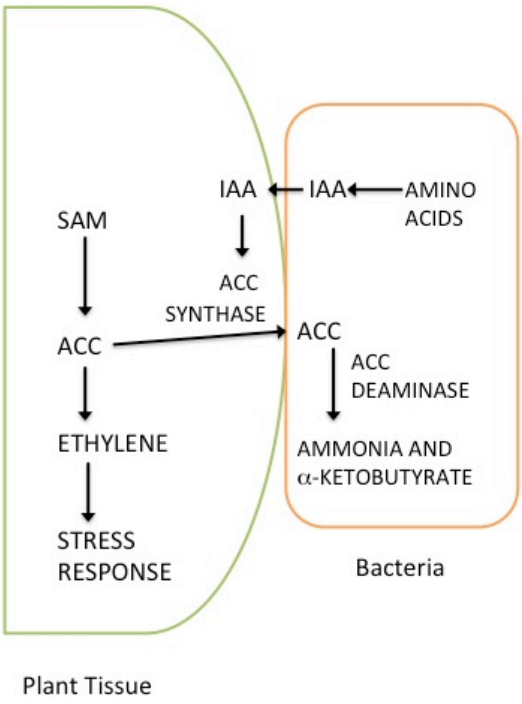


Fig. 1: How ACC deaminase-containing PGPB promotes plant growth (Glick, 2015).

1.3.2. Ethylen

When drought and water deficiency are induced, other chemical signals such as ethylene are synthesized, so that ethylene is regarded as a stress hormone (Chen *et al.,* 2013A). It is a gaseous hydrocarbon phytohormone (Dodd *et al.,* 2010) that is involved in several physiological roles in different phases of plant growth (Gamalero & Glick, 2012). As well, it can enhance or inhibit plant growth as a response to biotic or abiotic stress depending on the plant species and the hormone concentration (Gamalero & Glick, 2012; Dodd *et al.,* 2010). Moreover, during seed germination, ethylene stimulates the breaking of the seed's dormancy. In contrast, ethylene can inhibit root elongation if its levels remain high after seed germination (Glick*,* 2004). Moreover, ethylene affects plants in several ways; for example, tissue differentiation, flowering initiation, and fruit ripening (Glick*,* 2004). Under most conditions, ethylene is considered as a plant growth inhibitor. Therefore, to increase shoot growth and yield under drought stress, the ACC levels and ethylene production should be lowered. In addition, increasing ethylene production may lead to increasing amounts of ABA biosynthesis while decreasing ethylene production by ACC deaminase-containing bacteria can lead to a reduction in ABA levels (Jiang *et al.,* 2012).

1.3.3. IAA

Indole-3-acetic acid is the most common and well characterized of the auxins, which are a class of phytohormones (Glick*,* 1995) that are produced by bacteria, plants and fungi (Duca *et al.,* 2014; Patten & Glick, 1996). This phytohormone stimulates plant growth in the short term (cell elongation) and the long term (cell division and differentiation) (Glick*,* 1995;

Duca *et al.,* 2014). It also promotes secondary plant cell wall thickening and increases the size of xylem cells (Duca *et al.,* 2014). It is important to distinguish between the auxin that is synthesized by the plant as a response to PGPB, and the auxin that is synthesized by PGPB (Glick*,* 1995). The amount of IAA that is produced by PGPB affects the growth of the plants that are treated with an IAA-producing PGPB. For example, when comparing a mutant strain of the PGPB *Azospirillum brasilense,* which produces very low levels of IAA with the wild-type strain, the formation of lateral roots on wheat seedlings is no longer promoted. In contrast, a mutant strain of *Pseudomonas fluorescens* BSP53a that over-produces IAA inhibited the root development of cherry tree cuttings, while it stimulated the root development of blackcurrant softwood cuttings (Dubeikovsky *et al.,* 1993).

Soil bacteria, endophytic, marine and cyano- bacteria are examples of different bacteria that have the ability to produce IAA. Lower concentrations of IAA stimulate the formation of root hairs and primary roots. However, high concentrations of IAA can often inhibit the growth of primary roots. As a result, the level of endogenous plant IAA and the plant tissues' sensitivity to IAA determine whether the added bacterial IAA has positive or negative effects on the test plants. For instance, IAA that is produced by wild-type *Pseudomonas fluorescens* CHA0 enhanced the growth of cucumbers while the IAA that is produced by an IAA-overproducing mutant of this strain inhibited cucumber growth (Beyeler *et al.,* 1999). Thus, bacterial IAA has negative impacts on plants that already produce optimal levels of endogenous IAA and the addition of bacterial IAA could lead to plant senescence; however, if the plant endogenous IAA levels are suboptimal, the bacterial IAA can stimulate plant growth. On the other hand, reducing the virulence of pathogenic

IAA-producing bacteria can be a consequence of mutagenesis, which causes the pathogen to lose the ability to produce IAA. In addition, in some situations, IAA can protect bacteria from environmental stress (Surico *et al.,* 1985).

1.3.4. ABA

The plant's shoots in drying soil modify their physiology by producing a chemical signaling compound called abscisic acid (ABA) in roots; this compound is then transported to the shoots (Dodd *et al.,* 2008). There are two different ways for a plant to obtain ABA: it may be synthesized by leaves, or transported through the xylem from the roots (Chen *et al.,* 2013 A). ABA is regarded as a signal molecule that is produced by plants to make them more resistant to water deficiency. Moreover, ABA plays an important role in stomatal closure as a response to drought and other abiotic stresses (Chen *et al.,* 2013 A). Various microorganisms have the ability to synthesize ABA including strains of *Azospirillum brasilense* and many phytopathogenic fungi (Belimov *et al.,* 2013). Bacterial biosynthesis of ABA increases under osmotic stress. As a result, many endophytic bacteria that produce ABA are isolated from plants such as *Prospis strombulifera* (a shrub in the legume family) that are grown under conditions of extreme salinity (Belimov *et al.,* 2013). Moreover, when bacteria such as *Azospirillum* spp. that produce ABA are used to inoculate maize in drying soil, the bacteria can stimulate maize growth (Belimov *et al.,* 2013).

1.3.5. Siderophores

The amount of iron that is available to support plant and bacterial growth is typically very low compared to the abundance of iron in the soil as the fourth most common element on the earth (Glick, 2012). However, most iron in nature is present in soil as hydroxides, oxyhydroxides, and oxides (Gamalero & Glick, 2012). Plants and microorganisms such as rhizospheric bacteria and fungi compete for iron, because it is essential for the growth of these organisms (Glick, 2012). Thus, some bacteria facilitate the provision of iron for plants and other organisms by producing low molecular weight siderophores. These molecules have an extremely high affinity for the low abundant Fe^{+3} (Glick, 2012; Gamalero & Glick, 2012). In contrast, biocontrol PGPB effectively compete with pathogenic bacteria and fungi because siderophores that are produced by pathogenic bacteria and fungi typically have a much lower affinity for Fe^{+3} than the siderophores that are produced by PGPB (Glick, 2015). About 20% of characterized microbial siderophores have been identified as various types of pyoverdines. Pyoverdines are yellow-green fluorescent pigments that are produced by different strains of *Pseudomonas* spp. and other genera (Gamalero & Glick, 2012).

According to Glick (2015) some strains of *Pseudomonas* spp. have been observed to take up siderophores by using some of the five to twenty different bacterial outer membrane receptors that these bacteria possess. Using siderophores has many benefits for the plant's growth; for example, they can use ferric-siderophores as a source of iron, and also they can grow under limited iron conditions (Glick, 2012). The plant can use the iron after releasing it from the bacterial siderophores; furthermore, plants often use less iron than many microorganisms (Glick, 2015).

1.3.6. Trehalose

Trehalose may be defined as "a non-reducing disaccharide, an α, α-1,1-glucoside, consisting of two molecules of α-glucose, that is widely distributed in nature" (Glick, 2012). Many organisms have the ability to produce trehalose including bacteria, fungi, plants, insects, and invertebrates. Moreover, trehalose stability is very high and it is resistant to both acidity and high temperature. As a result, trehalose can protect plants against many stresses such as drought, salinity, and high temperature. In addition, trehalose has the ability to prevent some protein degradation under various ranges of temperature stresses (Glick, 2012). Treating plants with PGPB that have been genetically engineered to overproduce trehalose has affected many plants positively such as bean and maize, making them more drought resistance and increasing their biomass production. For example, strains of *Azospirillum brasilense* that have been modified to overproduce trehalose are more effective in facilitating plant growth under drought conditions than is the wild-type bacterium (Rodríguez-Salazar*, et al.,* 2009). Trehalose has osmoprotective role in the plant. It has been found that there is an increase in the plant's nitrogen-fixing nodules as a result of increasing in trehalose level; in addition, a decrease in trehalose production leads to decreases in the plant's nodule number (Glick, 2015).

There are two questions to be addressed in this study. The first one is can PGPB that possess both ACC-deaminase and trehalose protect Canola against drought stress? The bacterial strains *Pseudomonas* sp. UW4 (Duan *et al.,* 2013) and *Pseudomonas fluorescens* YsS6 (Rashid *et al.,* 2012) have the ability to produce ACC deaminase enzyme, but not their ACC deaminase minus mutants. In contrast, both the wild type strains and mutant strains

have the ability to produce trehalose. These strains will be used to help address the first question.

The second question is do endophytes with same activity as rhizospheric PGPB behave any differently than rhizospheric PGPB? *Pseudomonas* sp. UW4 (Duan *et al.,* 2013) and its ACC deaminase minus mutant (Li *et al.,* 2000) will be used as representative rhizospheric bacteria, and *Pseudomonas fluorescens* YsS6 (Rashid *et al.,* 2012) and its ACC deaminase minus mutant (Ali *et al.,* 2014) will be used as representative as endophytic bacteria to help answer this question .

2. Materials and Methods

2.1. Characterization of the bacterial strains

2.1.1. Bacterial strains

The bacterial strains that were used in this study include *Pseudomonas* sp. UW4 (Glick 1995; Duan *et al.,* 2013) and its ACC deaminase minus mutant (Li *et al.,* 2000), and *Pseudomonas fluorescens* YsS6 (Rashid *et al.,* 2012) and its ACC deaminase minus mutant (Ali *et al.,* 2014). Strain UW4 was isolated from the rhizosphere of reeds growing on the north campus of the University of Waterloo (Glick *et al.,* 1995). This rhizospheric plant growth-promoting bacterium produces IAA, siderophores and ACC deaminase and has been used in a number of studies where it has been shown to promote plant growth (Duan *et al.,* 2013). Strain YsS6 is an endophytic plant growth-promoting bacterium that was isolated from a rhizospheric soil from France kindly provided by Dr. Yvan Moenne-Loccoz of the University of Lyon, France (Rashid *et al.,* 2012). Strain YsS6 also produces IAA, siderophores and ACC deaminase; it has previously been shown to promote the growth of tomatoes in the presence of high levels of salt (Ali *et al.,* 2014) and to slow the wilting of flowers (Ali *et al.,* 2012).

2.1.2. Bacterial growth

The bacterial strains were stored as glycerol stocks at -80°C. The stored strains were streaked onto tryptic soy agar (TSA) (BactoTM; Becton, Dickinson and company; Sparks, MD, USA). Following incubation overnight at 30°C, 15 ml of tryptic soy broth (TSB)

(BactoTM; Becton, Dickinson and Company; Sparks, MD, USA) was inoculated with a single colony and grown overnight on a water bath shaker at 150 rpm at 30°C.

2.1.3. ACC deaminase assay

ACC deaminase activity was measured using the protocol described by Penrose & Glick (2003). For all strains, following overnight growth of a single colony from TSA streaked plates in 15 ml TSB medium at 30°C, the culture was centrifuged twice in an Eppendorf centrifuge 5810R (Hamburg, Germany) for 10 min at 8000 rpm, and after each centrifugation step the cells were washed with 5 ml DF salts minimal medium. One liter of DF salts minimal medium consisted of 990 ml of DF salts (4 g/L KH2PO4, 6 g/L Na2HPO4, 2 g/L gluconic acid, 2 g/L citric acid, autoclaved at 121^0C for 20 min), 100 µl trace elements (100 mg/L H3BO3,112 mg/L MnSO4•H2O, 1.25 g/L ZnSO4•7H2O, 782 mg/L CuSO4• 5H2O, 100 mg/L MoO3, autoclaved at 121^0C for 20 min), ~ 830 µl of 1M autoclaved $MgSO_4$, 100 µl of 10 g/L autoclaved $FeSO_4$•$7H_2O$, and 10 ml of 0.2 g/ml glucose (Penrose & Glick, 2003). After washing the cells with 5 ml DF salts minimal medium, the pellet was resuspended in 7.5 ml of DF salts minimal medium with 3 mM of filter-sterilized ACC, and incubated on a shaking water bath at 150 rpm at 30°C for 24 h. Then, the cultures were centrifuged twice in an Eppendorf centrifuge 5810R (Hamburg, Germany) for 10 min at 8000 rpm and washed after each centrifugation with 0.1 M Tris-HCl pH 7.6 in 1.5 ml microcentrifuge tubes. Next, the supernatants were removed and the pellets were resuspended in 400 µl 0.1M Tris-HCl, pH 8 and 20 µl of toluene was added before the mixture was vortexed for 30 sec at maximum speed. Following the addition of 50 µl of

lysate dispensed into two centrifuge tubes, one with 5 ml 0.5 M ACC and one with no ACC (as a sample negative control) and including Tris buffer +ACC as a negative control, the mixture was vortexed for approximately 5 sec. Following incubation of the reaction mixture for 30 min at 30°C, 500 ml of 0.56M HCl was added and the mixture was vortexed again for 5 sec before being centrifuged at 14,000 rpm for 5 min in an Eppendorf microcentrifuge (model#5417C; Hamburg, Germany). Lastly, 500 μl of supernatant was added to 13 x 100 mm glass test tubes with 400 μl 0.56M HCl and 150 μl DNP reagent (0.2% 2,4-dinitrophenylhydrazine in 2N HCl) and vortexed for 5 sec and then incubated at 30°C for 30 min. The absorbance of the samples was measured using a spectrophotometer at 540 nm wavelength after adding 1 ml 2N NaOH and vortexing for 5 sec. The absorbance readings of the samples were compared to a standard curve of α-ketobutyrate between 0.05 and 0.5 μmol. The standard curve was prepared by diluting 100 mM α-ketobutyrate stock 10x with 0.1 M Tris buffer pH8.0 (Table 1).

Table 1: ACC deaminase standard curve dilutions of α-ketobutyrate between 0.05 and 0.5 μmol diluted in 0.1 M Tris buffer, pH 8.0

μmoles of α-ketobutyrate	μl of α-ketobutyrate 100 mM stock solution	μl 0.1 M Tris buffer
0.05	5	495
0.1	10	490
0.2	20	480
0.3	30	470
0.4	40	460
0.5	50	450

2.1.4. IAA production assay

The ability of bacterial strains to produce IAA was measured based on a protocol that was described by Rashid et al. (2012). Each strain was incubated overnight in 5 ml DF salts minimal medium. Then, a 20 µl aliquot of the overnight culture was added to 5 ml of DF salts minimal medium with different concentrations of L-tryptophan (i.e., 0, 200, or 500 µg/ml). Following incubation for 48 h at temperature 30°C in a shaking water bath, the cells were centrifuged at 5,500 rpm for 10 min. A 1 ml aliquot of the supernatant of each sample was mixed with 4 ml of Salkowski's reagent (stock solution of 150 ml of concentrated H_2SO_4, 250 ml of distilled H_2O, 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_2O$), incubated for 20 min at room temperature, and the absorbance measured at 535 nm. The absorbance of the samples was compared to a standard curve that included IAA concentrations ranging from 0 µg/ml to 40 µg/ml (Table 2). To prepare 100 µg /ml of IAA stock solution, 10 mg of IAA powder was dissolved in 200 µl of 100% ethanol then mixed with 90 ml of sterile water and warmed gently to 70°C for a few min to remove residual ethanol, then adjusted to a final volume of 100 ml with sterile water.

Table 2.: Dilutions for IAA standard curve

μl IAA stock solution*	ml of water + 4 ml of Salkowski's reagent	Final concentration of IAA (μg/ml)
0	1 ml of water	0 μg/ml
20	980 μl of water	2 μg/ml
40	960 μl of water	4 μg/ml
80	920 μl of water	8 μg/ml
100	900 μl of water	10 μg/ml
200	800 μl of water	20 μg/ml
400	600 μl of water	40 μg/ml

* 100 μg /ml of IAA stock solution

2.1.5. Siderophore production assay

This assay was done qualitatively using chrome azurol S (CAS) agar as described by Louden et al. (2011) following growth of the bacterial cells in M9 minimal medium overnight (M9 salts per 1 liter: 64 g $Na_2HPO_4 \bullet 7H_2O$, 15g KH_2PO_4, 2.5 g NaCl, 5 g NH_4Cl. To prepare M9 minimal medium: 200 ml M9 salts, 2 ml 1M $MgSO_4$, 20 ml 20% glucose, 100 µl 1M $CaCl_2$, adjusted to a final volume of 1 liter with water). Five µl from each strain was added to the CAS agar plates and incubated for 3 days at 30°C. When bacteria that can produce siderophores that bind to iron are grown on the CAS agar, the siderophores remove the iron from the CAS indicator dye and the medium changes color from blue to orange.

2.1.6. Trehalose production assay

For this assay, each strain was grown overnight at 30°C in 19 ml of M9 minimal medium with 0.8 M NaCl. Then, the pellet was extracted by centrifuging in an Eppendorf centrifuge 5810R (Hamburg, Germany) for 10 min at 8000 rpm, resuspended in 0.5 ml of water, and incubated at 95°C for 20 min. The concentration of trehalose was measured using a trehalose assay kit (Megazyme International; Ireland) (Djonovic´ *et al.,* 2013). In this assay the absorbance at wavelength 340 nm was used for the measurement of blank, standard, and sample (Table 3) using a spectrophotometer and samples in a 96-well clear flat-bottomed plastic microplate. The standard was a single point standard. Following mixing after 5 min, the absorbance of the solutions were read at 340 nm. The trehalose concentration was calculated using the following equation:

$$g/L = \Delta A_{sample} / \Delta A_{standard} \times g/L \text{ standard} \times F$$

where F= dilution factor.

Table 3.: Trehalose measurements of blank, standard, and sample (Trehalose assay kit, Megazyme International; Ireland)

	Blank	Sample	Standard
Distilled water	220 µl	200 µl	200 µl
Sample solution	-	20 µl	-
Standard solution	-	-	20 µl
Solution 1 (buffer)	20 µl	20 µl	20 µl
Solution 2 ($NADP^+$/ATP)	10 µl	10 µl	10 µl
Suspension 3 (HK/G-6-PDH)	2 µl	2 µl	2 µl
Mixed and read the absorbance of the solutions (A1) after 5 min and then the reaction was started by adding:			
Suspension 4 (trehalase)	2 µl	2 µl	2 µl

2.1.7. Protein measurement

Bacterial protein was measured using the BioRad protein assay (Bradford, 1976). The samples were prepared in duplicate using the same protocol for preparing samples as with the ACC deaminase assay, but after the incubation and centrifugation, the supernatants were removed and the pellets were resuspended in 400 ml 0.1M Tris-HCl, pH 8, and 20 ml of toluene was added then the mixture vortexed for 30 sec at maximum speed. The lysate was then diluted in a microcentrifuge tube (26.5 μl lysate + 173.5 μl 0.1M Tris-HCl). Following this, 200 μl 0.1N NaOH was added and the mixture was vortexed for about 5 sec, then the samples were boiled for 10 min. For the standard curve, 10 mg/ml BSA stock was used to prepare triplicate standards from 200 to 1000 μg/ml (using distilled H_2O to dilute the standards; Table 4).

To start the assay, 8 μl of sample or standard was mixed with 792 μl H_2O + 200 μl BioRad protein dye reagent concentrate. Then, the above-mentioned components were incubated for 5 min and decanted into cuvettes and the absorbances were read at 595 nm.

Table 4.: Protein standard curve dilutions

Standard μg/ml	μl of BSA (10 mg/ml)	μl of H_2O
200	20	980
400	40	960
600	60	940
800	80	920
1000	100	900

2.2. Effect of PGPB on canola plant growth under drought stress

2.2.1. Plant growth

The model plant used in this study was canola. The canola plant belongs to *Brassicaceae* and is a cultivated widespread plant, so it can be adapted to wide ranges of temperatures in different areas such as North America, Europe, Asia, and Australia (Daun *et al.,* 2011). Canola seeds are considered as a spice and, more importantly, as a source of vegetable oil. The seeds have variation in size, color, chemical components, and morphology (Daun *et al.,* 2011). Canola was chosen because of the previous success in ameliorating the effects of various stresses in canola with PGPB in the lab.

Canola plants were grown in the greenhouse in in small plastic pots (7.8 cm x 6.5 cm) in potting soil (Sunshine mix # 4, SunGro; Jack Van Klaveren Ltd., Ancaster, Ontario). The Sunshine mix contains Canadian Sphagnum peat moss, Perlite, dolomitic limestone, a wetting agent and RESLIENCE (a silicon based amendment).

Canola plants were grown for one month, and then they were subjected to different levels of drought (0, 6, 8, and 10 days). In all experiments, measurements were taken of shoot fresh weight, shoot dry weight, shoot protein concentration, leaf chlorophyll concentration, and shoot trehalose concentration. For each treatment, at least 10 plants were utilized. In addition, the entire experiment was performed three times.

Canola seeds were treated with the bacterium *Pseudomonas* sp. UW4 (Duan *et al.,* 2013) and its ACC deaminase minus mutant (Li *et al.,* 2000), and *Pseudomonas fluorescens* YsS6 (Rashid *et al.,* 2012) and its ACC deaminase minus mutant. These bacteria were grown overnight at 30°C in 15 ml TSB medium, then centrifuged to obtain the pellet which then was resuspended and diluted with sterile water to an OD_{600} of 0.25. The sterilized canola

seeds (seeds were surface sterilized in 10% bleach (Magnifique 610-N) in water for 5 min followed by thorough rinsing with sterile water) were treated with this bacterial suspension for 1 h either at room temperature or 30°C. After the 1 h incubation, these seeds were planted in the soil in the pots directly.

2.2.2. Shoot fresh and dry weight

The plant fresh weights were measured immediately after the plants were harvested. The shoot dry weights were measured following incubation of the plant shoots at 37° for 72 h.

2.2.3. Shoot protein concentration

Based on Jones *et al.,* (1988) the shoot protein concentration was measured using the Bradford assay method (1976). Aliquots of 100 mg fresh weight of plant tissues were ground in liquid nitrogen with mortar and pestle. Then 1 ml of 0.1 N NaOH (pH 12.8) was added. The samples were vortexed for approximately 3 s and left at room temperature for ~30 min. Samples were remixed for 3 s and centrifuged for 5 min at high speed (>5000 x g) using an Eppendorf centrifuge 5417C (Hamburg, Germany). The supernatants were remixed for approximately 3 s, the three replicate aliquots (100 µl) of each sample were prepared and 100 µl of 0.1 N NaOH was used as a blank. The samples were added to 18 x 150 mm glass test tubes with 5 ml of 1:4 diluted Bradford dye reagent (Bio-Rad Laboratories), which was modified by adding 3 mg/ml soluble polyvinylpyrollidone (PVP) (Sigma pharmaceutical grade; MW ~40,000). Lastly, after 15 min, 1 ml of each replicate was transferred to a cuvette and the absorbance was recorded against the dye reagent/NaOH blank at 595 nm.

2.2.4. Leaf chlorophyll concentration

In small glass bottles (18 x 150 mm) with tight caps, one g of fresh leaves was incubated in the dark at 4^{o}C for 48 h with 5 ml of N,N-dimethylformamide. The absorbance of the resulting solution was read at 663 nm and 645 nm. Then the amount of total chlorophyll was calculated using the following formula (Ali *et al.,* 2014):

Total chlorophyll = $0.5 \times (20.2 \times A_{645} + 8.02 \times A_{663})$.

2.2.5. Shoot trehalose concentration

Using the trehalose microplate assay procedure (Megazyme International Ireland, 2014), 0.020 ml of sample solution was added to 0.200 ml of distilled water, 0.020 ml of buffer solution, 0.020 ml 0f $NADP^{+}$/ATP solution, and 0.002 ml of HK/G-6PDH solution. Following mixing in a 96-well, clear flat-bottomed microplate, the absorbance was read at 340 nm (A_1). In each instance, the reaction was started by the addition of 0.002 ml of Trehalase, and again the reaction contents were mixed and the absorbance was read at 340 nm (A_2). Then the amount of trehalose was calculated using the following formula:

$g/l = (\Delta A_{sample} / \Delta A_{standard}) X$ g/L standard X F ,

where $\Delta A = A_1 - A_2$ and F = dilution factor

3. Results

3.1. ACC deaminase

3.1.1. ACC deaminase standard and quantification in bacterial strains

The standard solution contains 100 mM α-ketobutyrate diluted with 0.1M Tris-HCl pH8. This solution was used to prepare standards from 0.05 to 0.5 μmols α-ketobutyrate, in duplicate. These diluted samples were used to prepare a standard curve for ACC deaminase activity (Fig. 2).

ACC deaminase activity was measured for all bacterial strains that were used in this study including *Pseudomonas* sp. UW4 and its ACC deaminase minus mutant, and *Pseudomonas fluorescens* YsS6 and its ACC deaminase minus mutant. As expected, the highest levels of ACC deaminase activity were seen in the wild-type strains (UW4 and YsS6), while the ACC deaminase minus mutant strains had much lower levels of ACC deaminase activity (Fig.3) because in these the ACC deaminase gene (*acdS*) has been replaced with an ACC deaminase gene with a tetracycline resistance gene that has been inserted within its coding region (Li *et al.,* 2000). As a result, these strains should not be able to produce ACC deaminase.

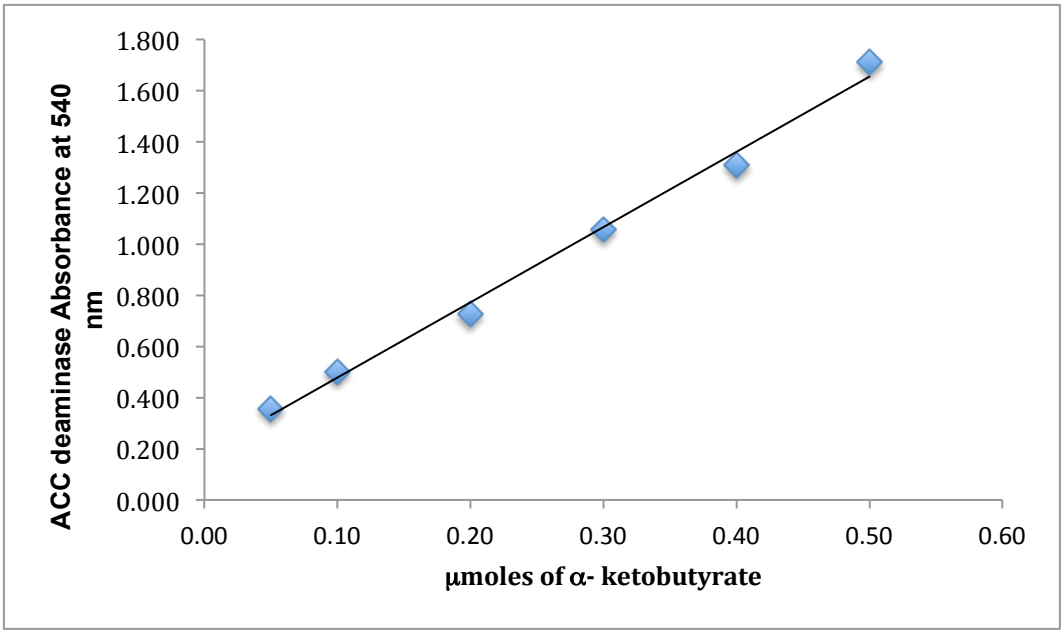


Fig. 2: The ACC deaminase standard curve. Equation: $y = 0.0294x + 0.1843$, $R^2 = 0.993$

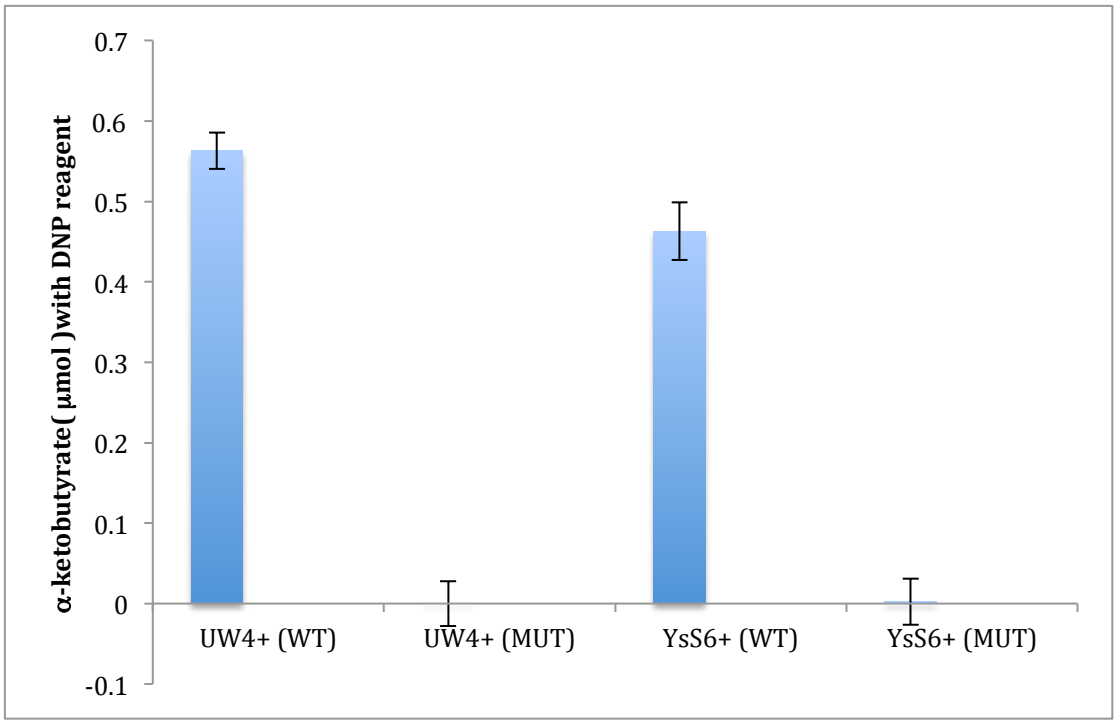


Fig. 3: The ACC deaminase level in bacterial strains *P. fluorescens* YsS6 wild-type, *P. fluorescens* YsS6 ACC deaminase minus mutant , *P.* sp. UW4 wild-type, and *P.* sp UW4 ACC deaminase minus mutant. The abbreviations WT and MUT denote wild-type and mutant, respectively.

3.2. IAA production assay

3.2.1. IAA standard and quantification in bacterial strains

The IAA standard curve (Fig. 4) was measured with an IAA stock solution that contains 10 mg of IAA with 200 μl of 100% ethanol and 90 ml of sterile water, warmed to 70°C to remove the ethanol and adjusted to a final volume of 100 ml with sterile water. Then concentrations were prepared from 0 μg/ml to 40 μg/ml (from 1 ml of water + 4 ml of Salkowski's reagent to 400 μl of IAA stock solution + 600 μl of water + 4 ml of Salkowski's reagent).

The ability of the bacterial strains, grown in the presence of 500 μg/ml L-tryptophan, to produce IAA was measured using Salkowski's reagent. This measurement showed that the YsS6 ACC deaminase minus mutant strain had a higher level of IAA than either of the wild-type strains (Fig. 5). This difference appears clearly between YsS6 wild-type and its mutant, while there is only a small difference in IAA production between the UW4 wild-type and its mutant (in this case a decrease in IAA synthesis).

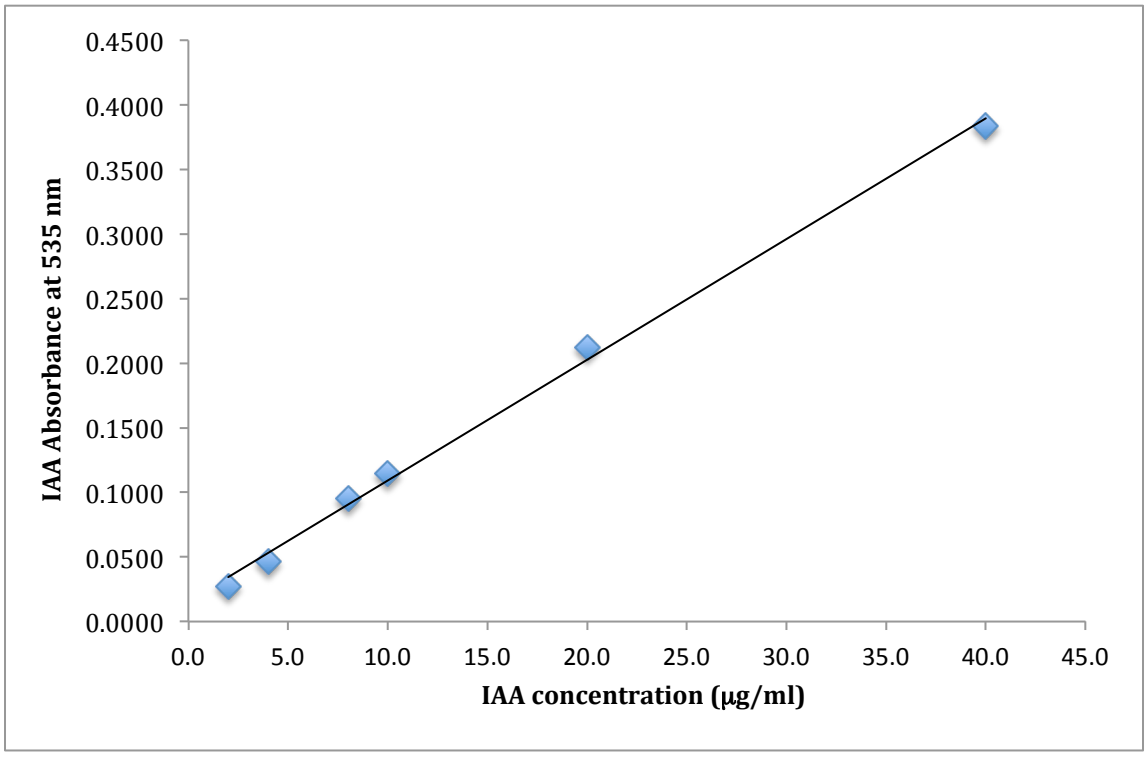


Fig. 4: The IAA standard curve. Equation: $y = 0.0093x + 0.0157$; $R^2 = 0.997$

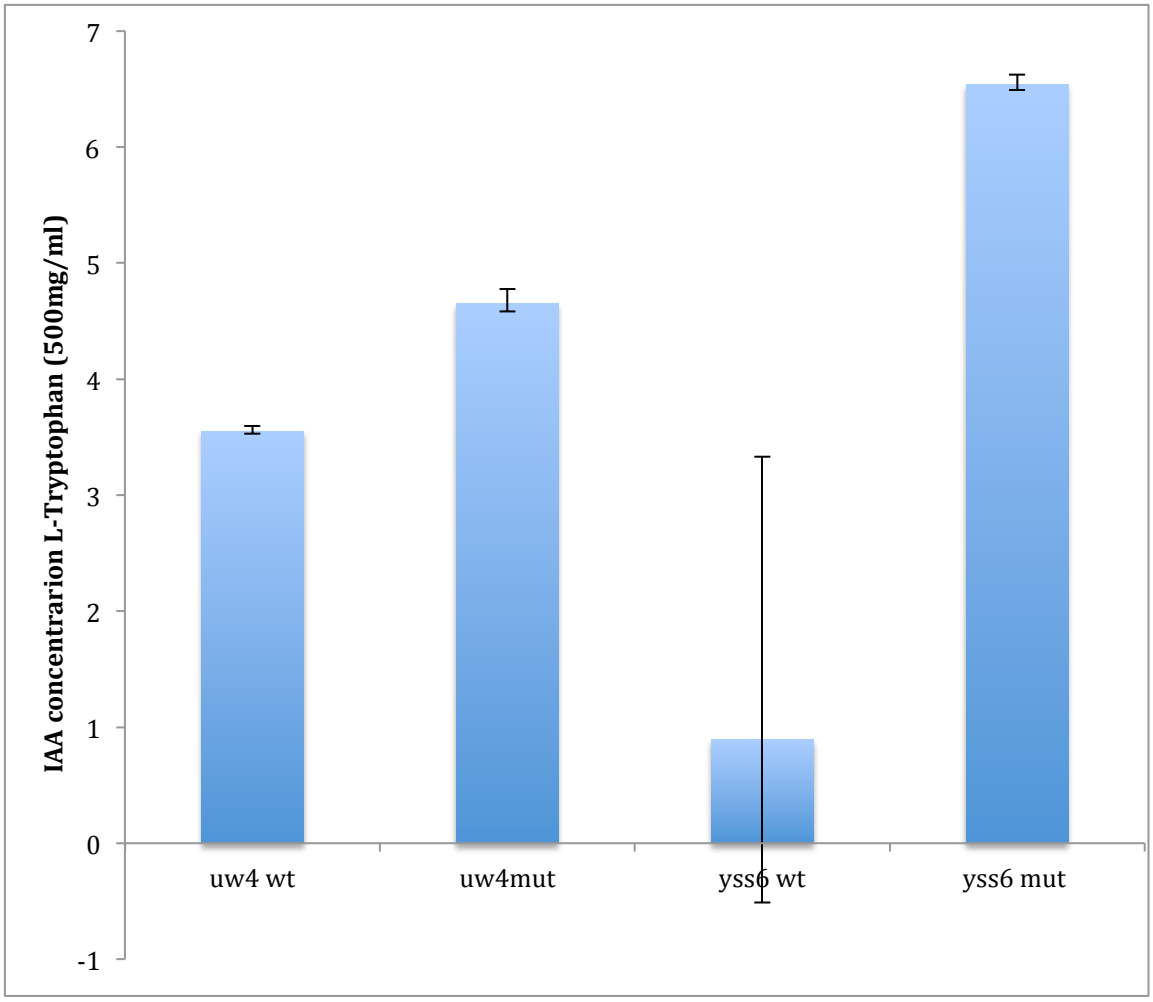
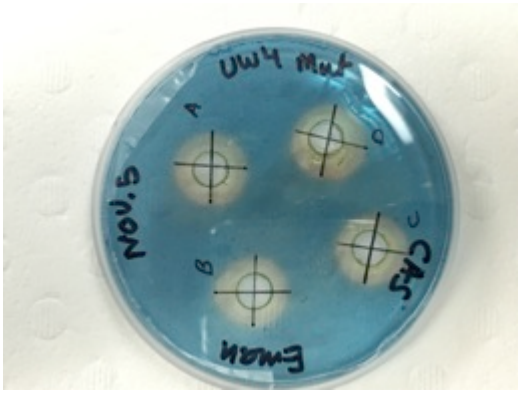


Fig. 5: The IAA production of bacterial strains grown in the presence of 500 µg/ml L-tryptophan including bacterial strains *P. fluorescens* YsS6 wild-type, *P. fluorescens* YsS6 ACC deaminase minus mutant, *P.* sp. UW4 wild-type, and *P.* sp. UW4 ACC deaminase minus mutant. This experiment was repeated 3 times. The measurements include standard errors.

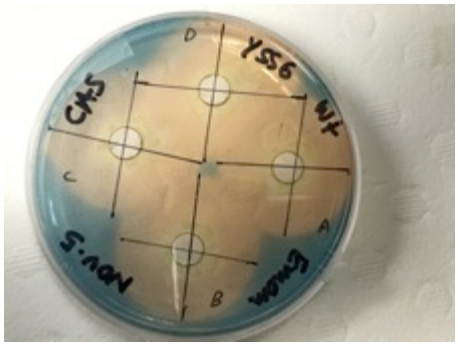
3.3. Siderophore production assay

Using chrome azurol S (CAS) agar, bacterial production of siderophores can be estimated. Siderophores remove the iron from the CAS indicator dye to which it is bound initially and, as a consequence, the medium changes color from blue to orange. Based on this, *P. fluorescens* YsS6 wild-type has the highest level of production of siderophores compared with the other three bacterial strains. In addition, UW4 levels do not change appreciably when comparing the wild-type and the mutant while YsS6 levels are significantly decreased in the mutant compared to the wild-type (Fig. 6).

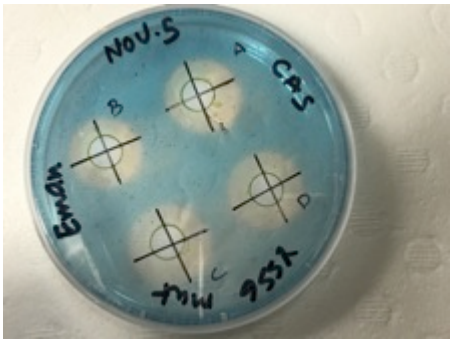
A: *P.* sp. UW4 wild-type



B: *P.* sp UW4 ACC deaminase minus mutant



C: *P. fluorescens* YsS6 wild-type



D: *P. fluorescens* YsS6 ACC deaminase minus mutant

Fig. 6: The siderophore production of bacterial strains (A) *P.* sp. UW4 wild-type, (B) *P.* sp. UW4 ACC deaminase minus mutant, (C) *P. fluorescens* YsS6 wild-type, and (D) *P. fluorescens* YsS6 ACC deaminase minus mutant. The bacterial strains were all grown on plates for the same amount of time (3 days at 30°C)

3.4. Trehalose production assay

The UW4 mutant had the highest level of trehalose production compared to the other three strains (Fig.7), while the UW4 wild-type had the lowest production of all of the strains. Given the high amount of variability in the trehalose measurements, the only significant difference in trehalose production between these four strains is the lower level of the UW wild-type strain (0.1205 g/l) compared to the other three strains.

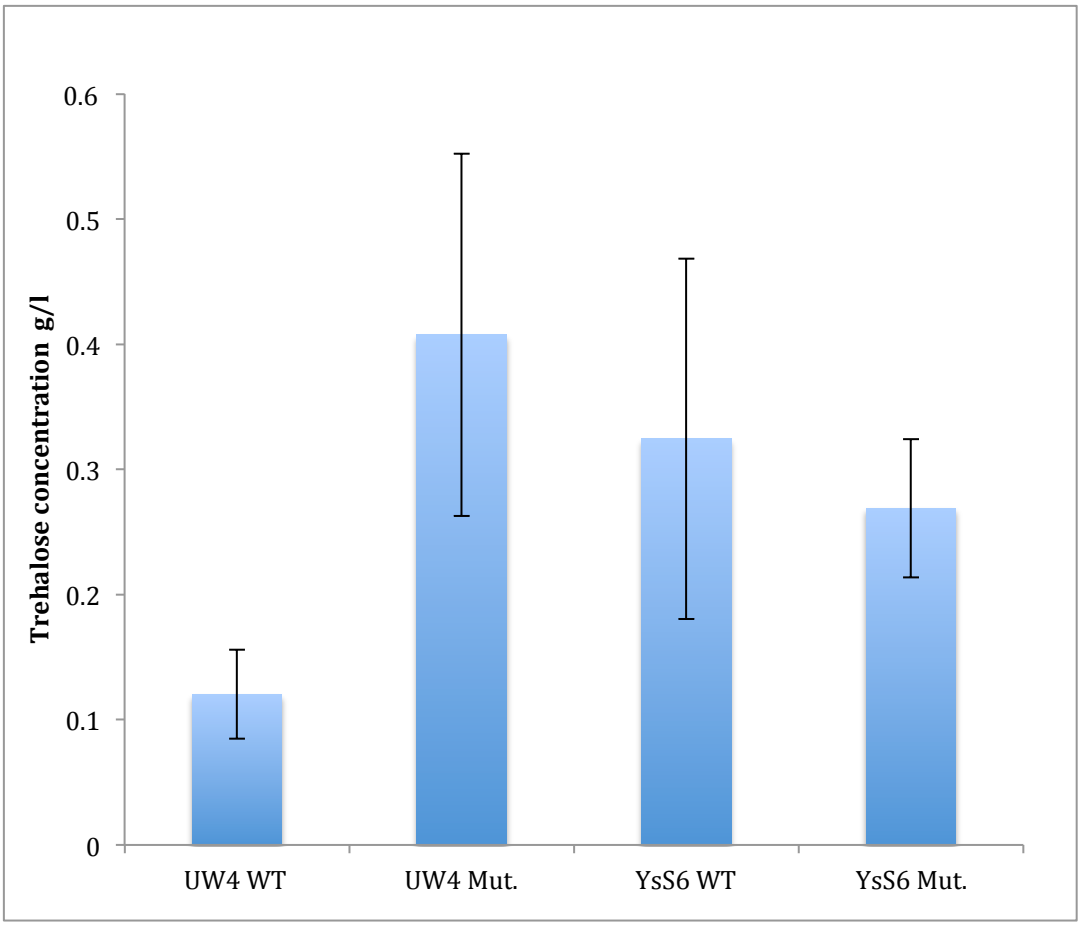


Fig. 7: The trehalose production level in g/l of the bacterial strains *P. fluorescens* YsS6 wild-type, *P. fluorescens* YsS6 ACC deaminase minus mutant, *P.* sp. UW4 wild-type, and *P.* sp. UW4 ACC deaminase minus mutant. This experiment was repeated 6 times. Error bars represent standard errors.

3.5. Protein measurement

3.5.1. Protein standards and bacterial measurement

Bacterial protein was measured using the BioRad protein assay (Bradford, 1976). A 10 mg/ml BSA stock solution was used to prepare the standards in triplicate from 200 to 1000 μg/ml (using distilled H_2O to dilute the standards) (Fig. 8).

The bacterial protein content of UW wild-type and mutant strains and YsS6 wild-type and mutant strains were measured. Wild-type UW4 had a significantly higher protein content than the UW4 mutant strain, and the wild-type YsS6 strain had a slightly higher protein content than its mutant (Fig. 9).

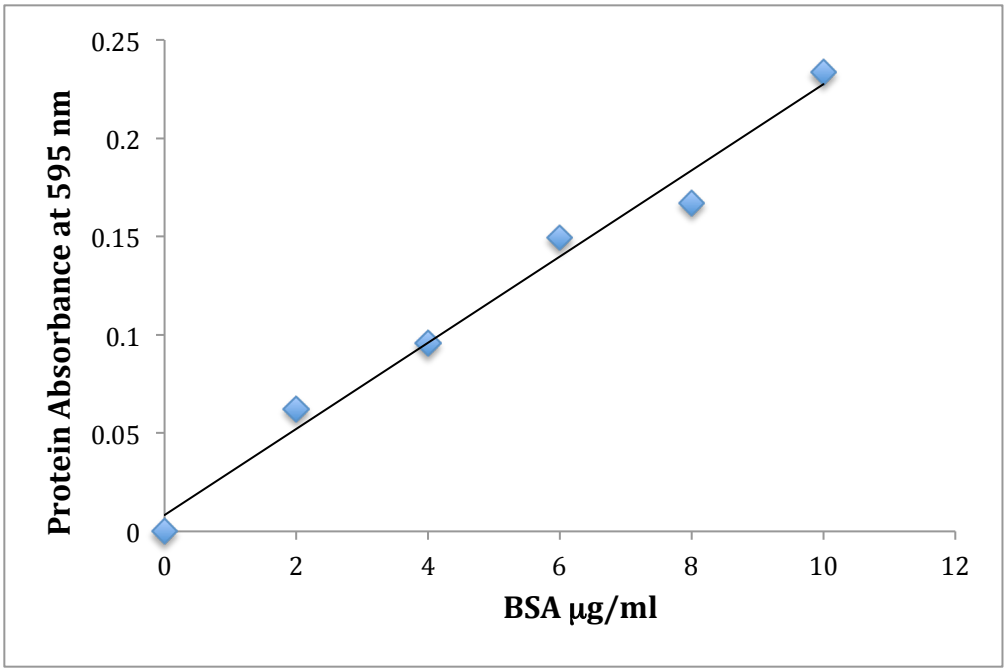


Fig. 8: The protein standard curve**.** Equation: $y = 0.0207x + 0.0175$; $R^2 = 0.975$

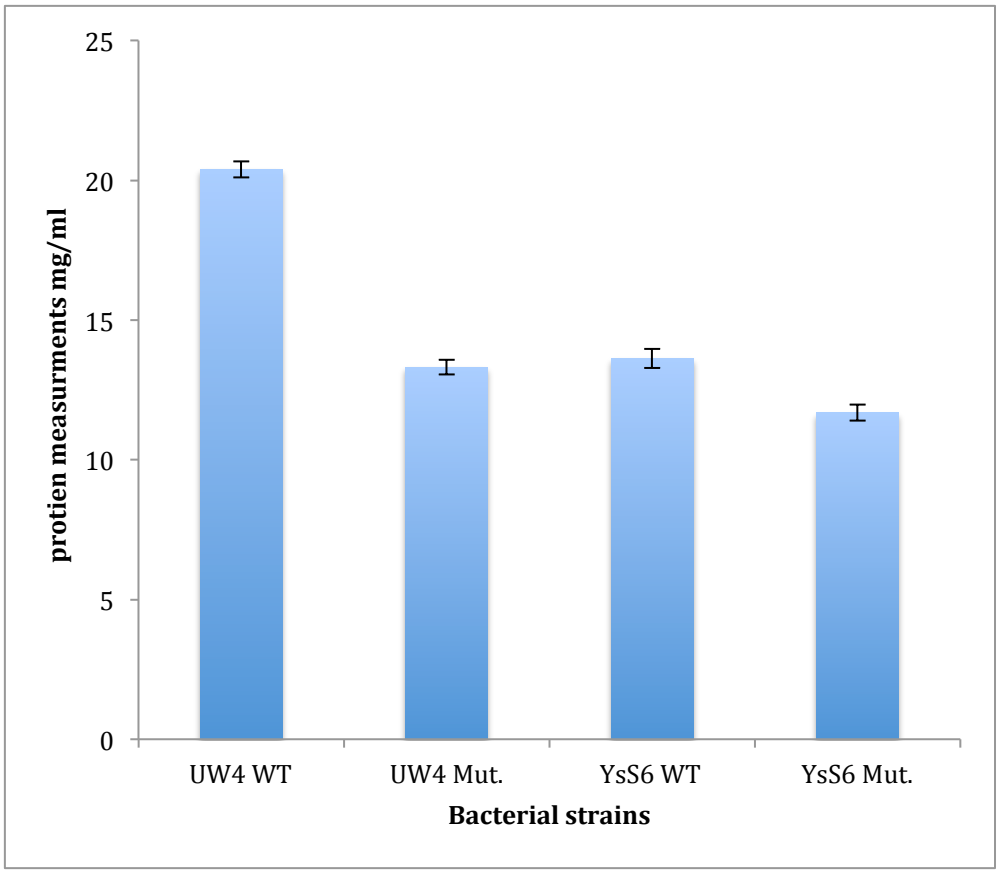


Fig. 9: The protein production level of bacterial strains *P. fluorescens* YsS6 wild-type, *P. fluorescens* YsS6 ACC deaminase minus mutant, *P.* sp UW4 wild-type, and *P.* sp UW4 ACC deaminase minus mutant. This experiment was repeated 4 times. The measurements include standard errors.

3.6. Effect of PGPB on canola plant growth under drought stress

Canola plants were grown in the greenhouse in small plastic pots (7.8 cm x 6.5 cm) in potting soil. They were grown for one month and watered normally, and then subjected to different levels of drought i.e., 0, 6, 8, or 10 days. Ten plants were utilized for each treatment. The treatments included the bacterium wild-type *Pseudomonas* sp. UW4 and its ACC deaminase minus mutant, and *Pseudomonas fluorescens* YsS6 and its ACC deaminase minus mutant (Figs. 10-13).

Fig. 10: The effect of bacterial strains on the growth of canola plants under different levels of drought stress. A-1: Canola plants treated with YsS6 wild-type with 0 days of drought (daily watering; positive control), A-2: Canola plants treated with YsS6 wild-type with 6 days of drought, A-3: Canola plants treated with YsS6 wild-type with 8 days of drought, and A-4: Canola plants treated with YsS6 wild-type with 10 days of drought.

A-1

A-2

A-3

A-4

Fig. 11: The growth of canola plants that treated with bacterial strain YsS6 mutant under different levels of drought stress. B-1: Canola plants treated with YsS6 ACC deaminase minus mutant strain with 0 day of drought (daily watering; positive control); B-2: Canola plants treated with YsS6 ACC deaminase minus mutant strain with 6 days of drought; B-3: Canola plants treated with YsS6 ACC deaminase minus mutant strain with 8 day of drought; and B-4: Canola plants treated with YsS6 ACC deaminase minus mutant strain with 10 days of drought.

B-1

B-2

B-3

B-4

Fig. 12: The effect of different levels of drought stress on the growth of canola plants treated with bacterial strain UW4 mutant. C-1: Canola plants treated with strain UW4 ACC deaminase minus mutant with 0 days of drought (daily watering; positive control); C-2: Canola plants treated with strain UW4 ACC deaminase minus mutant with 6 days of drought; C-3: Canola plants treated with strain UW4 ACC deaminase minus mutant strain with 8 days of drought; and C-4: Canola plants treated with strain UW4 ACC deaminase minus mutant with 10 days of drought.

C-1

C-2

C-3

C-4

Fig. 13: The growth of canola plants under different levels of drought stress and treated with bacterial strain UW4 wild-type. D-1: Canola plants treated with UW4 wild-type strain with 0 days of drought (daily watering; positive control); D-2: Canola plants treated with UW4 wild-type strain with 6 days of drought; D-3: Canola plants treated with UW4 wild-type strain with 8 days of drought; and D-4: Canola plants treated with UW4 wild-type strain with 10 days of drought.

D-1

D-2

D-3

D-4

3.6.1. Shoot fresh weight

Subsequent to drought and bacterial treatments, shoot fresh weights were measured immediately after the plants were harvested (Table 5). While it is clear that increasing the number of days without water, from 0 to 10 days, significantly decreased the fresh weights in all cases, none of the bacterial treatments yielded results that were significantly different from all of the other treatments which means that increased drought in all cases means decreased biomass. This result can also be seen qualitatively in Figs. 10-13.

Table 5. The measurements of shoot fresh weight for canola plants that were treated with bacterial strains *P. fluorescens* YsS6 wild-type, *P. fluorescens* YsS6 ACC deaminase minus mutant, *P.* sp. UW4 wild-type, and *P.* sp. UW4 ACC deaminase minus mutant in different drought levels; Positive controls were treated with bacteria and watered daily. Ten plants were used for each treatment; this experiment was repeated 3 times. The measurements include standard errors.

Addition	0 days drought (g)	6 days drought (g)	8 days drought (g)	10 days drought (g)
UW4 wild-type	14.38±6.7	7.85±0.7	5.29±3.2	1.86±0.2
UW4 mutant	12.6±3.5	7.95±1.01	3.54±2.1	1.94±0.16
YsS6 wild-type	14.88±7.1	8.52±1.83	4.27±2.47	1.19±0.24
YsS6 mutant	15.19±8.9	8.06±1.5	5.43±3.77	1.23±0.47

3.6.2. Shoot dry weight

The shoot dry weights were measured following incubation of the plant shoots at 37°C for 72 h. Similar to what was observed with the fresh weight measurements, increasing the number of days without water, from 0 to 10, significantly decreased the dry weights in all cases. However, none of the bacterial treatments yielded results that were significantly different from any of the other treatments (Table 6).

Table 6. : The shoot dry weight average measurements of canola plants under different drought levels and treated with bacterial strains *P. fluorescens* YsS6 wild-type, *P. fluorescens* YsS6 ACC deaminase minus mutant, *P.* sp. UW4 wild-type, and *P.* sp. UW4 ACC deaminase minus mutant. Ten plants were used for each treatment and the experiment was repeated 3 times. The measurements include standard errors.

Addition	0 days drought (g)	6 days drought (g)	8 days drought (g)	10 days drought (g)
UW4 wild-type	2.46±1.14	1.67±0.36	1.36±0.52	1.10±0.14
UW4 mutant	2.14±0.71	1.52±0.30	1.02±0.30	1.26±0.20
YsS6 wild-type	2.49±1.24	1.72±0.34	1.36±0.68	0.83±0.27
YsS6 mutant	2.20±1.24	1.43±0.23	1.34±0.59	0.76±0.17

3.6.3. Shoot protein concentration

When the shoot protein concentration was measured, in the plants treated with UW4 WT under 0 and 6 days of drought had higher protein content than the protein content in the plants treated with UW4 wild-type under 8 and 10 days of drought. The protein concentration in the plants treated with UW4 mutant and 0, 6, and 10 days of drought did not exhibit any significant differences, however the UW4 mutant treatment under 8 days of drought was slightly lower than controls. The protein concentration in the plants treated with YsS6 wild-type was similar to UW4 treatments, except for the measured amount of protein was lower for the 0 day control. In contrast, in the plants treated with YsS6 mutant the 0 day samples were higher than the drought treatments (Fig.14). This may reflect a decrease of water content within the plant, and not significant changes in the total amount of protein in the plant tissue.

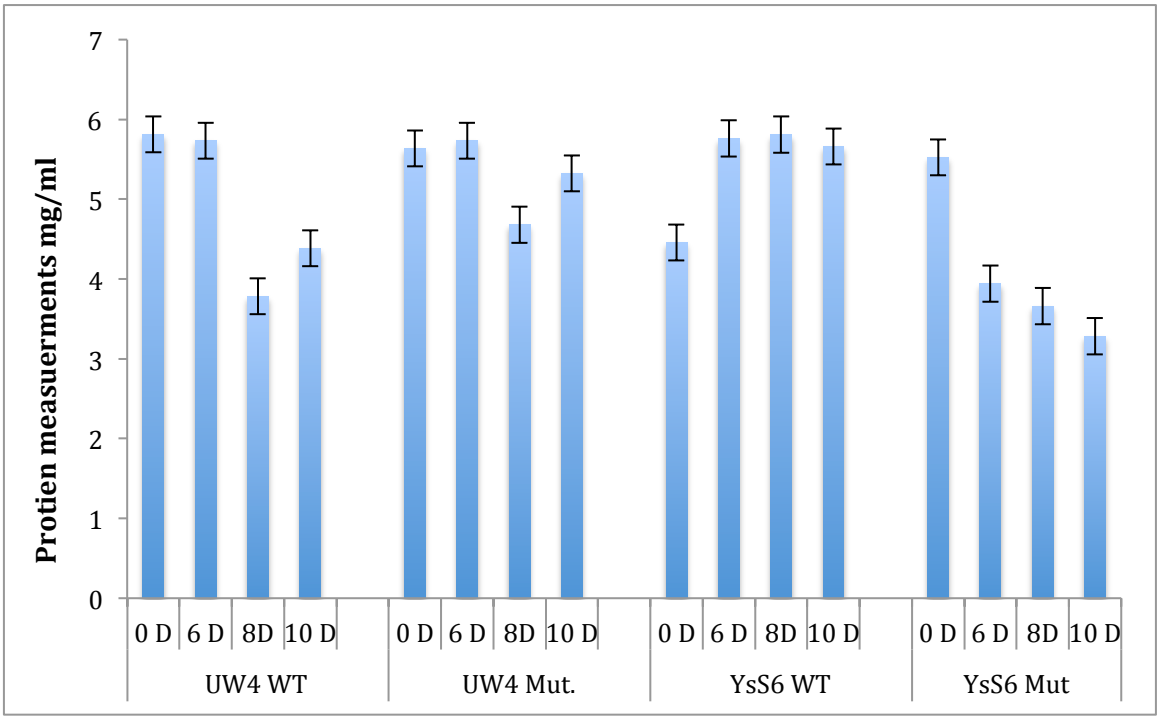


Fig. 14: The shoot protein measurements for canola plants that were treated with bacterial strains *P. fluorescens* YsS6 wild-type, *P. fluorescens* YsS6 ACC deaminase minus mutant, *P.* sp. UW4 wild-type, and *P.* sp. UW4 ACC deaminase minus mutant at different drought levels which included: control in which there was no drought, A1 in which there was six days of drought, A2 in which there was eight days of drought, and A3 in which there was ten days of drought. Error bars represent estimates of standard error.

3.6.4. Leaf chlorophyll concentration

The leaf chlorophyll concentration of canola plants was measured with N,N-dimethylformamide in the dark for 48 h. For the most part, there are no significant differences between any of the measurements. The highest chlorophyll concentration was in the plant that treated with UW4 wild-type under 0 days of drought (40.60 mg/g), while the lowest was in the plant that treated with YsS6 mutant under 10 days of drought (12.17 mg/g) (Fig. 15).

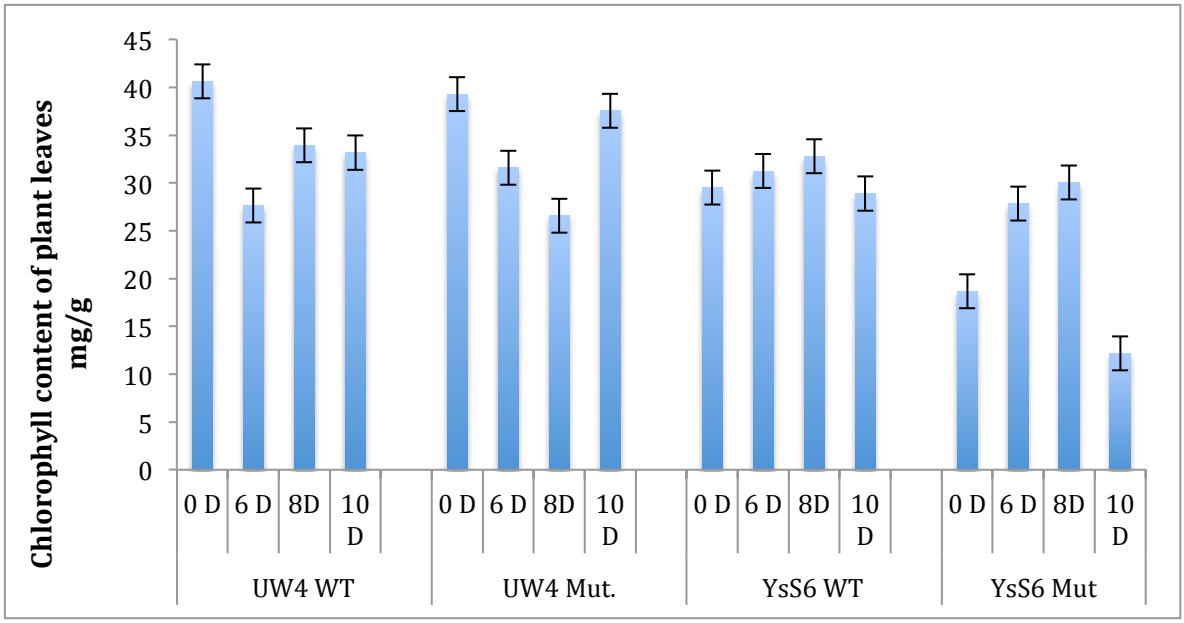


Fig. 15: The leaf chlorophyll concentration measurements of Canola plants that were treated with bacterial strains *P. fluorescens* YsS6 wild-type, *P. fluorescens* YsS6 ACC deaminase minus mutant, *P.* sp. UW4 wild-type, and *P.* sp. UW4 ACC deaminase minus mutant in different drought levels which included: control in which there was no drought, A1 in which there was six days of drought, A2 in which there was eight days of drought, and A3 in which there was ten days of drought. This experiment was repeated 2 times. Error bars are estimates of standard error.

3.6.5. Shoot trehalose concentration

When shoot trehalose concentrations were measured (Fig.16), the highest level of shoot trehalose concentration was observed in the plants treated with the UW4 mutant strain following 6 days of drought, while, the other plants were treated with both bacterial strains under different drought conditions had no statistically significant differences between each other (the highest was the plant were treated with UW4 wild-type under 6 days of drought with 6.03 g/l, and the lowest for the plant were treated with YsS6 mutant under 6 days of drought with 1.58 g/l)

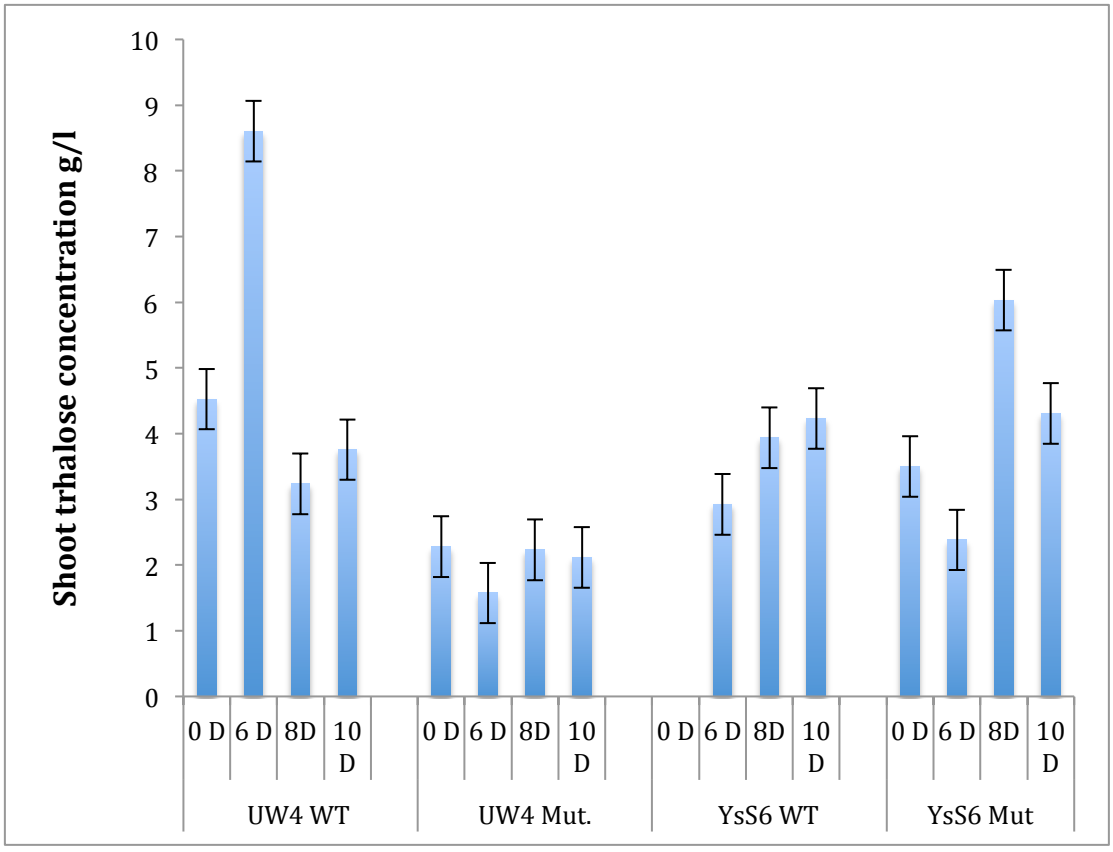


Fig. 16: The shoot trehalose concentrations of plants that were treated with bacterial strains *P. fluorescens* YsS6 wild-type, *P. fluorescens* YsS6 ACC deaminase minus mutant, *P.* sp. UW4 wild-type, and *P.* sp. UW4 ACC deaminase minus mutant. This experiment was repeated 3 times. Errors bars are estimates of standard error.

4. Discussion

In agricultural lands, drought and water stress are considered as serious plant growth concerns. Due to these problems, the water stress decreases the production of food on a global basis significantly (Naveed *et al.,* 2014; Glick, 2013). Plant biomass decreases are the result of a host of metabolic changes that are caused by drought (Bresson *et al.,* 2014). Production of ethylene that results from this water stress significantly inhibits plant growth (Belimov *et al.,* 2009). Inoculation of plants impacted by drought stress with PGPB can potentially help these plants to better tolerate drought. PGPB that contain the enzyme ACC deaminase may help the plants by decreasing their ethylene levels and thereby promoting plant growth (Belimov *et al.,* 2013). There are two different types of bacteria that interact with plants. Rhizospheric bacteria that are isolated from the plant root surface and its adjacent area, and endophytic bacteria that are isolated from internal plant tissues (Dodd *et al.,* 2010). In the present study, *Pseudomonas* sp. strain UW4 and its ACC deaminase minus mutant were used as typical plant growth-promoting rhizospheric bacteria while *Pseudomonas fluorescens* strain YsS6 and its ACC deaminase minus mutant were used as typical plant growth-promoting endophytic bacteria.

4.1. Characterization of the bacterial strains

All four of the bacterial strains that were utilized in these experiments were characterized biochemically and physiologically. In the first instance, it was confirmed that both of the ACC deaminase mutants (i.e. derived from strains UW4 and YSs6) had lost the high level of ACC deaminase activity possessed by the wild-type versions of these strains (Fig. 3). However, while strain UW4 and its ACC deaminase minus mutant produced similar

levels of IAA and siderophores, IAA production was dramatically increased and siderophore production was significantly decreased in the ACC deaminase minus mutant of strain YsS6 compared to its wild-type levels (Figs. 5 and 6). Thus, the ACC deaminase mutation in strain UW4 had no effect on the ability of the mutant strain to produce IAA (Li *et al.,* 2000). In fact, IAA plays a role in the survival and functioning of the bacteria. Duca *et al.*, (2014) have pointed out that IAA may play an important role in protecting the bacterium itself against environmental stresses; it enhances bacteria to be more adapted and resistant to various environmental stresses.

Most soil PGPB produce siderophores to limit the iron availability for other microorganisms and to compete with pathogens. In optimal growth conditions, bacteria that grow in iron-deficient environments are expected to sequester sufficient amounts of iron (Hussien & Joo, 2014). That means, most siderophores-producing PGPB strains in soil are highly competitive against pathogens. Thus, they can be used as biocontrol agents and at the same time promote plant growth by providing iron to the plant. In addition, siderophores enhance induced systemic resistance (ISR) in the plant (Sukweenadhi *et al.,* 2015; Ahmad & Kibret, 2014).

The results that were observed for strain YsS6 are similar to what was previously observed for another endophytic bacterial strain, *Burkholderia phytofirmans* PsJN (Sun *et al.,* 2009). In the case of strain PsJN, the observed altered physiology (i.e. changes to IAA and siderophore levels) was attributed to a six- to eight-fold increase in the expression of RpoS, the stationary phase sigma factor, in the ACC deaminase minus mutant of strain PsJN (Sun *et al.,* 2009). The reason why a mutation in ACC deaminase, a non-essential gene in strain PsJN, causes such a dramatic change in the expression of the stationary phase sigma

factor is unclear. However, the data presented here suggest that YsS6, another endophytic bacterium behaves similarly to PsJN and differently from UW4, a rhizospheric bacterium. It would be of interest to examine other ACC deaminase-containing bacterial endophytes to see whether they behave similarly to strains YsS6 and PsJN.

Interestingly, strains UW4 and it ACC deaminase minus mutant behaved differently from strain YsS6 and its ACC deaminase minus mutant when assessing other traits in addition to those mentioned above. Thus, wild-type strain UW4 contained a much higher level of cellular protein but lower trehalose than its ACC deaminase minus mutant (Figs. 7 and 9). On the other hand, both strain YsS6 wild-type and its ACC deaminase minus mutant strains had similar (low) levels of cellular protein and similar (high) levels of trehalose (Fig. 6 and 8). These data are consistent with the notion that mutating ACC deaminase in rhizospheric UW4 results in different changes to the host bacterial cellular physiology than does mutating ACC deaminase in endophytic YsS6. The high amounts of trehalose that were previously detected in bacterial strains grown in minimal medium with 0.8 M NaCl (this medium is considered to be a stressed medium) were interpreted as indicating that bacterial trehalose is synthesized as a response to stress and the trehalose protects the parental microorganism under these stress conditions (Goddijn & Dun, 1999).

4.2. Effect of the bacterial strains on canola plants

Based on previous experiments with the above-mentioned bacterial strains, it was predicted that the two wild-type bacterial strains but their ACC deaminase minus mutants would promote the growth of plants under drought stress. Thus for example, Li *et al.,* (2013) reported, that flooded canola plants that were treated with wild-type strain UW4 had

significantly greater root length and protein expression levels of selected proteins compared to canola plants that were treated with the UW4 mutant strain under gnotobiotic conditions because of the ability of the wild-type to produce ACC deaminase. In addition, both wild-type strains YsS6 and UW4 but not their respective ACC deaminase minus mutants were previously found to dramatically protect tomato and canola plants, respectively, from growth inhibition by the presence of high salt in the growth medium (Cheng *et al.,* 2007; Ali *et al.,* 2014).

In the experiments reported here, shoot fresh and dry weights were measured after the plants were harvested. These results definitively show that in all cases increasing the number of days that the plants went without water, from 0 to 10, as expected, significantly decreased both the fresh and dry weights (Tables 5 and 6). Unfortunately, however, contrary to expectation, none of the bacterial treatments yielded results that were statistically significantly different from all of the other bacterial treatments. A perusal of the plant data indicates that there is a large standard error in all of the plant measurements. Thus, it would appear that reliable and statistically significant data likely requires a much larger number of samples. For example, each of the plant measurements reported here included only ten plants. On the other hand, it is standard operating procedure to use measurements of from 50-60 separate seedlings to assess canola root elongation in growth pouches, with these experiments being repeated two to three times (Penrose & Glick, 2003).

Again, in previous experiments with pea plants that were subjected to drought, 41% of plants un-inoculated with ACC-containing bacteria displayed reduced shoot biomass while only 18% of inoculated plant shoots showed reduced biomass (Arshad *et al.,* 2008). As Belimov *et al.,* (2005) stated, there is a relationship between ACC deaminase enzyme

activity in vitro and the effect of bacteria containing this enzyme on promoting plant growth. For a plant exposed to drought, the ACC deaminase enzyme leads to a decrease in the plant ethylene levels and stimulates the plant's shoot and root growth, and also enhances the plant tolerance to the different biotic and abiotic ethylene-inducing stresses (Belimov *et al.,* 2005; Chen *et al.,* 2013 A; Jiang *et al.,* 2012; Glick, 2013).

IAA can stimulate ACC synthesis by promoting the transcription of ACC synthase (Li *et al.,* 2000). However, in the experiments reported here, there is no evidence that increased ACC synthesis played any role in the observed results. The IAA production and secretion levels determine the effect of IAA in the plant growth either positively or negatively, for example; *P. flourescens* CHA0 wild-type produces IAA and promotes cucumber growth, while an IAA-overproducing mutant strain of this bacterium inhibits cucumber growth (Beyeler *et al.,* 1999). The impact of an IAA-producing bacterium on plant growth is significantly affected by the level of IAA that exists within a host plant prior to the addition of the bacterium. If the IAA level in the plant is suboptimal, it will be increased by the bacterial IAA to more optimal levels and thereby promote plant growth. On the other hand, if the endogenous plant level of IAA is already optimal, the addition of bacterial IAA will affect the plant growth negatively (Duca *et al.,* 2014).

In previous studies, the plant chlorophyll content has been reported to be decreased under drought stress as a response of stomatal closure and decreases in photosynthesis that are parts of drought tolerance. This tolerance is induced by ABA that lowers the transpiration rate and decreases the plant's loss of water (Kim *et al.,* 2012). In addition, stomatal closure could be affected by the reduction of interference between ABA and ethylene thus stimulating induced systemic tolerance (IST) in the plant by non-pathogenic gram-negative

ACC-containing bacteria such as strains UW4 and YsS6. IST lowers the ethylene level in the plant roots as a response against drought stress (Kim *et al.,* 2012).

Ali *et al.,* (2014) have noted that, under salinity stress, tomato plants treated with strain YsS6 WT had higher chlorophyll content than tomato plants treated with the YsS6 mutant strain and tomato plants not treated with any bacteria. According to Naveed *et al.,* (2014), maize plants treated with the endophytic ACC deaminase-containing strain PsJN under drought conditions increased the chlorophyll content compared to the untreated control plants under the same conditions. A similar result was published by Wang *et al.,* (2012) who found that the chlorophyll content increased in cucumber plants treated with a mixture of *Bacillus cereus* AR156*, Bacillus subtilis* SM21*, and Serratia sp.* XY21 compared to the untreated control plant under drought stress.

Plants often respond to abiotic stresses by accumulating some compounds such as trehalose (Cortina, & Culiáñez-Macià, 2005). In plant-bacterial interactions, most of the trehalose that is synthesized by bacteria is located in the host plant cells' cytoplasm, with the presence of trehalose increasing the plant's tolerance to drought stress (Fernandez *et al.,* 2010). In present study, the plant shoot trehalose concentrations were not very high except for plants were treated with the UW4 Wild-type strain following 6 days of drought. These results are opposite of what was found in a previous study by El-Bashiti *et al.,* (2005) who observed that trehalose was accumulated in high amounts in wheat plants under drought stress conditions compared to control conditions.

In flowering plants, trehalose is typically present in very low amounts, although it has a role in drought tolerance as a stress molecule (Zhou *et al.,* 2013; Almeida *et al*., 2005). During a biotic stress, sucrose takes over trehalose's role in protecting plants, thus the

accumulation of trehalose in some higher vascular plants may be low under abiotic as well as biotic stress conditions (Goddijn & Dun, 1999). However, the presence of validamycin A, which is a trehalase inhibitor could help the plants that possess it to accumulate trehalose in low amounts (Zhou *et al.,* 2013; Goddijn & Dun, 1999). Moreover, the low levels of trehalose that have been detected in some plants may alter sugar and starch metabolism within the plant leading to sugar synthesis instead of leading to osmoprotection and stress tolerance (Cortina, & Culiáñez-Macià, 2005). In addition, sucrose cleavage might be more helpful for the plant under stress due to the high free energy that can result from sucrose hydrolysis and microorganisms are generally better able to utilize sucrose than trehalose, which is advantageous for plant-microbial interactions (Wingler, 2002).

5. Conclusions

Plant growth-promoting bacteria (PGPB) that contain the enzyme (1-aminocyclopropane-1-carboxylate) ACC deaminase *Pseudomonas* sp. UW4 (a rhizospheric PGPB) (Duan *et al.,* 2013) and *Pseudomonas fluorescens* YsS6 (an endophytic PGPB) (Rashid *et al.,* 2012) and their respective (1-aminocyclopropane-1-carboxylate) ACC deaminase minus mutants behave differently than each other with the biochemical assays, which means the rhizospheric bacteria same activity as rhizospheric PGPB act differently than rhizospheric PGPB. Moreover, in this study, it is not proven that the PGPB that possess both ACC-deaminase and trehalose but not their ACC deaminase minus mutant can protect Canola against drought stress.

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