“A Study on the Beneficial Effects of Rhizobacteria on Physiological Status, Proline Metabolism and Endogenous Phytohormone Accumulation in *Arabidopsis thaliana* Under Water-stress”

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CERTIFICATE

This is to certify that the thesis entitled **“A Study on the Beneficial Effects of Rhizobacteria on Physiological Status, Proline Metabolism and Endogenous Phytohormone Accumulation in *Arabidopsis thaliana* Under Water-stress”** was submitted by **Daipayan Ghosh, ID. No. 2013PHXF0400H** for the award of Ph. D. degree of the Institute embodies original work done by him under my supervision.

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Abstract

Although the physiological benefits of plant growth promoting rhizobacteria (PGPR) to plants are well reported, the precise mechanisms that make these PGPR such wonderful friends of plants under adverse environmental conditions are still under investigation. We have investigated the role of a drought tolerant PGPR strain, *Pseudomonas putida* GAP-P45 in ameliorating the negative impacts of water-stress in *Arabidopsis thaliana* and have studied some aspects of the underlying mechanisms of such beneficial plant-PGPR interaction. We have studied the effects of *P. putida* GAP-P45 on (a) morpho-physiological characteristics (b) proline metabolism and (c) phytohormone accumulation in *A. thaliana* under water-stress. Plants inoculated with *P. putida* GAP-P45 exhibited characteristics of water-stress mitigation by improvement of morpho-physiological parameters such as better growth, increased fresh weight, enhanced plant water content, reduction in primary root length, enhanced chlorophyll content in leaves and increased accumulation of endogenous proline content, when compared to non-inoculated water-stressed plants. We observed that *P. putida* GAP-P45 alleviated the effects of water-stress in *A. thaliana* by causing drastic changes in proline metabolic gene expression at different time points post stress induction. Quantitative real-time analysis of proline metabolic gene expression in inoculated plants under water-stress showed a delayed but prolonged upregulation of genes involved in proline biosynthesis, i.e., *ornithine-Δ-aminotransferase* (*OAT*), *Δ1- pyrroline-5-carboxylate synthetase1* (*P5CS1*), *Δ1-pyrroline- 5-carboxylate reductase (P5CR)*, as well as proline catabolism, i.e., *proline dehydrogenase1 (PDH1)* and *Δ1-pyrroline- 5-carboxylate dehydrogenase (P5CDH)*. We also analyzed the impact of this PGPR strain on the activity of proline metabolic enzymes encoded by the aforementioned genes and observed that inoculation of *A. thaliana* with *P. putida* GAP-P45 under water-stress modulated the proline

metabolic enzyme activity in a similar pattern as the proline metabolic genes, stated earlier. Since any observed physiological status of plants either under stress or normal conditions is directly correlated to the accumulation, distribution and cross-talk between major phytohormones, we were interested to study the modulation of accumulation of four major endogenous hormones (abscisic acid, auxin, cytokinin and gibberellic acid) in *A. thaliana*, separately, in the roots and shoots, mediated by *P. putida* GAP-P45 inoculation under water-stress. We observed that, while water stress increased the accumulation of abscisic acid and decreased the content of auxin and cytokinin in shoots and roots; the level of gibberellic acid decreased in shoots but increased in roots due to stress. Inoculation with *P. putida* GAP-P45 under water stress effectively reversed the trends of phytohormone accumulation, making their levels similar to the non-stressed, non-inoculated control plants. This happened despite there being no change in the water-potential of the medium due to *P. putida* GAP-P45 inoculation. We also observed that the pattern of phytohormones secreted by the PGPR varied depending on composition of nutrient media and culture conditions.

Our observations on the modulation of proline metabolic gene expression and enzyme activity point towards transcriptional and translational regulation of proline metabolism and enhancement of proline turnover rate in *A. thaliana* by *P. putida* GAP-P45 inoculation under water-stress. It can be concluded that *P. putida* GAP-P45 stimulates not only enhanced proline accumulation, but also its concomitant degradation, thus modulating proline homeostasis in *A. thaliana* under water-stress. From the observations on phytohormone accumulation we conclude that *P. putida* GAP-P45 alleviates water-stress in *A. thaliana* by altering the endogenous hormone accumulation and re-distribution in both roots and shoots without causing any change to the water-potential of the medium.

CHAPTER 1

Introduction and review of literature

1.1 <u>Climate change and drought</u>

Prediction studies based on the current statistical records have estimated the world's total population at 9.8 billion by 2050 (Nations and Affairs 2017). Increasing global population and their rising living standards have created a continuous, ever-increasing demand for the steady supply of staple food all over the world (Godfray et al. 2010). Growing competition for land, water, and energy, in addition to the overexploitation of forests, grasslands and natural water bodies have affected our ability to produce enough food to meet the demand of the current population of 7.6 billion (Godfray et al. 2010; Watson et al. 2017). So, it is anticipated that, in the near future, the ratio of supply and demand of food will decrease at a rate faster than the present. It is also predicted that this demand for accelerated food production will be maximum in the rapidly developing countries like India. Development of new cultivars with enhanced crop yield, better utilization of the available agricultural land, better management of the available resources including water conservation and reduced yield gap are now the major focus of the scientific community all over the world. (Gouda et al. 2018). Two of the toughest hurdles in the way of increased crop yield are climate change and global warming, which negatively impact the gross production and yield of crops across the globe (Khanal et al. 2018). The major causes for climate change have been identified as massive deforestations, emission of greenhouse gases and burning of fossil fuel since 1850 which has affected the climate by the means of massive reduction in rainfall (10-15% of annual rainfall), below-average precipitation and increase in mean temperature (0.1-1.3 ºC) in the tropical countries including India (Lawrence and Vandecar 2015). As the maximum percentage of annual gross crop production in India depends mostly on the annual rainfall (rest is irrigation dependent), climate change and global warming have drastically affected annual food supply by means of crop destruction due to scarcity of sufficient water (Khanal et al. 2018). As an obvious result of these environmental factors, lowering of soil water table have been observed for last three decades, leading to greater incidents of drought stress in the tropical regions around the globe. Drought stress alone is responsible for almost 70 % of total crop damage in the tropical and subtropical countries (Akram et al. 2013; Kuwayama et al. 2018). The multiple aspects and magnitude of drought stress can be classified as 1) meteorological drought (long-term deficiency of precipitation) 2) agricultural drought (insufficiency of water for crop production or plant growth) 3) pedological (shortage of soil-water storage) 4) hydrological drought (deficiency of water-flow to meet the needs of water-supply) 5) socioeconomic drought (cumulative effect of other types of drought on demand and supply of economically important goods) (Hao and Singh 2015). However, it is hard to identify the specific pattern of drought stress that affects the agricultural system of a tropical or subtropical region, as in most cases, the observed effects are the cumulative outcome of all of the above. Agricultural drought is mainly assessed by the moisture content of the soil and the percentage of humidity. However, other co-varying factors such as, crop species, crop rotation time, plant phenology, soil texture, rooting system of the crop etc. are also equally important to develop strategies to combat drought-induced loss of crop yield (Daryanto et al. 2017). Since the turn of this century, drought has drastically affected the Western and Southern Indian agriculture, with severe drought observed at least once every 2-3 years. Our neighboring countries like Pakistan, Nepal, Bangladesh and Sri Lanka have also suffered severe drought challenges in last five decades (Miyan 2015). The observed effects have encompassed the threat to food security and population health, depleted water resources, annual surface run off, biodiversity conservation, hydroelectric power generation and livelihood (Miyan 2015).

1.2 Effects of drought on plants

According to classical plant physiology, drought is a type of osmotic stress which results from a decrease in soil water potential below a level that adversely affects water uptake by roots (Taiz, L. and Zeiger 2003). Effects of drought stress on plants at physiological and molecular levels have been summarized in Fig. 1.1. Drought stress disrupts normal physiological status and morphological traits of plants by adversely affecting cellular water-potential (and thus, cell turgidity), fresh weight, biomass accumulation (particularly shoot growth), water content, nutrient transport from root to shoot etc. At cellular levels, unavailability of water disrupts bilayer structure of cell membrane by causing membrane porosity, displacement and disruption of protein structures (which ultimately affect integrity and selectivity of membranes), collapse cellular compartmentalization and impose osmotic imbalance. Due to extreme dehydration of the cytoplasm under prolonged drought stress, concentrations of the cellular electrolytes (sodium, potassium and chlorides) increase and their leakage from the cell inhibits normal metabolic pathways; cytosolic and organelle proteins lose their stability and undergo denaturation, ultimately causing cell death (Mahajan and Tuteja 2005). It also damages chlorophyll content by photo-oxidation, causes membrane deterioration by inducing reactive oxygen species and restricts growth and development caused due to protein degradation (Smirnoff 1993; Sgherri et al. 2000; Caravaca et al. 2005). Decrease in intracellular CO_2 concentration induces massive ROS (such as, superoxide, hydrogen peroxide, hydroxyl radicals) production due to over-reduction of the electron transport chain intermediates and transfer of electrons to oxygen at photosystem I, ultimately causing photo-oxidation (Boyer et al. 1997; Meyer and Genty 1998). Major damage to the chloroplast membrane is caused by the oxidative stress induced by drought, as ROS causes extreme de-esterification and peroxidation of membrane lipids, leading to protein misfolding and DNA mutation (Bowler et al. 1992). Rapid drop in humidity and movement of dry air mass in the environment due to drought conditions cause increase in vapor pressure gradient between leaf and ambient environment, increasing transpiration rate (Mahajan and Tuteja 2005). Activity of the key photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) decreases under drought stress due to lower CO_2 concentrations, which ultimately limits photosynthetic efficacy of the plants (Carmo-Silva et al. 2012). Diminished photosynthetic machinery under extreme and prolonged drought conditions adversely affects the normal growth kinetics, development and biomass accumulation of plants. Effects of drought stress on hampered growth kinetics have been reported and characterized in many important crop species such as, rice, wheat, maize, barley etc. (Kasim et al. 2013).

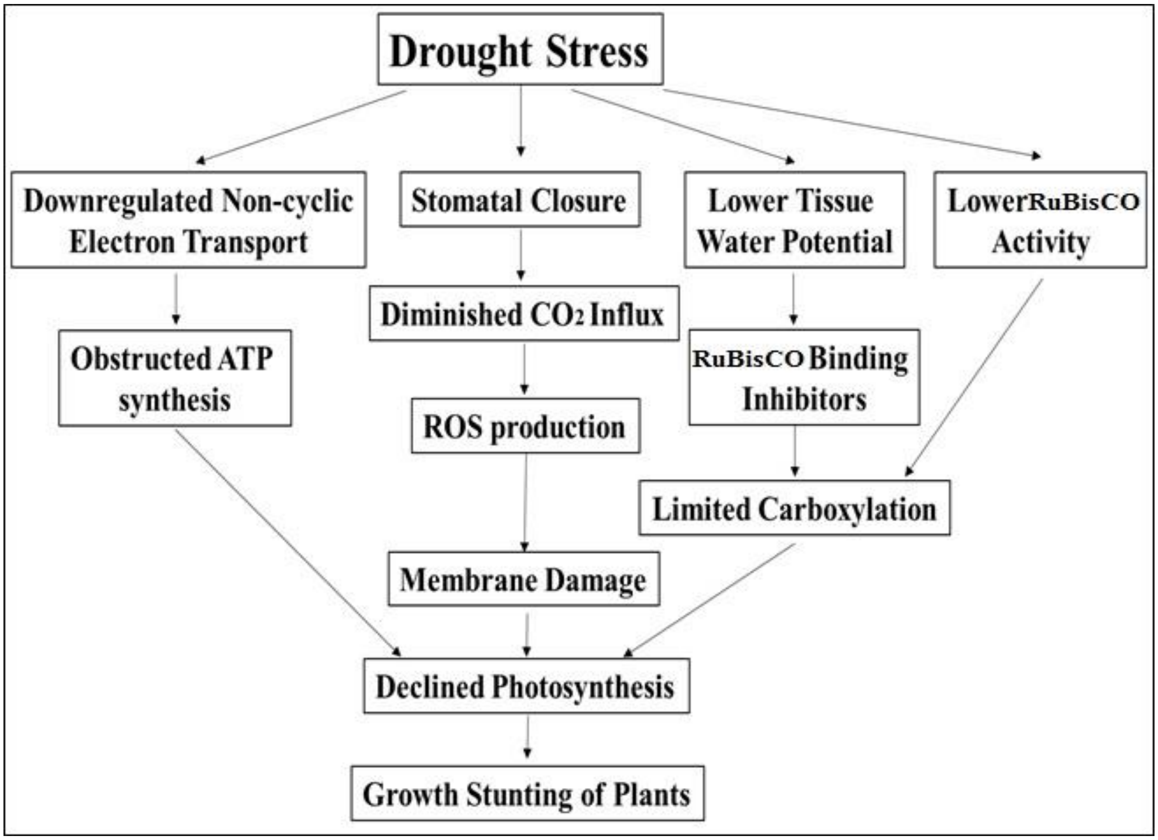


Fig. 1.1 Effects of drought stress on plants at physiological and molecular levels.

1.3 Responses of plants to drought stress

To abate the detrimental effects of drought stress, plants have evolved several complex defense mechanisms involving morphological, physiological and biochemical alterations such as, suppression of shoot biomass accumulation, minimization of water loss by stomatal closure, induction of root growth, delayed or accelerated flower development etc. (reviewed by Szabados and Savouré 2010). Some of these mechanisms are elaborated below:

1.3.1 Stomatal closure and osmotic adjustment:

Drought stress induces stomatal closure of the plants as a primary and immediate response to water scarcity to prevent transpirational water loss, which causes depletion in intercellular CO_2 concentration and metabolic instability due to reduced leaf water content (Chaves et al. 2003). Hydropassive closure of stomata is caused by direct evaporation of water from guard cells of leaves without any metabolic involvement. Stomatal closure induced by alteration of ionic fluxes and metabolites in the guard cells is termed as hydroactive closure (Mahajan and Tuteja 2005). One of other earliest responses of plants to drought is cellular osmotic adjustment by accumulation of several compatible osmolytes such as sugar alcohols (sorbitol), amino acids (proline), amino acid derivatives (glycine betain) etc., proline being the most important of all (Kavi Kishor et al. 2005b; Szabados and Savouré 2010; Zlatev and Lidon 2012; Krasensky and Jonak 2012; Reddy et al. 2015; Ghosh et al. 2017). Accumulation of these solutes at high concentration in cells prevents exosmosis of water by decreasing cellular water potential, thus sustaining turgor pressure (Krasensky and Jonak 2012; Liang et al. 2013; Reddy et al. 2015). The role of free proline (a proteinogenic amino acid) as an important compatible osmolyte is well established. It is well reviewed that the cellular concentration of proline increases from 20%, under non-stressed conditions, to 80% under osmotic stress, of the total free amino acid pool in several plant species (Yancey et al. 1982; Kavi Kishor et al. 2005a; Choudhary et al. 2005; Sharma and Verslues 2010; Liang et al. 2013). Similar observations have been made under salt and cold stress as well (Kaplan et al. 2007; Sharma and Verslues 2010). High proline accumulation in response to abiotic stress has been positively correlated with maintenance of optimum cell turgor pressure, cytosolic pH and intracellular redox potential (Kavi Kishor et al. 2005a; Verbruggen and Hermans 2008; Sharma and Verslues 2010; Liang et al. 2013; Ben Rejeb et al. 2014; Reddy et al. 2015). Other than functioning as an osmo-protectant, proline plays important roles in energy yielding as $FADH_2$ and NAD(P)H (Hare and Cress 1996; Szabados and Savouré 2010), stabilization of protein structure as molecular chaperons (Szabados and Savouré 2010; Liang et al. 2014), maintenance of cellular nitrogen content (Wu 2003), generation of reactive oxygen species (Székely et al. 2008; Liu et al. 2012), cellular reprogramming and development (Funck et al. 2012; D'Aniello et al. 2015) etc. Proline has been reported to prevent bacteria (Zhang et al. 2015) and plants (Szabados and Savouré 2010) from oxidative damage under abiotic stress conditions. However, elicitation of these endogenous defense mechanisms of plants under moderate or extreme drought conditions do not provide tolerance for a long enough time period to maintain normal metabolic processes for growth and development. Compatible osmolytes other than proline, such as, glycine-betaine (GB), mannitol, sorbitol and trehalose, have been well reported to be upregulated and accumulated in plant cells under drought stress. GB accumulates mainly in chloroplast, as it helps maintain photosynthetic efficiency by protecting thylakoid membrane during stress and other osmolytes have been reported to accumulate mainly in the leaf tissues (reviewed by Chen and Murata 2002; Ashraf and Foolad 2007). The amount of free soluble sugars in drought increases due to the hydrolysis of the starch which help in adjusting osmotic potential in leaves and roots of the plants (Verbruggen and Hermans 2008; Ahemad and Kibret 2014). Both the approaches, (1) production of transgenic plants with the capability of over-producing these compatible solutes and (2) exogenous application of these osmolytes to plants under drought stress conditions, have been successful in imparting stress tolerance to the plants, mainly rice, mustard, *Arabidopsis* and tobacco (reviewed by Chen and Murata 2002; Ashraf and Foolad 2007).

1.3.2 Modulation of phytohormones:

Modulation in the pool of major phytohormones (auxins, cytokinins, gibberellins, abscisic acid, ethylene, brassinosteroids etc.) under drought stress and redistribution in different tissues in response to different environmental stresses ultimately dictate sensitivity or tolerance of plants to the exerted stress (Dobra et al. 2010; Sreenivasulu et al. 2012; Fahad et al. 2015b). Abscissic acid is the key stress-signaling hormone and is reported to confer tolerance to plants especially under drought and salinity stress as an immediate response but up to a certain limit (Wasilewska et al. 2008; Ding et al. 2016; Sah et al. 2016). Under water stress, initially ABA is synthesized and accumulated in the roots and is then transported to the shoots with increasing stress conditions (Waadt et al. 2014; Hu et al. 2016). Rapid accumulation of ABA under drought activates a cascade of signaling pathways and modifies gene expression specific for drought adaptation which in turn causes stomatal closure and reduced leaf expansion (Wilkinson et al. 2012; O'Brien and Benková 2013). Auxins, mainly IAA is the most widely reported multi-functional phytohormone for normal growth and development as well as for coordinating regulatory responses of plants under both biotic and abiotic stress conditions (Dodd et al. 2010; Kazan 2013; Llanes et al. 2016). According to the proposed model given by Shi et al. (2014), auxins mediate drought stress tolerance by improving root structure architecture, leaf water uptake, metabolic homeostasis, ROS detoxification and inducing a plethora of stress related genes. Cytokinins (CK) are termed as master regulators of plant growth and development because of their involvement in cell division, apical dominance, leaf senescence, shoot differentiation, vascular differentiation, chloroplast biogenesis and all other aspects of plant growth (Nishiyama et al. 2011; Kang et al. 2012) as well as in abiotic stress response (O'Brien and Benková 2013). Under water stressed conditions, endogenous cytokinin content has been reported to decrease in shoots due to decreased biosynthesis and transport of cytokinins from roots to shoots (Llanes et al. 2014). This, coupled with reported increases in ABA content leads to higher ABA/CK ratio and finally, accelerated leaf senescence (Riefler et al. 2006; Nishiyama et al. 2011). Gibberellins are involved in a number of developmental and physiological processes in plants such as seed germination, seedling emergence, stem and leaf growth, floral induction, flower and fruit growth, promotion of root growth and root hair abundance (King and Evans 2003; Yamaguchi 2008). There are increasing evidences of involvement of GAs in abiotic stress responses. It is speculated that water stress markedly decreases endogenous GA content by inhibiting biosynthesis or enhancing degradation in plant tissues (Rood et al. 2000; Yang et al. 2001; Achard et al. 2006).

1.4 Plant growth promoting rhizobacteria (PGPR)

Plant growth promoting rhizobacteria (PGPR) constitute a group of soil bacteria that colonize the surface of plant roots and are well known to contribute positively towards alleviation of abiotic stress in plants (Hayat et al. 2010; Saharan and Nehra 2011; Bhattacharyya and Jha 2012; Liu et al. 2013; Timmusk et al. 2014; Bishnoi 2015; Ngumbi and Kloepper 2016). Based on their interaction with plant root cells, PGPR are classified into 2 types: iPGPR (symbiotic) and ePGPR (free living). Species of *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, *Sinorhizobium*, and *Mesorhizobium* are symbiotic nitrogen-fixing bacteria while free living bacteria belong to species of *Azospirillum*, *Enterobacter*, *Klebsiella*, *Bacillus* and *Pseudomonas* (Ahemad and Kibret 2014). Inoculations of plants under drought conditions with various strains of PGPR have been reported to improve biomass production via root and shoot growth, enhance nutrient uptake, increase chlorophyll content and impose resistance against pathogenic microbes (Dimkpa et al. 2009; Hayat et al. 2010; Saharan and Nehra 2011; Salomon et al. 2014). Promotion of plant growth under drought stress by PGPR inoculation involves an array of mechanisms which can be broadly classified into 2 categories: 1) direct mechanisms and 2) indirect mechanisms.

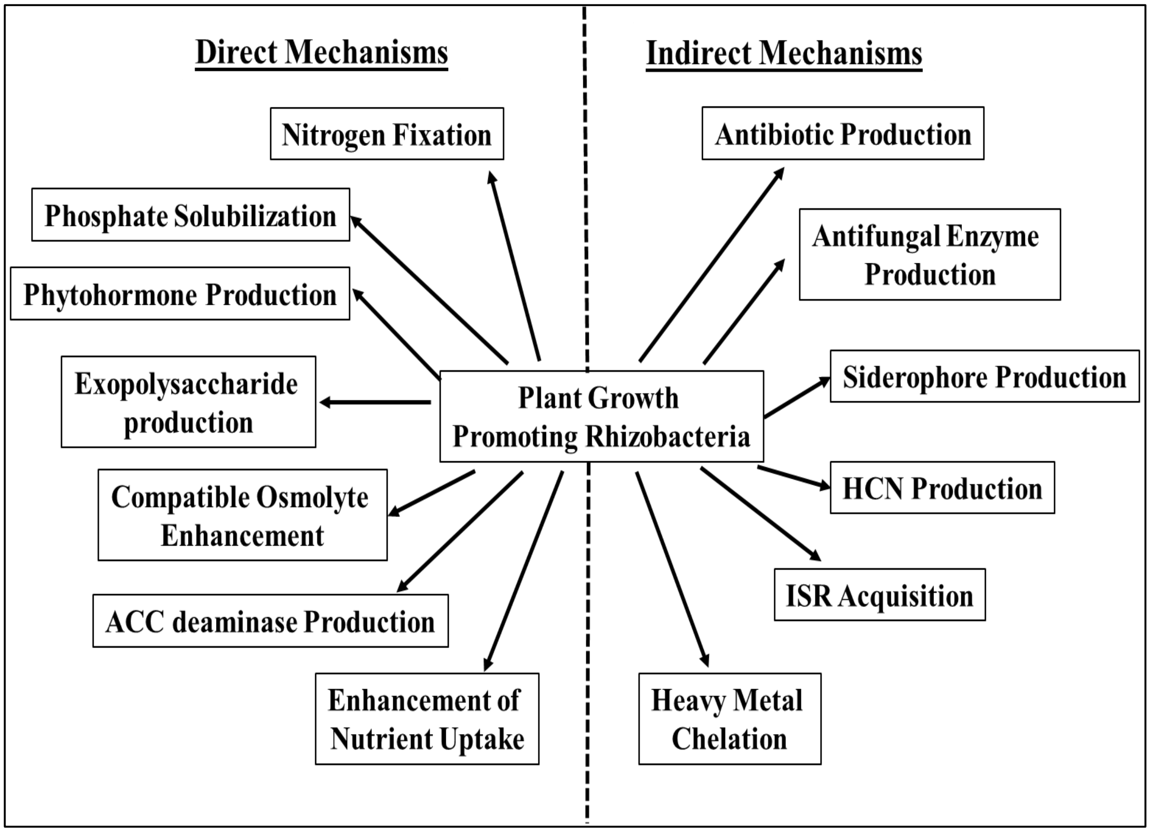


Fig. 1.2 Different mechanisms of plant growth promotion activities showed by PGPR.

1.4.1 Direct mechanisms:

PGPR affect plant growth directly through nitrogen fixation, phosphate solubilization, and production of exopolysaccharides, compatible osmolytes like proline, phytohormones like auxin, cytokinin, gibberellins and abscisic acid and also production of 1-aminocyclopropane-1-carboxylate deaminase (ACC) to alleviate ethylene levels in plants (Saharan and Nehra 2011; Bhattacharyya and Jha 2012; Ahemad and Kibret 2014).

(1) Nitrogen fixation

Nitrogen is one of the most vital nutrients required for plant growth. Plants cannot uptake nitrogen from air and therefore, they need to be converted into plant usable forms. Nitrogen fixers are of two types: symbiotic (such as, *Rhizobium* and *Frankia*) and non-symbiotic (such as, *Azospirillum*, *Azotobacter*, *Acetobacter* and *Azoarcus*) microbes. Symbiotic microbes fix about 80 % of total amount of fixed nitrogen and the rest is done by non-symbiotic ones. They convert atmospheric nitrogen to nitrite and then to nitrate. This is brought about by enzyme complex nitrogenase produced by *nif* gene. Use of nitrogen-fixing bacteria as bio-fertilizer and bio-enhancer may reduce the over-application of harmful chemical fertilizers.

(2) Phosphate solubilization

Like nitrogen, phosphorus is one of the most important macronutrients required by plants. The limited bioavailability of phosphorus from soil retards plant growth. Proper solubilization of phosphorus in the soil in the form of orthophosphates is essential for the proper growth of plants. Utilization of phosphate solubilizing bacteria (PSB) as bio-enhancer can be a promising alternative to the nutritional challenges faced by the plants under drought stress (Rodríguez et al.

2007). PGPRs exudate organic acids that solubilize inorganic phosphate into acids, available in soil for the plants to uptake (Gyaneshwar et al. 2002). *Bacillus, Rhizobium* and *Pseudomonas* among bacteria and *Aspergillus, Penicillium* among fungi are reported to be the most potent phosphate solubilizers (Rivas et al. 2006).

(3) Exopolysaccharide (EPS) production

Exopolysaccharides (EPS) are high molecular weight polymers of carbohydrate and protein residues that can be homopolysaccharides as well as heteropolysaccharides (Rossi et al. 2012). Secretion of exopolysaccharides by rhizobacteria has been reported to help them colonize and form biofilms that protect them from dessication (Sandhya et al. 2009, 2010b; Rossi et al. 2012). Secretion of EPS by beneficial soil bacteria under water-stress provides advantage not only to the bacteria itself but also to the plants as EPS helps better aggregation of soil particles which enhances nutrient uptake by plant roots under water deficit conditions. During drought condition, EPS produced by PGPR causes increase in the growth of roots and shoots.There are studies which prove that the secreted EPS by PGPRs like *Azospirillum* (Bashan et al. 2004), *Pseudomonas* and *Bacillus* spp. (Sandhya et al., 2009; Sandhya et al., 2010; Vardharajula et al., 2011) cause changes in the soil structure, root associated soil/ root ratio and the macroaggregate properties which allows plants to grow better and expand their root system, leading to easy uptake of water and nutrients. (Ahn et al. 2007). It has also been reported that EPS secreted by the *Pseudomonas* spp. forms hydrophilic biofilm on the surface of roots thus preventing root hardening (Rolli et al. 2015). Since EPS contains lipids and polysaccharides, it can act as an emulsifier and quench free radicals, protecting plants from the harmful effects of ROS (Dimitrova et al. 2013). It has been observed that under stress conditions, composition of

EPS changes, containing higher amount of glucose, rhamnose, mannose, trehalose that enhance the properties of EPS for better water retention ability (Tewari and Arora 2014).

(4) Production of proline and other compatible osmolytes

Similar to the responses of plants to osmotic stress conditions, drought-tolerant rhizobacteria accumulate compatible osmolytes in the cytosol that help them maintain cellular osmotic balance, helping in the maintenance of subcellular structures, cellular pH, redox potential, free radical scavenging etc., thus overcoming the detrimental effects of abiotic stresses (Szabados and Savouré 2010; Ben Rejeb et al. 2014; Surender Reddy et al. 2015; Vurukonda et al. 2016; Anjum et al. 2017). Researchers have found that PGPRs accumulate compatible osmolytes not only within bacterial cells, but also enhance the biosynthesis and accumulation within the plant tissues, mainly leaves (Sandhya et al. 2010a; Grover et al. 2011). Increase in the production of osmolytes in plants such as, proline, sorbitol, glycine-betaine etc. by PGPR creates a concentration gradient between soil and root cells which causes the water to move from soil to roots thus increasing water uptake capacity of plants under drought (Ait Barka et al. 2006; Kohler et al. 2008). As discussed earlier in this chapter that drought induces proline accumulation in plant cells, these bacteria may lead to further upregulation of proline biosynthetic pathway so as to maintain high proline levels and thus, cell water status in plants. This helps to prevent the degradation of membrane and proteins due to drought stress (Szabados and Savouré 2010; Sandhya et al. 2010b; Vardharajula et al. 2011; Pii et al. 2015).

(5) Production of phytohormones

There are numerous studies that report the beneficial effects of using soil microbes that have the ability to produce phytohormones and thus help in alleviating drought stress effects in plants.

It has been observed that inoculation of plants with IAA-producing PGPR, increased root surface area and number of root tips, thus enhancing the uptake of nutrients and water (reviewed by Mantelin and Touraine, 2003). Such changes in the root architecture increase leaf water content and increased water potential, as found when *Azospirillum* was inoculated with wheat enhanced plant growth under water stress (Arzanesh et al. 2011). In another study, Kang et al., (2014a) reported increased levels of endogenous GAs in cucumber plants inoculated with PGPR strains like *Burkholdera cepacia SE4*, *Promicromonospora* spp. SE188 and *Acinetobacter calcoaceticus* SE370. Similarly, maize (Cohen et al. 2009) and wheat (Creus et al. 2004) plants survived under drought stress when inoculated with GA producing PGPR, *Azosprillum lipoferum*. When cytokinin-producing PGPR *Micrococcus luteus* chp37 was inoculated with maize plants under water stress conditions, it improved the physiology such as, enhanced shoot and root biomass, increased photosynthetic pigments etc. (Raza F and Faisal 2013). Improved shoot biomass was found when cytokinin producing *Bacillus subtilis* was inoculated with lettuce under water stress conditions (Arkhipova et al. 2007). In a study by Salomon et al., (2014), inoculation of ABA producing *B. licheniformis* and *P. fluorescens* improved growth of grapevine under drought conditions. In another study, elevated levels of ABA in *Arabidopsis* that lead to decreased leaf transpiration were found on inoculation with PGPR *Phyllobacterium brassicacearum* strain STM196 thus alleviating osmotic stress (Bresson et al. 2013).

(6) Secretion of 1-aminocyclopropane-1 carboxylate (ACC) deaminase

Ethylene is a senescence causing hormone which, among other things, promotes leaf abscission. During high stress, ethylene levels increase tremendously, leading to senescence, chlorosis, and abscission and may lead to a significant inhibition of plant growth and survival (Jalili et al. 2009; Ahemad and Kibret 2014; Kumari et al. 2016). Ethylene is synthesized from 1-

aminocyclopropane-1 carboxylate (ACC) via the enzyme ACC oxidase (Nascimento et al. 2018). Some PGPRs are found to secrete ACC deaminase which degrades ACC, producing F-ketobutyrate and NH_3, which can be utilized by the bacteria as a source of nitrogen (Glick 2005; Saharan and Nehra 2011). It is known that application of PGPRs as bio-inoculants which are able to produce ACC deaminase under drought stress conditions enhances plant survival and development under water-limitation by enhancing root growth (Belimov et al. 2009; Glick 2014). Inoculation of tomato and pepper plants with *Achromobacter piechaudi* containing ACC deaminase activity conferred tolerance under drought stress. ACC deaminase producing strain *Variovorax paradoxus* has been reported to enhance drought tolerance in pea plants (Belimov et al. 2009).

1.4.2 Indirect mechanisms:

Indirect mechanisms include those mechanisms that enable PGPRs to act as biocontrol agents, thereby, indirectly helping the plants.

(1) Production of antibiotics, lytic enzymes and hydrogen cyanide (HCN)

PGPRs have been reported to secrete a wide range of antibiotics such as, 2,4-diacetyl phloroglucinol and phenazine in the vicinity of roots in order to prevent growth of pathogenic microbes around the rhizosphere (Saharan and Nehra 2011). Some important strains of PGPR reported to prevent pathogen infections are: *Pseudomonas fluorescens* TDK1, *Arthrobacter*, sp., *Pseudomonas putida* UW4, *Bacillus subtilis, Pseudomonas fluorescens* MSP-393, *Pseudomonas aeruginosa* Sha8, *Bacillus luciferensis* KJ2C12, *Pseudomonas fluorescens* CHA0 and Arbuscular Mycorrhizal fungi (Mayak et al. 2004; Haas and Défago 2005; Barriuso et al. 2008; Saharan and Nehra 2011). Some PGPR produce enzymes that include chitinases, cellulases, 1,3-

glucanases, proteases, laminarinases and lipases that can lyse a portion of the cell walls of many pathogenic fungi (Bhattacharyya and Jha 2012; Beneduzi et al. 2012; Ahemad and Kibret 2014). Gram-negative *Pseudomonas fluorescens, Pseudomonas aeruginosa*, *Chromobacterium violaceum* and a wide variety of *Bacillus* strains produce hydrogen cyanide as a secondary metabolite, which is volatile in nature. It suppresses growth of various microorganisms, weeds and shows negative effects on growth and development of plants as well (Jalili et al. 2009; Saharan and Nehra 2011; Raza F and Faisal 2013). However, HCN production by PGPRs have been considered to be an indirect beneficial characteristic as the amount produced in the soil prevents pathogenic attack and, most of the times, is too low to affect growth and development of the host plant.

(2) Production of siderophores

Iron is an essential element for cellular growth and metabolism in all organisms. It is present in ferric ion form in soil that cannot be assimilated by plants and microorganisms (Ahemad and Kibret 2014). Siderophores are small iron chelating molecules (such as, enterobactin, desferrioxamine B etc.) produced and utilized by bacteria. Siderophores bind ferric ions with higher affinity than any other pathogenic bacteria or fungi, depriving them from essential growth requirement of iron (Saharan and Nehra 2011). A ferric-siderophore complex is recognized by specific membrane receptors of bacteria (Bhattacharyya and Jha 2012; Beneduzi et al. 2012). Siderophores play an important role in plant development by facilitating uptake of iron by plants (Saharan and Nehra 2011; Numan et al. 2018). Siderophore producing bacteria belong to genera *Rhizobium*, *Bradyrhizobium*, *Pseudomonas*, *Serratia* and *Streptomyces* (Sandhya et al. 2010b; Beneduzi et al. 2012; Numan et al. 2018).

(3) Induced Systemic Resistance (ISR)

Inoculation of plant roots with PGPRs have been reported to activate signaling cascades that lead to acquisition of resistance to numerous pathogenic organisms by the host plant. This phenomenon in plants is known as induced systemic resistance (ISR) that occurs when plants activate their defense mechanisms in response to infection by a pathogenic agent (Dimkpa et al. 2009; Hayat et al. 2010; Saharan and Nehra 2011). Immune system of plants respond in two different ways: (1) response to the virulence factors imposed by infection of pathogenic organisms (called systemic acquired resistance or SAR), (2) memory-based recognition and response to the elicitor molecules secreted by non-pathogenic PGPRs that ultimately confers resistance to other pathogens or abiotic stresses which constitutes ISR (Dimkpa et al. 2009; Saharan and Nehra 2011). ISR involves salicylic acid, jasmonic acid and ethylene signaling within the plant and these hormones stimulate the host plant’s defense responses to a range of pathogens (Saharan and Nehra 2011; Ahemad and Kibret 2014). Numerous PGPR strains such as, *Pseudomonas fluorescens* ENPF1, *Pseudomonas chlororaphis* BCA, *Bacillus* sp., *Paenibacillus polymyxa*, *Bacillus subtilis* GBO3, *Bacillus amyloliquefaciens* IN937a, have been reported by researchers to confer ISR to a variety of host plant species (Timmusk and Wagner 1999; Dimkpa et al. 2009; Hayat et al. 2010).

1.4.3 Role of PGPR in drought stress tolerance

Our research interest is predominantly based on PPGPR-mediated amelioration of drought/osmotic stress in plants. Several groups of researchers have reported amelioration of detrimental effects of drought stress in plants due to inoculation with wide varieties of PGPR strains and have identified a number of major mechanisms playing important roles behind plant-

PGPR interactions, such as, secretion of phytohormones by PGPRs directly or indirectly modulating endogenous hormonal concentrations in plants (Vacheron et al. 2013; Liu et al. 2013), modulation of endogenous proline content and upregulation of proline metabolic gene expression in plants (Sandhya et al. 2010a; Ghosh et al. 2017), nitrogen fixation, phosphate solubilization, ACC deaminase production etc. (reviewed by Forni et al. 2017). However, the molecular basis of these physiological observations have not been hitherto reported.

Some examples of various PGPR strains reported to ameliorate drought stress in a wide variety of host plants are listed in Table 1.1 below:

Table 1.1 Amelioration of drought/osmotic stress in host plant by PGPR inoculation

Bacterial Inoculate	**Stress**	**Plant Species**	**Reference**
Azospirillum brasilense	Drought	Common bean (*Phaseolus vulgaris*)	(German et al. 2000)
Azospirillum brasilense	Drought	Wheat (*Triticum aestivum*)	(Pereyra et al. 2012)
Azospirillum sp.	Drought	Wheat (*Triticum aestivum*)	(Creus et al. 2004)
Azospirillum sp.	Drought	Rice *(Oryza sativa*)	(Ruíz-Sánchez et al. 2011)
Azospirillum sp.	Osmotic stress	Wheat (*Triticum aestivum*)	(Pereyra et al. 2006)
Azospirillum brasilense	Osmotic stress	Wheat (*Triticum aestivum*)	(Creus et al. 1998)
Pseudomonas putida	Drought	Maize (*Zea mays*)	(Sandhya et al. 2010b)
Pseudomonas sp.	Drought	Asparagus (*Asparagus officinalis*)	(Liddycoat et al. 2009)
Pseudomonas mendocina	Drought	Lettuce (*Lactuca sativa*)	(Kohler et al. 2008)
Pseudomonas oxyzihabitans	Drought	Potato (*Solanum tuberosum*)	(Belimov et al. 2015)
Pseudomonas putida	Osmotic stress	*Arabidopsis thaliana*	(Ghosh et al. 2017)
Pseudomonas cholorophis	Drought	*Arabidopsis thaliana*	(Cho et al. 2008)
Pseudomonas putida	Drought	Chickpea (*Cicer arietinum*)	(Kumar et al. 2016)
Bacillus subtilis	Drought	*Arabidopsis thaliana*	(Zhang et al. 2010)

Table 1.1 Amelioration of drought/osmotic stress in host plant by PGPR inoculation (cont..)

Bacterial Inoculate	Stress	Plant Species	Reference
Bacillus sp.	Drought	Lettuce (*Lactuca sativa*)	(Arkhipova et al. 2007)
Bacillus subtilis	Osmotic stress	*Arabidopsis thaliana*	(Zhang et al. 2010)
Bacillus megaterium	Osmotic stress	Maize (*Zea mays*)	(Marulanda et al. 2010)
Bacillus sp.	Osmotic stress	Pepper (*Capsicum annuum*)	(Sziderics et al. 2007)
Bacillus thuringiensis	Drought	Wheat (*Triticum aestivum*)	(Timmusk et al. 2014)
Bacillus licheformis	Drought	Pepper (*Capsicum annuum*)	(Lim and Kim 2013)
Bacillus cereus *Bacillus subtilis* *Serratia sp.*	Drought	Cucumber (*Cucumis sativa*)	(Wang et al. 2012)
Bacillus amyloliquefaciens	Drought	Chickpea (*Cicer arietinum*)	(Kumar et al. 2016)
Bacillus amyloliquefaciens	Drought	Tomato (*Solanum lycopersicum*)	(Selvakumar et al. 2018)
Paenibacillus polymyxa	Drought	*Arabidopsis thaliana*	(Timmusk and Wagner 1999)
Bacillus subtilis	Drought	*Platycladus orientalis*	(Liu et al. 2013)
Ensifer meliloti Mediterranense	Drought	Bean (*Phaseolus vulgaris*)	(Mnasri et al. 2007)
Phyllobacterium brassicacearum	Drought	*Arabidopsis thaliana*	(Bresson et al. 2013)
Burkholderia phytofirmans *Enterobacter sp.*	Drought	Maize (*Zea mays*)	(Naveed et al. 2014)
Curvularia proturberata	Drought	*Solanum lycopersicum*	(de Zelicourt et al. 2013)
Serratia sp. *Aerococcus sp.*	Drought	Wheat (*Triticum aestivum*)	(Bangash et al. 2013)
Achromobacter xylosoxidans	Drought	Potato (*Solanum tuberosum*)	(Belimov et al. 2015)
Azorhizobium caulinodans	Drought	Wheat (*Triticum aestivum*)	(Weyens et al. 2009)
Rhizobium tropici *Paenibacillus polymyxa*	Drought	Common bean (*Phaseolus vulgaris*)	(Figueiredo et al. 2008)
Bradyrhizobium elkanii	Drought	Flat crown (*Albizia adianthifolia*)	(Swaine et al. 2007)
Arthrobacter sp.	Osmotic stress	Pepper (*C. annuum*)	(Sziderics et al. 2007)

1.5 Gaps in Existing Research

The review above elucidates tremendous potential of PGPRs to be utilized as eco-friendly alternatives to chemical fertilizers, pesticides, weedicides etc. They can also be utilized as cheaper alternatives to huge investments for water supply in agricultural fields affected by drought stress and increasing demand of high quality germplasms of crops for better stress tolerance (Gouda et al. 2018). As mentioned before, two of the most important modes of action shown by PGPR to impart drought tolerance to plants include: (1) enhanced accumulation of compatible osmolytes (mostly proline) in plant cells due to inoculation with various PGPR strains under drought stress (Sandhya et al. 2010a; Ghosh et al. 2017), (2) modulation of endogenous phytohormone levels in plants by the PGPRs capable of secreting different phytohormones in root rhizosphere (Ali et al. 2009; Liu et al. 2013). However, there is still lack of cumulative evidence on the precise molecular basis of drought stress mitigation in plants by PGPR inoculation. Based on the literature survey on the above-mentioned mechanisms and our hypothesis on the available information on plant-PGPR interaction, we have identified some gaps in existing research:

- ➢ Increased proline accumulation in leaves under drought stress is one of the early drought responses of plants. However, this response provides tolerance to stress up to a certain limit of drought intensity and exposure. Though several groups have reported that inoculation with PGPR further enhances proline accumulation in plants under drought/osmotic stress, there are no reports on how the proline metabolic genes are regulated in plants due to PGPR inoculation with the progression of drought/osmotic stress in a time-dependent manner.

- Whether PGPR-mediated modulation of expression of the proline metabolic genes at transcriptional levels corroborate the regulations at translational levels of the enzymes encoded by these genes are still elusive.
- Modulations in the endogenous level of phytohormones in plants under any biotic or abiotic stress, ultimately dictate sensitivity or tolerance of the plant to the exerted stress. There are several reports on the amelioration of drought/osmotic stress in plants due to PGPR inoculation that are capable of secreting phytohormones (mainly IAA, ABA, GA and cytokinins) in root rhizosphere under various stressed conditions. However, the dynamics of PGPR-mediated time-dependent modulations of the above-mentioned major phytohormones in root as well as in shoot level, have hitherto not been unveiled.

In this study, we focused on the elucidation of the molecular basis of the plant-PGPR interactions involved in enhancement of proline accumulation in plants and regulation of the dynamics of phytohormone accumulation in root and shoot by phytohormone-secreting PGPR. We chose the model plant *Arabidopsis thaliana* wild type (ecotype Columbia-0) as the host plant for bacterial inoculation as, it is an extremely useful model system for molecular studies (the complete genome map and different mutants in several pathways are available). Out of 6 potent PGPR strains (*Pseudomonas aeruginosa* PM389, *Pseudomonas aeruginosa* ZNP1, *Bacillus endophyticus* J13, *Bacillus tequilensis* J12, *Pseudomonas putida* GAP-P45, *Pseudomonas putida* AKMP7) procured from different repositories (part of materials and methods in individual chapters), we selected *Pseudomonas putida* GAP-P45 as the choice of strain for the mechanistic studies.

The PGPR strain, *Pseudomonas putida* GAP-P45 used in our study, was originally isolated from root rhizosphere of sunflower plants in semi-arid zones of Hyderabad, India by

Sandhya et al., (2009). This drought tolerant strain was reported to ameliorate detrimental effects of drought stress in maize and sunflower by increasing root and shoot length, root and shoot biomass, relative water content, leaf water potential and minimizing electrolyte leakage of inoculated plants (Sandhya et al. 2009, 2010a). This strain was also observed to produce exopolysaccharide, HCN, ammonia, siderophores and phytohormones such as, IAA, GA and cytokinin; traits that qualify GAP-P45 as a potential PGPR (Sandhya et al. 2010b). Bacterization of maize seeds with GAP-P45 enhanced drought tolerance of the inoculated plants by improving physiological and biochemical parameters as compared to the non-inoculated stressed plants. Elevated accumulation of cellular proteins, amino acids, proline, total soluble sugar and starch were observed in case of inoculated stressed plants. Sandhya et al., (2010) reported significantly lower activity of the antioxidant enzymes (APX, CAT, GPX) in GAP-P45 inoculated plants as compared to non-inoculated plants under drought.

1.6 Scope and objectives of work

Based on the gaps of existing research data available, we decided to investigate the molecular basis of plant-PGPR interaction by studying the following objectives:

- Testing the cross-compatibility and plant growth promoting efficacy of the selected strain, *Pseudomonas putida* GAP-P45 on *Arabidopsis thaliana* Col-0 in soil free experimental conditions by observation on morpho-physiological parameters such as, fresh weight, dry weight, plant water content, root length, root structure architecture, chlorophyll content and proline content of *Arabidopsis thaliana* inoculated with GAP-P45 under water-stress, induced by 25 % PEG.

- Studying the effect of *Pseudomonas putida* GAP-P45 inoculation on the expression pattern of the genes involved in proline metabolic pathway in *Arabidopsis thaliana* under water-stress in a time-dependent manner and activity of the enzymes encoded by those genes under similar stressed condition with PGPR inoculation.
- Studying the concentrations of phytohormones secreted by *Pseudomonas putida* GAP-P45 in plant growth media (available for the inoculated plants) and the dynamics of endogenous phytohormone-pool modulation in *Arabidopsis thaliana* at both root and shoot level under water-stress, in a time-dependent manner.
- Characterization of four other rhizobacterial strains (*Pseudomonas aeruginosa* PM389, *Pseudomonas aeruginosa* ZNP1, *Bacillus endophyticus* J13 and *Bacillus tequilensis* J12) isolated from arid soils by different groups in India and study on their drought mitigating impact on *Arabidopsis thaliana*.

CHAPTER 2

Effect of *Pseudomonas putida* GAP-P45 on morphological and physiological status of *Arabidopsis thaliana* under water-stress

2.1 INTRODUCTION

The adverse effects of drought have been elaborated in the previous chapter. As mentioned, to combat the alarming problem of drought, researchers all over the world have focused on developing new strategies to generate plants that are capable of alleviating the adverse effects of stresses and maintain total yields. One of those strategies that has gained immense limelight and interest of the scientific community is the use of PGPR that are capable of enhancing plant growth and physiological status under both normal and/or stressed conditions. To reiterate, there are several reports on the observed effects of PGPR inoculation in abating drought stress in many plant species, though exact molecular mechanisms induced by the PGPRs are still elusive.

Before performing any molecular studies on plant-bacterial interaction, the selected strain *Pseudomonas putida* GAP-P45 had to be tested for its cross-compatibility and plant growth promoting effects on *A. thaliana* under in vitro experimental conditions. So, we performed physiological studies such as, morpho-physiological observations on plant health, fresh weight, dry weight, plant water content, root length, root structure architecture, chlorophyll content and proline content of *A. thaliana* seedlings post water-stress induction and GAP-P45 inoculation(Ghosh et al. 2017).

2.2 MATERIALS AND METHODS

2.2.1 Germination and growth of *Arabidopsis thaliana*:

Standard protocols were employed for the routine growth and maintenance of *A. thaliana*. Seeds in a 2 mL microcentrifuge tube, were surface sterilized by adding 1 mL of 0.02 % $HgCl_2$ and vigorously inverting the tube for 1 min, followed by removal of $HgCl_2$ by washing the seeds with autoclaved distilled water twice. Then 1 mL of 70 % ethanol was added to the seeds and the tube was repeatedly inverted for another 1 min. Finally, traces of ethanol were removed by washing the seeds with autoclaved milli Q (Millipore) water for 5 times and seeds were stratified in the dark at 4 °C for 3 days. After stratification, seeds were sown on autoclaved square pieces of stainless-steel meshes (0.01 inch wire diameter, 0.015 inch clear opening) placed on Petri plates containing half strength, sterile Murashige and Skoog (MS) medium (Sigma) (Murashige and Skoog 1962) with 0.6 % agar (Sigma) and 1 % sucrose (Sigma) (modified from Zhang et al. 2010). Seeds germinated on the meshes within 2 days of incubation of the plates containing seeds in a plant growth chamber under controlled environment at 22(±1) °C and 16/8 h light/dark cycle with about 10,000 LUX light intensity (approximately 150 $\mu molm^{-2}s^{-1}$) at 50-70 % relative humidity. The seedlings were allowed to grow for next 7 days up to four-leaves stage before being transferred to Magenta boxes for the experiments. After the incubation, meshes containing 5-7 seedlings each were transferred to Magenta boxes with half strength MS-agar media, each Magenta box contained 4 of these meshes.

2.2.2 Water-stress induction and PGPR inoculation:

Water-stress induction was done by transferring 7-day-old seedlings (4-leaved stage) to Magenta boxes containing MS-agar medium (with 1% sucrose) supplemented with 25% polyethylene glycol (PEG-6000) (van der Weele 2000). Media was prepared by PEG-infusion method i.e. 16 mL of MS-agar media was overlaid with 10 mL of 80% PEG-6000 and incubated for 48 h. After incubation, the overlay was decanted completely. We observed that approximately 4 g of PEG is retained by the MS-agar media in each Magenta box which makes it ~25% PEG-infused MS-agar media. Before starting an experiment, *P. putida* GAP-P45 was grown overnight in LB (Bertani 1951) broth in a shaking incubator at 28 °C to an $O.D._{600}$ of 0.6-

0.8, sub-cultured and re-grown to the same O.D. and used for inoculating the plants. Prior to inoculation, bacterial cells were centrifuged and re-suspended in autoclaved, distilled water. Half of the control and water-stress induced plants were subjected to bacterial inoculation by the addition of 200 µL of this aqueous suspension to the respective Magenta boxes. Thus, there were four experimental sets namely: 1. No-treatment controls (NT) 2. Non-stressed, GAP-P45 inoculated (NS+I) 3. Water-stressed, non-inoculated (WS+NI) 4. Water-stressed, GAP-P45 inoculated (WS+I). For each experiment, Magenta boxes were used in triplicate (as mentioned before, each Magenta box contained 4 meshes, each with 5-7 seedlings). All experiments were repeated at least once, and, where needed, twice. To monitor the growth of *P. putida* GAP-P45 in Magenta boxes throughout the experiment, a loop-full of culture from the surface of inoculated MS-agar media (with or without PEG) at 2, 4 and 7 days post treatment, was scraped, streaked onto LB-agar plates and incubated at 28 °C for 12 h. In order to rule out any contamination, similar action was performed from non-inoculated media as well. In order to prove that any drought-mitigation observed is not due to bacterial inoculation in general, a separate set of experiments replacing GAP-P45 with the common laboratory strain *E. coli* DH5-α was also performed, using the same conditions as with GAP-P45. Water potential of the media of all four treatments were measured after 7 days post water-stress induction and GAP-P45 inoculation to observe any plausible change in water potential due to the bacterial growth on MS-agar media. The values of measured water potential are given below (mean ± SE of 3 replicate samples):

1. NT: -0.66 ± 0.028 MPa 2. NS+I: -0.67 ± 0.012 MPa
3. WS+NI: -1.96 ± 0.034 MPa 4. WS+I: -1.93 ± 0.048 MPa

In order to assess if the 200 µL water (present in the inoculum) made any difference to the water potential of the medium, the MS-agar media with or without PEG supplementation and with or without addition of 200 µL water were subjected to water-potential measurements using PSYPRO water potential system (Wescor Inc.) in a separate experiment. The values are given below:

1. MS-agar (control): -0.62 (±0.029) MPa
2. MS-agar with 200 µL water: -0.6 (±0.031) MPa
3. PEG supplemented MS -agar medium: -2.17 (±0.046) MPa
4. PEG supplemented MS-agar medium with 200 µL water: -2.16 (±0.049) MPa

Thus, it is evident that addition of 200 µL water or growth of GAP-P45 itself in MS-agar media (with or without PEG) did not cause any significant change in water-potential of the stressed or non-stressed media.

2.2.3 Physiological studies on plant responses to *Pseudomonas putida* GAP-P45 inoculation under water-stress:

Physiological studies were performed to assess the impact of the PGPR strain on water-stress alleviation of *A. thaliana* at different time-points (2 days, 4 days and 7 days) post treatments. Experiments included observations on overall plant health, fresh weight (FW), dry weight (DW), plant water content (PWC) of whole seedlings along-with primary root length and chlorophyll content of leaves. For measurement of FW, 60 seedlings from three replicate Magenta boxes, (20 seedlings from each box) were harvested. Following FW measurements, the seedlings were incubated at 80°C for 48 h for measurement of DW. Plant water content was measured both on FW and DW basis, by using the formulae:

$\text{PWC}_{\text{(DW basis)}} = [(\text{FW}-\text{DW})/\text{DW}] \times 100$ and $\text{PWC}_{\text{(FW basis)}} = [(\text{FW}-\text{DW})/\text{FW}] \times 100$ (Turner 1981).

In order to measure primary root length, plants with intact roots were placed on a glass plate, the tap root was straightened and the secondary roots separated using a fine needle. Length of the primary roots was measured using a centimeter ruler. A modification from the method of Hu et al. (2013) was used for the extraction and estimation of chlorophyll pigment from leaves of *A. thaliana* subjected to all treatments. Leaf samples (40 mg) were placed in a graduated tube containing 10 mL of 80 % buffered acetone (80 mL of acetone made up to 100 mL with 20 ml of 2.5 mM sodium phosphate buffer, pH 7.8). The leaves were incubated in the solvent in dark at 4 ^{o}C with occasional shaking to accelerate the extraction of the pigments. At the appropriate time of estimation, the extract was filtered to remove leaf pieces. The Chlorophyll content was spectrophotometrically analyzed in the filtrate at 663 nm and 646 nm for chl a and b respectively. Total chlorophyll content was assessed using the formula: Chl (a+b) = 7.49*A663 + 18.21*A 646 (Barnes et al., 1992).

2.2.4 Accumulated free proline content in plants under water-stress and *Pseudomonas putida* GAP-P45 inoculation:

Proline estimation was done in whole seedlings at 2, 4 and 7 days post treatment, using the method of Bates et al., 1973. Whole seedlings (100 mg) were homogenized in 5 mL of 3% aqueous sulphosalicylic acid and the homogenate was collected by filtering through Whatman no. 2 filter paper. Filtrate (2 mL) was treated with 2 mL glacial acetic acid and 2 mL of acid ninhydrin (warm 1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL of 6 M phosphoric acid). The samples were incubated in a boiling water bath for 1 h and reaction was terminated by placing the reaction tubes on ice. To this reaction mixture, 4 mL toluene was added and stirred well for 20-30 sec. Chromophore containing toluene layer was separated and warmed to room temperature. Absorbance was read at 520 nm using a UV-visible spectrophotometer, blanked with toluene. Different concentrations of an aqueous solution of L-proline were used to plot a standard curve of absorbance vs. concentration and concentrations of proline in plant samples were extrapolated from it.

2.3 RESULTS

2.3.1 Plant growth under water-stress:

Prior to the treatments, all plants exhibited similar growth and developmental phenotypes (Fig. 2.1 A-D). GAP-P45 inoculation under well-watered conditions did not cause any visible change in the size/growth of the plants at any time period of the study- E vs. F at day 2, I vs. J at day 4 and M vs. N at day 7 in Fig. 2.1. By day 2 post transfer to PEG supplemented medium, the PEG treated, non-inoculated plants exhibited significant growth stunting as opposed to the all other treatments (Fig. 2.1 G, K, O). Under PEG-treated conditions, GAP-P45 inoculated plants exhibited much better growth as opposed to the non-inoculated ones (Fig. 2.1 H vs. G at day 2, L vs. K at day 4 and P vs. O at day 7). With progression of days, the PEG treated, non-inoculated plants exhibited gradual decline in health while those inoculated with GAP-P45 exhibited much better tolerance to dehydrating conditions. The LB-agar plates used for monitoring bacterial growth showed discrete growth of GAP-P45 at all 3 time points of the study, while no contamination was detected in the non-inoculated medium (Fig. 2.2). No drought mitigation was observed with *E.coli* DH5-α inoculation as negative control (Fig. 2.3). Rather, this led to deterioration in plant health even under non-stressed conditions, probably because of the competition between the bacteria and the plants for nutrients.

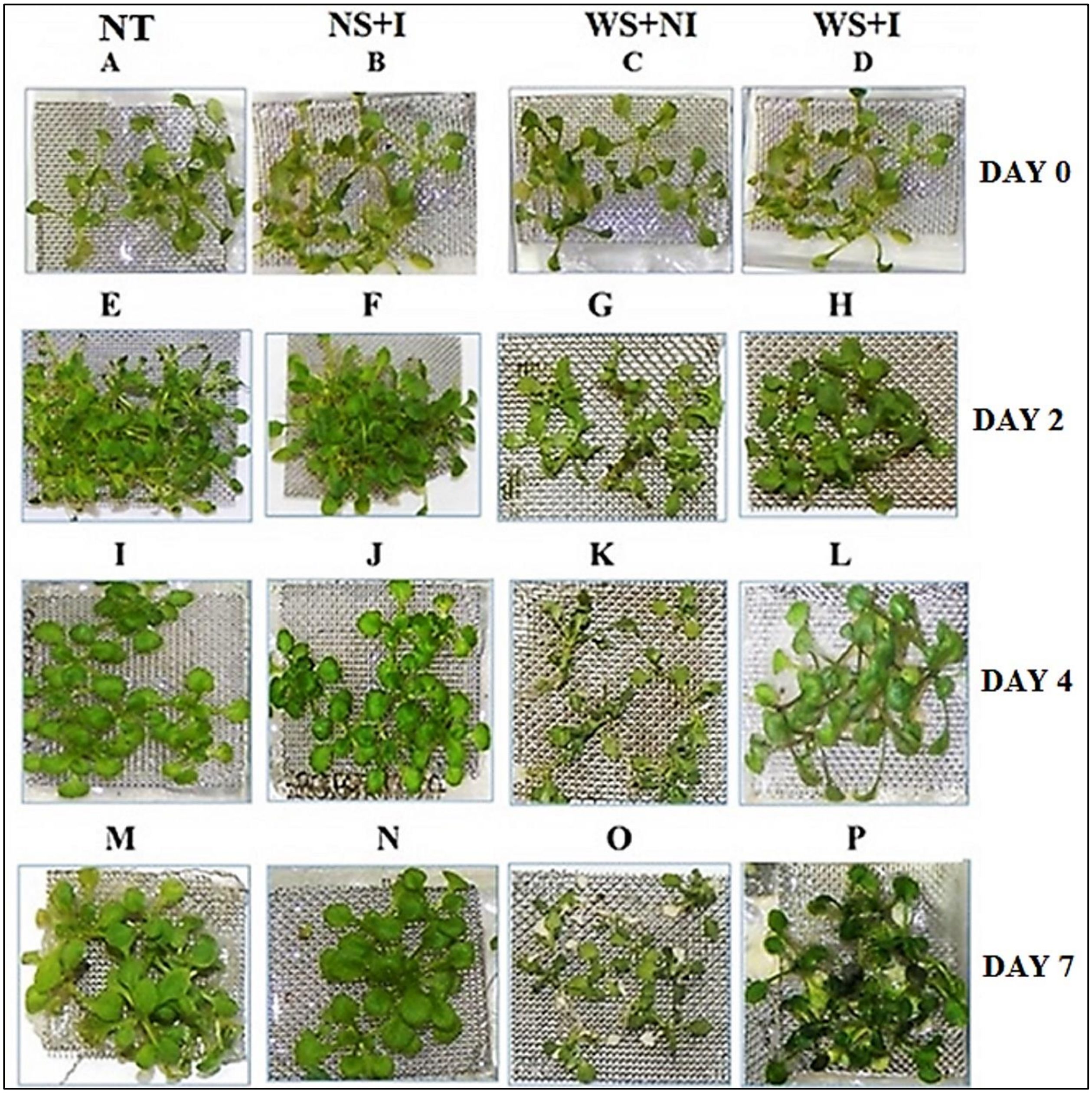


Fig. 2.1 Plant growth and development following *Pseudomonas putida* GAP-P45 inoculation in *Arabidopsis thaliana* under water-stress (25% PEG). While figures in **A-D** represent a period, just before treatments, **E-H** represents day 2; **I-L**, day 4 and **M-P**, day 7 post treatments. NT-no treatment controls; NS+I-non-stressed, inoculated; WS+NI-water-stressed, non-inoculated; WS+I-water-stressed, inoculated.

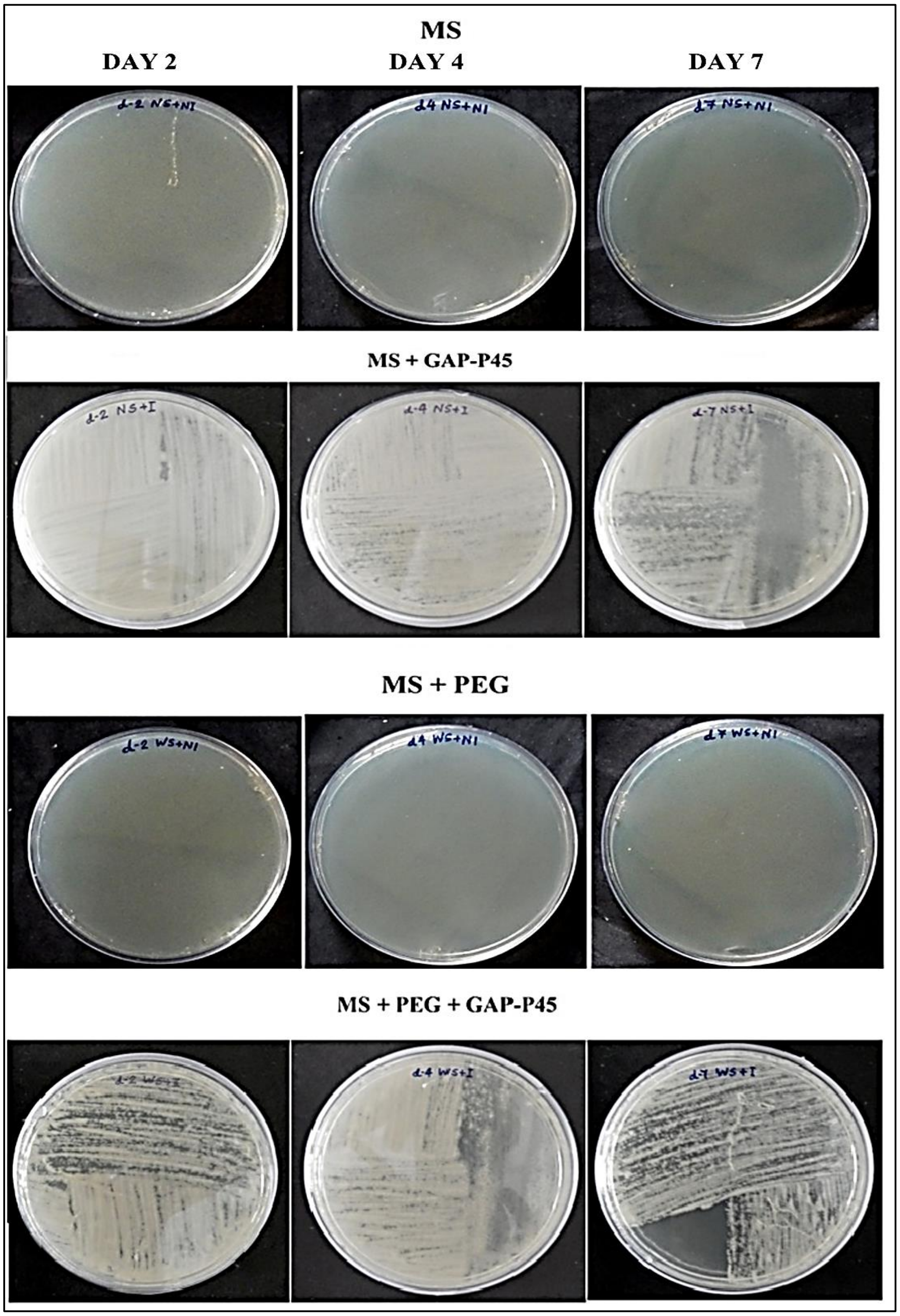


Fig 2.2 Monitoring the growth of *Pseudomonas putida* GAP-P45 throughout the experiment (day 2, day 4 and day 7) in all four treatment conditions.

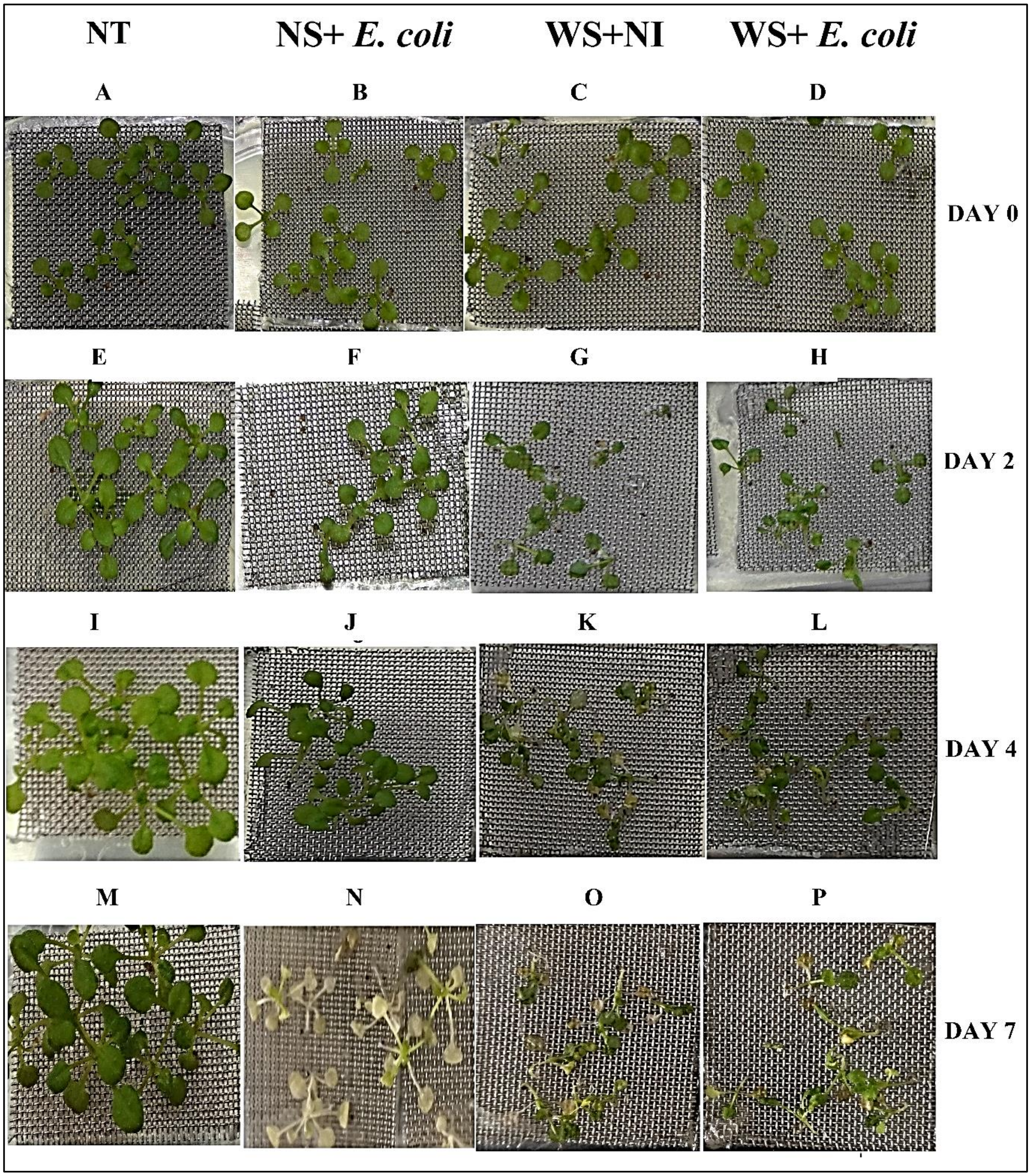


Fig 2.3 Observations on plant growth and development due to *E. coli* inoculation (as non-PGPR control) of *Arabidopsis thaliana* under water-stress (25% PEG). While figures in A-D represent a period, just before treatments, E-H represents day 2; I-L, day 4 and M-P, day 7 post treatments i.e. no treatment controls (NT), non-stressed inoculated (NS+*E. coli*), water-stressed non-inoculated (WS+NI) and water-stressed inoculated (WS+ *E. coli*).

2.3.2 Fresh weight, dry weight and plant water content:

In order to quantify the impact of *Pseudomonas putida* GAP-P45 on plant water status, we analyzed plant fresh weight (FW), dry weight (DW), and plant water content (PWC) at all time-periods of this study. As can be seen from Fig. 2.4 A and B, GAP-P45 inoculation of *Arabidopsis* under non-stressed condition did not significantly change FW and DW when compared to control plants. Drastic reduction in FW and DW was observed in case of the water-stressed, non-inoculated plants from day 2 to day 7. However, both FW and DW of water-stressed plants increased significantly on GAP-P45 inoculation at all three time periods of study. Plant water content (PWC) was calculated, both on DW and FW basis (Turner 1981). PWC (DW basis) and PWC (FW basis) followed similar trends in that the water-stressed, non-inoculated plants recorded the lowest PWC among all the treatments at all time points of the study with the progression of the severity of water-stress (Fig 2.4 C and D). The PEG treated, GAP-P45 inoculated plants exhibited significantly higher PWC, both on DW and FW basis, as opposed to the water-stressed, non-inoculated plants.

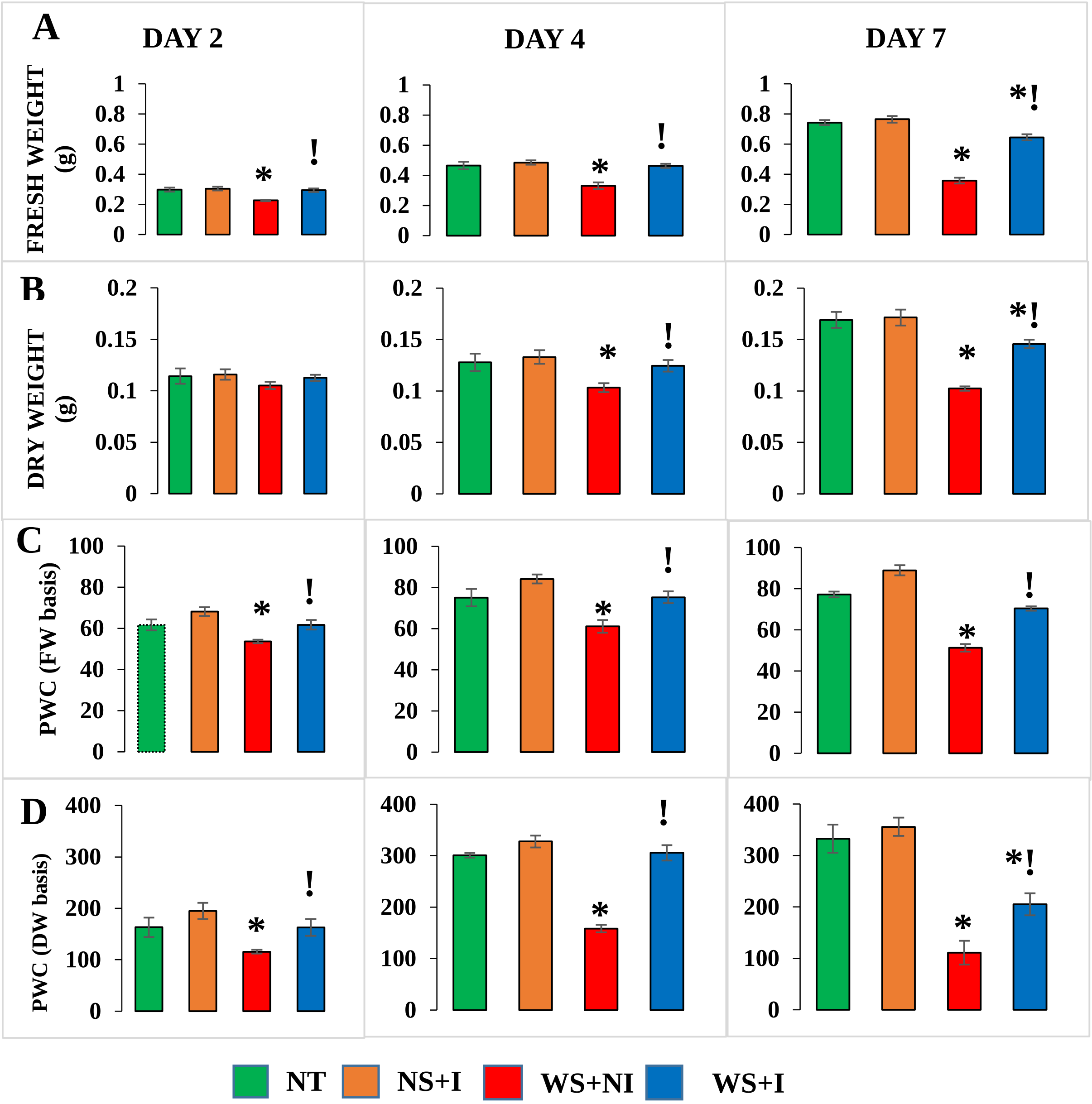


Fig. 2.4 Physiological studies on the impact of *Pseudomonas putida* GAP-P45 on water-stress amelioration in *Arabidopsis thaliana*. Fresh weight 'A', dry weight 'B' and plant water content 'C'- fresh weight basis and 'D' – dry weight basis of whole seedlings day 2, day 4 and day 7 post treatments. Each bar represents mean ± SE of 60 replicate plants. * indicates significant difference ($p \leq 0.05$) in data between NT and any other treatment within a particular day of analysis. ! indicates significant difference ($p \leq 0.05$) in data between WS+I and WS+NI samples within a particular day of analysis.

2.3.3 Chlorophyll content:

Chlorophyll content followed a similar trend as PWC (Fig. 2.5). While on day 2, marginal enhancement was seen in chlorophyll content by GAP-P45 inoculation without water-stress, on day 4 and day 7, GAP-P45 treatment under normal conditions did not cause any significant change in the chlorophyll content. As expected, PEG-treatment caused a significant decrease in chlorophyll content on all days of study, while GAP-P45 inoculation under water-stress, significantly elevated the chlorophyll content in leaves.

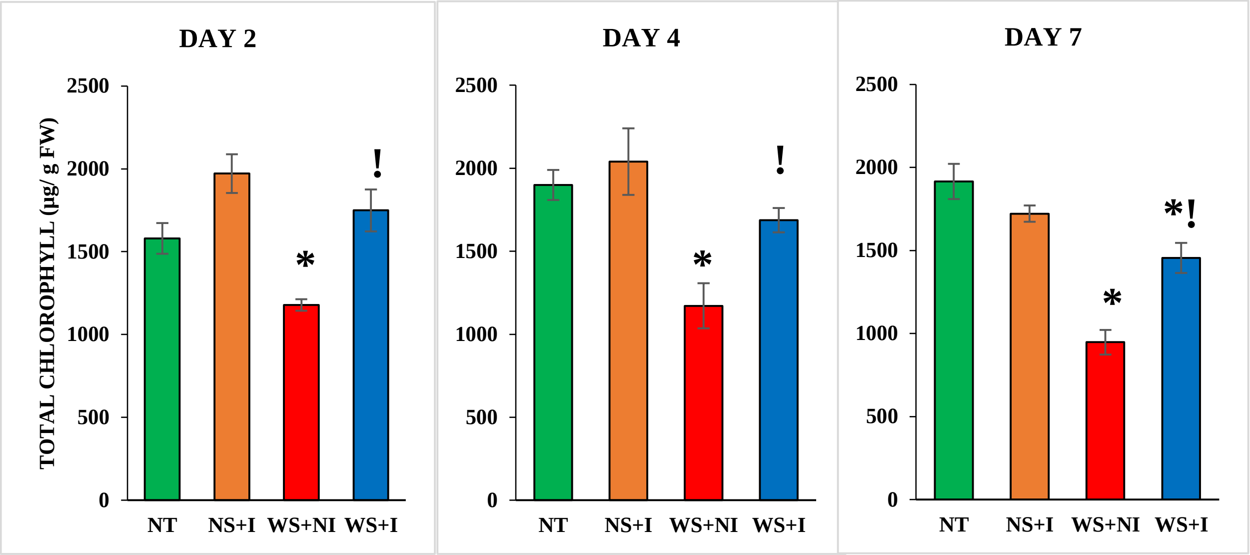


Fig. 2.5 Chlorophyll content of *Arabidopsis thaliana* on day 2, day 4 and day 7 post treatments. Each bar represents mean ± SE of 6 replicate sets, each with 40 mg leaf sample. * indicates significant difference (p≤0.05) in data between NT and any other treatment within a particular day of analysis. ! indicates significant difference (p≤0.05) between WS+I and WS+NI samples within a particular day of analysis.

2.3.4 Primary root length and root structure architecture (RSA):

It has been reported that length of the primary root tends to increase under drought conditions in many plants (Pace et al. 1999; Jacobs et al. 2004; Grossnickle 2005). Commensurate with these findings, we also observed similar trends in our plants (Fig. 2.6 and 2.7) at all time-periods of the study. Just before application of treatments (day 0), all plants showed similar root length (Fig. 2.6 and 2.7). Water-stressed, non-inoculated plants exhibited the highest primary root length and enhanced branching at day 2, day 4 and day 7 while in case of water-stressed, GAP-P45 inoculated plants, primary root length and branching pattern were similar to control plants at most time periods of the study. Except for day 7 where GAP-P45 treatment caused a small dip in primary root length under non-stressed condition, there was no significant difference in root length between control and GAP-P45 treated (without PEG) plants.

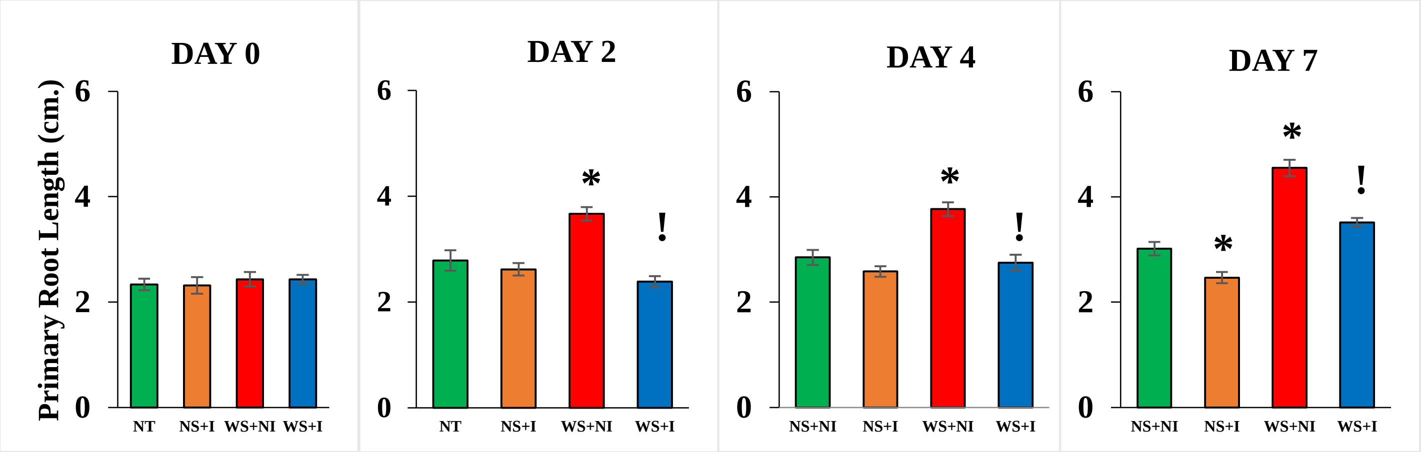


Fig. 2.6 Primary root length of *Arabidopsis thaliana* on day 2, day 4 and day 7 post treatments. Each bar represents mean ± SE of 10 replicate plants, taken from 3 Magenta boxes. * indicates significant difference ($p \leq 0.05$) in data between NT and any other treatment within a particular day of analysis. ! indicates significant difference ($p \leq 0.05$) between WS+I and WS+NI samples within a particular day of analysis.

Fig. 2.7 Observation on primary root length of one replicate plant from each of the four treatments. Day 0 represents the time period just before treatments (the respective treatments in the X-axis indicate sets of plants which were thereafter transferred to Magenta boxes for those treatments).

2.3.5 Proline content:

In order to analyze the level of proline accumulation under dehydrating conditions, proline content of the plants was measured in all treatments and at all time periods of this study. As can be seen from Fig. 2.8, PGPR inoculation alone (without water-stress) did not cause any change in proline content of the plants, as compared to the controls. It was observed that, post water-stress induction, both non-inoculated and inoculated plants exhibited enhanced proline levels, as compared to the controls. Gradual, time-dependent increase in proline levels were seen in both these treatments from day 2 to day 7. Under water-stress, higher proline content was observed in the non-inoculated plants as compared to the inoculated plants, on day 2 and day 4 post treatments. At day 7, however, proline levels were similarly induced in both these treatments. This indicates that inoculation with GAP-P45 delayed proline accumulation in plants under water-stress. The non-stressed plants (non-inoculated as well as inoculated) exhibited minor increase in proline levels from day 2 to day 7.

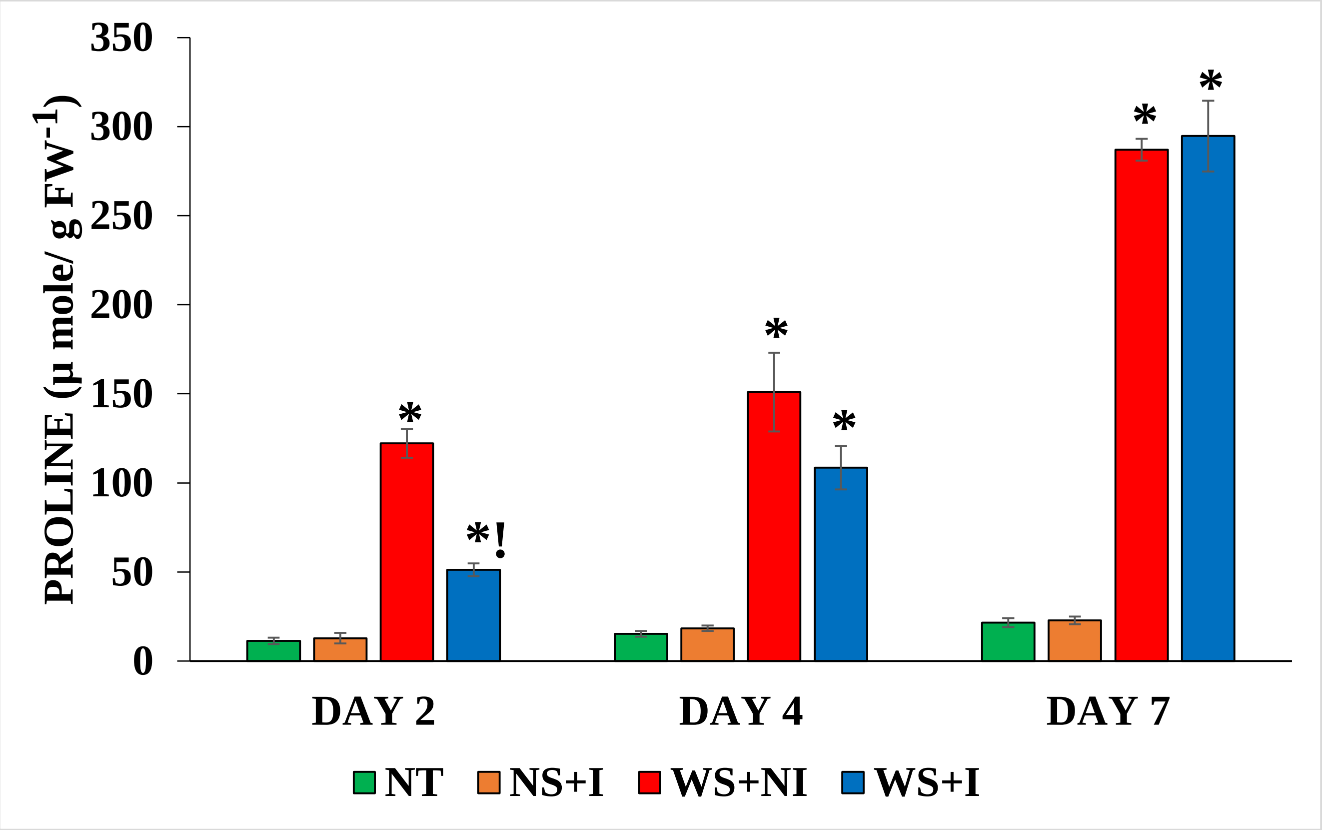


Fig. 2.8 Content of accumulated free proline in *Arabidopsis thaliana* on day 2, day 4 and day 7 post treatments. Each bar represents mean ± SE of 6 replicate samples (each sample represents 100 mg of seedlings from one Magenta box). * indicates significant difference ($p \leq 0.05$) in data between NT and any other treatment within a particular day of analysis. ! indicates significant difference ($p \leq 0.05$) between WS+I and WS+NI samples within a particular day of analysis.

2.4 DISCUSSION

As mentioned earlier, the strain of PGPR used in this study (*Pseudomonas putida* GAP-P45) has previously been shown to confer drought tolerance to maize and sunflower (Sandhya et al., 2010a, 2009). Since there was no report on the impact of this strain on *A. thaliana* under drought conditions, we conducted experiments to ensure that the strain enhances water-stress tolerance in *A. thaliana* with respect to growth and physiological status of the plants. This study was carried out *in vitro* using PEG 6000 as inducer of water-stress by lowering water potential of MS-agar medium (the effects of which is similar, but may not be identical to that of water deprivation in soil). In our study we showed that inoculation of *A. thaliana* seedlings with *P. putida* GAP-P45 ameliorated the negative effects of water-stress in PEG-infused MS-agar medium as compared to non-inoculated stressed plants. As evident from Fig. 2.1, during water-stress, plant health declined rapidly soon after transferring the plants to non-inoculated PEG-infused medium. Observations on fresh weight, dry weight and plant water content of the non-inoculated stressed plants after 2, 4 and 7 days post treatment (Fig. 2.4) indicated towards stress-induced growth stunting of shoot and showed more susceptibility of these plants to water-stress in the absence of PGPR inoculation. However, the seedlings inoculated with GAP-P45 under the same intensity of water-stress, showed better morpho-physiological status with respect to shoot growth and increased fresh weight, dry weight and plant water content despite the fact that GAP-P45 inoculation did not change the water potential of the medium. Under non-stressed condition GAP-P45 did not show any significant improvement of morphological and physiological status of the inoculated plants evident from the similar FW, DW and PWC of these plants as compared to the no-treatment controls. There are several reports where researchers have argued that PGPRs are more effective in stimulating growth and development of the stressed plants under drought conditions rather than under well-watered control conditions (Chanway and Holl 1994; Timmusk and Wagner 1999; Sandhya et al. 2010a; Rubin et al. 2017). Sandhya et al., (2010) also reported GAP-P45 enhanced biomass accumulation in maize plants under drought condition when compared to plants inoculated under non-stressed condition. Growth on the LB-agar plates inoculated with a loop-full of culture from the inoculated Magenta boxes (both non-stressed and water-stressed) at all time points of analysis, confirmed the tolerance and steady growth of GAP-P45 under water-stress in PEG-infused MS-agar medium (Fig. 2.2). Diminished health of *A. thaliana* seedlings inoculated with laboratory strain of *E. coli* DH5α under similar experimental setup, clearly indicated that the observed amelioration of water-stressed in case of GAP-P45 inoculated plants was not merely due to addition of suspension culture of any random bacterial strain (Fig. 2.3).

Water-stress mitigation and better physiological status observed in *A. thaliana* seedlings in our study could be the effect of production and secretion of phytohormones (auxin, cytokinin and gibberellic acid) by GAP-P45 in the medium as characterized by Sandhya et al., (2010). There are reports on alleviation of water-stress effects in various plant species by PGPRs capable of producing phytohormones (Cohen et al. 2009; Liu et al. 2013; Kang et al. 2014b; Khan et al. 2016; Selvakumar et al. 2018). However, whether these bacteria-secreted phytohormones are taken up by the plants or it induces some other endogenous signaling cascades in the inoculated plants remains to be seen and can be part of the future scope of this study. The strain GAP-P45 was also characterized as a producer of exopolysaccharides, siderophores and ammonia (Sandhya et al. 2010b), which are important plant growth promoting traits that can help *A. thaliana* seedlings withstand the exerted water-stress. Reduction in plant size and increase in root-to-shoot ratio are common strategies of plants under water-stress to minimize stress injury and water consumption (Boyer 1985; Tardieu et al. 2011; Bresson et al. 2013). Similar trends of enhanced elongation and branching of roots were also observed in non-inoculated water-stressed plants in our study (Fig. 2.6 and 2.7). However, GAP-P45 inoculated seedlings showed significantly lower root elongation and branching under water-stress, indicating that stress senescence was much lower in these plants. Photosynthetic efficiency of plants is an important aspect of plant growth and development under both non-stressed and stressed conditions. Water-stress affects the photosynthetic machinery of plants by causing upregulation of abscisic acid, stomatal closure and degradation of chlorophyll. Decrease in chlorophyll content is one of the typical symptoms of deteriorated plant health under water-stress caused due to oxidative stress, which in turn induces photo-oxidation of pigments and chlorophyll degradation (Munné-Bosch and Alegre 2004; Santos 2004; Anjum et al. 2017). In our study, we also observed dramatic decrease in chlorophyll content of the water-stressed non-inoculated plants at all time point of analysis, which can be directly correlated to the diminished health of the plants under this treatment. On the contrary, plants inoculated with GAP-P45 exhibited significantly higher amount of chlorophyll content despite exertion of similar water-stress (Fig. 2.5). Many researchers have previously reported that inoculation with PGPR such as, *Pseudomonas*, *Bacillus*, *Azospirillum*, *Burkholderia* etc. enhanced chlorophyll content and increased photosynthetic efficiency in plants as compared to non-inoculated drought stressed plants (Tank and Saraf 2010; Heidari and Golpayegani 2012; Patel et al. 2012; Tallapragada et al. 2016; Tyagi et al. 2017).

As discussed in the introduction section, rapid accumulation of free proline in the cells to lower cellular water potential, is reported as an immediate response of plants under water-stress which helps plants to prevent further water loss by transpiration. One of the mechanisms by which PGPRs impart water-stress tolerance to the plants, is by upregulating proline biosynthetic genes and inducing proline accumulation in the plant cells (Porcel and Ruiz-Lozano 2004; Kohler et al. 2008, 2009; Heidari and Golpayegani 2012; Tallapragada et al. 2016). The strain *P. putida* GAP-P45 had also been reported to enhance proline accumulation in maize plants under drought stress to maintain cellular water status and protect membranes and proteins from degradation (Sandhya et al. 2010a). In our study, we observed that under water-stress both inoculated and non-inoculated *A. thaliana* seedlings accumulated higher amount of free proline as compared to the no-treatment control plants. However, at day 2 post treatment level of accumulated proline in water-stressed GAP-P45 inoculated plants were significantly lower than non-inoculated ones. This observation indicated delayed stress senescence in case of inoculated plants due to PGPR inoculation. Similar levels of accumulated proline content observed in both non-inoculated and inoculated plants under water-stress at day 4 and day 7 indicated delayed but steady upregulation of proline biosynthesis in inoculated plants due to GAP-P45 inoculation.

2.5 CONCLUSION

1. As can be seen from Fig. 2.1 to 2.8, this PGPR strain *Pseudomonas putida* GAP-P45 has shown cross-compatibility and profound positive impact on water-stress tolerance of *A. thaliana.*
2. Inoculation with GAP-P45 under water-stress enhanced plant growth and morphology by modulating root structure architecture, fresh weight, dry weight, plant water content, chlorophyll content and proline accumulation in *A. thaliana*.

These experiments were necessary to establish the compatibility of GAP-P45 with *A. thaliana* for osmotic-stress amelioration before moving on to the molecular experiments. While many more assays could be done for quantitative estimation of drought alleviation by PGPR, we chose a subset of these experiments because our main focus was on gene expression analysis and only wanted to establish that GAP-P45 indeed exhibited drought ameliorating responses in *Arabidopsis* before moving on to the molecular analyses. The observed pattern of proline accumulation in plants inoculated with GAP-P45 under water-stress further interested us to investigate the PGPR mediated time-dependent regulation pattern of the proline metabolic genes in *A. thaliana*. We also analyzed the activity and specific activity of the enzymes coded by these genes to correlate the transcriptional and post transcriptional regulation of proline metabolism, elaborated in the following chapter.

CHAPTER 3

Modulation of proline metabolic gene expression and enzyme activity in *Arabidopsis thaliana* under water-stress by *Pseudomonas putida* GAP-P45

3.1 INTRODUCTION

As mentioned in the previous chapters, an important cellular mechanism by which plants abate water-stress is through internal osmotic adjustment, by accumulating several compatible osmolytes in their cells to prevent water loss (Kavi Kishor et al. 2005a; Szabados and Savouré 2010; Zlatev and Lidon 2012; Krasensky and Jonak 2012; Reddy et al. 2015). Such compatible solutes include sugar alcohols (such as sorbitol), amino acids (such as proline) and amino acid derivatives (such as glycine betaine). These compatible solutes help decrease cell water potential, thus preventing the exosmosis of water, enabling the sustenance of turgor pressure and ensuring the continuation of plant metabolic activity and therefore, growth and productivity (Zlatev and Lidon 2012; Krasensky and Jonak 2012; Liang et al. 2013; Reddy et al. 2015).

Proline being the most important compatible osmolyte, its accumulation in different tissues in plants, both under stressed and non-stressed conditions and proline homeostasis during plant growth and development have been well studied (Kavi Kishor and Sreenivasulu 2014). However, evidences from certain studies on proline metabolism under osmotic stress in *A. thaliana* have critically challenged the conventional hypothesis of "more proline leads to better tolerance". Experiments using *A. thaliana* mutants for proline metabolism (*p5cs1* and *pdh1*) under dehydration stress revealed that, not just accumulation of proline, but its simultaneous catabolism is also required for better growth and development under water-stress (Sharma et al. 2011; Bhaskara et al. 2015).

The first committed step in proline biosynthesis is the production of glutamate-γ-semialdehyde (GSA), which gets converted into Δ^1-pyrroline-5-carboxylate (P5C), the immediate precursor of proline. Glutamate-γ-semialdehyde can be made either from ornithine via the enzyme ornithine aminotransferase (OAT) in mitochondria (Delauney et al., 1993; Sharma and Verslues, 2010; Liang et al., 2013; Sharma et al., 2013) or from glutamate via P5C synthase (P5CS) (Savouré et al., 1995; Székely et al., 2008; Mattioli et al., 2009; Sharma and Verslues, 2010) in the cytosol and chloroplast. Many studies have reported the upregulation of *OAT* gene under salt and osmotic stress in *A. thaliana* or increased tolerance via *OAT* overexpression (Roosens et al. 1998; Roosens et al. 2002; Wu 2003; Armengaud et al. 2004; Sharma and Verslues 2010).On the contrary, it has been reported by Funck et al., (2008) via mutant analysis that OAT does not take part in proline biosynthesis under drought stress conditions in *A. thaliana*. The enzyme P5CS is reported to catalyze the rate-limiting step in proline biosynthetic pathway and has a bifunctional activity, i.e. that of both γ-glutamyl kinase and glutamic- γ-semialdehyde dehydrogenase (reviewed by Kavi Kishor et al., 2005; Krasensky and Jonak, 2012). The enzyme P5C reductase (P5CR) is responsible for the conversion of P5C to proline (Sharma et al., 2011; Funck et al., 2012; Giberti et al., 2014). The catabolism of proline (i.e. its conversion to glutamate) involves two important oxidation steps (1) proline is oxidized to form P5C by the enzyme proline dehydrogenase (PDH) (Funck et al., 2010; Sharma and Verslues, 2010; Sharma et al., 2011) and (2) P5C is then oxidized to produce glutamate by the enzyme Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH) (Deuschle et al. 2004; Sharma and Verslues 2010; Rizzi et al. 2015).

Drought mediated regulation of the proline metabolic genes is well established. It is well reported that dehydration conditions upregulate gene expression leading to proline biosynthesis (Zhang et al. 1997; Choudhary et al. 2005; Szabados and Savouré 2010; Sharma and Verslues 2010; Sharma et al. 2011; Liang et al. 2013; Bhaskara et al. 2015; Reddy et al. 2015). The genes *P5CS1* (Yoshiba et al., 1995; Hong et al., 2000), *P5CR* (Zhang et al. 1997) and *OAT* (Hare and

Cress 1996) are reported to be upregulated as a form of primary response of *A. thaliana* to dehydration stress. The enzyme P5CS1 has been shown to play the rate-limiting role in increased proline biosynthesis from glutamate (Székely et al. 2008). The other orthologue of this enzyme, P5CS2 majorly functions in growth and development and biotic stress responses of *A. thaliana* (Fabro et al. 2004; Toka et al. 2010), but has not been reported to play any role in dehydration stress tolerance (Székely et al. 2008; Mattioli et al. 2009). Proline catabolic genes, *PDH1* and *P5CDH* have mostly been reported to be downregulated under drought stress conditions (Verbruggen et al. 1996; Borsani et al. 2005; Verslues et al. 2007; Sharma and Verslues 2010), although, Bhaskara et al., (2015); Fabro et al., (2004); Kaplan et al., (2007) have shown simultaneous upregulation of both proline biosynthetic and catabolic genes under dehydration conditions indicating the importance of proline turnover in plants during drought for better survival. Sharma et al., 2011 have reported tissue-specific upregulation of *PDH1* in plants under drought stress. The orthologue *PDH2* was reportedly un-induced by drought (Sharma and Verslues 2010).

After obtaining data from morpho-physiological studies, we proceeded towards conducting molecular studies focusing on *P. putida* GAP-P45 mediated regulation of the expression of proline metabolic genes under dehydrating conditions in a time-dependent manner. We also measured the activities and specific activities of the related enzymes encoded by the aforementioned genes involved in the proline metabolic pathway, to investigate the transcriptional regulation of these genes under water-stress with/ without *P. putida* GAP-P45 inoculation. Our observations indicate that, under dehydrating conditions, *P. putida* GAP-P45 not only up-regulates the expression of proline biosynthetic genes but also concomitantly up-regulates the expression of the genes that function in proline catabolism (i.e. its conversion to glutamate and ornithine), thus possibly enabling enhanced proline turnover (Ghosh et al. 2017). Based on our results, we hypothesized that one of the ways in which this strain imparts water-stress tolerance to *A. thaliana* is via modulation in proline metabolic gene expression and transcriptional regulation of the respective enzymes. We also hypothesized that not only accumulation, but simultaneous catabolism of accumulated proline was induced by the PGPR *Pseudomonas putida* GAP-P45 under water-stress resulting in higher proline turnover in the plants in comparison to the non-inoculated water-stressed plants (Ghosh et al. 2017, 2018b).

3.2 MATERIALS AND METHODS

3.2.1 Plant growth, maintenance and treatments:

Procedures involved in germination, growth, maintenance of *A. thaliana* seedlings; induction of water-stress; inoculation of seedlings with Pseudomonas putida GAP-P45 and treatment conditions have previously been discussed in details in chapter 2.

3.2.2 Gene expression studies:

For gene expression analysis, seedlings were harvested at different time periods (2, 4 and 7 days) post water-stress induction and GAP-P45 inoculation. Total RNA isolation was done from whole seedlings by TRIZOL reagent (Invitrogen). The plant samples (200 mg for each treatment), were homogenized in liquid nitrogen and 2 mL of TRIZOL was added to each tube and incubated for 5 min. following this, 400 µL of chloroform was added to the homogenized sample, mixed by inverting slowly, incubated for 5 min and centrifuged at 12000 ×g for 10 min. The colourless supernatant was collected in a fresh micro-centrifuge tube each and 1 mL of isopropanol was added. The mixture was then incubated for 10 min and centrifuged at 12000 ×g for 10 min at 4 ºC. the supernatant was discarded and the obtained pellet of total RNA was washed with 70 % ethanol twice and dried. Finally, the obtained total RNA was dissolved in 50 µL of 1x TE buffer, treated with DNase I, quantified using a nanodrop and stored at -20 ºC. cDNA synthesis was performed using Superscript III Reverse Transcriptase (Invitrogen) starting with 2 µg of total RNA. Semi-quantitative PCR was performed using appropriate primers (table 3.1) used by Sharma and Verslues (2010), for the following genes - *OAT, P5CS1, P5CR, PDH1* and *P5CDH.* The obtained qualitative expression pattern of these genes (evident from the band-intensity of the amplicons in agarose gel) were corroborated using quantitative real-time PCR (Step One Plus, Applied Biosystems, USA) using a SYBR green PCR master mix (Invitrogen). All the primers were tested at different concentrations (ranging from 50-250 nM per reaction) with the serially diluted cDNA (ranging from 0.08-250 ng, using 1:5 incremental dilution factor) by generating standard curves with regression value closest to 0.999 in real-time PCR to determine optimum primer and template concentration for further relative quantification. Amplification of desired cDNA segment at all concentrations of templates, without interference of false positive signals due to noise or primer-dimer, were ensured by analyzing the melt-curves generated in real-time PCR. Melt-curve is the determination of melting temperature (T_m) of the amplicon following PCR. Formation of primer dimers or any non-specific amplifications usually exhibit a lower T_m value than the desired amplicon. Presence or absence of primer-dimers and any other non-specific products/ contamination were confirmed by the amplification of the NTC (no treatment control) without addition of template cDNA. Standard curves and melt-curves of all the genes of interest are collectively shown in Fig. 3.1 and Fig. 3.2. Gene expression analysis was done using relative quantification by the $\Delta\Delta C_T$ method (Applied Biosystems). *β-ACTIN2* was used as endogenous control (Sharma and Verslues 2010) and gene expression was quantified relative to the non-stressed, non-inoculated controls (reference control). The presence of genomic DNA contamination was ruled out using P5CS1 primers which amplifies an intron-spanning product from genomic DNA template which has a larger size (550 bp) as opposed to the template obtained from amplification of cDNA (135 bp). The formulae used to calculate the $\Delta\Delta C_T$ and relative quantification values are shown below:

Step 1: $\Delta C_T = C_T$ of the target genes - C_T of the endogenous control (within same treatment).

Step 2: $\Delta\Delta C_T = \Delta C_T$ of the target gene in treatment - ΔC_T of the target gene in reference sample.

Step 3: Relative quantification (RQ) = $2^{(-\Delta\Delta CT)}$

Where, C_T = threshold cycle for amplification; Target gene = proline metabolic genes; Endogenous control = *β-ACTIN2*; Treatment = NS+I/ WS+NI/WS+I; Reference sample = NT.

Table 3.1: Oligonucleotide sequences of the proline metabolic genes used for semi-quantitative and quantitative PCR reactions (Sharma and Verslues, 2010).

Genes	**Forward Primer**	**Reverse Primer**
β-ACTIN2	5’-GATTCAGATGCCCAGAAGTCTTGT-3’	5’-TGGATTCCAGCAGCTTCCAT-3’
OAT	5’-TCCCGACGGTTACTTGAAAGC-3’	5’-CAGGACGAATTTCTTCCCAATCAC-3’
P5CS1	5’-GCAAAGTTGGACTATCCAGCAG-3’	5’-CTTGGTCCACCATACAAAGTGAC-3’
P5CR	5’-TAGCAATTGAAGCTTTAGCCGATG-3’	5’-ACCATCGTTGCAGCTCCAAG-3’
PDH1	5’-AGCTGCCAAATCTTTACCAACATC-3’	5’-GCTTCCATGAGAGTTTGAAGTTCG-3’
P5CDH	5’-GTTCCTGGCACGGTCTTTTG-3’	5’-CTGAAGTAGTGGAATCTCAAGTGG-3’

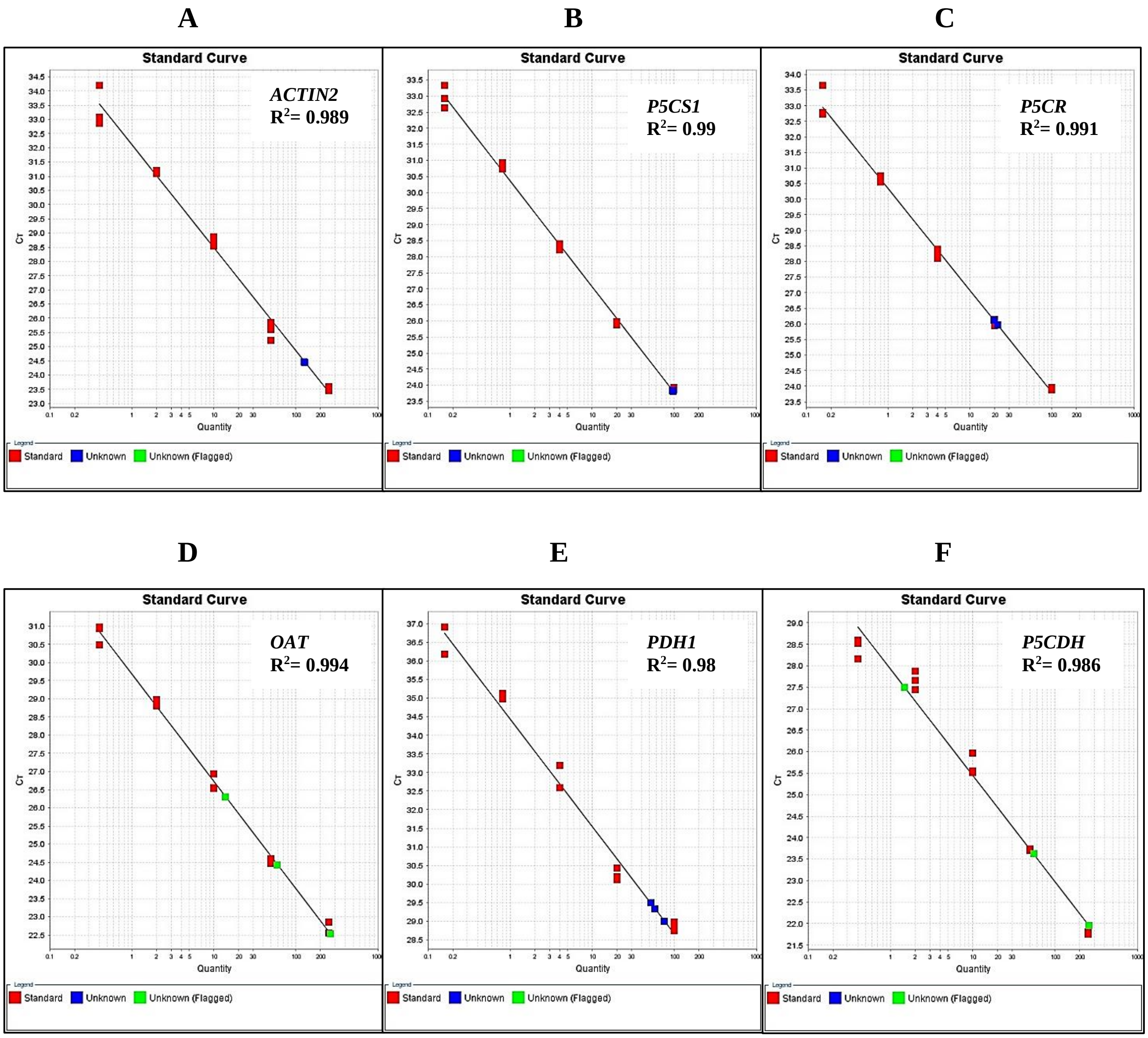


Fig. 3.1 Generation of standard curves of all the gene primers to optimize the primer and template concentration for the relative quantification of expression of the genes of interest in real-time PCR analysis. (A) *ACTIN2* (B*) P5CS1* (C) *P5CR* (D) *OAT* (E*) PDH1* (F) *P5CDH*. 5 Standard concentrations of cDNA were prepared by serial dilution and optimum concentrations of primers were used.

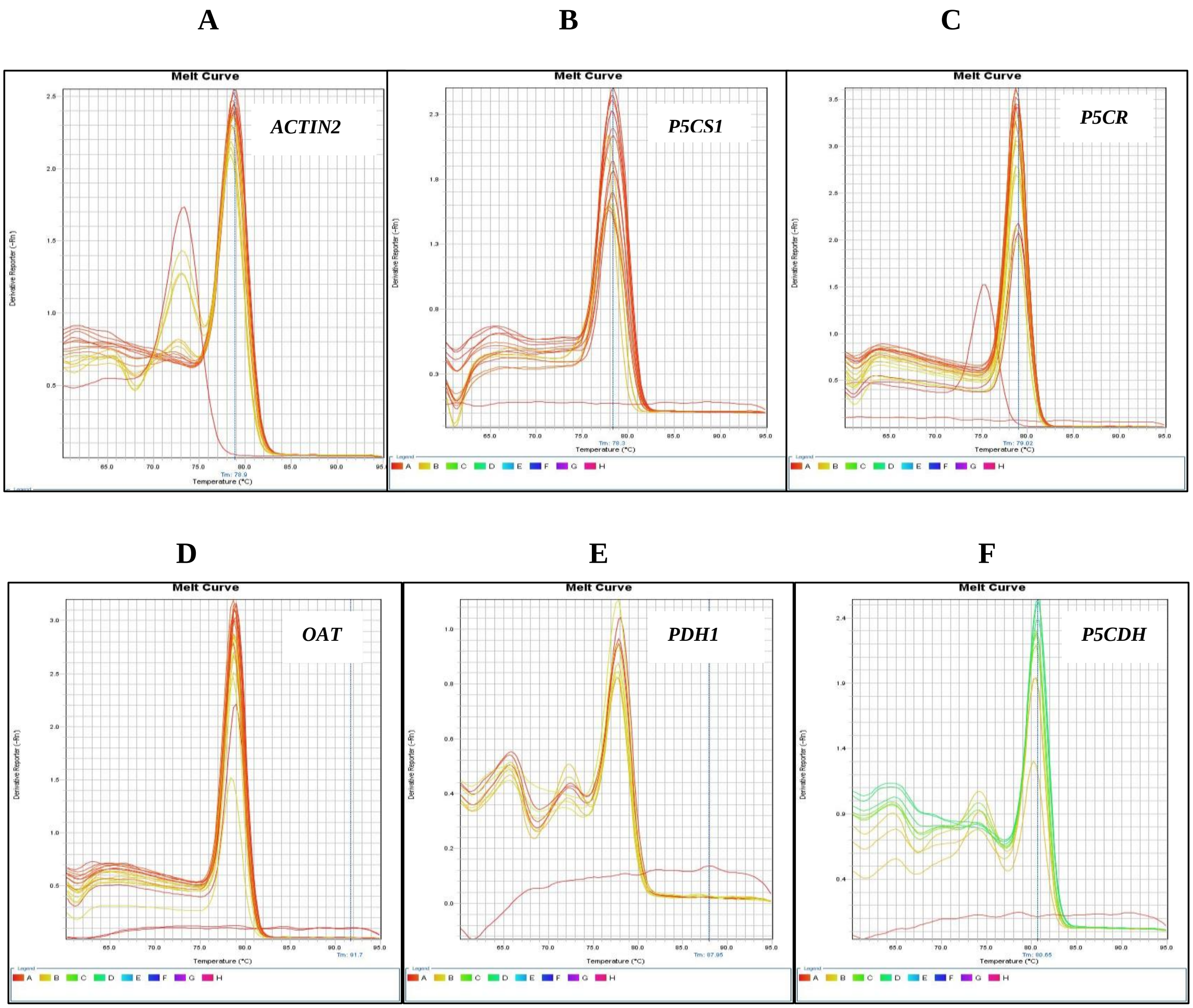


Fig. 3.2 Generation of melt curves of all the gene products to determine absence of any non-specific amplification of template DNA which can interfere with the relative quantification of expression of the genes of interest in real-time PCR analysis. (A) *ACTIN2* (B*) P5CS1* (C) *P5CR* (D) *OAT* (E*) PDH1* (F) *P5CDH*. ACTIN2 and P5CR primers showed primer-dimer in (A) and (C) but the T_m values were much lesser than the actual amplicon.

3.2.3 Activity of proline metabolic enzymes:

Extraction and measurement of activity of the enzyme P5CS was carried out following the protocol given by Wang et al. (2011). Seedlings (500 mg) from each replicate Magenta box were homogenised in extraction buffer composed of 100 mM Tris-Cl (pH 7.5), 10 mM β-mercaptoethanol, 10 mM $MgCl_2$ and 1 mM PMSF and centrifuged at 4° C for 20 min at 10,000 × g. The enzyme assay was performed by adding appropriate amount of crude enzyme extract (supernatant) to the reaction buffer containing 100 mM Tris-Cl (pH 7.2), 25 mM $MgCl_2$, 75 mM Na-glutamate, 5 mM ATP and 0.4 mM NADPH. The reaction velocity was measured as the rate of NADPH consumption (extinction coefficient of NADPH is 6220 M^{-1} cm^{-1}), monitored as the decrease in absorbance at 340 nm as a function of time (Filippou et al. 2014).

P5CR activity was measured at 37°C as the P5C-dependent oxidation of NADPH with a slight modification of the protocol reported by Forlani *et al.*, 2015. The assay mixture was composed of 50 mM Tris-Cl, pH 7.75, 0.5 mM NADPH, 2 mM DL-P5C and 1 mM $MgCl_2$ in a final volume of 1 ml. Due to the unavailability of commercially manufactured DL-P5C, it was synthesized by the method given by Williams and Frank, 1975.In the absence of pyridine nucleotide, the proline oxidase catalyzed the oxidation of L-proline to L-P5C. A mixture containing 150 µmoles of L-proline, 200 µmoles of Tris-Cl (pH 8.5), 50 µmoles of $MgCl_2$ and 20 units of proline dehydrogenase (purified from seedling samples) were prepared, incubated at 37 ºC in a shaking incubator for 60 min and after incubation the enzyme was denatured with 1 mL 10 % trichloroacetic acid. The reaction mixture was then centrifuged at 16000 ×g to remove denatured protein. Isolation of L-P5C was carried out passing the supernatant through a DOWEX 50 column and stored in 1 M HCl at 4 °C. Appropriate amount of plant crude extract was added to the pre-warmed mixture, and the decrease in absorbance at 340 nm was determined for up to 3 min by continuous monitoring of the sample against blanks from which P5C had been omitted. Activity was determined from the initial linear rate of decrease in absorbance up to one minute with an interval of 10 seconds (extinction coefficient for NADPH is 6220 M^{-1} cm^{-1}).

Extraction and estimation of OAT activity was performed following the measurement of the amount of P5C produced using the o-aminobenzaldehyde method developed by Kim et al., (1994). Enzyme was extracted in buffer containing 100 mM potassium-phosphate, pH 8.0, 0.2 mM pyridoxal phosphate, 1 mM EDTA, 10 mM β-mercaptoethanol. The reaction mixture containing 100 mM potassium-phosphate buffer, pH 8.0, 35 mM L-ornithine, 5mM α-ketoglutarate, 0.05 mM pyridoxal phosphate, 5 mM o-aminobenzaldehyde and appropriate amount of crude plant extract was incubated at 37°C for 30 minutes and the reaction was stopped by adding 0.5 ml of 10% trichloroacetic acid. Absorbance was measured at 440 nm.

Enzyme extraction and measurement of activity of PDH was carried out following slight modification of the method described by (Monteoliva et al. 2014). The extraction buffer (50 mM Tris–HCl buffer (pH 7.4), 7 mM $MgCl_2$, 3 mM EDTA, 1 mM DTT, 600 mM KCl, 1mM PMSF, and 10 mM β-mercaptoethanol) was added to the plant samples and homogenized, followed by centrifugation at 12,000 xg for 20 min. The reaction mixture contained 100 mM Na_2CO_3-$NaHCO_3$ buffer, pH 10.3, 10 mM NAD, 20 mM L-proline, and the enzyme extract, where proline was used to initiate the reaction. The reference cuvette contained all the solutions except NAD. The activity was calculated using the extinction coefficient of NADPH as 6220 M^{-1} cm^{-1}.

P5CDH activity was measured following the method described by Forlani et al. (1997) with a slight modification, by monitoring NADH formation at 340 nm (extinction coefficient of NADH as 6220 M^{-1} cm^{-1}) at 37 °C. Extraction buffer contained 25 mM Hepes-KOH buffer pH

7.5, 0.5 mM DTT, 1 mM $MgCl_2$ and 1 mM PMSF. Assays were performed using Tris-HCl, pH 7.5, 2 mM NAD^+, 50 mM potassium phosphate (pH 7.5), 25 mM $MgCl_2$, 2 mM DL-P5C and an appropriate amount of crude plant protein extract.

3.2.4 Statistical analysis:

Wherever applicable, statistical analysis was performed by student's t-test (level of significance, $p \leq 0.05$) using Microsoft Excel (2016). As mentioned previously, each experiment was performed with at least three replicate Magenta boxes, each containing about 20 seedlings, distributed in 4 steel meshes and each experiment was performed at least three times.

3.3 RESULTS

3.3.1 Gene expression analysis:

We analyzed the GAP-P45 mediated, time-dependent modulation of the expression of all important genes in the proline metabolic pathway, in response to water-stress treatment. The candidate genes analyzed can be broadly classified into proline biosynthetic genes (*OAT, P5CS1* and *P5CR*) and proline catabolic genes (*PDH1* and *P5CDH*). As can be seen from Fig. 3.3 and Fig. 3.4, except for minor inductions in a few genes at certain time periods (the biosynthetic genes on day 7 and *P5CDH* on day 2), GAP-P45 treatment under non-stressed conditions did not cause any significant change in the expression of any of the above mentioned genes. Fluctuations were observed, though, in expression levels of all genes analyzed post stress induction with or without PGPR inoculation as compared to the non-stressed plants.

On day 2 post transfer to PEG supplemented medium, the expression of all three biosynthetic genes were highest in water-stressed, non-inoculated plants as opposed to all other treatments (Fig. 3.3). In these plants, water-stress increased the expression of *OAT*, *P5CS1* and

P5CR genes by 8 fold, 13 fold and 6 fold respectively, with respect to the non-stressed, non-inoculated controls. Maximum expression was thus shown by *P5CS1* out of the three genes. In the water-stressed, inoculated plants, however, the same genes exhibited significantly lower increase in expression, i.e. 2 fold, 4 fold and 3-fold increase respectively, relative to the non-stressed, non-inoculated controls. By day 4 post treatments, the expression of these genes had not changed much in the water-stressed, non-inoculated plants. However, by day 4, the expression of *OAT*, *P5CS1* and *P5CR* in water-stressed, inoculated plants had surpassed that of the water-stressed, non-inoculated plants, with the most dramatic increase seen in the expression of the *P5CS1* gene. On day 7 water-stressed, GAP-P45-inoculated plants exhibited consistent high expression of *OAT*, *P5CS1* and *P5CR* with minor fluctuations from day 4 data, whereas water-stressed non-inoculated plants exhibited substantial decrease in the expression of *P5CS1* and *P5CR* as opposed to day 2 and day 4. Thus, comparing across the different time-periods of study, we can see an overall reduction in the expression of proline biosynthetic genes in the PEG-treated, non-inoculated plants from day 2 and day 7. On the other hand, the water-stressed, inoculated plants exhibited an overall (and strong) increase in expression of the same from day 2 to day 7 post treatments. Trends of expression of these genes were similar to the observed data acquired in semi-quantitative PCR reactions at all three time points of analysis (Fig. 3.5).

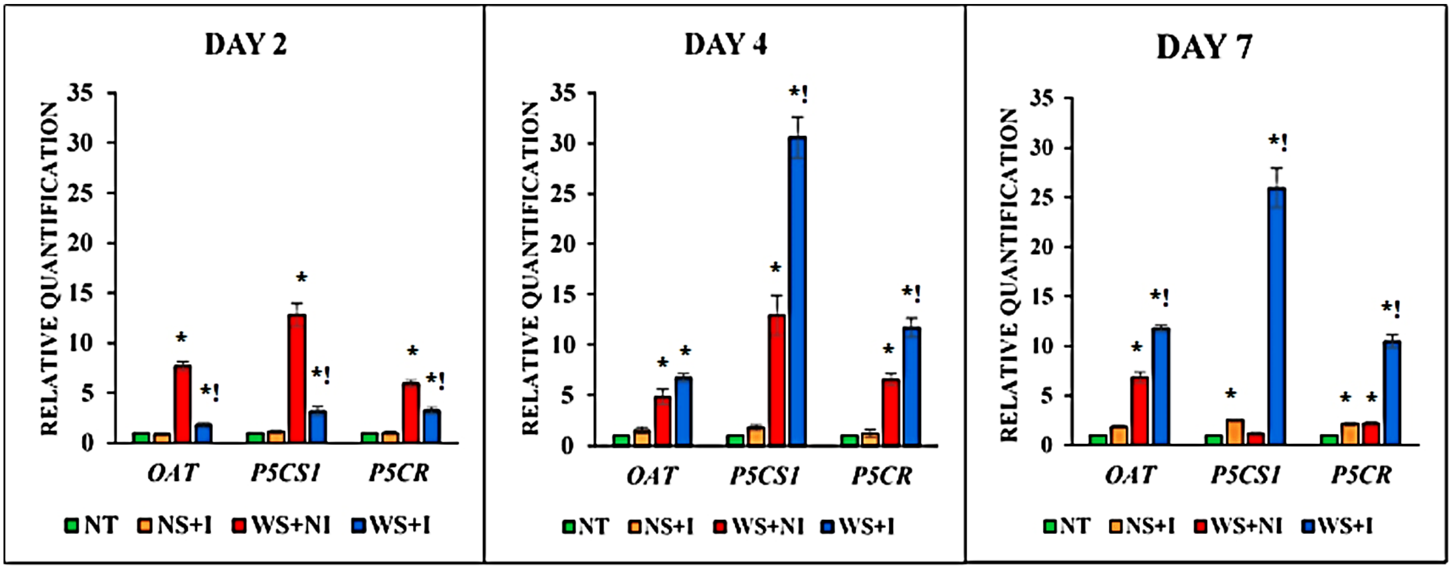


Fig. 3.3 Quantitative RT-PCR analysis of the genes involved in proline biosynthesis in *Arabidopsis thaliana* on day 2, day 4 and day 7 post treatments. Each bar represents mean ± SE of 6 replicate samples (each sample represents 200 mg of seedlings from one Magenta box). "*" indicates significant difference ($p \leq 0.05$) in data between NT and any other treatment within a particular day of analysis. "!" indicates significant difference ($p \leq 0.05$) between WS+I and WS+NI samples within a particular day of analysis. Statistical analysis was done to compare data between two different treatments by student's t-test (two-tailed analysis, $p \leq 0.05$).

As far as the catabolic genes (*PDH1* and *P5CDH*) are concerned (Fig. 3.4), on day 2 post treatments, their expression in the water-stressed, non-inoculated plants were similar to the non-stressed, non-inoculated controls; but significantly higher in the water-stressed, inoculated plants (about 3fold increase as opposed to the controls). A partially time-dependent decrease was observed in the expression of these genes in the water-stressed, non-inoculated plants, while an overall increase (about 5 fold) in their expression was observed in the water-stressed, inoculated plants. Before performing quantitative RT-PCR, changes in gene expression was observed qualitatively using semi-quantitative PCR. As can be seen from Fig. 3.5, the data corroborates well with the quantitative RT-PCR data.

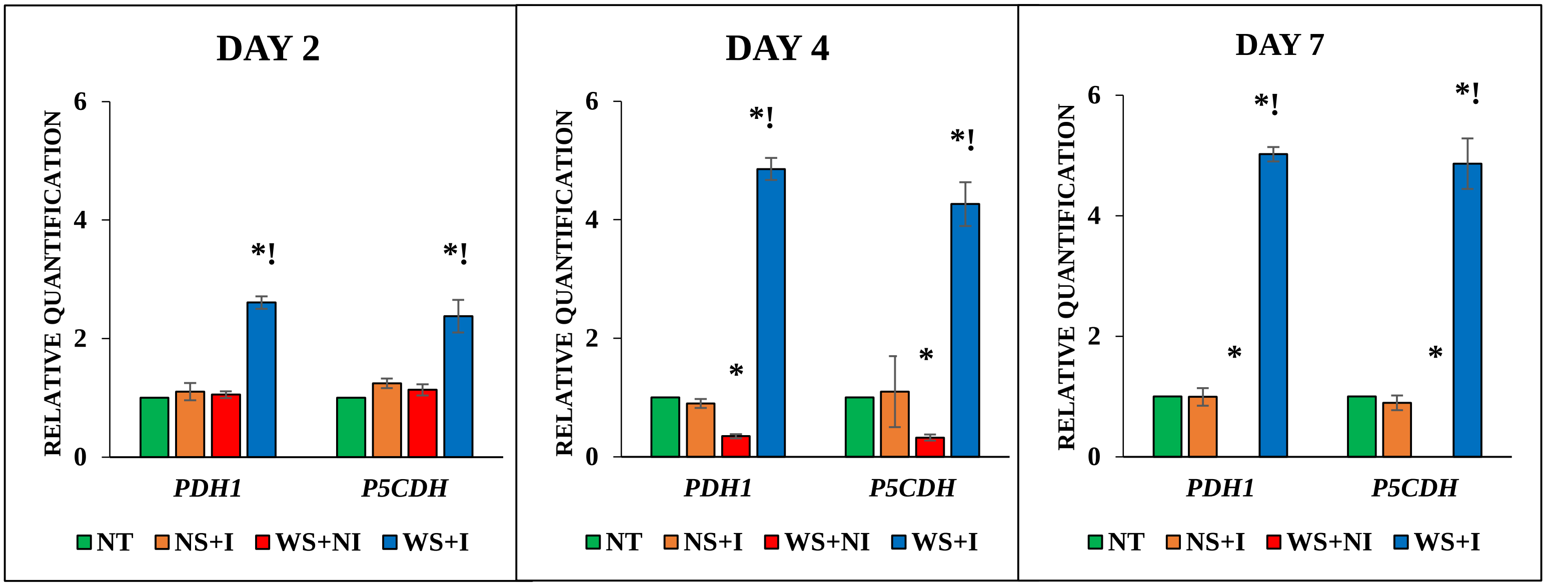


Fig. 3.4 Quantitative RT-PCR analysis of the genes involved in proline catabolism in *Arabidopsis thaliana* on day 2, day 4 and day 7 post treatments. Each bar represents mean ± SE of 6 replicate samples (each sample represents 200 mg of seedlings from one Magenta box). "*" indicates significant difference ($p \leq 0.05$) in data between NT and any other treatment within a particular day of analysis. "!" indicates significant difference ($p \leq 0.05$) between WS+I and WS+NI samples within a particular day of analysis. Statistical analysis was done to compare data between two different treatments by student's t-test (two-tailed analysis, $p \leq 0.05$).

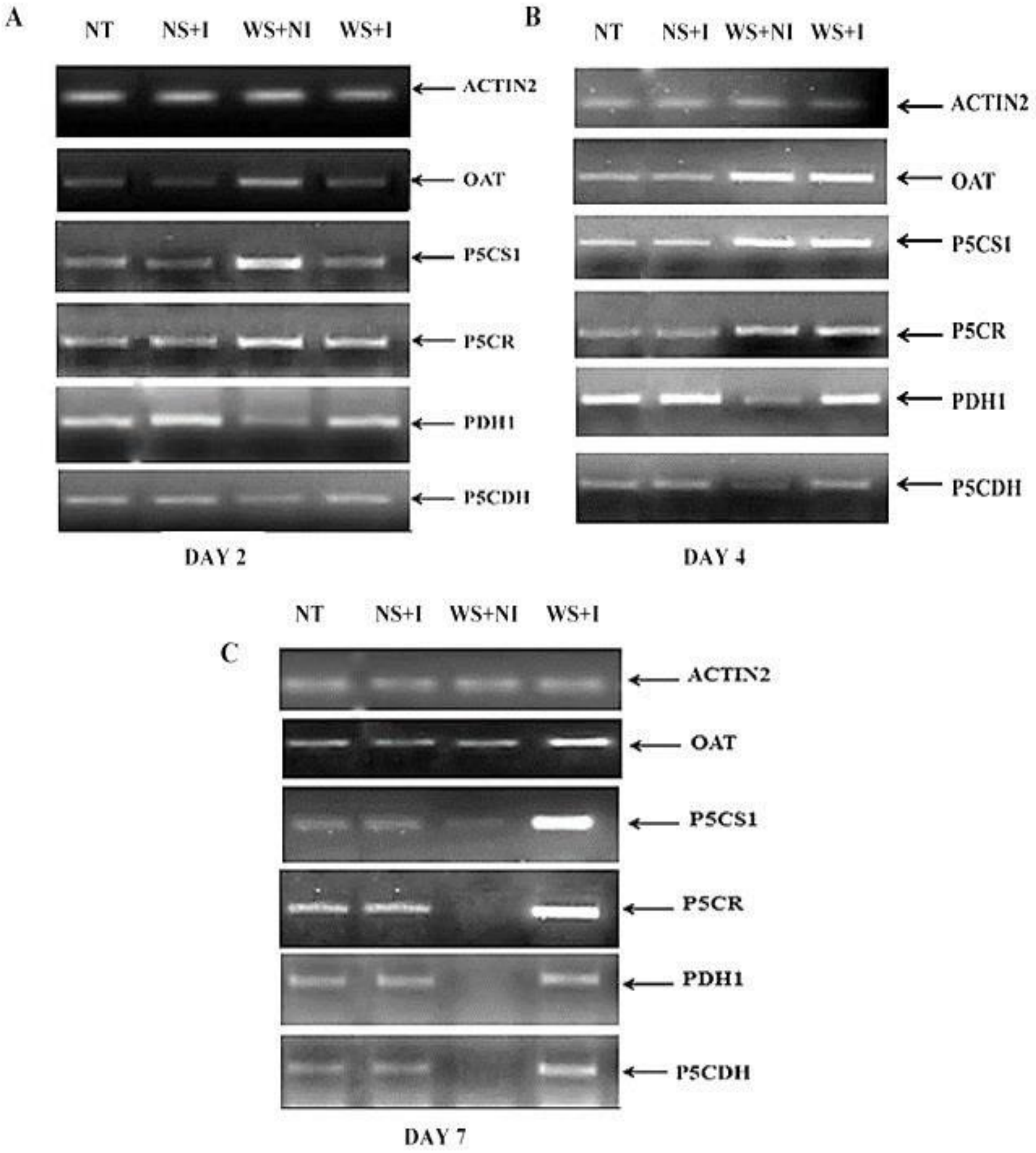


Fig. 3.5 Semi quantitative PCR analysis of expression pattern of the proline biosynthetic (*P5CS1*, *P5CR*, *OAT*) and catabolic (*PDH1*, *P5CDH*) genes post water-stress induction and *Pseudomonas putida* GAP-P45 inoculation. (A) day 2 (B) day 4 (C) day 7 post treatment.

3.3.2 Activity and Specific activity of proline metabolic enzymes:

The biosynthetic enzymes

P5CS: As can be seen from Fig. 3.6 A, on day 2 post treatments, the P5CS activity was highest in the water-stressed plants as opposed to all other treatments. On day 2, in the other treatments, P5CS activity was similar. On day 4, the activity of P5CS remained fairly similar to day 2 in the non-stressed plants, while a significant increase in its activity was seen in the stressed plants, as opposed to day 2. In the water-stressed, inoculated samples, a sharp surge (about 3-fold) was seen, surpassing that of the water-stressed plants. Trends and quantities on day 7 were similar to those of day 4, except that a drop in activity of P5CS was observed in the water-stressed, non-inoculated plants. Trends in specific activity (U mg^{-1} protein) of P5CS in the four treatments were almost identical to its activity (U mg^{-1} FW) on all days in all treatments (Fig. 3.6 B).

P5CR: The activity of P5CR (Fig. 3.7 A) also exhibited similar trends as P5CS. Like P5CS, activity of P5CR was initially induced by dehydration and further up-regulated by GAP-P45 inoculation under water-stress with progression of days. Trends in specific activity (U mg^{-1} protein) of P5CR were almost identical to its activity (U mg^{-1} FW) among the four treatments on all days (Fig. 3.7 B).

OAT: For the most part, OAT exhibited similar trends as the other two proline biosynthetic enzymes (Fig. 3.8 A). As with the two other biosynthetic enzymes, on day 2, the water-stressed, non-inoculated plants exhibited the highest activity of OAT, while, on day 4 and day 7, GAP-P45 inoculation caused a significant increase in the activity of OAT in the water-stressed plants. When compared across days, it was found that, except for water-stressed, inoculated, the activity of OAT did not change much in any other treatment from day 2 to day 7. Trends in specific activity (U mg^{-1} protein) of OAT were almost identical to its activity (U mg^{-1} FW) on all days in all treatments (Fig. 3.8 B).

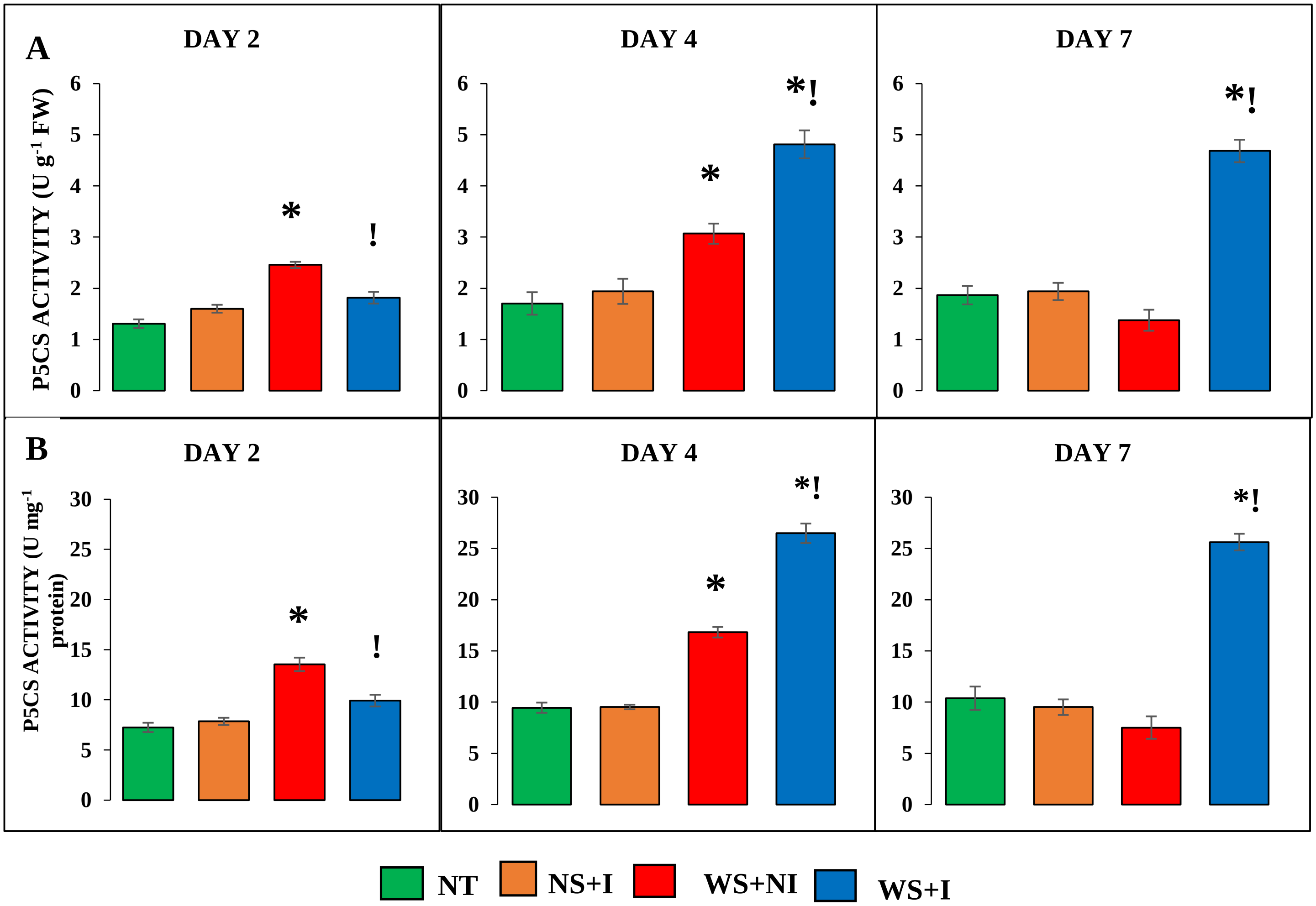


Fig 3.6 Activity of proline biosynthetic enzyme Δ^1-pyrroline-5-carboxylate synthetase (P5CS) in *Arabidopsis thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 treatment under normal and water stress induced conditions compared to untreated controls. Activity of P5CS expressed as **(A)** units (U) g^{-1} FW, U= µ mole NADPH oxidized min^{-1}; **(B)** U mg^{-1} protein (specific activity). Each bar represents mean (±) S.E of 6 independent replicates of each treatment. "*" represent significant difference ($p \leq 0.05$) of all treatments from the untreated

control. “!” represent significant difference (p≤0.05) between water stressed and water stressed + GAP-P45 treated seedlings.

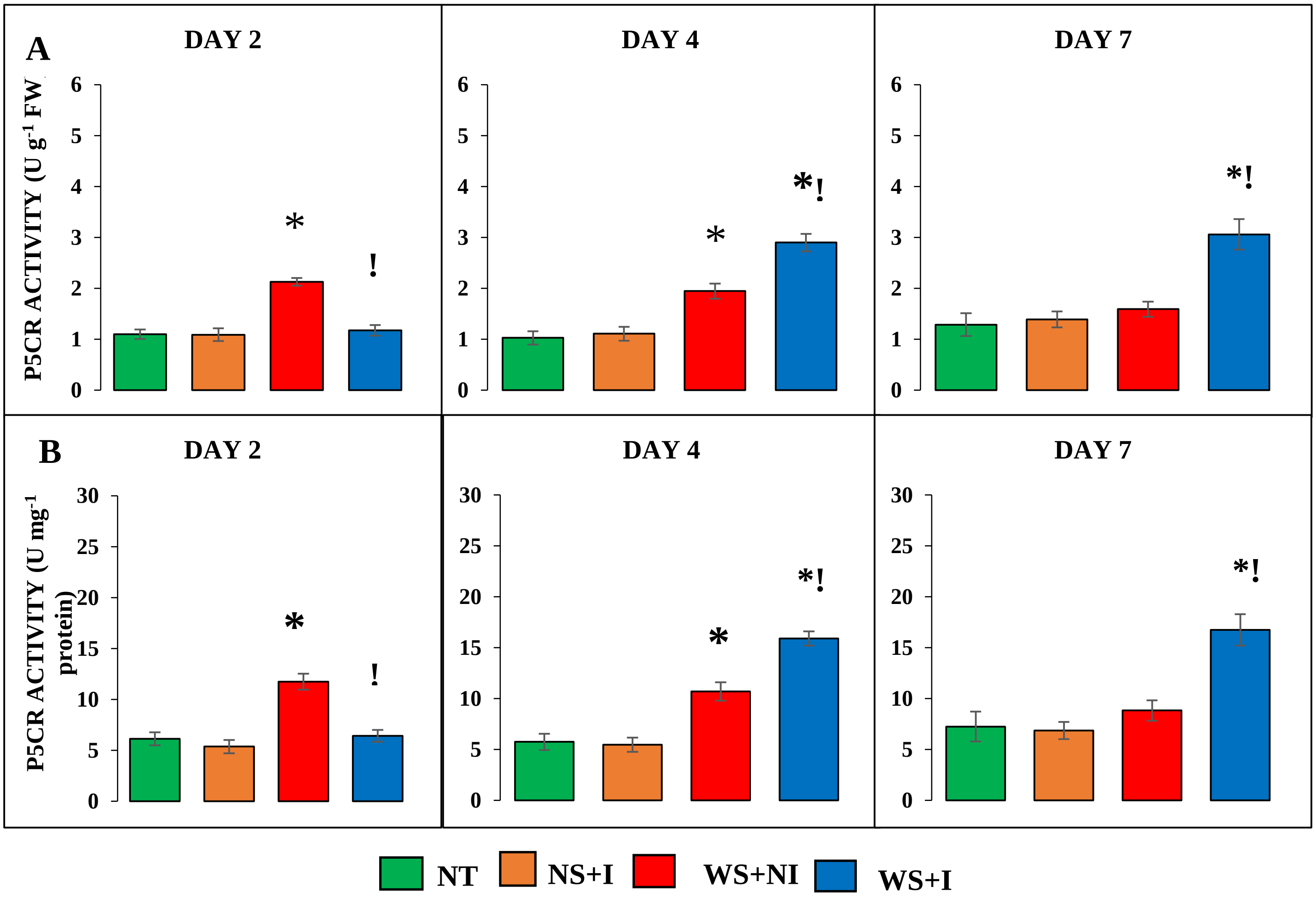


Fig 3.7 Activity of proline biosynthetic enzyme Δ^1-pyrroline-5-carboxylate reductase (P5CR) in *Arabidopsis thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 treatment under normal and water stress induced conditions compared to untreated controls. Activity of P5CR expressed as **(A)** U g^{-1} FW, U= µ mole NADPH oxidized min^{-1}; **(B)** U mg^{-1} protein (specific activity); Each bar represents mean (±) S.E of 6 independent replicates of each treatment. “*” represent significant difference (p≤0.05) of all treatments from the untreated control. “!”

represent significant difference ($p \leq 0.05$) between water stressed and water stressed + GAP-P45 treated seedlings.

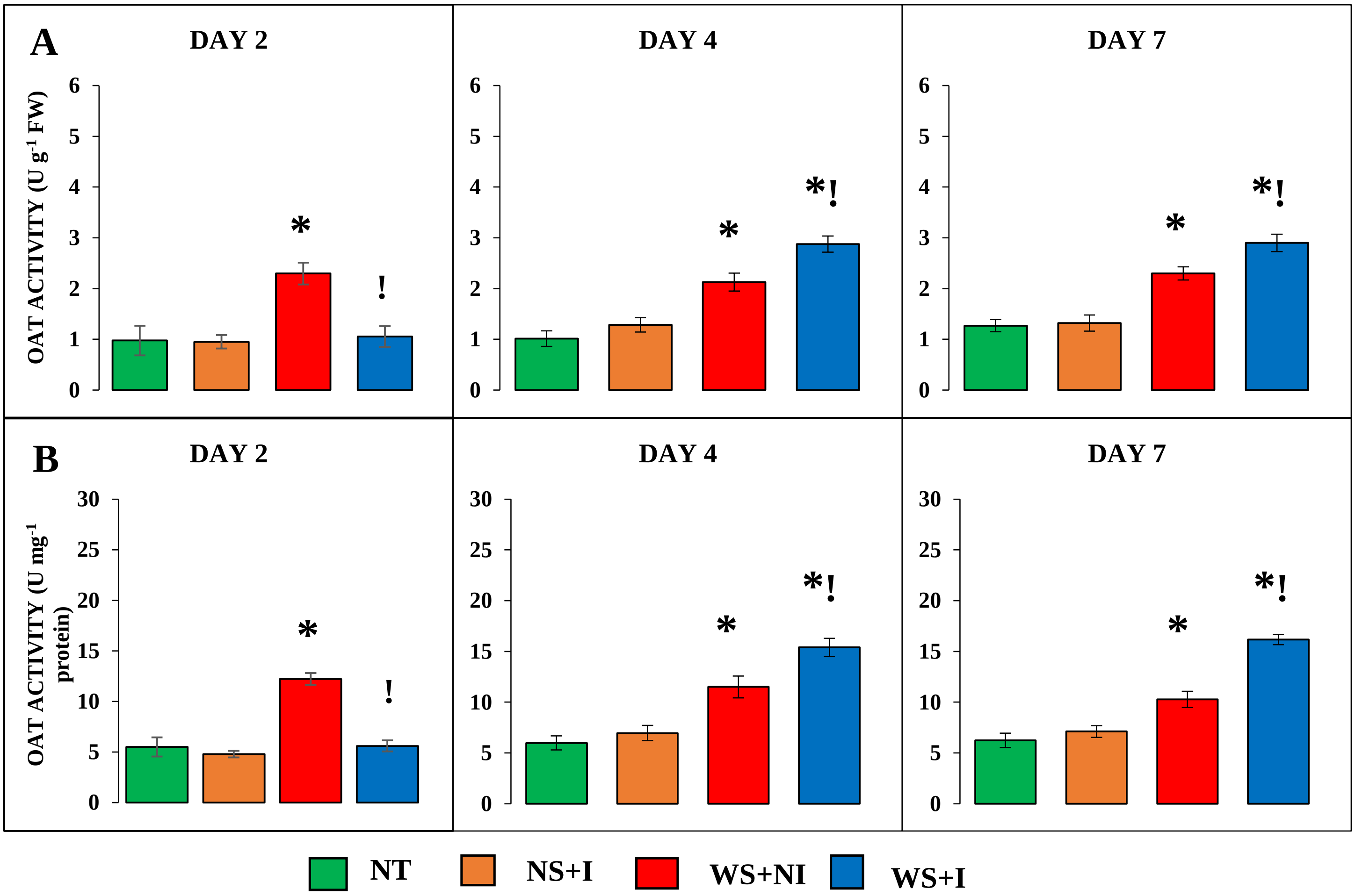


Fig 3.8 Activity of proline biosynthetic enzyme ornithine-Δ-aminotransferase (OAT) in *Arabidopsis thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 treatment under normal and water stress induced conditions compared to untreated controls. Activity of OAT expressed as **(A)** units (U) g^{-1} FW, U= μ mole P5C formed min^{-1}; **(B)** U mg^{-1} protein (specific activity). Each bar represents mean of U of enzyme activity (±) S.E of 6 independent replicates of each treatment. "*" represent significant difference ($p \leq 0.05$) of all treatments from the untreated control. "!" represent significant difference ($p \leq 0.05$) between water stressed and water stressed + GAP-P45 treated seedlings.

The catabolic enzymes

PDH: On day 2, the activity of proline dehydrogenase was similar in all treatment, except water-stressed + GAP-P45 in which there was a significant increase in proline dehydrogenase activity on day 2, as opposed to the other three treatments (Fig. 3.9 A). On day 4 and day 7, the activity of proline dehydrogenase remained constant in the non-stressed plants, with or without GAP-P45 treatment. However, a marked reduction was seen in the activity of this enzyme in the water-stressed plants without GAP-P45 treatment, when compared to all other treatments. Proline dehydrogenase activity, however, kept on steadily increasing in the water-stressed, GAP-P45 treated plants from day 2 to day 7. Trends in specific activity (U mg^{-1} protein) of PDH were almost identical to its activity (U mg^{-1} FW) on all days in all treatments (Fig. 3.9 B).

P5CDH: Trends in P5CDH activity and specific activity were almost identical to that of proline dehydrogenase on all days and in all treatments (Fig. 3.10 A). Trends in specific activity (U mg^{-1} protein) of P5CDH were almost identical to its activity (U mg^{-1} FW) on all days in all treatments (Fig. 3.10 B).

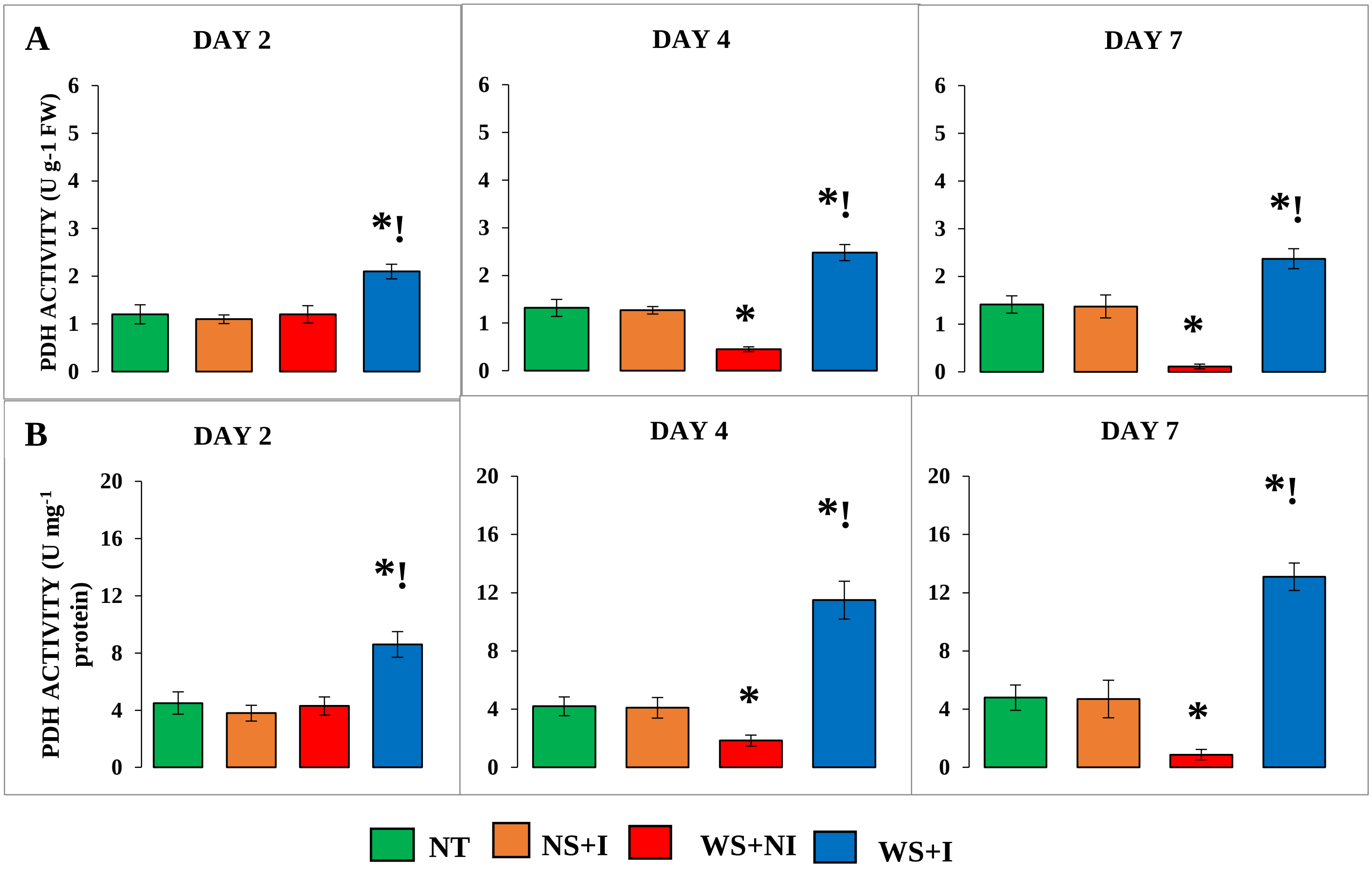


Fig 3.9 Activity of proline catabolic enzyme proline dehydrogenase (PDH) in *Arabidopsis thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 treatment under normal and water stress induced conditions compared to untreated controls. Activity of PDH expressed as **(A)** U g^{-1} FW, U= μ mole NADPH formed min^{-1}; **(B)** U mg^{-1} protein (specific activity). Each bar represents mean (±) S.E of 6 independent replicates of each treatment. "*" represent significant difference ($p \leq 0.05$) of all treatments from the untreated control. "!" represent significant difference ($p \leq 0.05$) between water stressed and water stressed + GAP-P45 treated seedlings.

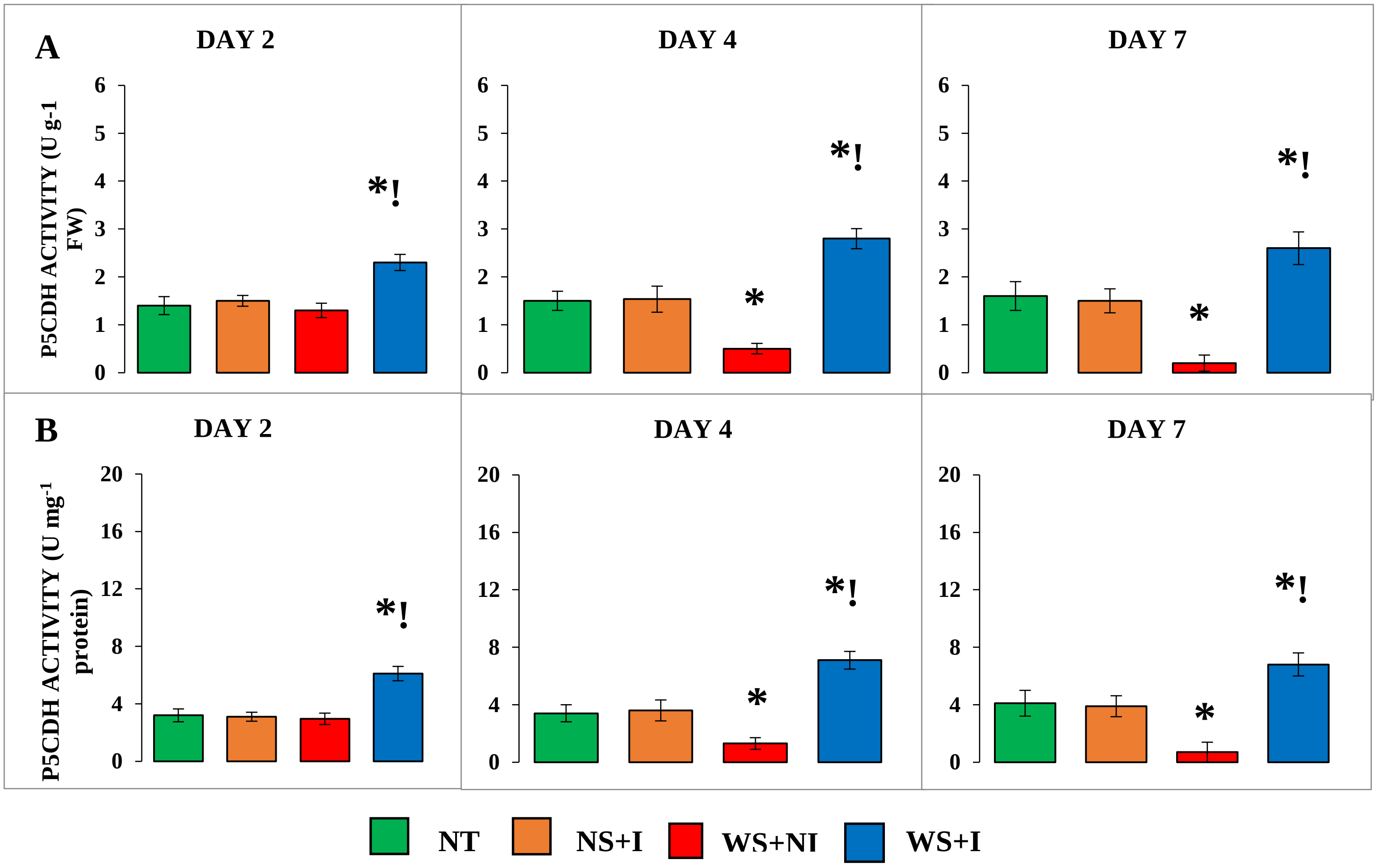


Fig 3.10 Activity of proline catabolic enzyme Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH) in *Arabidopsis thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 treatment under normal and water stress induced conditions compared to untreated controls. Activity of P5CDH expressed as **(A)** U g^{-1} FW, U= µ mole NADPH formed min^{-1}; **(B)** U mg^{-1} protein (specific activity); Each bar represents mean (±) S.E of 6 independent replicates of each treatment. "*" represent significant difference ($p \leq 0.05$) of all treatments from the untreated control. "!" represent significant difference ($p \leq 0.05$) between water stressed and water stressed + GAP-P45 treated seedlings.

3.4 DISCUSSION

As previously mentioned, we wanted to study the PGPR mediated modulation in the expression of genes and activity of the respective enzymes involved in proline metabolic pathway (*OAT*, *P5CS1*, *P5CR*, *PDH1* and *P5CDH*) in *A. thaliana*, under water-stress and correlate them with stress-induced proline accumulation. Our objective was to observe the regulation pattern of these genes under PEG-induced water-stress with GAP-P45 inoculation. Though increased proline accumulation with PGPR inoculation under dehydration conditions has already been reported in several studies, the aspects of regulation of both proline biosynthetic and catabolic genes with respect to PGPR has not been explored up to now.

In our study, under water-stress, proline accumulation followed an increasing trend from day 2 to day 7 in both, non-inoculated and inoculated plants (Fig. 2.8). However, data showed that GAP-P45 inoculation delayed proline accumulation under water-stress. This delay was an intriguing phenomenon which deserved investigation. It could be argued that any of such PGPR-mediated delayed response to drought can be a consequence of enhanced hydration of the media by PGPR themselves. However, in our case, we observed no change in the water potential of the media following GAP-P45 inoculation under PEG-treated conditions. Hence, we hypothesized that the delayed proline accumulation is a more specific response caused by the bacterium. This hypothesis needed to be tested through in-depth studies. We also observed that, although, there was enhanced proline accumulation under water-stress in both non-inoculated and inoculated plants, amelioration of the stress effect was better in inoculated plants vs. non-inoculated ones. This observation can be explained by critically examining the gene expression data (Fig. 3.3 and 3.4) which not only correlated well with proline accumulation data (Fig. 2.8), but also provided interesting details into the pattern of regulation in the expression of the proline metabolic genes brought about by GAP-P45 inoculation under osmotic stress conditions. By day 2 post treatment, the PEG-treated, non-inoculated plants exhibited 5 to 15-fold surge in the expression of proline biosynthetic genes (expression of *P5CS1* being the highest) relative to the non-stressed, non-inoculated plants, whereas, PEG-treated, GAP-P45-inoculated plants recorded only 2-4 fold elevation in the expression of these genes. These data correlated well with the proline accumulation at day 2. By day 4 post treatment, the PEG-treated, non-inoculated plants did not show much change in the expression of the biosynthetic genes (*OAT, P5CS1* and *P5CR*), though at day 7, an overall downregulation in the expression of *P5CS1* and *P5CR* was observed in these plants. However, the PEG-treated, inoculated plants exhibited several-fold increase in the expression of the same genes at day 4 and maintained almost the same level of expression at day 7 post treatment, with *P5CS1* (which codes for an important and rate-limiting enzyme of the proline biosynthetic pathway) exhibiting the highest expression level. Hence, by day 7, while proline levels were similarly induced in both, non-inoculated and inoculated plants under water-stress, the gene expression patterns were different in both these treatments. While the PEG-treated non-inoculated plants had undergone a marked downregulation in the expression of proline biosynthetic genes (except *OAT*), the water-stressed, inoculated plants exhibited a sustained upregulation of all three genes. One can argue that downregulation of proline biosynthetic genes in water-stressed, non-inoculated plants at day 7 could suggest accumulated proline -mediated feed-back inhibition of *P5CS1* (Hong et al., 2000; Sharma and Verslues, 2010; Reddy et al., 2015) consequently leading to down-regulation of *P5CR*. However, such a feedback inhibition is not seen in the water-stressed, inoculated plants in spite of similar surge in proline content. This suggested that GAP-P45 inoculation could somehow be suppressing the feedback inhibition caused by proline accumulation. Conversely, other than feedback inhibition, specific osmotic-stress responsive factors might be contributing towards the downregulation of these genes under water-stress. The expression pattern of the two proline catabolic genes (*P5CDH* and *PDH1*) exhibited some similarities with that of the biosynthetic genes. There was an overall, time-dependent downregulation in the expression of *P5CDH* and *PDH1*in the PEG treated non-inoculated plants, whereas in the PEG treated, GAP-P45 inoculated plants, there was an overall induction in the expression of both of these catabolic genes from day 2 to day 7. High amount of proline accumulation in spite of downregulated *P5CS1* and *P5CR* in PEG-treated non-inoculated plants at day 7 could be the combined consequences of upregulated *OAT* and downregulated *PDH1* and *P5CDH*. Hence, we speculated that *OAT* might play an important role in proline biosynthesis in our non-inoculated plants under water-stress. As mentioned previously, there are reports on *OAT* upregulation under salt and osmotic stress in *A. thaliana* and enhanced stress tolerance via *OAT* overexpression (Roosens et al. 1998; Roosens et al. 2002; Wu 2003; Armengaud et al. 2004; Sharma and Verslues 2010). However, mutant analysis studies by Funck et al., (2008) have established that OAT does not take part in proline biosynthesis under drought stress conditions in *A. thaliana*. Although our observations indicate towards the former reports and contradicts the later, specific experiments should be done to elucidate the specific role of upregulated *OAT* in our study.

Sustained induction of the proline catabolic genes, *PDH1* and *P5CDH* at day 4 and day 7 in the water-stressed, inoculated plants suggests that, although, both inoculated and non-inoculated plants accumulate proline under water-stress, there is probably higher proline turnover in the former vs. the later. While, conventionally, abiotic stress amelioration in plants is associated with enhanced proline accumulation, several reports suggest that enhanced proline turnover could be a key player in this phenomenon (Ghosh et al. 2018b). As mentioned in the introduction, this has been established with studies using *p5cs1* and *pdh1* mutants of *A. thaliana* (Sharma et al. 2011 and Bhaskara et al. 2015) as well as studies showing simultaneous upregulation of both proline biosynthetic and catabolic genes under dehydration conditions (Sharma et al., 2011; Bhaskara et al., 2015; Fabro et al., 2004; Kaplan et al., 2007). Our observations on stress alleviation by GAP-P45 treated plants i.e. better morpho-physiological status under water-stress than that of PEG-treated non-inoculated plants, substantiate the hypothesis that increased proline turnover via simultaneous upregulation of both biosynthetic and catabolic genes, is more important for better sustenance of *A. thaliana* under water-stress than mere accumulation of proline. Observations on the activity and specific activity of the biosynthetic and catabolic enzymes coded by these aforementioned genes corroborated well with the gene expression data confirming that GAP-P45 inoculation of *A. thaliana* seedlings under water stress condition modulates proline turnover in plants at both transcriptional and post-transcriptional levels (Fig. 3.6 to 3.10). In the enzyme activity study, we observed that:

(a) GAP-P45 inoculation under non-stressed conditions did not cause any change in the activities of proline metabolic enzymes.

(b) The activities of proline biosynthetic enzymes (P5CS, P5CR and OAT) were upregulated under water-stress as opposed to non-stressed plants.

(c) GAP-P45 inoculation under water-stress caused further upregulation of their activities.

(d) The proline catabolic enzymes (PDH and P5CDH) on the other hand recorded a decrease in activities under water-stressed non-inoculated conditions.

(e) GAP-P45 inoculation, under water-stress, however, increased the activities of these enzymes to levels higher than all other treatments.

(f) As with proline biosynthetic enzymes, GAP-P45 inoculation under non-stressed conditions did not cause any change in the activities of the proline degrading enzymes either.

3.5 CONCLUSION

1. GAP-P45 inoculation initially delayed proline accumulation in *A. thaliana* and enhanced the expression of both proline biosynthetic as well as catabolic genes under water-stress in a partially time-dependent manner, possibly leading to enhanced proline turnover. Kinetics of proline biosynthesis and degradation (i.e. its conversion to glutamate and/or ornithine) will have to be studied in order to gain greater insight into this process.
2. As time of exposure to dehydration increased, all drought treated plants, i.e., non-inoculated and GAP-P45-inoculated, accumulated enhanced proline, but in the inoculated plants, proline catabolism possibly kept pace with its biosynthesis, leading to enhanced turnover of proline. This indicates that the particular strain of PGPR used in this study, stimulates not only enhanced proline accumulation, but also its concomitant degradation, thus modulating proline homeostasis under drought conditions.
3. The enhanced proline degradation could be a cause or an effect of better drought tolerance in the inoculated plants.

CHAPTER 4

Dynamics of endogenous hormone accumulation in *Arabidopsis thaliana* by phytohormone secreting rhizobacterium *Pseudomonas putida* GAP-P45 under water-stress

4.1 INTRODUCTION

One area of plant-PGPR interaction that has invited a lot of attention is the capability of PGPR to produce major phytohormones such as auxins (mainly indole-3-acetic acid; IAA), gibberellins (GAs), cytokinins (CKs) and abscisic acid (ABA) (Saharan and Nehra 2011; Vacheron et al. 2013; Vejan et al. 2016; Gouda et al. 2018; Numan et al. 2018). Since phytohormones are plant growth regulators and are involved in all phenomena related to plant growth, development and stress tolerance, hence, the obvious hypothesis is that the uptake and utilization by plants of PGPR-secreted phytohormones is a key player in such beneficial interactions.

There are several reports on alleviation of abiotic stresses in plants by PGPR that produce phytohormones. It is reported that these bacteria modulate hormonal balance in host plants which confers tolerance to abiotic stresses (reviewed by Fahad et al. 2015b; Figueiredo et al. 2016). Marulanda et al., (2009) reported an increase in the biomass as well as the IAA concentration in clover when the seeds were treated with the PGPR strains like *Pseudomonas putida* and *Bacillus megaterium*. A beneficial species of fungi, *Trichoderma virens* inoculated with *A. thaliana*, changed root architecture by modulation in the IAA concentration (Contreras-Cornejo et al. 2009). Similar changes in the root architecture system due to the production of IAA by PGPR was found in the studies of Mantelin and Touraine (2003), helping the plants in rapid uptake of water from the soil under drought conditions. Pereyra et al., (2012) have observed that, on inoculation of wheat seedlings with *Azospirillum* under osmotic stress, there were some morphological changes in the xylem architecture which was due to the upregulation of indole-3-pyruvate decarboxylase gene and increased IAA production. Similarly, drought mitigation in wheat due to enhanced root growth and lateral root formation by *Azospirillum* was reported by

Arzanesh *et al.* (2011). Inoculation of GA secreting PGPR, *P. putida* H-2-3 enhanced the growth characteristics of soyabean plants under drought condition (Kang et al. 2014b). Augmented growth of cucumber plants was reported both under salinity and drought conditions when inoculated with *Burkholdera cepacia SE4*, *Promicromonospora spp. SE188 and Acinetobacter calcoaceticus SE370* which increased the internal levels of GA in the plants (Kang et al. 2014a). Mitigation of drought stress in maize plants were observed by Cohen *et al.* (2009), when ABA and GA secreting *Azospirillum* spp. was inoculated with the plants. Leaf transpiration in *Arabidopsis* was reported to reduce due to increased ABA levels under osmotic stress upon inoculation of *Phyllobacterium* (Bresson et al. 2013). Enhanced shoot and root growth in *A. thaliana* through modified root architecture and endogenous hormonal signaling was reported when inoculated with *Phyllobacterium brassicacearum* STM196 (Contesto et al. 2010; Galland et al. 2012; Bresson et al. 2013). Significant increase in shoot and root biomass and chlorophyll content in tomato plants were obtained by *Bacillus subtilis* LK14 inoculation which was characterised as an IAA producing PGPR (Khan et al. 2016). Selvakumar *et al.*, 2018 reported that inoculation with cytokinin-producing bacteria *Citricoccus zhacaiensis* and *Bacillus amyloliquefaciens* increased photosynthesis, transpiration, relative water content and yield in tomato plants under water stress.

Although several studies have reported the beneficial effects of phytohormone secreting PGPR on overall health, physiological status and endogenous level of few hormones in plants under water-stress, there is a lack of information on how a certain strain of PGPR can help plants ameliorate water-stress by modulating the accumulation and localization pattern of all four major endogenous hormones- ABA, IAA, trans-zeatin (tZ) and GA. Therefore, the major objective of this study was to investigate the impact of a phytohormone producing PGPR on the regulation of accumulation and re-distribution of the aforementioned four major phytohormones in *A. thaliana* under water-stress.

As reported earlier, the PGPR strain used in this study, *P. putida* GAP-P45, has been found to secrete the phytohormones such as, IAA, GA, cytokinins, under drought (Sandhya et al. 2009, 2010b). Hence, we wanted to study if GAP-P45 induces any time-dependent fluctuations in the concentrations of the four major phytohormones (ABA, IAA, tZ and GA) in *A. thaliana* under water-stress. Before looking into the endogenous dynamics of phytohormone synthesis and redistribution in the plants, we investigated if GAP-P45 secreted similar amounts and varieties of phytohormones when growing at 22 ºC in MS-agar media (since this is the type of media and conditions used for the interaction studies) as secreted in LB liquid media at 28 ºC. We have estimated endogenous phytohormone concentrations at three different levels- the growth media (MS-agar with or without PEG), roots and shoots of *A. thaliana* on inoculation with GAP-P45 under water-stress. As far as we know, this is the first report on such a comprehensive analysis of time dependent modulation of phytohormones in plants by a PGPR under water-stress (Ghosh et al. 2018a).

4.2 MATERIALS AND METHODS

4.2.1 Plant growth, maintenance, drought induction and PGPR inoculation:

The procedures followed for plant growth and maintenance and further treatments are described previously in chapter 2.

4.2.2 Bacterial growth and extraction of phytohormones secreted by *Pseudomonas putida* GAP-P45:

Phytohormones secreted by *P. putida* GAP-P45 were detected and quantified both in LB broth and in MS-agar media (inoculated with *A. thaliana*). In the former case, 50 mL of LB broth was used as control and another 50 mL was supplemented with 25% PEG to mimic water-stress. Each of these broth cultures were sub-cultured from a primary, overnight culture of GAP-P45 (O.D. $_{600}$ 0.8) to get a final O.D.$_{600}$ of 0.1. Then the cultures were incubated at 28 $^{\circ}$C at 150 ×g for 72 h. Extraction of secreted phytohormones was carried out with slight modification of the protocols described by Yasmin et al. (2017), Iqbal & Hasnain (2013) and Lee et al. (2012). The culture was centrifuged at 7197 ×g for 30 minutes at 4 $^{\circ}$C to remove bacterial cells. Supernatant was collected and acidified to pH 2.5 with concentrated HCl before phytohormone extraction with equal volume of ethyl acetate. Chloramphenicol (20 ng/mL working concentration) was used as internal standard (Xiong *et al.*, 2014) owing to the high costs and procurement difficulty involved of more conventional, heavy-isotope based internal standards. After an incubation at 10 ºC for 48 h, followed by centrifugation at 7197 ×g, the organic layer was seperated and evaporated using a vaccum rota-evaporator (Equitron Medica). The residue was dissolved in 500 µL of 80 % HPLC-grade methanol. For extraction of secreted phytohormones from MS-agar media), 5 mL of 80 % methanol was added to the residual media (both inoculated and non-inoculated) after harvesting of plant samples and the Magenta boxes were kept in a shaking incubator at 150 ×g for 24 h at 4 ^{o}C. After incubation, the mixture of methanol and MS-agar media from each Magenta box was centrifuged at 12000 ×g, the supernatant was collected, chloramphenicol was added, filtered with 22 µm syringe-filter and subjected to vacuum concentrator at 4 ^{o}C till the tubes were dry. Finally, the residues were reconstituted in 500 µL of 80 % methanol and subjected to further analysis.

4.2.3 Extraction of phytohormones from plant tissues:

At 2, 4 and 7 days post water-stress induction and GAP-P45 inoculation, 500 mg of shoot and root samples were collected separately and frozen immediately in liquid nitrogen. Roots of the inoculated plants were thoroughly rinsed several times with 50% ethanol and autoclaved distilled water to remove attached bacterial cells before freezing in liquid nitrogen. Extraction of phytohormones was carried out following the protocol by Almeida Trapp *et al.* (2014), and Saini *et al.* (2017). The frozen samples were ground to powder in liquid nitrogen and 1 mL of 80 % methanol was added to each sample along with chloramphenicol and incubated overnight in a shaking incubator at 150 ×g at 4 ^{o}C. Next day, the samples were centrifuged at 16000 ×g at 4 ^{o}C for 15 min and the supernatant was dried using a vacuum concentrator. Finally, the residues were reconstituted in 500 µL of 80 % methanol and subjected to further analysis.

4.2.4 Detection and quantification of phytohormones:

Chromatographic separation and quantification of the four major phytohormones in both plant and bacterial samples were performed by reversed-phase HPLC (Shimadzu) on a 4.6 × 150 mm C18 column (Optisil) with 5 µm pore size, following the method given by Górka and Wieczorek, (2017) with some minor modifications. The column was first washed with Millipore water and 80 % methanol and equilibrated with isocratic flow of acetonitrile and formic acid solution (pH-4.0) in a 27:77 (v/v) ratio, at a flow rate of 2.0 mL/min. and 25 ^{o}C column oven temperature (Górka and Wieczorek 2017). The same solvent system described above, was used as mobile phase for separation of phytohormones. Flow rate during separation was maintained at 1.5 mL/min., total run time for each sample was 17 min. and volume of sample injected was 10 μL. Detection was done using a photodiode array detector at a wavelength of 254 nm. Quantification was done by extrapolating the concentration of each phytohormone from its standard curve (area under the curve vs. concentration) prepared using commercially available phytohormones with a range of concentrations. Quantity of secreted phytohormones by GAP-P45 was expressed in LB broth as both ng/ mL culture media and µg/ g FW of the bacterial pellet after 72 h and in MS-agar media as ng/ mL media at day 2, day 4 and day 7. Quantity of the endogenous phytohormones in both roots and shoots of the seedlings were expressed as ng/g FW of plant tissue. In order to confirm the identity of phytohormones, an additional check was done by subjecting the samples to a mass spectrometer (LC/MS, Shimadzu) equipped with an ESI source. The samples were injected through direct syringe pump at a flow rate of 1.5 mL/min. Samples were scanned at both positive and negative Q3 scan modes (mass scan range m/z 10-400) with source voltage of 4.8 kV and scan speed of 7500 u/second. The capillary temperature and sheath flow rate (N_2) were 250^{0} C and 3 L/min for both scan modes.

4.2.5 Statistical analysis:

Statistical analysis was performed by student’s t-test (level of significance, $p \leq 0.05$) using Microsoft Excel (2016). As mentioned previously, each experiment was performed with at least three replicate Magenta boxes, each containing about 20 seedlings, distributed in 4 steel meshes and each experiment was performed at least twice.

4.3 RESULTS

4.3.1 Phytohormones secreted by *Pseudomonas putida* GAP-P45:

As mentioned in materials and methods, quantity of secreted phytohormones by GAP-P45 was analyzed under two separate growth conditions (1) from bacteria growing in LB broth (the conventional growth medium for most bacteria) and (2) from bacteria growing in MS-agar medium (in the presence of plants), with or without PEG treatment. As reported earlier (Sandhya et al. 2009, 2010b), in broth culture at ambient temperature of 28 °C, GAP-P45 secreted auxin (IAA), cytokinin (trans-zeatin; tZ) and gibberellic acid (GA) under both non-stressed and water-stress induced conditions, though, significant reductions in quantities of secreted phytohormones was observed under water-stress. We wanted to test whether we observe the same trends or not under similar growth conditions since our methods of phytohormone extraction, detection and quantification were different from the methods used by the aforementioned authors. Therefore, we performed the analysis in LB medium without the intervention of plants. Our observations on the trends of secreted phytohormones by this strain followed the same pattern as reported by Sandhya et al., (2010, 2009). Under non-stressed condition GAP-P45 produced higher amount of IAA, cytokinin and GA as compared to water-stress and production of GA was found to be highest among all the phytohormones under both stressed and non-stressed conditions (Fig. 4.1).

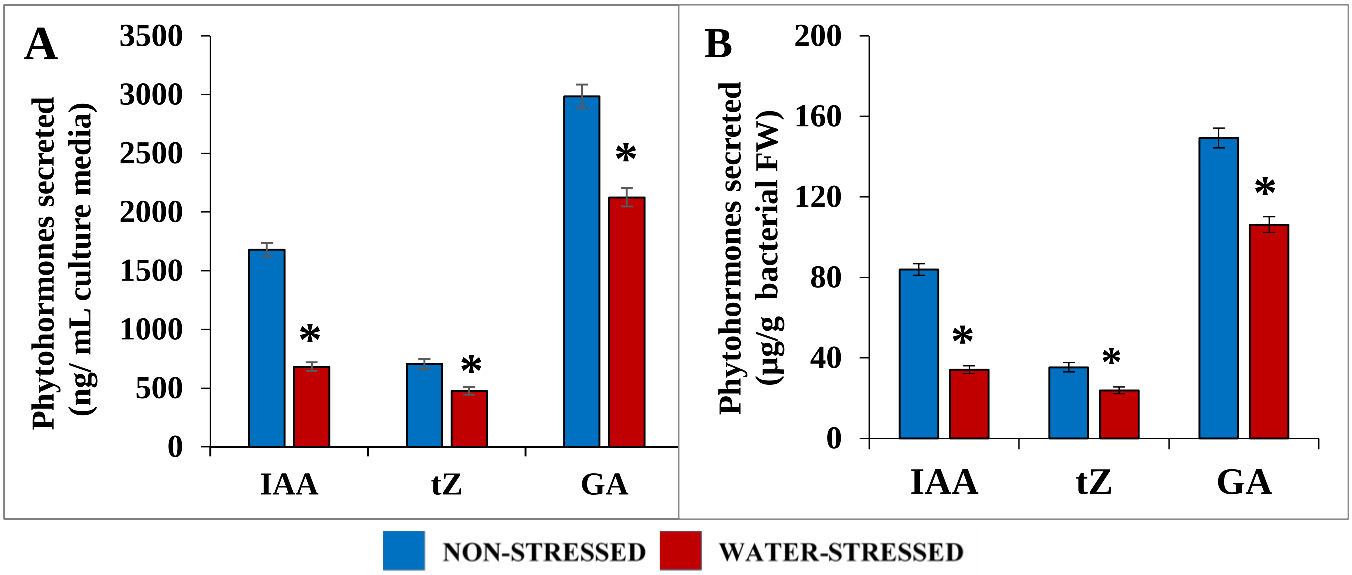


Fig. 4.1 Quantification of phytohormones secreted by *Pseudomonas putida* GAP-P45 in LB broth medium at 28 ºC after 72h of incubation. **A)** on the basis of ng/ mL culture media **B)** on the basis of µg/ g fresh weight of bacterial pellet. Each bar represents mean (±) S.E of 6 independent replicates of each treatment. "*" represent significant difference (p≤0.05) in quantity of phytohormones between non-stressed and water-stressed treatments.

However, a true picture of the amount of phytohormones that is actually accessible to the plants can be obtained only when the media containing plants inoculated with the bacteria are analyzed. Therefore, we also measured the quantities of secreted phytohormones by this bacterium in MS-agar medium under inoculated conditions with *A. thaliana* at 2, 4 and 7 days post treatment with or without water stress. As can be seen, through RP-HPLC, distinct and discernible peaks could be observed only for IAA and tZ (Fig. 4.2). No distinct and quantifiable peak was detected in RP-HPLC for GA in MS-agar medium at any time point of analysis. As with broth cultures, in PEG-supplemented MS-agar also, significant decrease in quantity of secreted phytohormones was observed on 2, 4 and 7 days post treatment as opposed to PEG-free, inoculated MS-agar media (Fig. 4.2). As can be seen, per mL of culture media, almost 50-100 fold higher quantities of phytohormones were observed in LB media vs. MS-agar media. Also, IAA concentrations were higher than tZ concentrations in the LB media while the opposite trends were seen in the MS-agar media. No phytohormones were detected in the media containing non-inoculated controls (NT) and water-stressed non-inoculated (WS+NI) plants, indicating that none of the phytohormones detected in the media were secreted by the plant roots.

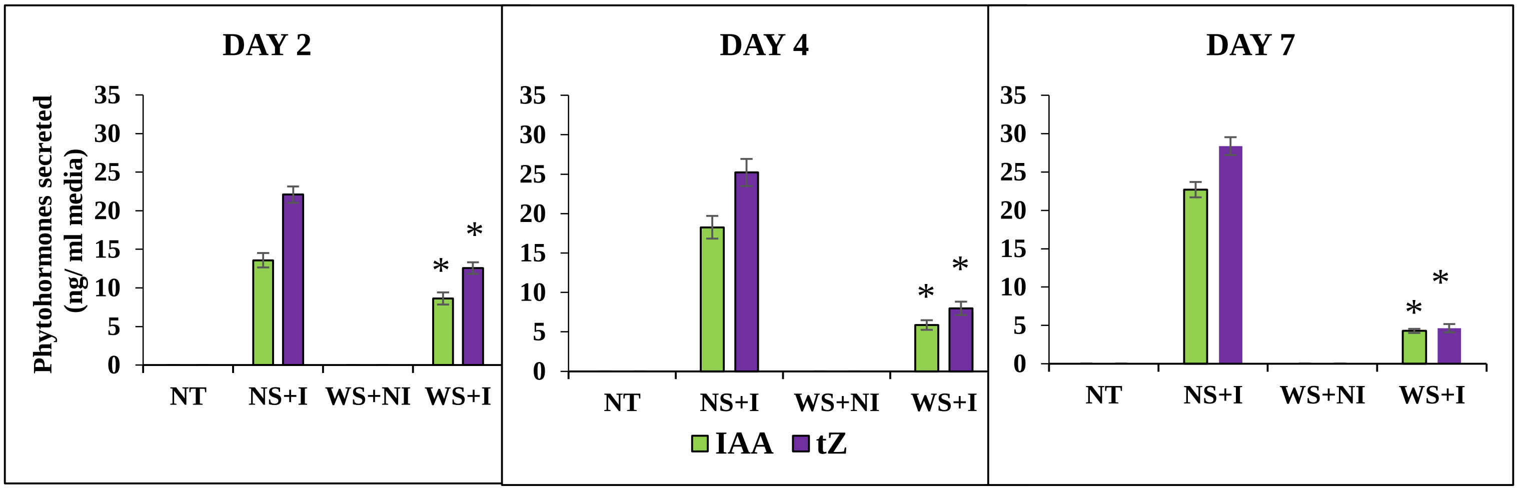


Fig. 4.2 Quantification of phytohormones secreted by *Pseudomonas putida* GAP-P45 in MS-agar medium at 22 ºC after 2, 4 and 7 days of incubation. Each bar represents mean (±) S.E of 6 independent replicates of each treatment. "*" represent significant difference ($p \leq 0.05$) in quantity of each phytohormones between non-stressed and water-stressed treatments. NT: no-treatment control, NS+I: non-stressed inoculated, WS+NI: water-stressed non-inoculated, WS+I: water-stressed inoculated.

4.3.2 Impact of *Pseudomonas putida* GAP-P45 on the endogenous levels of phytohormones in shoots and roots of *Arabidopsis thaliana* under normal and water-stressed conditions:

Abscisic acid (ABA): As evident from Fig. 4.3 A, under water-stress, endogenous levels of ABA in shoot increased drastically in both inoculated (WS+I) and non-inoculated (WS+NI) plants throughout 2, 4 and 7 days post treatments, when compared to non-stressed plants, both non-inoculated (NT) and inoculated (NS+I). However, the accumulation of ABA in water-stressed, inoculated plants was much lower (~2.5-fold lower) than water-stressed, non-inoculated plants. Inoculation of plants with GAP-P45 under non-stressed conditions had no effect on endogenous levels of ABA when compared to the no-treatment controls. Similar pattern of ABA accumulation was observed in root samples of the same plants in all four treatments and on all days of analyses (fig. 4.3 B). Level of accumulated ABA was highest in non-inoculated, water-stressed roots, while GAP-P45 inoculation under water-stress caused a decrease in the ABA levels. It is also interesting to note that, a time-dependent, gradual increase in ABA levels was seen, both in the shoots as well as roots, in water-stressed samples, whether inoculated or not. No such time-dependent fluctuations were seen in the non-stressed plants, with or without GAP-P45 inoculation. For any given treatment and at any given time point, higher ABA levels were recorded in the shoots than in the roots.

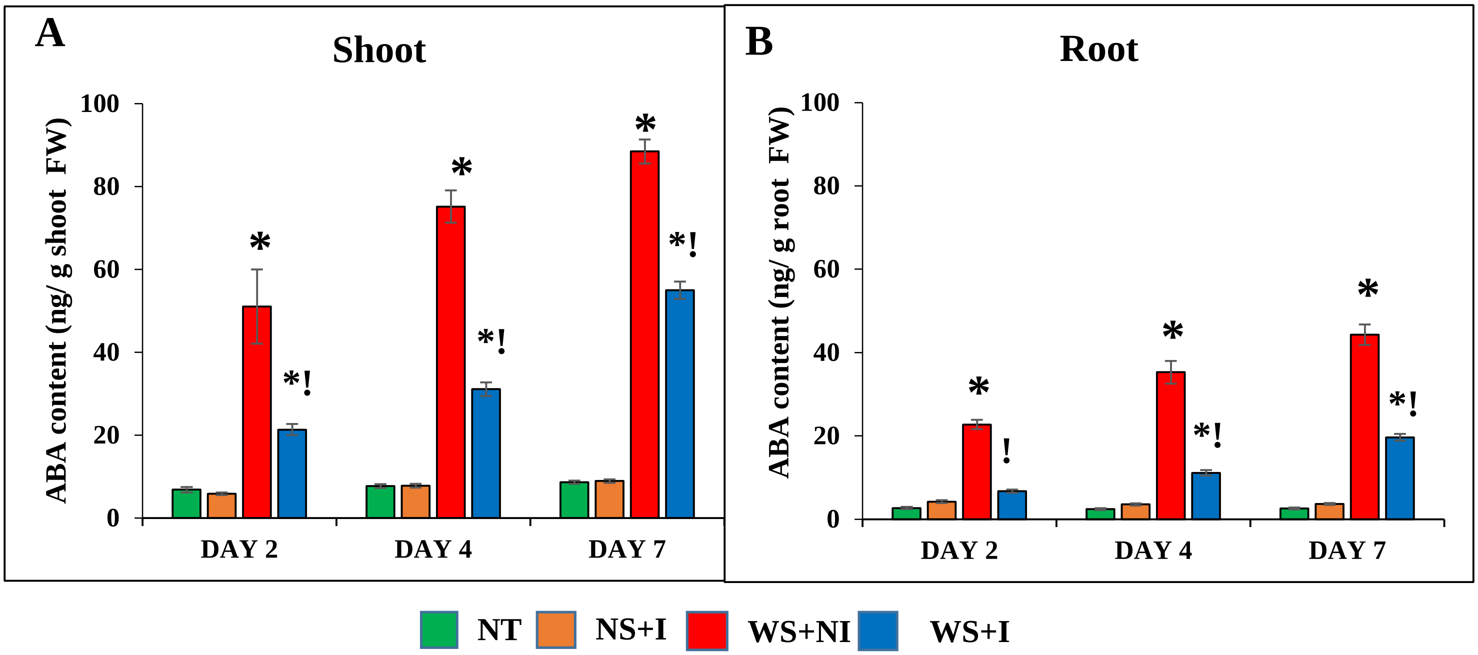


Fig. 4.3 Effect of *Pseudomonas putida* GAP-P45 inoculation on endogenous levels of abscisic acid (ABA) in *Arabidopsis thaliana* **A)** shoots and **B)** roots after 2, 4 and 7 days of treatment. Each bar represents mean (±) S.E of 6 independent replicates of each treatment. "*" represent significant difference ($p \leq 0.05$) of all treatments from the no-treatment control. "!" represent significant difference ($p \leq 0.05$) between water-stressed non-inoculated and water-stressed + GAP-P45 inoculated samples.

Auxin (IAA): Much like ABA, overall, the endogenous levels of IAA were higher in the shoots than in the roots on all days of analysis. In the non-stressed plants, GAP-P45 inoculation (NS+I) did not cause much change in endogenous IAA levels in the shoots but did cause an increase in the same at day 4 and day 7 in the roots (Fig. 4.4 A, B). Water-stress without inoculation (WS+NI) caused a sharp, significant drop in the levels of IAA, both in the shoots as well as in roots, with GAP-P45 (WS+I) inoculation elevating the IAA levels on all days of analysis. On some cases, (day 2 in shoots, day 2 and day 4 in roots), this elevation brought up the IAA levels to almost as much as those in the non-treated control samples. Unlike ABA, where a time-dependent increase was seen, in case of IAA, gradual, time-dependent reduction in endogenous levels were observed under water-stress, in both inoculated as well as non-inoculated plants, in shoot as well as in root samples. This decrease was much more pronounced in the water-stressed, non-inoculated plants vs. water-stressed, inoculated plants. However, it is interesting to note that there was an overall increase in the levels of IAA in non-stressed inoculated plants from day 2 to day 7 in both roots and shoots.

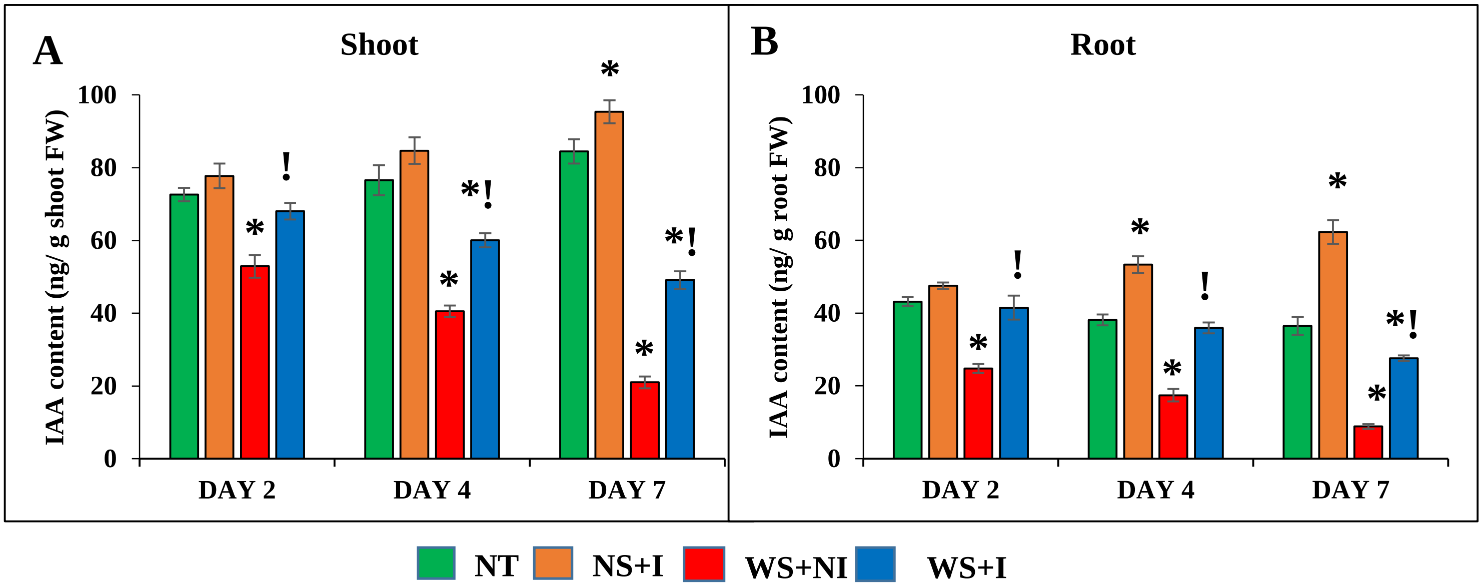


Fig. 4.4 Effect of *Pseudomonas putida* GAP-P45 inoculation on endogenous levels of auxin (IAA) in *Arabidopsis thaliana* **A)** shoots and **B)** roots after 2, 4 and 7 days of treatment. Each bar represents mean (±) S.E of 6 independent replicates of each treatment. "*" represent significant difference (p≤0.05) of all treatments from the no-treatment control. "!" represent significant difference (p≤0.05) between water-stressed non-inoculated and water-stressed + GAP-P45 inoculated samples.

Trans-zeatin (tZ): Endogenous content of tZ in roots were somewhat higher than in shoots in case of all the four treatment conditions for a given day of analysis. Inoculation of *A. thaliana* with GAP-P45 under non-stressed condition (NS+I) significantly increased endogenous tZ content in shoots at all three time points of the study. However, GAP-P45 inoculation had no effect on root endogenous tZ levels under the same conditions (Fig. 4.5 A and B). Similar to IAA, water-stress without inoculation (WS+NI) resulted in a decrease in endogenous tZ levels in both shoots and roots, whereas, inoculation with GAP-P45 under water-stress (WS+I) increased tZ levels in shoots and roots at all time points of analysis. While time-dependent gradual decrease in tZ levels from day 2 to day 7 was observed in shoots and roots of both inoculated and non-inoculated plants under water stress, a gradual increase in tZ content was observed in non-stressed inoculated shoots.

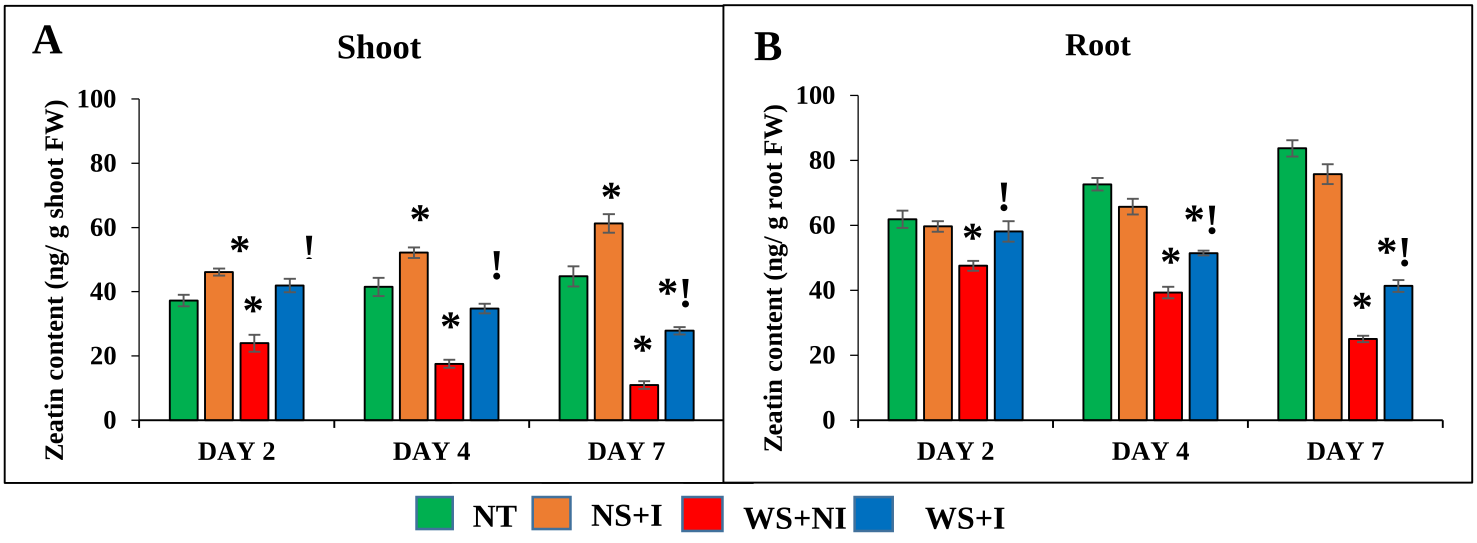


Fig. 4.5 Effect of *Pseudomonas putida* GAP-P45 inoculation on endogenous levels of cytokinin (zeatin) in *Arabidopsis thaliana* **A)** shoots and **B)** roots after 2, 4 and 7 days of treatment. Each bar represents mean (±) S.E of 6 independent replicates of each treatment. "*" represent

significant difference ($p \leq 0.05$) of all treatments from the no-treatment control. "!" represent significant difference ($p \leq 0.05$) between water-stressed non-inoculated and water-stressed + GAP-P45 inoculated samples.

Gibberellic acid (GA): Significant reduction in endogenous GA content was observed in shoots in water-stressed, non-inoculated (WS+NI) *A. thaliana* seedlings across all days of analysis as compared to the other three treatments (Fig. 4.6 A). However, inoculation of GAP-P45 to the water-stressed plants (WS+I) significantly elevated endogenous GA levels in shoots. On the contrary, endogenous GA content in roots of non-inoculated plants under water-stress (WS+NI) were observed to be much higher than that in the other treatments throughout the study. GAP-45 inoculation under water stress (WS+I) caused a decrease in the levels of root GA. While a time-dependent decrease in accumulated GA levels was observed in case of the shoots (Fig. 4.6 A), a time-dependent increase was seen in the roots of the treated plants under water-stress without inoculation (Fig. 4.6 B). Inoculation with GAP-P45 under non-stressed conditions (NS+I) did not show any alteration in shoot GA content of *A. thaliana*, however, at day 4 and day 7, a significant drop in endogenous GA levels were observed in roots of the treated plants.

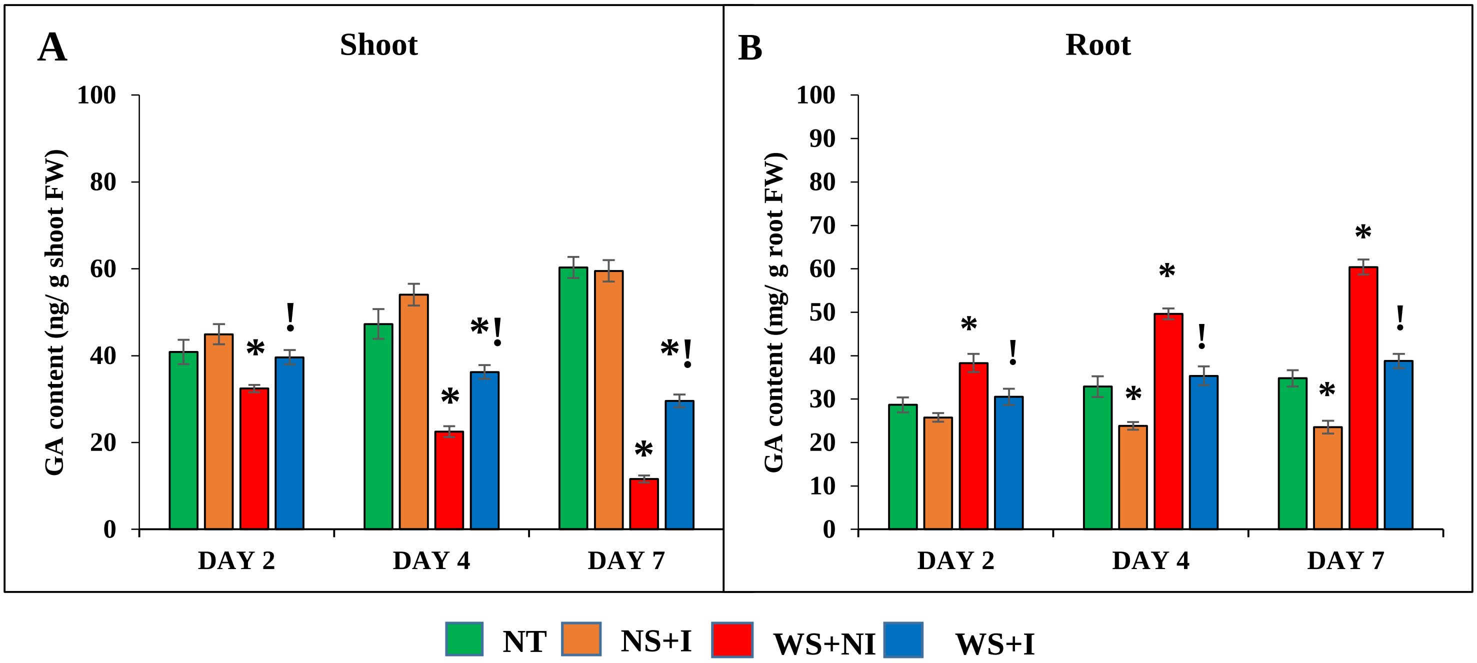


Fig. 4.6 Effect of *Pseudomonas putida* GAP-P45 inoculation on endogenous levels of gibberellic acid (GA) in *Arabidopsis thaliana* **A)** shoots and **B)** roots after 2, 4 and 7 days of treatment. Each bar represents mean (±) S.E of 6 independent replicates of each treatment. “*” represent significant difference ($p \leq 0.05$) of all treatments from the no-treatment control. “!” represent significant difference ($p \leq 0.05$) between water-stressed non-inoculated and water-stressed + GAP-P45 inoculated samples.

4.4 DISCUSSION

As mentioned before, it is hypothesized that an important mechanism by which PGPR improve sustainability of plants under water-stress is by secretion of phytohormones in the vicinity of roots and causing alteration in endogenous hormonal status in plants (Dodd et al. 2010; Barnawal et al. 2017). However, whether these modulations occur due to uptake of bacterial phytohormones by plants or due to alteration in plant's endogenous hormone metabolism induced by bacteria, or a combination of both, still remains unclear. Additionally, comprehensive insights into the modulation of endogenous plant hormones by PGPR are missing. In our previous studies, we have reported that inoculation with *P. putida* GAP-P45 ameliorated the adverse effects of water-stress in *A. thaliana* by increasing fresh weight, dry weight, plant water content, chlorophyll content and proline turnover (Ghosh et al. 2017) and by modulating plant polyamine metabolism (Sen et al. 2018). Since any observed physiological status of plants either under stress or normal conditions is directly correlated to the accumulation, distribution and cross-talk between major phytohormones, we were interested to study the modulation of the content of four major hormones in *A. thaliana*, separately, in the roots and shoots, mediated by *P. putida* GAP-P45 inoculation under water-stress. We attempted to correlate our observations from this study with the phenotypic and physiological changes reported by us previously in *A. thaliana* with *P. putida* GAP-P45 inoculation under water-stress (Ghosh et al., 2017).

4.4.1 Effect of nutrient media, growth conditions and inoculation with *Arabidopsis thaliana* on the pattern of phytohormone production by *Pseudomonas putida* GAP-P45:

The strain *P. putida* GAP-P45 used in our study, which was characterized as a producer of IAA, cytokinin and GA under both non-stressed and stressed conditions in LB broth culture at 28 ºC for 72 h (Fig. 4.1 and Sandhya et al. 2010), secreted only IAA and cytokinin in MS-agar media when inoculated with *A. thaliana* plants at 22 ºC (Fig. 4.2). Additionally, while auxin (IAA) content was higher than the cytokinin (tZ) content in the LB broth, this trend was mostly reversed in the MS-agar medium (Fig. 4.2). These observed differences in phytohormone secretion by the same bacterial strain could be attributed to different growth conditions and media composition between the LB broth cultures and the MS-agar containing plant medium. In fact, the nature and amount of phytohormones secreted by the bacteria could well be influenced by the plants in the vicinity of the bacterium. Hence, a PGPR may not behave the same way upon inoculation with plants under stressed environment, as it does in ambient culture conditions, specific for bacterial growth. Therefore, the fact that the bacterium is secreting certain types of phytohormones in certain quantities in broth culture does not guarantee secretion of same phytohormones at similar concentrations when inoculated with plants in plant growth media or soil. However, in most studies involving PGPR characterization, an extrapolation of the accessibility of PGPR-secreted phytohormones to plants is made based on observed phytohormone secretion under broth conditions. Here we show that such an extrapolation may be erroneous. In this study, we carried out identification and quantification of phytohormones secreted by GAP-P45 in MS-agar medium in the vicinity of *A. thaliana* roots, which depicts the more realistic scenario of plant-microbial interaction. However, decrease in secreted phytohormone quantity under water-stress was similar in both the culture conditions. Following the measurement of phytohormone levels in media, we proceeded to measure the endogenous phytohormone content in the plants at two different levels- the roots and shoots. The four phytohormones chosen for this study were ABA, IAA, tZ and GA because of their involvement in major physiological and developmental processes in plants.

4.4.2 Modulation of endogenous levels of major hormones in *Arabidopsis thaliana* by *Pseudomonas putida* GAP-P45 under water-stress:

Responses of plants to drought stress conditions and the array of metabolic and physiological changes orchestrated for abating the exerted stress are predominantly regulated by phytohormones via complex, interconnected networks of signaling cascades (Gupta et al. 2017; Rasool et al. 2018). Perception of water-stress triggers signal transductions that affect ongoing baseline pathways involved in normal growth and development of plants and modulate biosynthesis or catabolism of endogenous hormones. In this study, we report the modulation of endogenous concentrations of the four major hormones in *A. thaliana* under water-stress due to phytohormone-secreting *P. putida* GAP-P45 inoculation. As mentioned before, we have previously reported that this strain imparted better water-stress tolerance to the inoculated plants vs. non-inoculated ones as evident from improved plant morphology and physiology (Ghosh et al. 2017). As can be seen from Fig. 4.3-4.6, the trends of phytohormone accumulation in water-stressed non-inoculated plants i.e. increase in ABA content in shoot and root, decrease in IAA and tZ content in shoot and root, decrease in GA content of shoots and increase in GA content of roots, were reversed by GAP-P45 inoculation, bringing the hormonal levels closer to the non-stressed, non-inoculated controls. These observations indicate two possible broad mechanisms by which GAP-P45 could be imparting water-stress tolerance to the *A. thaliana*- (1) increasing the water-potential of the media through enhanced water retention, thus reducing the degree of stress on plants, or (2) initiating complex signaling cascades in plants by secreting messenger molecules that alter endogenous hormone metabolism to withstand water-stress, reduce stress mediated injury and sustain growth. To investigate these possibilities, we measured the water-potential in both non-inoculated and inoculated water-stressed media. We did not observe any improvement in water-potential of PEG-infused MS-agar media post inoculation with GAP-P45 as compared to non-inoculated ones, which means that, both, inoculated and non-inoculated plants treated with PEG are experiencing identical water-stress from the dehydrated media. This indicates that the observed modulations in endogenous hormonal levels in plants were not a repercussion of simply an alteration (increase) in water potential of the GAP-P45 inoculated media, but precisely regulated by molecular signal transduction cascades initiated by the bacteria, which delayed stress senescence in the inoculated plants. Our observations on fluctuations in phytohormone content also help us better understand some of the underlying mechanisms behind the morpho-physiological observations reported in our previous study (Ghosh et al., 2017).

Dramatic increase in ABA biosynthesis and transport into leaves is one of the various early adaptive responses of plants to water-stress which induces stomatal closure, preventing further water loss. However, this hampers photosynthetic efficiency of plants, inhibits shoot growth and prevents auxin and cytokinin biosynthesis (Dodd et al. 2010; Todaka et al. 2017). As can be seen from Fig. 4.3, under non-stressed conditions, GAP-P45 did not cause any change in the levels of ABA either in roots or shoots of *A. thaliana*. This can be expected since ABA modulation is primarily a stress related phenomenon. Also, as expected, water-stress (without inoculation) dramatically increased ABA levels in shoots and roots. We observed lower concentration of accumulated ABA in shoots and roots of GAP-P45 inoculated water-stressed plants as compared to non-inoculated stressed ones (Fig. 4.3). Generally, low ABA implies that the plant is not experiencing enough water-stress to enhance its ABA levels. As mentioned before, since an increase in water-potential of the medium is ruled out, the lowering of ABA in stressed, inoculated plants suggests an internal mechanism of water-stress amelioration which precedes the signal for enhanced ABA biosynthesis in the roots and subsequent transport to the leaves. We have previously reported that under water-stress, GAP-P45 enhanced biosynthesis of polyamines and turnover of proline metabolism in *A. thaliana*, which was anticipated to be a cause of water-stress amelioration in the inoculated plants (Ghosh et al. 2017; Sen et al. 2018). It is known that proline and (arguably) polyamines are important osmo-regulators that help in preventing dehydration of plant cells under water-limiting conditions (Szabados and Savouré 2010; Hayat et al. 2012; Liu et al. 2015). These mechanisms could be helping the plant cells in preventing water loss so that the stomatal closure signal is not required anymore, resulting in lower production and hence, lower re-distribution of ABA (from roots to shoots). Contrary to its role as shoot growth inhibitor, ABA induces lateral root development under drought stress (Sharp and LeNoble 2002; De Smet et al. 2006; Dodd et al. 2010). In our previous study, we observed highest root length and lateral root branching in water-stressed, non-inoculated seedlings (Ghosh et al. 2017), which could possibly be the effect of high levels of accumulated ABA in the roots. Overall lower ABA levels in roots vs. shoots could indicate greater transportation of ABA from roots to shoots under stressed conditions (Hu et al. 2016). The slightly lower ABA levels in the roots vs. shoots of non-stressed plants could point towards higher rate of basal level of ABA biosynthesis in shoots than in roots for normal development of plants (McAdam et al. 2016). The gradual, time-dependent increase in ABA levels under water-stress in both non-inoculated and inoculated seedlings could be a repercussion of longer duration of the stress.

Among different auxins, IAA is the most well-studied phytohormone involved in overall development and abiotic stress tolerance in plants. While water-stress negatively affects IAA biosynthesis and accumulation in plant tissues, exogenous application or endogenous auxin-overproduction has been reported to confer tolerance to water-stress in many plant species (Du et al. 2013; Kim et al. 2013). This correlates well with our observations of drastic overall decrease in shoot and root IAA content in non-inoculated, water-stressed plants on all days of analysis. It also correlates with the fact that, while there is no change (shoots) or marginal change (roots) in the IAA levels of non-stressed, non-inoculated controls from day 2 to day 7, a gradual decrease in IAA content was seen in both roots and shoots of water-stressed, non-inocualted plants from day 2 to day 7. In other words, with increase in time of exposure to water-stress, the IAA content decreased in both, the roots as well as the shoots. (Fig 4.4). However, the water-stressed induced decrease in IAA content as opposed to the non-stressed controls was reversed in both shoots and roots of plants inoculated with the auxin producing GAP-P45 under water-stress. We also observed that under non-stressed conditions, GAP-P45 inoculation mostly did not modulate the endogenous IAA content in shoots (except for day 7), however, elevated IAA accumulation was observed in the roots at day 4 and day 7. This observation points to a possible mechanism of water-stress amelioration of *A. thaliana* by GAP-P45. Auxins play contrasting roles in normal growth and development of shoot and root in plants. While lower IAA content under water-stress inhibits shoot growth and leaf water status (Ali et al. 2009; Shi et al. 2014), such low IAA levels can induce primary root elongation in *A. thaliana* (Spaepen et al. 2007; Remans et al. 2008; Shi et al. 2014). Hence, observation on IAA levels in this study correlates well with our previously observed phenotypes i.e. similar shoot growth in both non-inoculated and inoculated non-stressed plants but slight decrease in primary root length and lateral root formation in inoculated ones (Ghosh et al. 2017). The overall decrease in shoot and root IAA content in non-inoculated, water-stressed plants from day 2 to day 7 (Fig 4.4) can be correlated with the time-dependent inhibition of shoot growth but induced primary root elongation in *A. thaliana* seedlings observed in our previous study (Ghosh et al. 2017). There are several reports on enhancement of drought tolerance in plants inoculated with IAA-secreting PGPRs and the endogenous levels of IAA in plants have been observed to be correlated with the amount of IAA secreted by the PGPRs (Ali et al. 2009; Dodd et al. 2010). However, there is still no evidence which confirm that the elevated IAA level observed in these plants are due to uptake of IAA produced by the PGPRs. We speculate that higher accumulation of IAA in shoot and root of inoculated plant whether stressed or non-stressed, indicate two possible mechanisms: either IAA secreted by GAP-P45 in the media was taken up by the roots and transported to the shoot or bacterial IAA stimulated certain signaling cascade to upregulate endogenous IAA biosynthesis in *A. thaliana*.

Cytokinins are involved in plant growth, development and abiotic stress tolerance as they stimulate cell division, tissue expansion, cell enlargement and stomatal conductance. Trans-zeatin (tZ) being the most physiologically active of all cytokinins is also the most well-studied (Kang et al. 2012; O'Brien and Benková 2013; Llanes et al. 2014, 2016; Fahad et al. 2015a). Water-stress has been reported to decrease cytokinin concentration and transport from root to shoot and inhibit shoot development (Golan et al. 2016). Likewise, we also observed significant decrease in cytokinin levels in both shoots and roots in non-inoculated water-stressed *Arabidopsis* seedlings (Fig 4.5). This also correlated well with the diminished plant health and physiological status observed in our previous study at all three time points of analysis (Ghosh et al. 2017). Cytokinins play an antagonistic role to that of ABA as they induce opening of stomata and act as negative regulators of drought stress signaling (Tran et al. 2010; Zwack and Rashotte 2015). Intense cross-talk between ABA and cytokinin is one of the key aspects of drought stress perception in plants as upregulated ABA accumulation prevents cytokinin biosynthesis (Ha et al. 2012). Hence, reduced concentration of endogenous cytokinin, while increased levels of ABA in our water-stressed, non-inoculated plants increased ABA/CK ratio, further explaining the stressed phenotypes (Ghosh et al., 2017). As cytokinins have been reported to reduce primary root elongation but induce root meristem differentiation and root hair proliferation, reduction in accumulated tZ content may have contributed to the enhanced primary and secondary root development in water-stressed non-inoculated plants reported in Ghosh et al. (2017). Alleviation of drought stress in plants inoculated with cytokinin-producing PGPRs have been reported in many studies (Arkhipova et al. 2007; Liu et al. 2013; Selvakumar et al. 2018). Inoculation of plants with GAP-P45, a cytokinin producing PGPR used in our study, increased concentration of accumulated tZ in both shoots and roots under water-stress. Similar observation on elevated cytokinin accumulation due to PGPR inoculation was reported by other researchers (Liu et al. 2013). It is interesting to note that, without water-stress, GAP-P45 did cause a small upregulation in the cytokinin content of shoots but not in the roots. This could be attributed to differential redistribution of cytokinin from roots to shoots, since this hormone is made in the roots and transported to the shoots. On analyzing the trends, it looks like this difference is due to lower redistribution of tZ from roots to shoots of the non-stressed, non-inoculated controls. Conversely, since GAP-P45 is secreting tZ (Fig. 4.2), therefore, the observed elevation in endogenous tZ content in shoot and root of GAP-P45 inoculated plants under normal and water-stressed conditions might be the result of uptake of bacteria-secreted tZ by plants. Trends in time-dependent modulation of tZ were identical to that of IAA in all treatments.

One of the objectives of this study was to compare the concentrations of the phytohormones between MS-agar media, roots and shoots to be able to draw some inference about the dynamics of secretory (bacterial) and endogenous (plant) phytohormones in plant-PGPR interaction. As mentioned before, GAP-P45 secretes only IAA and tZ in MS-agar when inoculated to plants (Fig. 4.2). Also, the bacterium secreted higher amounts of tZ vs. IAA on most days of analysis and levels of these enzymes decreased under water-stress vs. normal conditions. When this is compared with the levels of IAA and tZ recorded in the roots (under inoculated conditions only), it can be seen that overall, the levels of tZ are higher in the roots than the levels of IAA in inoculated plants within a time period. However, the trends in the shoots are somewhat reversed. This observation is interesting and it can be speculated with some confidence that higher cytokinin vs auxin in the roots under inoculated conditions could be attributed to the accessibility and subsequent uptake of these hormones by the plant roots from the media. However, since hormone biosynthesis, degradation and transport are regulated by complex mechanisms, further experiments have to be done to confirm this hypothesis. In the shoots, however, the levels of IAA were found to be somewhat higher than the levels of tZ, possibly pointing towards higher transportation of IAA to the shoots than tZ.

Gibberellins play a crucial role in overall plant growth and development by inducing leaf expansion, primary and lateral root elongation, photosynthetic efficiency, sink strength of photosynthates and providing tolerance against abiotic stresses in diverse plant species (Yamaguchi 2008; Iqbal et al. 2011; Fahad et al. 2015a). In our study, GA is the only hormone that exhibited opposite patterns of accumulation in the shoots vs. the roots under water-stress, with or without GAP-P45 inoculation (Fig. 4.6). While water-stress without inoculation caused a dip in GA levels of shoots, the same was upregulated in the roots under water-stressed non-inoculated conditions. Similarly, while GAP-P45 inoculation under water-stress increased this level in shoots, the bacterium decreased the GA content of roots under water-stress, as opposed to water-stressed, non-inoculated plants. Decrease in endogenous GA concentration in plants under drought stress has been reported to restrict plant growth (Colebrook et al. 2014). Hence, the pattern of GA accumulation in water-stressed, non-inoculated plants vs. non-stressed, non-inoculated controls directly correlated with the phenotypic observation of diminished shoot growth but enhanced primary and secondary root growth in *A. thaliana* seedlings reported in Ghosh et al. (2017). This observation indicated towards rapid transport of GA from shoot to root in plants under water-stressed, non-inoculated conditions. Direct correlation between increased primary root length and enhanced accumulation of GA in roots have been reviewed by Dodd et al. 2010. However, increased content of endogenous GA observed in shoots of plants inoculated with GAP-P45 under water-stress resulted in enhanced tolerance to water-stress and better physiological status of these plants observed in Ghosh et al. 2017. Concentration of GA in roots of the plants inoculated with GAP-P45 under water-stress was observed to be similar to that of non-stressed non-inoculated plants resulting in similar primary root length observed in our previous study (Ghosh et al. 2017). Under non-stressed condition, plants inoculated with GAP-P45 did not show any significant improvement in shoot development, evident from similar concentration of GA accumulated in shoot observed in this study. However, reduction in endogenous GA level in roots of non-stressed inoculated plants at day 4 and day 7 inhibited primary root elongation without affecting plant health.

4.5 CONCLUSION

Overall, the outcome of this study can be summarized as follows:

1. Pattern of phytohormone production and secretion by PGPRs may vary depending on available source of nutrition, culture condition and presence of plants.
2. *P*. *putida* GAP-P45 downregulated endogenous ABA levels in *A. thaliana* under water-stress without changing water-potential of the media.
3. *P putida* GAP-P45 increased water-stress tolerance in *A. thaliana* by elevating IAA and tZ accumulation in shoots and roots.
4. Increase in endogenous GA content in shoot but decrease of the same in root tissue caused due to GAP-P45 inoculation improved physiological status of the plants under water-stress.
5. Uptake of bacterial phytohormones by plants might have enhanced endogenous IAA and tZ level in *A. thaliana* inoculated with GAP-P45 under water-stress.

CHAPTER 5

Testing the impact of four other drought-tolerant, potential PGPR strains on water-stress mitigation in *Arabidopsis thaliana*

5.1 INTRODUCTION

After having performed the aforementioned work on the interaction between *P. putida* GAP-P45 and *A. thaliana*, we wanted to extend this study to the other PGPR strains that we had procured. Hence, we proceeded to perform a comparative analysis of water-stress responsiveness and plant growth promotion (PGP) traits of different strains of drought tolerant, potentially PGPR isolates procured from different sources (mentioned in materials and methods), that have hitherto not been characterized based on the PGP characteristics. We studied the growth patterns and the viability of the four different PGPR strains: *Pseudomonas putida* PM389, *Pseudomonas putida* ZNP1, *Bacillus endophyticus* J13 and *Bacillus tequilensis* J12, under normal and water-stressed conditions. We also estimated the total cellular protein and free proline content of these strains both under non-stressed and water-stress as important parameters to check the ability of these strains to tolerate water-stress. Further, physiological studies were conducted to understand the differences in the PGP traits of the four strains. This includes the (1) quantification of the four major phytohormones (indole-3-acetic acid, zeatin, abscisic acid and gibberellic acid), secreted by the bacteria into the growth medium and (2) quantification of EPS secreted into the growth medium, under normal vs. water-stressed conditions. Finally, the stress mitigating properties of these bacteria were tested on *A. thaliana* under PEG-induced water-stress *in vitro* through the observations on morphological status of plant health and analysis of physiological parameters related to water-stress amelioration such as, fresh weight, dry weight and plant water content when inoculated with these strains individually. All strains exhibited plant growth promoting properties under water-stress, although the levels of secreted phytohormones and EPS varied from strain to strain under both non-stressed and stress conditions.

5.2 MATERIAL AND METHODS

5.2.1 Bacterial growth under water-stress:

The four bacterial strains mentioned previously were procured from two different sources. (1) *Pseudomonas aeruginosa* PM389 and *Pseudomonas aeruginosa* ZNP1 were generous gifts from Dr. Prabhat Nath Jha of BITS-Pilani, Pilani Campus. (2) *Bacillus endophyticus* J13 and *Bacillus tequilensis* J12 were commercially obtained from Indian Agricultural Research Institute, New Delhi (Yadav et al. 2015). These bacteria were isolated from arid and semi-arid root rhizosphere by researchers in the respective organizations mentioned above but have not been hitherto tested comprehensively for their drought-mitigating ability. The ability of these strains to grow under water-stress, induced by polyethylene glycol (PEG-6000) in the media, was tested both by measurement of O.D. at 600 nm (O.D.$_{600}$). Growth conditions used were: 28 ºC for *Pseudomonas aeruginosa* ZNP1 and 37 ºC for *Pseudomonas aeruginosa* PM389, *B. endophyticus* J13 and *B. tequilensis* J12 under moderate shaking (150 ×g). Overnight LB broth cultures (Bertani 1951) of all four bacterial strains at an O.D.$_{600}$ of 0.8 were sub-cultured to an O.D.$_{600}$ of about 0.1, followed by growth monitoring (O.D.$_{600}$ using a spectrophotometer) at an interval of 2 hours both under non-stressed and water-stressed conditions (induced by adding 25% PEG 6000 to the LB broth; Sandhya et al. 2009). For CFU/mL analysis, about 10 µL of culture was collected every two hours from each flask (both non-stressed and stressed) of sub-cultured bacterial culture growing under shaking conditions. The sample so collected was further diluted with LB broth and plated onto freshly prepared LB-agar plates which were incubated at their respective temperatures for 8-10 hours in order to get countable single colonies. Later, the colonies grown on the LB-agar plate for each strain at every time interval were counted using a colony counter, recorded and multiplied by respective dilution factors.

5.2.2 Cellular protein content:

Total cellular protein was determined by lysing the cell pellets obtained for all four bacterial strains from the 72 hours LB broth culture both under normal and water-stress conditions (Sandhya et al. 2010b). To the cell pellet of each bacterial strain, 5 mL of cell lysis buffer (140 mM NaCl, 2.7 $MgCl_2$, 1mM PMSF, DNase, 2.5 mM TrisCl, 0.01%Triton X-100, 0.2 mg/ml lysozyme) was added. Cell suspension was sonicated (SONICS Vibra-Cell) using following parameters: Pulse ON: 30 seconds, Pulse OFF: 10 seconds, number of cycles: 5. After sonication, cell lysate was centrifuged at 7197 x g for 15 minutes at 4ºC. Supernatant was collected and total protein was estimated by using Bradford reagent (Bradford 1976). To 50µl of protein supernatant, 1ml of Bradford reagent was added and mixed properly. Tubes were incubated for 20 minutes after which absorbance at 595 nm were recorded for all the samples. Blank was prepared by adding 1 ml of Bradford reagent to 50µl of cell lysis buffer. A standard curve was plotted by preparing different concentrations of bovine serum albumin and following similar protocol by using Bradford reagent.

5.2.3 Cellular proline content:

The free proline accumulated in bacterial cells was estimated using standard method of Bates et al. (1973). As mentioned previously, supernatant was obtained after lysis of cell pellet of each strain grown for 72 hours both under water-stressed and non-stressed conditions. This supernatant (1 mL) was treated with 2 mL of 3 % aqueous sulphosalicylic acid and tubes were incubated for 30 minutes. After 30 minutes, the solution was centrifuged at 7197 ×g for 20 minutes at 4 ºC. The pellet was discarded and 1 mL of supernatant so obtained was treated with 2 mL glacial acetic acid and 2 mL acid ninhydrin (warm 1.25 g ninhydrin in 30 mL glacial acetic acid, and 20 mL 6 M phosphoric acid). Tubes were incubated in water bath at 100 ºC for an hour after which the reaction was stopped by placing tubes in ice. To each reaction mixture, 4 mL of toluene was added and mixed well. A chromophore containing toluene layer was separated and the absorbance was measured at 520 nm using toluene as a blank. A standard curve was plotted using different concentrations of L-proline and following similar procedure. The concentration of free proline in bacterial cells was extrapolated from the standard curve.

5.2.4 Detection and quantification of phytohormones secreted by all four strains:

The four major phytohormones that were analyzed in this study are indole-3-acetic acid (IAA), trains-zeatin (tZ), abscisic acid (ABA) and gibberellic acid (GA). A slight modification of the protocols of Yasmin et al., (2017), Iqbal & Hasnain, (2013), Lee, Ka, & Song, (2012) was used to extract phytohormones secreted by bacterial strains. To begin with, the supernatant was collected by spinning down the bacterial cells from cultures grown for 72 hours at 28 ºC (*P. aeruginosa* ZNP1) and at 37 ºC (*P. aeruginosa* PM389, *B. endophyticus* J13 and *B. tequilensis* J12) respectively. Extraction and quantification of the phytohormones were done following the same method used for detecting phytohormones secreted by *Pseudomonas putida* GAP-P45 (explained in details in the previous chapter section 4.2.2 and 4.2.4).

5.2.5 Quantification of secreted Exopolysaccharide:

The drought tolerant bacterial strains were analyzed for their ability to produce exopolysaccharides (EPS) under non-stress and 25% PEG induced water-stress. For the extraction of EPS, the procedure from Sandhya, et al. (2010) was used. In brief, bacterial cultures grown for 72 hours were centrifuged at 7197 ×g for 30 minutes at 4 ºC. The supernatant was collected and two volumes of chilled absolute alcohol were added to it. The mixtures were kept overnight at 4 ºC, centrifuged at 7197 ×g for 30 minutes at 4 ºC and the precipitated EPS was further dissolved in 5mL of autoclaved Milli-Q water. The total carbohydrate content in the precipitated EPS was estimated by following the standard protocol of Dubois *et al.* (1956). To a 1 ml of the EPS solution obtained from each strain, 3ml of concentrated H_2SO_4 was added and vortexed for 30 seconds. The tubes were cooled by keeping in ice for 5 minutes and absorbance was recorded at 350 nm using a spectrophotometer (JASCO V-650). Distilled water was used as a blank. A standard curve was plotted for different concentrations of glucose (10 µg/ml – 50 µg/ml) vs absorbance at 350nm by following the same protocol as above. The carbohydrate content in the EPS of the four bacterial strains was extrapolated using the standard curve.

5.2.6 Growth of *Arabidopsis thaliana*, water-stress induction and PGPR inoculation:

The procedures involved in germination, growth, water-stress induction have previously been described in chapter 2. Before starting an experiment, the PGPR strains were grown overnight in LB broth in a shaking incubator at 28 °C or 37 °C (depending on the strain) to an $O.D._{600}$ of 0.6-0.8 and used for inoculating the plants. Prior to inoculation, bacterial cells were centrifuged, the supernatant was discarded and cells were re-suspended in autoclaved, distilled water. Half of the control (non-stressed) and water-stressed plants were subjected to bacterial inoculation by the addition of 200 µL of this aqueous suspension to the respective Magenta boxes. An equal amount of water was added to the other half (non-inoculated plants) in order to account for any changes in the medium due to the water in the inoculum. Thus, for each PGPR, there were four experimental sets namely: 1. No treatment controls (NT) 2. Non-stressed, inoculated (NS+I) 3. Water-stressed, non-inoculated (WS+NI) 4. Water-stressed, inoculated (WS+I). For each experiment, at least three replicate Magenta boxes were used, each Magenta box containing 4 meshes, each with 5-7 seedlings.

5.2.7 Physiological studies on plant responses to PGPR inoculation under water-stress:

Physiological studies were performed to assess the impact of the PGPR strains on water-stress alleviation of *A. thaliana* after 7 days post treatments as described in chapter 2. Experiments included observations on (1) morpho-physiological status and plant health; (2) fresh weight (FW); (3) dry weight (DW) and (4) plant water content (PWC) of whole seedlings. For measurement of FW, 60 seedlings from three replicate Magenta boxes, (20 seedlings from each box) were harvested. Following FW measurements, the seedlings were incubated at 80 °C for 48 hours for measurement of DW. Plant water content was measured both on FW and DW basis, as described in chapter 2.

5.3 <u>RESULTS</u>

5.3.1 Bacterial growth and viability under control and water-stressed conditions:

The growth curve experiments have been represented, both in terms of $O.D._{600}$ (Fig. 5.1) as well as CFU/mL (Fig. 5.2). As can be seen, in all four strains, there was significantly higher growth under non-stressed conditions as opposed to PEG-induced water-stress. From the O.D.$_{600}$ data, it is clear that, under control conditions, growth rate of all four strains slowed down significantly after 8-10 hours of incubation. The 25% PEG treated cells exhibited a slower growth rate as opposed to the control cells throughout the period of analysis. The CFU/mL data have thrown more light into the precise growth pattern of these cells. As can be seen, the CFU/mL data corroborate the O.D.$_{600}$ based finding that, for the most part, the water-stressed cells were growing at a much slower rate than the control cells. It also demonstrates actual growth retardation in all strains after 8-10 hours of growth. From Fig. 5.2, we can conclude that the maximum number of viable colonies were found in *P. aeruginosa* ZNP1 followed by *P. aeruginosa* PM389 under control conditions, at most time-points of analysis. However, under water-stress, the number of viable colonies were higher in *P. aeruginosa* PM389 as compared to all the strains. The DH5α strain of *E. coli* was used as a negative control for the above experiment. Under water-stress, DH5α did not grow at all.

5.3.2 Levels of endogenous proline and total protein content:

After analyzing bacterial growth under water-stress, we also wanted to test for the ability of these bacterial strains to modulate their endogenous proline and total protein content under water-stress. This was done to gain additional insights into the ability of these strains to withstand water-stress. Proline, a compatible osmolyte, is known to accumulate under abiotic stress (Sandhya et al. 2010b). Similarly, total cellular protein content is also known to be modulated under water-stress (Sandhya et al. 2010b). It was seen that while all strains have the ability to grow under water-stress to varying degrees (as indicated by the growth curve data), only in *B. endophyticus* J13 the amount of free proline was elevated under water-stress, whereas in all the other strains, the level of free proline was less under water-stress when compared to the non-stressed conditions (Fig. 5.3A). The total cellular protein after 72 hours was found to be high in case of *P. aeruginosa* PM389 and *B. endophyticus* J13 under PEG treated conditions. The amount of protein was less under PEG treated conditions for the other two strains as compared to control conditions (Fig. 5.3B).

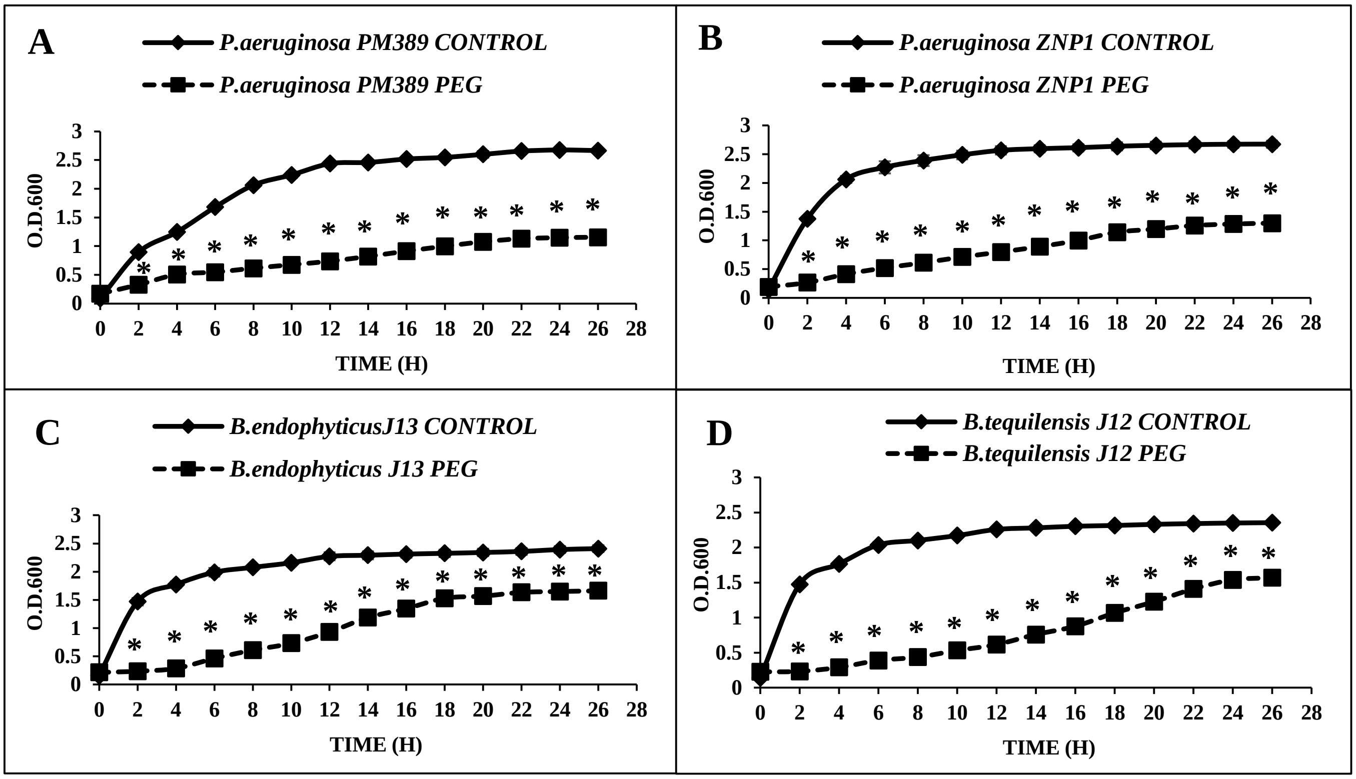


Fig. 5.1 Growth curve of the four bacterial strains both under non-stressed (control) and water-stressed (PEG) condition based on the O.D.600 nm at an interval of 2 hours. A) Growth curve of *P.aeruginosa* PM389, B) Growth curve of *P. aeruginosa* ZNP1, C) Growth curve of *B. endophyticus* J13 and D) growth curve of *B. tequilensis* J12. Each point represents mean ± SE of 6 replicate samples. '*' represents the significant difference between stressed vs. non-stressed values.

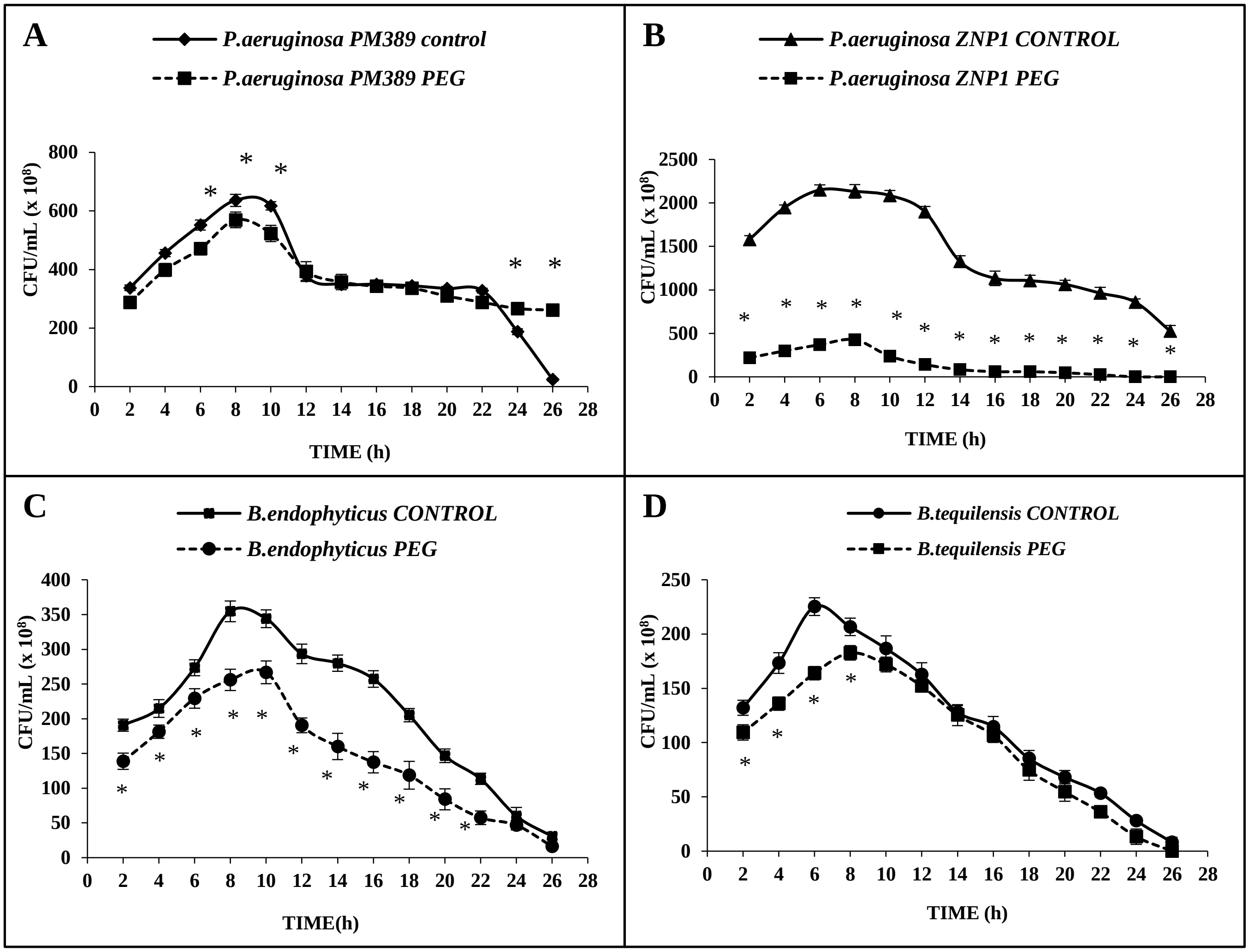


Fig.5.2 Analysis of viability of all four bacterial strains through CFU/mL both under non-stressed and water-stressed condition at an interval of 2 hours. A) *P. aeruginosa* PM389 (CFU/mL x 10^8), B) *P. aeruginosa* ZNP1 (CFU/mL x 10^8), C) *B. endophyticus* J13 (CFU/mL x 10^8) and D) *B. tequilensis* J12 (CFU/mL x 10^8). Each point represents mean ± SE of 6 replicate samples. '*' represents the significant difference between the water-stressed vs. non-stressed conditions.

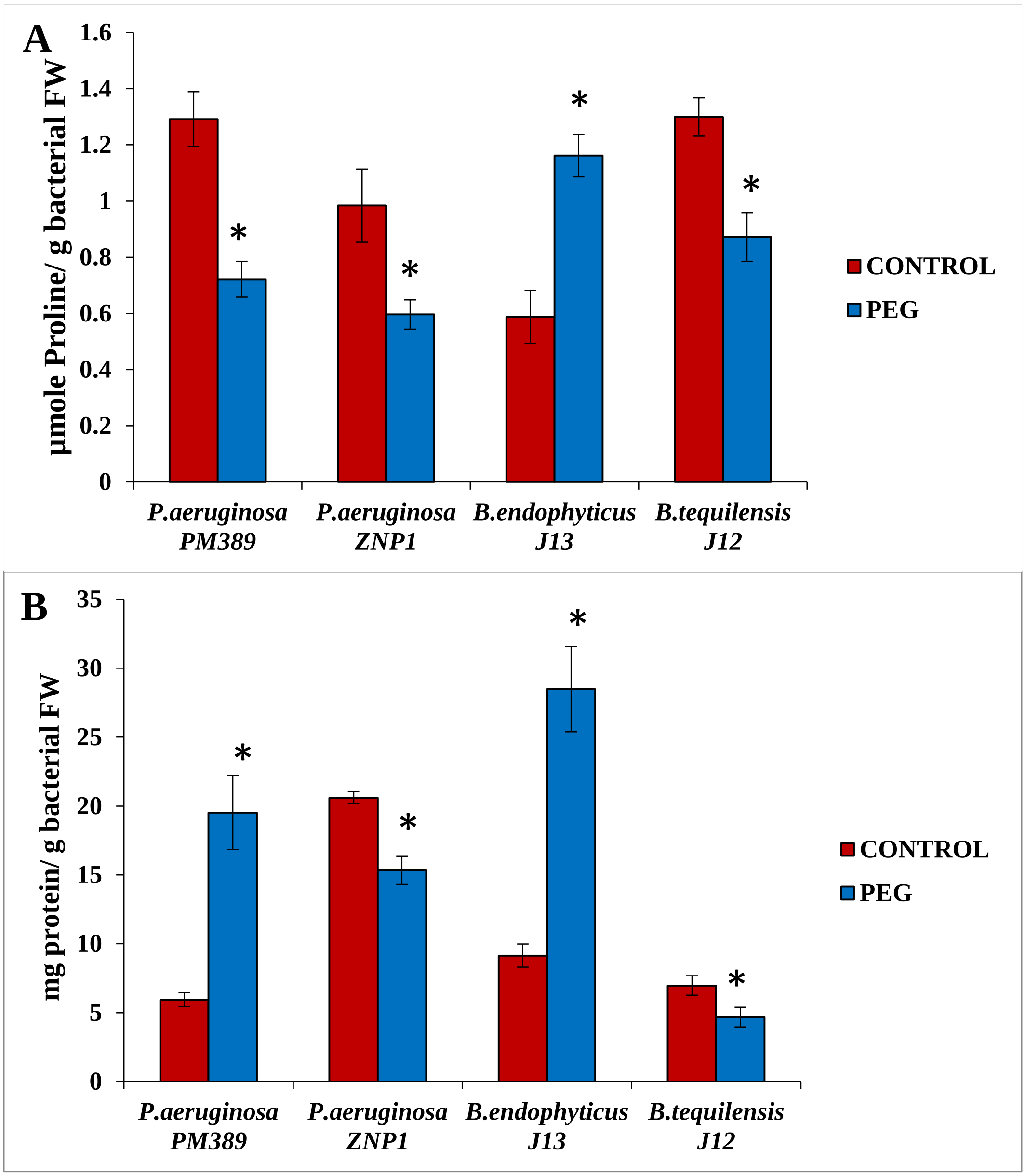


Fig. 5.3 Quantification of A) cellular free proline and B) cellular total protein in all four PGPR strains under non-stressed and water-stressed condition. Each bar represents mean ± SE of 6 replicate samples.'*' represents the significant difference between water-stressed vs. non-stressed conditions.

5.3.3 Levels of phytohormones secreted by the four bacterial strains:

As mentioned in materials and methods, an HPLC based method was used to detect and quantify the secretory phytohormones from the bacterial strains. Fig. 5.4 shows a representative chromatogram of the four commercial hormone standards and the phytohormones secreted by the *P. aeruginosa* PM389 under non-stressed conditions. The identity of peaks obtained by HPLC was confirmed using LC-MS (Fig. 5.5). Out of the four phytohormones of our interest, only three (IAA, tZ and GA) were detected in the bacterial supernatants, while ABA was not detected in case of any strain, both under non-stressed and water-stressed conditions. As can be seen from Fig. 5.6, water-stress caused either an increase or a decrease in the levels of phytohormones in all strains. Both *P. aeruginosa* strains secreted higher amounts of IAA (with ZNP1 exhibiting the highest levels) than the *Bacillus* strains under non-stressed conditions (Fig. 5.6A). Under water-stress, while there was a dramatic decrease in the IAA levels of the *P. aeruginosa* strains, while the *Bacillus* strains exhibited small but significant increases in secretory IAA. Level of secretory tZ was highest in *P. aeruginosa* PM389 under non-stressed conditions, while the other strains exhibited lower levels of secretory tZ under normal growth condition (Fig. 5.6B). While the *P. aeruginosa* strains exhibited a decrease in tZ levels under water-stress (this decrease being >6-fold in case of PM389), the *Bacillus* strains exhibited an increase in the same. This increase was higher in *B. endophyticus* J13 than in *B. tequilensis* J12. As far as GA is concerned, *B. tequilensis* J12 exhibited higher secretory GA than the other three strains under non-stressed conditions. Under water-stress, all four strains secreted more GA than under non-stressed conditions, however, this increase was higher in the *Bacillus* strains than in the *Pseudomonas* strains (Fig. 5.6C).

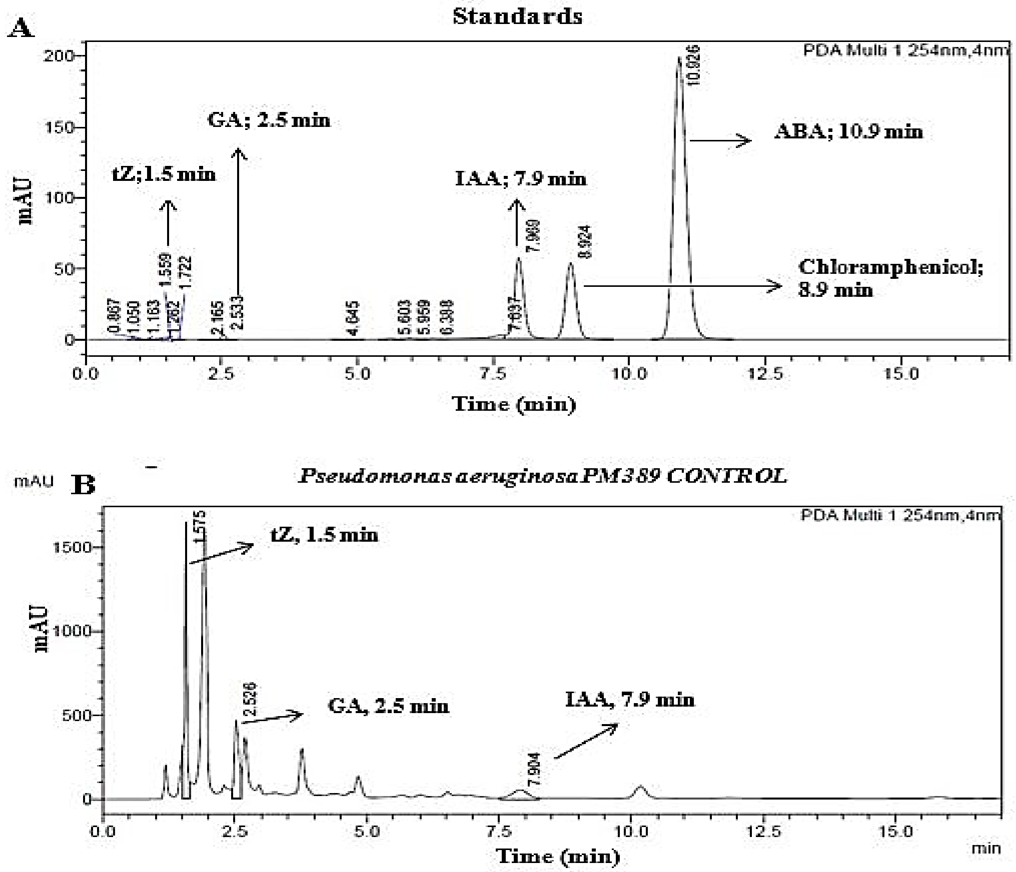


Fig. 5.4 Representative HPLC chromatogram of the four commercial hormone standards (A) and the phytohormones secreted by the *P. aeruginosa* PM389 under control condition (B).

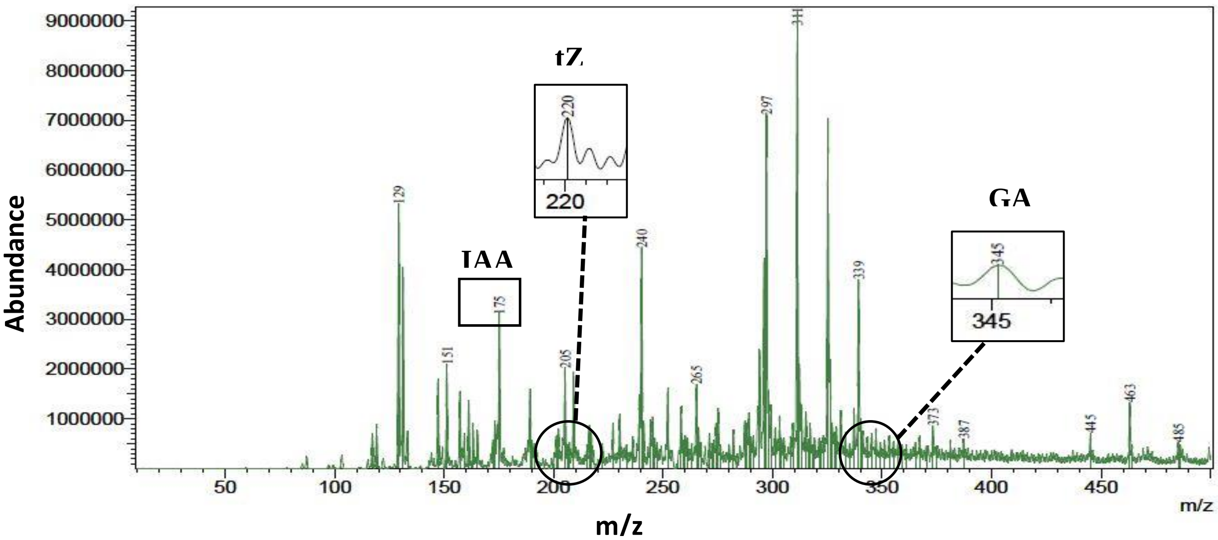


Fig. 5.5 Representative mass spectra of the phytohormones detected in *P. aeruginosa* PM389 samples under control condition.

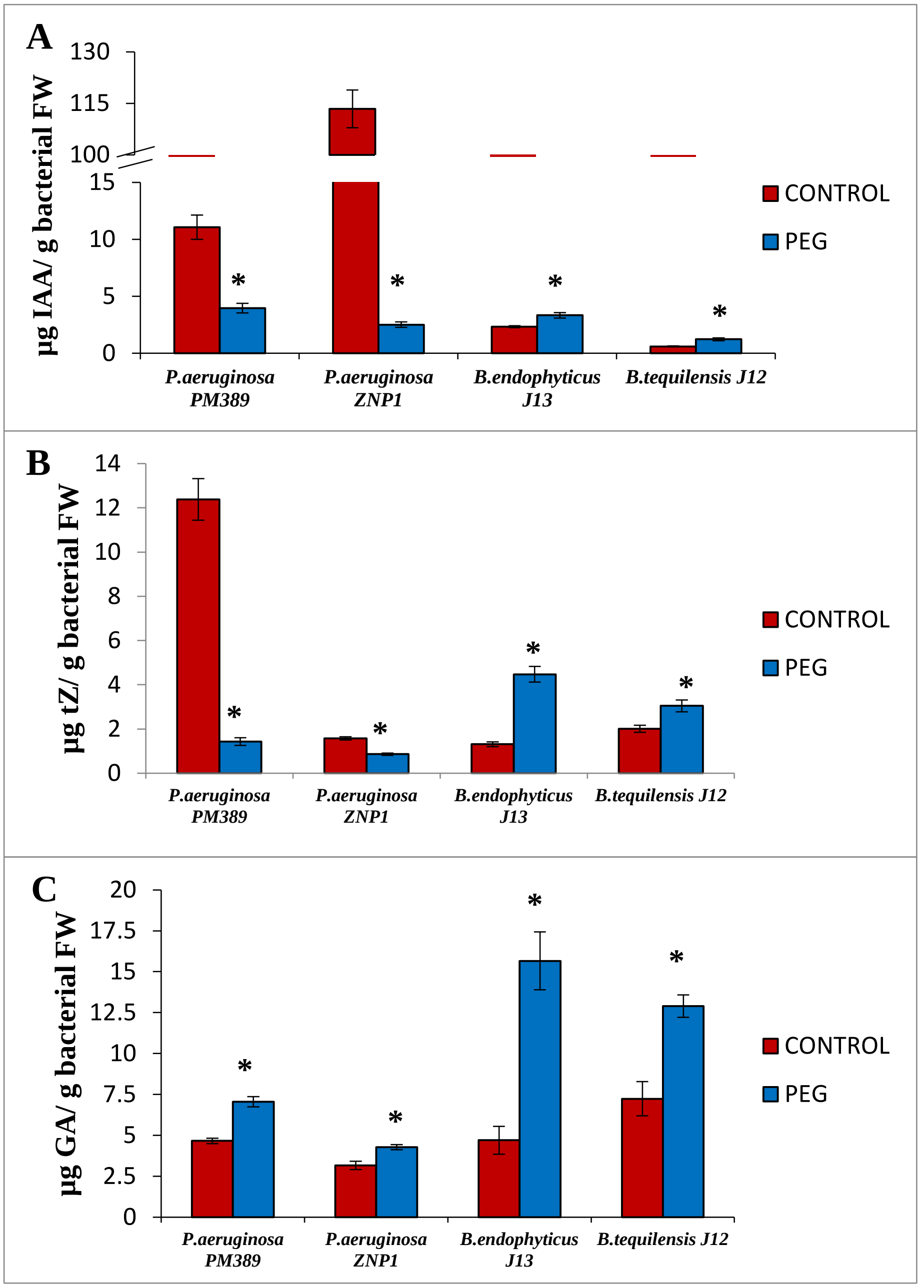


Fig. 5.6 Quantification of secreted A) indole-3-acetic acid (IAA), B) trans-zeatin (tZ) and C) gibberellic acid (GA) by the four bacterial strains both under non-stressed and water-stressed condition. Concentration of the secreted phytohormones are expressed as µg/ g bacterial FW. Each bar represents mean ± SE of 6 replicate samples. '*' represents the significant difference between water-stressed vs. non-stressed conditions

5.3.4 Levels of exopolysaccharides secreted by the bacterial strains:

Under non-stressed conditions, all four strains exhibited similar levels of secretory EPS (Fig. 5.7). Under water-stress, there was about 2.5-fold increase in the production of EPS by *P. aeruginosa* ZNP1 and about 1.5-fold increase in case of *B. endophyticus* J13. However, in case of *P. aeruginosa* PM389, the levels of secreted EPS were adversely affected by water-stress. No change in secreted EPS level was seen in case of *B. tequilensis* J12.

The trends of data obtained from the comparative analysis of all four bacterial strains to determine and quantify the PGP traits (Gupta A 2018 M.E. dissertation; **Ghosh, Gupta et al., under review)** are summarized in Table 5.1.

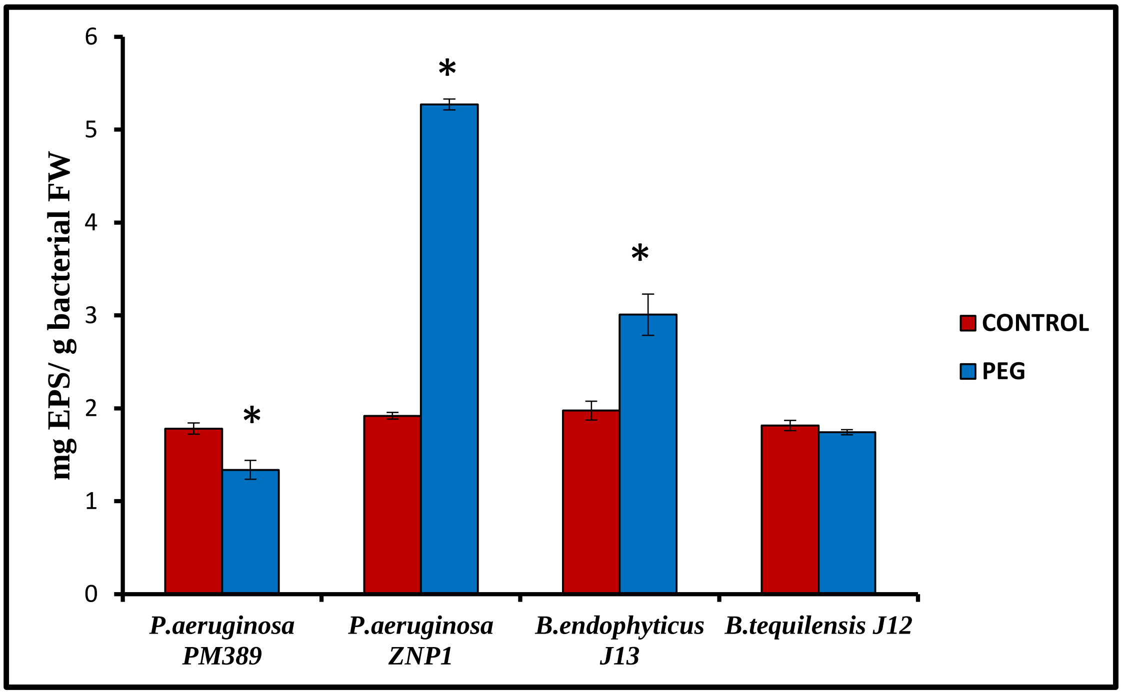
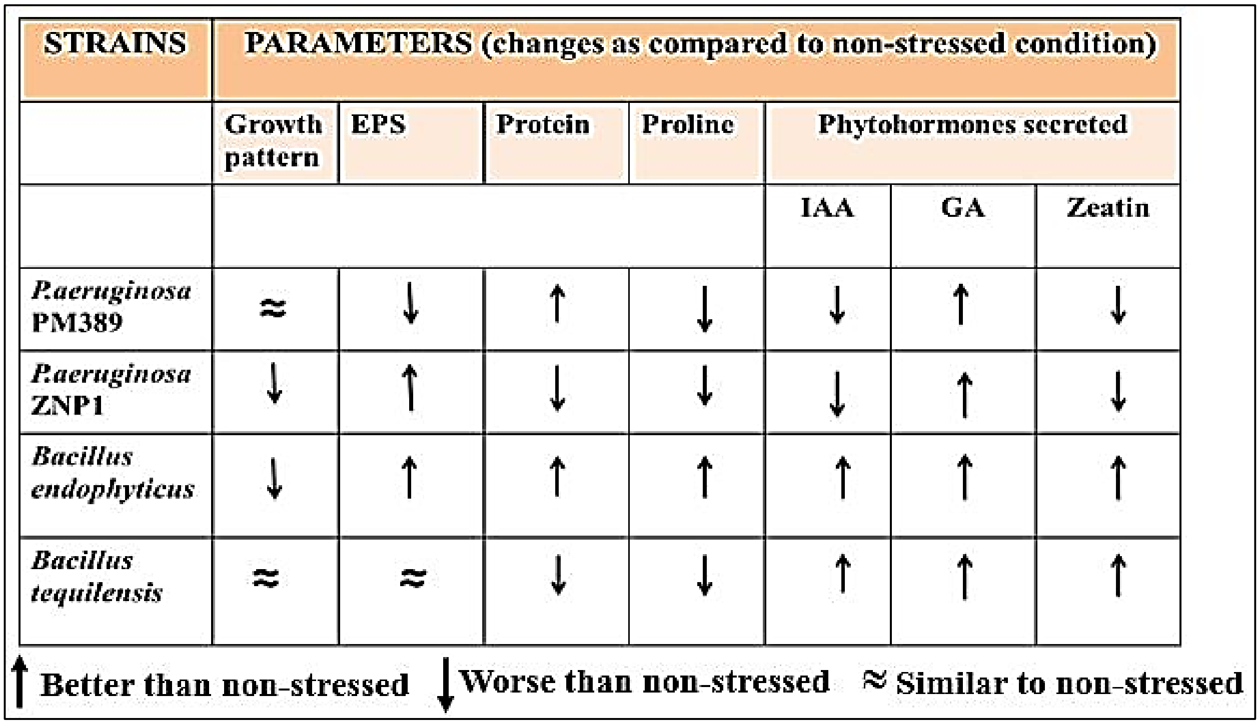


Fig. 5.7 Quantification of exopolysaccharide (EPS) expressed as mg/g bacterial FW secreted by the four potential PGPR strains under non-stress and water-stressed condition. Each bar represents mean ± SE of 6 replicate samples. '*' represent the significant difference between water-stressed vs. non-stressed conditions.

Table 5.1 Trend obtained from the comparative analysis of all four bacterial strains to determine and quantify the PGP traits under both non-stressed and water-stressed conditions.



STRAINS	**PARAMETERS (changes as compared to non-stressed condition)**						
	Growth pattern	**EPS**	**Protein**	**Proline**	**Phytohormones secreted**		
					IAA	**GA**	**Zeatin**
***P.aeruginosa* PM389**	≈	↓	↑	↓	↓	↑	↓
***P.aeruginosa* ZNP1**	↓	↑	↓	↓	↓	↑	↓
Bacillus endophyticus	↓	↑	↑	↑	↑	↑	↑
Bacillus tequilensis	≈	≈	↓	↓	↑	↑	↑

↑ Better than non-stressed ↓ Worse than non-stressed ≈ Similar to non-stressed

5.3.5 Plant growth under water-stress induction and PGPR inoculation:

Prior to the treatments (at day 0), all plants exhibited similar growth and developmental phenotypes in case of all experimental set ups with individual PGPR strain (Fig. 5.8). Inoculation with the aforementioned four potent PGPR strains under non-stressed conditions did not cause any visible enhancement of the morpho-physiological status of the plants after 7 days post treatments (Fig. 5.8). By day 7 post transfer to PEG supplemented medium, the water-stressed non-inoculated plants exhibited significant growth stunting as opposed to all other treatments. However, under PEG-treated conditions, plants inoculated with these four individual PGPR strains, exhibited much better growth and water-stress tolerance as opposed to the non-inoculated ones. These observations confirmed the beneficial effect of the four potent PGPR strains (*Pseudomonas putida* PM389, *Pseudomonas putida* ZNP1, *Bacillus endophyticus* J13 and *Bacillus tequilensis* J12) on water-stress amelioration in *A. thaliana* in soil-free system.

5.3.6 Fresh weight, dry weight and plant water content:

In order to quantify the impact of the PGPR strains on plant water status, we analyzed the fresh weight (FW), dry weight (DW), and plant water content (PWC) of the treated seedlings. As can be seen from Fig. 5.9 A and B, PGPR inoculation of *A. thaliana* under non-stressed condition did not significantly enhance FW (except for *P. aeruginosa* ZNP1) and DW when compared to control plants (NT). However, both FW and DW of water-stressed plants increased significantly on inoculation with all four PGPR strains seperately after 7 days of treatment. Plant water content (PWC) was calculated, both on DW and FW basis (Turner 1981). PWC (FW basis) and PWC (DW basis) followed similar trends in that the water-stressed, non-inoculated plants recorded the lowest PWC among all the treatments at all time points of the study (Fig. 5.10 A and B). The PEG treated, inoculated plants exhibited significantly higher PWC, both on DW and FW basis, as opposed to the water-stressed, non-inoculated plants.

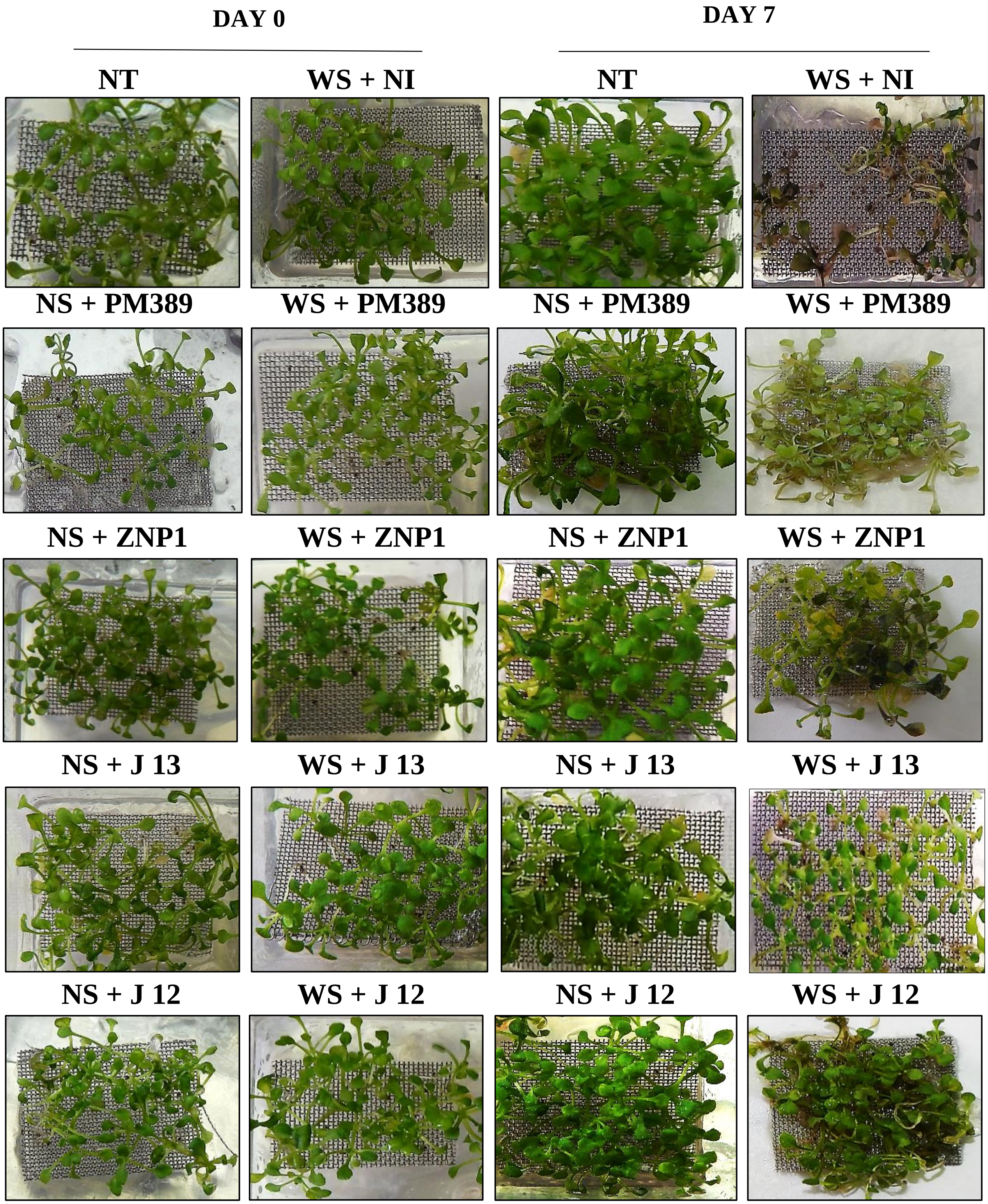


Fig. 5.8 Plant growth and development prior treatment (day 0) and at day 7 following inoculation of *A. thaliana* with all four PGPR strains separately under water-stress (25% PEG). NT: no-treatment control; WS+NI: water-stressed, non-inoculated; NS+PM389: non-stressed, *P.*

aeruginosa PM389 inoculated; WS+PM389: water-stressed, *P. aeruginosa* PM389 inoculated; NS+ZNP1: non-stressed, *P. aeruginosa* ZNP1 inoculated; WS+ZNP1: water-stressed, *P. aeruginosa* ZNP1 inoculated; NS+J13: non-stressed, *B. endophyticus* J13 inoculated; WS+J13: water-stressed, *B. endophyticus* J13 inoculated; NS+J12: non-stressed, *B. tequilensis* J12 inoculated; WS+J12: water-stressed, *B. tequilensis* J12 inoculated.

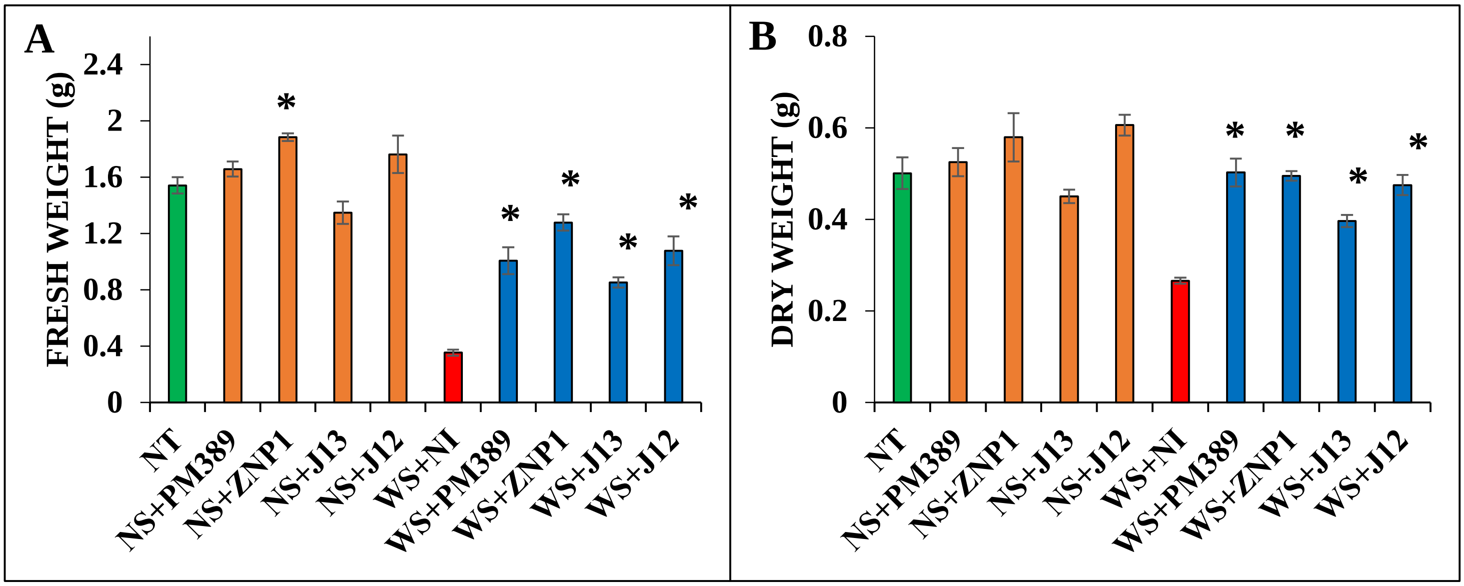


Fig. 5.9 Physiological studies on the impact of all four PGPR strains on water-stress amelioration in *Arabidopsis thaliana*. (A) Fresh weight and (B) Dry weight of whole seedlings 7 days post treatments. Each bar represents mean ± SE of 30 replicate plants. * indicates significant difference (p≤0.05) in data between non-inoculated and PGPR inoculated plants under both non-stressed and water-stressed condition.

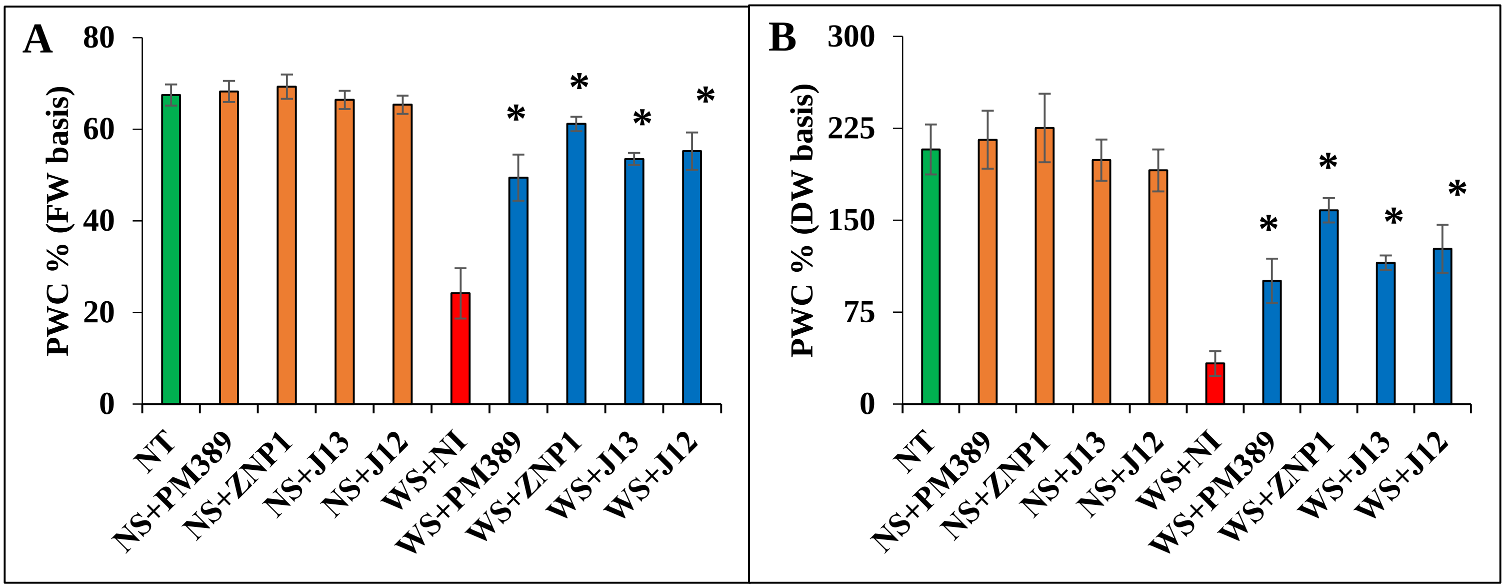


Fig. 5.10 Impact of all four PGPR strains on plant water content in *Arabidopsis thaliana* under non-stressed and water-stressed conditions. (A) PWC (Fresh weight basis) and (B) PWC (Dry weight basis) of whole seedlings 7 days post treatments. Each bar represents mean ± SE of 30 replicate plants. * indicates significant difference ($p \leq 0.05$) in data between non-inoculated and PGPR inoculated plants under both non-stressed and water-stressed condition.

5.4 DISCUSSION

5.4.1 Bacterial growth and responses under water-stress:

For any rhizobacterial strain to act as a potential drought-mitigating PGPR, it is a pre-requisite that it should be able to survive and grow under water-stress in order to mitigate effects of water-stress in their host plants. The objective of this experiment was to understand the ability of potential PGPR strains (*P. aeruginosa* PM389, *P. aeruginosa* ZNP1, *B. endophyticus* J13 and *B. tequilensis* J12) to sustain in PEG-supplemented MS-agar media and mitigate water-stress in *A. thaliana*. Polyethylene glycol induces water-stress in bacterial cells by decreasing the availability of surrounding water molecules to the cells. Hence, it is but expected that a dehydrating agent like PEG will slow down the growth rate of bacteria in the medium (Sandhya et al. 2010b). From Fig. 5.1 and 5.2, it is evident that all four bacterial strains were able to survive and multiply under water-stress, albeit their growth rate was, in general, slower than their non-stressed counterparts. Evidently, both *Pseudomonas* strains were able to proliferate better and hence survive better than the *Bacillus* strains under water-stress as they exhibited higher number of viable colonies when compared to the *Bacillus* strains (Fig. 5.1 and 5.2).

In order to get some insight into the mechanisms of drought tolerance by these bacteria, we analyzed their endogenous free proline content under water-stress. Soil bacteria under abiotic stress conditions are known to increase the endogenous concentrations of compatible osmolytes (such as proline, glycine betaine, trehalose etc.) within themselves as a part of protection mechanism which prevent membrane damage and provides stability to proteins under water limiting condition (Sandhya et al. 2010b; García et al. 2017). Since proline is one of the most well-reported compatible osmolytes, its accumulation under water-stress is a good indication of the drought tolerating ability of bacteria. Among all strains, *B. endophyticus* is the only strain that demonstrated enhanced proline accumulation under water-stress as opposed to the non-stressed bacterial cells (Fig. 5.3A). It is interesting to note that the other strains did not exhibit enhanced proline accumulation under water stress and yet survived the dehydrating impacts of 25% PEG 6000 as is evident from their growth curves (Fig. 5.1). It is possible that these strains could be accumulating one or more of the other compatible osmolytes which remains to be investigated. Total cellular protein content in bacteria has been reported to reduce under water-stress, which indicates the degenerative effects of water-stress (Sandhya et al. 2010b). It has been suggested that under stress condition bacterial cellular proteins are used to produce exopolysaccharides which in turn protects the bacterial population from the detrimental effects of water-stress (Roberson and Firestone 1992). In case of cellular protein while *P. aeruginosa* PM389 and *B. endophyticus* J13 recorded increased levels under stress, *P. aeruginosa* ZNP1 and *B. tequiliensis* J12 showed lower cellular protein accumulation, which can be an indication of the utilization of cellular proteins in producing polysaccharides secreted by these PGPR strains (Fig. 5.7). In case of *Pseudomonas aeruginosa* strains, the inverse relation between total cellular protein content and secreted exopolysaccharides was quiet evident, though *Bacillus* strains did not follow similar pattern suggesting that, response to water-stress in case of different bacteria may vary at physiological and molecular levels.

5.4.2 Bacterial secretions under water-stress:

Among the various plant growth promoting traits exhibited by drought tolerant PGPR strains, secretion of phytohormones is an important characteristic. Phytohormones are secreted as secondary metabolites by beneficial rhizobacteria (Rademacher 1994). Major classes of phytohormones like auxins, gibberellins, abscisic acid and cytokinins play an important role in providing tolerance to plants against abiotic and biotic stress. As seen in Fig. 5.6, all four strains studied here secrete phytohormones (auxin, cytokinin and gibberellin), both under non-stressed as well as water-stressed conditions. In our study, levels of secretory IAA were extremely high under non-stressed conditions in *P. aeruginosa* ZNP1 as compared to other PGPR strains (Fig. 5.6A). High IAA production by ZNP1 under normal conditions can possibly have two effects when inoculated with plants: (1) it may help enhance shoot growth, provided levels of cytokinins remain low (as it is important to maintain an optimum auxin/ cytokinin ratio) (Schaller et al. 2015) or (2) it may become deleterious to root system leading to stunted growth of the roots since high amount of IAA activates ACC synthase resulting in ethylene formation (Stepanova et al. 2005; Shah et al. 2017). Under water-stress all four strains were found to produce IAA which besides contributing to general plant growth may also help in water-stress tolerance (Marulanda et al. 2009; Iqbal and Hasnain 2013). *Bacillus* strains secreted somewhat higher IAA under water-stress while *Pseudomonas* strains produced lower IAA under water-stress. *Bacillus* strains were found to produce high levels of cytokinin, while the *Pseudomonas* strains recorded lower levels of cytokinin under water-stress (Fig. 5.6B). In our study, under water-stress all these strains were found to produce high levels of gibberellin, the *Bacillus* strains recording the highest amount of all (Fig. 5.6C). It is reported that under drought condition, production of phytohormones by PGPRs tend to decrease significantly (Sandhya et al. 2009, 2010b). however, in our study, enhanced secretion of IAA, tZ and GA by the *Bacillus* strains and higher production of GA by the *Pseudomonas* strains under water-stress indicate novel observations and immense potentials of these four strains as PGPRs.

It has been reported that inoculation of plants with high EPS-producing rhizobacteria under stress condition stimulates plant growth through improved nutrition uptake, increase in relative water content, root and shoot biomass etc. (Sandhya et al., 2009; Vurukonda et al., 2016). In our study. *P. aeruginosa* ZNP1 and *B. endophyticus* were found to generate high amount of EPS under water-stress indicating their improved ability to colonize in soil thereby positively influencing plant-microbe interaction (Sandhya et al. 2009). On the other hand, under stressed conditions *P. aeruginosa* PM389 and *B. tequilensis* either produced lower or similar amount of EPS respectively, as compared to their non-stressed counterparts.

5.4.3 Impact of PGPR strains on plant responses to water-stress:

When inoculated with *A. thaliana* seedlings in MS-agar media under both non-stressed and water-stressed condition, all the four PGPR strains exhibited beneficial effects on plant health, biomass accumulation and plant water content of the inoculated plants imparting tolerance to water-stress after 7 days of treatment (Fig. 5.8-5.10). This could have been the effect of the phytohormones IAA, tZ and GA secreted by the PGPR strains that enhance plant growth and stress-tolerance. However, whether these bacterial phytohormones secreted in the media are transported into the roots or the exogenous phytohormones induce specific stress-responsive signaling cascades in the plants, is still not unveiled. Exopolysaccharides secreted by the PGPRs could have positive effects on the plant health, as EPS enhances better colonization of bacteria on the roots and form a protective layer around the roots that help plants reduce stress-induced injury of the roots, maintenance of better water status and better absorption of nutrients by the roots. In our study, *P. aeruginosa* ZNP1 exhibited highest amount of secreted exopolysaccharides under water-stress which could have enhanced the root colonization efficiency of this strain, as the plants inoculated with *P. aeruginosa* ZNP1 under water-stress exhibited highest fresh weight and plant water content (Fig. 5.9 and 5.10).

5.5 CONCLUSIONS

1. From the data obtained in our study on bacterial growth and cell viability, all four strains *P. aeruginosa* PM389, *P. aeruginosa* ZNP1, *B. endophyticus* J13 and *B. tequilensis* J12 were found to be drought tolerant.
2. The ability of these bacterial strains to secrete phytohormones under water-stress suggests their possible contribution to ameliorate the adverse effects of water-stress in plants.
3. While *Bacillus* strains secreted higher amount of IAA and tZ under water-stress, *Pseudomonas* strains exhibited lower concentration of these phytohormones as compared to non-stressed conditions.
4. It was interesting to observe higher production of GA by all the strains under water-stress as compared to non-stressed conditions.
5. *P. aeruginosa* ZNP1 and *B. endophyticus* J13 exhibited higher production of EPS under water-stress which indicate probability of better root colonization by these bacteria. Observations on the morpho-physiological health, biomass accumulation and plant water status of *A. thaliana* seedlings inoculated with all four strains separately, confirmed the PGP characteristics and proved their beneficial effects in imparting water-stress tolerance to the seedlings.

CHAPTER 6

Summary and Conclusions

Based on the studies performed on plant physiology, proline metabolic gene expression, enzyme assays and dynamics of four major phytohormones in *Arabidopsis thaliana*, inoculated with PGPR strain *Pseudomonas putida* GAP-P45 under water-stress and characterization of four potent PGPR isolates, following conclusions can be drawn on the mechanisms of plant-PGPR interactions:

6.1 Amelioration of water-stress in *Arabidopsis thaliana* by *Pseudomonas putida* GAP-P45:

- Better morpho-physiological status, and enhanced physiological parameters such as, fresh weight, dry weight, plant water content, chlorophyll content and proline content of *A. thaliana* seedlings inoculated with *Pseudomonas putida* GAP-P45 under water-stress as compared to non-inoculated plants were observed in our study.
- These phenomena confirmed cross-compatibility and beneficial effects of the selected PGPR strain, delayed stress-senescence in the plants due to PGPR inoculation and amelioration of water-stress effects by this strain in *A. thaliana*.

6.2 *Pseudomonas putida* GAP-P45 enhances proline turnover in *Aarabidopsis thaliana* under water-stress:

- Accumulation of proline under water-stress due to upregulation of biosynthetic genes and down regulation of catabolic genes has been reported to ameliorate drought stress in a wide variety of plant species studied. However, our observation on the time-dependent regulation of both proline biosynthetic and catabolic genes and activity assay of the enzymes coded by these genes in *A. thaliana* inoculated with GAP-P45 under water-stress, indicated that not only accumulation, but also concomitant degradation of accumulated proline under stressed condition is responsible for PGPR mediated water-stress amelioration.

- Upregulation of both biosynthetic and catabolic genes and enzymes in the inoculated plants, thus increasing the rate of proline turnover and modulating proline homeostasis in plant cells, are key players for amelioration of water-stress in inoculated plants.

6.3 Dynamics of endogenous hormone accumulation in *Arbidopsis thaliana* by *Pseudomonas putida* GAP-P45:

- Alleviation of water-stress in *A. thaliana* was achieved by time-dependent modulation of endogenous levels of four major hormones (ABA, IAA, tZ and GA) in plants due to inoculation with phytohormone-producing PGPR strain *P. putida* GAP-P45.
- Downregulation of ABA levels in both roots and shoots and enhanced GA level in shoots but decrease in roots of the inoculated plants as opposed to non-inoculated stressed seedlings were observed, though GAP-P45 did not secrete ABA or GA in the plant growth media. This phenomenon indicated PGPR mediated molecular regulation of the endogenous ABA and GA metabolism in plants.
- Enhanced concentration of endogenous IAA and tZ in both roots and shoots of the inoculated plants under water-stress as compared to non-inoculated stressed seedlings, could be due to direct uptake of these phytohormones secreted by GAP-P45 in the media by plants or PGPR induced precise modulations of the endogenous metabolism of IAA and tZ.

6.4 Plant growth promotion by other potential PGPR strains:

- *Pseudomonas aeruginosa* PM389, *Pseudomonas aeruginosa* ZNP1, *Bacillus endophyticus* J13 and *Bacillus tequilensis* J12, four arid-rhizosphere isolates exhibited plant growth promoting traits such as: tolerance to extreme water-stress, production of exopolysaccharides for root colonization and production of phytohormones (IAA, tZ and GA).
- Observations on better morpho-physiological status, fresh weight, dry weight and plant water content of *A. thaliana* seedlings due to inoculation of these bacteria under water-stress confirmed the potentials of these afore-mentioned bacteria as PGPR.

CHAPTER 7

Specific contribution and future perspective

7.1 SPECIFIC CONTRIBUTION OF THIS WORK

- First report on the PGPR-mediated time-dependant regulation of proline metabolic gene expression in *A. thaliana* under water-stress.
- First evidence that not only accumulation but concomitant degradation of accumulated proline, thus enhancing proline turn-over under dehydration condition plays a pivotal role in PGPR-mediated alleviation of water-stress in *A. thaliana*.
- First report on the dynamics of endogenous regulation of four major phytohormones (IAA, tZ, ABA and GA) in *A. thaliana* due to PGPR inoculation leading to water-stress tolerance.
- Characterization of four hitherto uncharacterized arid-rhizospheric bacterial isolates based on plant growth promoting traits and testing their ability to ameliorate water-stress effects in *A. thaliana*.

7.2 FUTURE PERSPECTIVE BASED ON THE KNOWLEDGE OBTAINED

- Use of ABA biosynthetic and ABA insensitive mutant *A. thaliana* under the same experimental conditions to determine whether the observed phenomenon of enhanced proline turnover is ABA dependent or independent.
- IAA and tZ biosynthetic and utilization mutant plants of *A. thaliana* can be inoculated with *Pseudomonas putida* GAP-P45 under the same experimental conditions or uptake inhibitors of these hormones can be applied in the media to determine whether plants directly uptake phytohormones secreted by PGPR from the media or bacterial phytohormones induce modulation of endogenous hormone levels in plants.

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List of Publications

1. **Ghosh D**, Sen S, Mohapatra S (2017) Modulation of proline metabolic gene expression in *Arabidopsis thaliana* under water-stressed conditions by a drought-mitigating *Pseudomonas putida* strain. Ann Microbiol 67:655–668 . doi: 10.1007/s13213-017-1294-y
2. **Ghosh D***, Sen S*, Mohapatra S (2018) Drought-mitigating *Pseudomonas putida* GAP-P45 modulates proline turnover and oxidative status in *Arabidopsis thaliana* under water stress. Ann Microbiol 68:579–594 . doi: 10.1007/s13213-018-1366-7. * Equal contribution.
3. **Ghosh D**, Gupta A, Mohapatra S (2018) Dynamics of endogenous hormone regulation in plants by phytohormone secreting rhizobacteria under water-stress. Symbiosis 1–14 . doi: 10.1007/s13199-018-00589-w
4. Sen S, **Ghosh D**, Mohapatra S (2018) Modulation of polyamine biosynthesis in *Arabidopsis thaliana* by a drought mitigating *Pseudomonas putida* strain. Plant Physiol Biochem 129:180–188 . doi: 10.1016/J.PLAPHY.2018.05.034
5. Shah DA, Sen S, Akula S, **Ghosh D**, et al (2017) An auxin secreting *Pseudomonas putida* rhizobacterial strain that negatively impacts water-stress tolerance in Arabidopsis thaliana. Rhizosphere 3:16–19 . doi: 10.1016/J.RHISPH.2016.11.002

Manuscripts under revision

6. "A comparative analysis of exopolysaccharide and phytohormone secretions by four drought-tolerant rhizobacterial strains and their impact on water-stress mitigation in *Arabidopsis thaliana*"- **Daipayan Ghosh**, Anshika Gupta, Sridev Mohapatra. Under revision (World Journal of Microbiology and Biotechnology).

Conference Proceedings

1. “PGPR- mediated modulation of proline metabolic gene expression in *Arabidopsis thaliana* under drought stress”- 3rd International Plant Physiology Congress, 11-14 December, 2015, JNU, New Delhi.
2. “Modulation of proline metabolic gene expression in *Arabidopsis thaliana* under water-stressed conditions by a drought mitigating *Pseudomonas putida* strain”- International Conference on Plant Developmental Biology and 3rd International Arabidopsis Meeting, 12-16 December, 2017, NISER, Bhubaneshwar.

Biography of the Supervisor

Dr. Sridev Mohapatra, Assistant Professor in Biological Sciences Department, has been with Birla Institute of Technology and Science, Pilani, Hyderabad Campus, India since 2012.He obtained his Ph.D. degree from University of New Hampshire in 2008 and M.Sc. from Utkal University, Bhubaneswar, India in 2001. He had served as Post-Doctoral research associate in Texas Tech. University, Lubbock, Texas, U.S.A. and University of Texas at Arlington, Arlington, Texas, USA.

Dr. Sridev Mohapatra's research focus is in plant biotechnology, plant-microbial interaction under abiotic and biotic stress conditions, molecular signaling mechanisms underlying PGPR-mediated amelioration of stresses and their biotechnological applications. Beginning his professional career in 2008, Dr. Sridev Mohapatra has almost 10 years of academic experience in the field of Plant Biotechnology. He has published over 10 research papers with good citations in reputed international journals and conferences. He has served as areviewer for many international journals. Currently, his group is engaged in elucidating the precise signaling mechanisms and regulation patterns involved in PGPR-mediated abiotic stress tolerance in plants. He has successfully completed/currently investigating research projects sponsored by DST, DBT and BITS-Pilani.

Biography of the Candidate

Mr. Daipayan Ghosh is a full time Ph. D. student at BITS-Pilani Hyderabad Campus under the supervision of Dr. Sridev Mohapatra in the Department of Biological Sciences. He has obtained his Master's degree (M. Sc.) in Biotechnology from Presidency College, University of Calcutta, India in 2012 and started his research as a research fellow in 2013. He is well versed in various microbial, plant molecular, analytical and protein expression techniques. He has good number of publications and awards to his credit and has presented his work in several national and international conferences. Currently, his career interests are focused on the development of stress-tolerant crop varieties by transgenic technology based on the understanding of plant-microbial interactions.

APPENDICES