"Impact of Plant Growth Promoting Rhizobacteria on Plant Polyamine Metabolism Under Water Stress: Novel Insights into Plant-Rhizobacterial Interaction"

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CERTIFICATE

This is to certify that the thesis entitled **"Impact of Plant Growth Promoting Rhizobacteria on Plant Polyamine Metabolism Under Water Stress: Novel Insights into Plant-Rhizobacterial Interaction"** and submitted by **Sunetra Sen** ID No **2013PHXF0002H** for award of Ph.D. of the Institute embodies original work done by him/her under my supervision.

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Abstract

Plant growth promoting rhizobacteria (PGPR) are a diverse group of beneficial soil bacteria that help plants in growth and development, as well as stress mitigation. A group of aliphatic amines called polyamines are present in all organisms. In plants, they are involved in many cellular processes due to their poly-cationic nature. Polyamines are known to play important roles in abiotic stress amelioration. Here, we report the impact of a free living, drought-mitigating rhizobacterial strain, *Pseudomonas putida* GAP-P45 on the polyamine metabolism of *Arabidopsis thaliana* under water stress. Firstly, morpho-physiological investigation clearly showed significant improvement in growth parameters (phenotypic observations, fresh weight, plant water content etc.) of *A. thaliana* with GAP-P45 inoculation under water-stress. Secondly, GAP-P45 inoculation in *A. thaliana* under water-stress significantly impacted polyamine metabolism. At the transcript level, it caused significant inductions in the expression of polyamine biosynthetic (*ADC, AIH, CPA, SPDS, SPMS, SAMDC)* as well as catabolic genes (*CuAO1-3, PAO1-5)* at different time points of our study. GAP-P45 inoculation under water-stress also increased the activity of the putrescine catabolic enzyme, copper amine oxidase. Putrescine and spermidine accumulated in *A. thaliana* in response to GAP-P45 inoculation under water-stress as compared to the water-stressed seedlings without inoculation. Our results point towards transcriptional and post transcriptional regulation of polyamine metabolism in *A. thaliana* in response to GAP-P45 under water stress. Thirdly, inoculation with GAP-P45 under water stress decreased the levels of reactive oxygen species and significantly lowered the activity of antioxidant enzymes in *A. thaliana* seedlings across the time points of our study. All the observations made in this study positively correlated with *P. putida* GAP-P45 mediated water stress tolerance and improved phenotype of *A. thaliana*.

Chapter 1

Introduction and Review of Literature

1.1.1 Global climate change, environmental stresses and their impact on agriculture

The world population is rapidly increasing and is predicted to reach approximately 10 billion by 2050. Coupled with a steady population growth, the competition for sustainable crop output is expected to aggravate in the coming years (Godfray et al. 2010b, a; Gornall et al. 2010; Tilman et al. 2011; A. Nastis 2012; Godfray and Garnett 2014). The projected scenario demands the agricultural crop production to grow by at least 2–3 % every year to ensure adequate supply for human, livestock and industrial use. Unfortunately, this presents a major challenge what with the ever increasing population, global climate change and corresponding decline in the availability of fertile land and water for agriculture, especially in the developing countries, including India (Mall et al. 2007; Pathak et al. 2012; Kumar et al. 2016). According to experts, in India, climate change has about 4-9 % impact on agriculture every year. As agriculture's contribution to India's GDP is about 15 %, climate change presumably causes about 1.5 % loss in GDP. Besides, by 2030, about 6-10% loss in the yield of staple food crops, such as wheat and rice is predicted. This loss is attributed to the lack of available resources and agricultural inputs, growing demand for irrigation etc. (Mall et al. 2007; Kumar and Gautam 2014; Anand and Khetarpal 2015; Kumar et al. 2016).

In India, agriculture is mostly rain- fed as 50-55% of the total cultivated areas lack efficient irrigation facilities, thereby making water availability the most important factor/ constraint in agricultural crop production. In the last couple of decades, urbanization, rapid industrialization and other developmental initiatives have led to tremendous water usage and consequently a growing demand for the same. Besides, the water cycle in many parts of India has changed drastically due to agricultural practices, such as, variable cropping and land use systems, groundwater exploitation, irrigation, drainage etc. (V. et al. 2012; Kumar and Gautam 2014; Udmale et al. 2014; Kumar et al. 2016). Crop plants are constantly exposed to a repertoire of environmental stresses. Environmental stresses can disrupt cellular structures and impair important physiological processes in crop plants (Cramer et al. 2011; Pereira 2016; Pandey et al. 2017a; Ahanger et al. 2017). Environmental factors predominantly include extremes of temperatures (heat, cold, chilling), drought (reduced precipitation, drying winds), and salinity that adversely affect plant growth and development, and thus crop productivity. However, drought is considered the single most devastating environmental stress, which decreases crop productivity more than any other environmental stress (Strzepek and Boehlert 2010; Elliott et al. 2013; Nelson et al. 2014). India has faced 23 large scale drought episodes since 1891 to 2009 (Kumar and Gautam 2014) and the situation is expected worsen in the near future. A step towards addressing these issues is to identify mechanisms that can be developed into cost- effective and sustainable value-added technologies. Scientists all over the world have thus focused on developing "climate smart crops" (Anand and Khetarpal 2015) through multidisciplinary approaches to be employed towards crop improvement and sustainable doubling of crop productivity.

1.2 A closer look at the physiological and biochemical impact of drought stress on plants

Agricultural drought can be defined as the lack of sufficient moisture required by a plant for normal growth and development during its lifecycle (Mannocchi et al. 2003). This condition arises due to a continuous reduction in rainfall / precipitation (meteorological drought) coupled with a demand for higher evapo-transpiration (Mishra and Cherkauer 2010). Fundamentally, it is a condition marked by diminished soil water primarily leading to decreased water absorption by roots (Jaleel et al. 2009; Farooq et al. 2009a, b, 2012; Zlatev and Lidon 2012). Drought stress adversely affects plant growth and development, thus slowing the crop growth rate and biomass accumulation. This ultimately leads to devastating crop losses worldwide. The physiological and biochemical implications of drought stress on plants have been illustrated in **Fig. 1.1**.

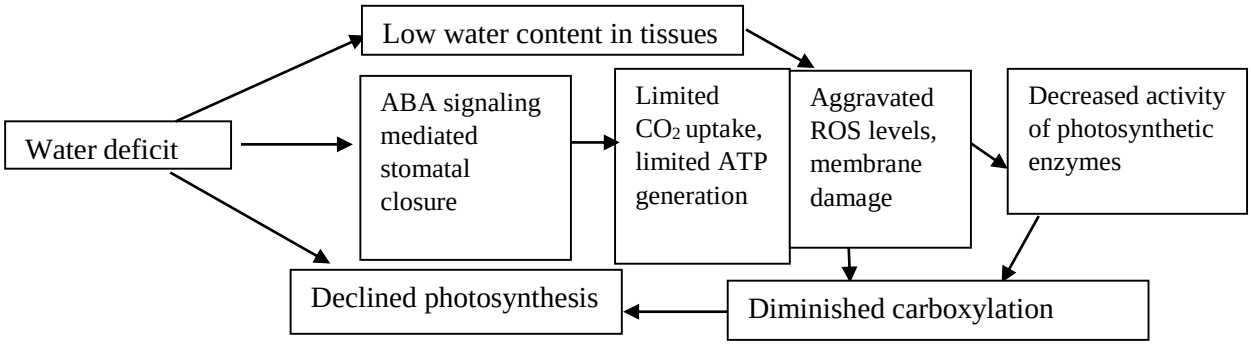

Fig 1.1 Multitude of effects manifested in plants during drought stress (Zlatev and Lidon, 2012)

Drought stress impacts a plant right from the stage of cell growth. Cell growth is accomplished through cell division, cell enlargement and differentiation. Under drought stress conditions, the turgor pressure of the cells decrease significantly, thereby inhibiting cell elongation processes. This occurs when water flow from the xylem to the surrounding elongating cells is interrupted due to severe water deficiency (Nonami 1998; Anjum et al. 2011; Jaiphong et al. 2016; Murtaza et al. 2016). The decrease in tissue water content due to reduced water uptake therefore results in turgor loss. This further results in disorganization of membranes, denaturation and loss of activity of proteins etc. Similarly, drought stress may also decline photo-assimilation processes and the availability of metabolites required for cell division. This leads to an impairment of mitosis, cell elongation and expansion which adversely affects leaf size, stem elongation and root proliferation, disturbs stomatal oscillations, plant water and nutrient relations etc. Overall, these effects may lead to inhibition of photosynthesis, metabolic dysfunction, and structural damage eventually contributing to declined growth, reduced fertility, and premature senescence in plants (Jaleel et al. 2009; Farooq et al. 2009a; Anjum et al. 2011, 2017; Krasensky and Jonak 2012).

The manifestations of drought stress in plants is perceived at various stages of plant growth and development as reviewed by several authors (Jaleel et al. 2009; Farooq et al. 2009a; Zlatev and Lidon 2012). Drought stress has been reported to severely reduce germination in crop plants by affecting the germination potential, hypocotyl length, shoot and root fresh and dry weights etc. Other aspects of crop yield, such as starch biosynthesis and assimilate partitioning affecting grain filling, seed formation and flower production are also known to be impacted in water-stressed crops. Plant water relations determined through various parameters like relative water content, leaf water potential, stomatal conductance, rate of transpiration, leaf temperature etc. are hugely impacted by water deficit. The ratio between water consumed and dry matter produced is termed as water-use efficiency at the whole-plant level (Blum 2005). In the event of water deficit, water-use efficiency is also reduced significantly since the plant growth is inhibited to a greater extent. Water deficit has a huge impact on the acquisition of nutrients by the root and their transport to shoots and thus limits total nutrient uptake in plant tissues, leading to decreased absorption of the inorganic nutrients and further compromising their metabolism in plant tissues (Blum 2005; Franks et al. 2015; Ruggiero et al. 2017). Prolonged period of water deficit disrupts photosynthesis in plants and is associated with alterations in carbon and nitrogen assimilation. This results from a decrease in leaf expansion, impaired photosynthetic machinery, premature leaf senescence etc. leading to reduced food production in plants (Anjum et al. 2011, 2017; Zlatev and Lidon 2012; Krasensky and Jonak 2012; Kaur and Asthir 2017). Drought-induced stomatal closure is a crucial response generated in plants that limits CO_2 uptake by leaves making them more susceptible to photo-damage (Daszkowska-Golec and Szarejko 2013; Tombesi et al. 2015; Pirasteh-Anosheh et al. 2016) thereby causing a progressive decline in photosynthesis. Other important components of the photosynthetic machinery that are damaged or altered due to drought stress include the photosynthetic pigments and enzymes and other proteins (Pinheiro and Chaves 2011; Zargar et al. 2017; Wang et al. 2018) all of which significantly diminish crop yield (Anjum et al. 2011; Zlatev and Lidon 2012; Golldack et al. 2014). Another important aspect that inhibits plant growth and photosynthetic capacity is the imbalance in oxidative status of plants (Va Hideg et al. 2000; Foyer and Shigeoka 2011). Water deficit in the rhizosphere (soil adhering to roots) leads to an increased rate of respiration in the roots of plants leading to an imbalance in the utilization of carbon resources thereby decreasing ATP production which in turn leads to excessive generation and accumulation of reactive oxygen species (ROS). They include superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O_2$). These are partially reduced or activated forms of atmospheric oxygen (O_2) produced as a result of aerobic metabolism in life forms. This further translates into oxidative stress, impacting proteins, membrane lipids and other cellular components leading to deleterious effects in plants (Cruz de Carvalho 2008; You and Chan 2015).

1.3 Plant adaptation strategies and drought- tolerance mechanisms/responses in plants

Plants, due to their sessile nature, are most frequent and obvious victims of diverse environmental stresses, as previously stated. Hence, nature has equipped plants with a variety of stress tolerance mechanisms in order to cope with these challenges. Plants undergo a plethora of cellular or molecular alterations like quick induction of specific genes, leading to responses such as accumulation of organic solutes, stomatal closure etc. as important physiological adaptations to most abiotic stresses. Drought stress reprograms the transcriptional, post-transcriptional and metabolic processes in plants, therefore causing morpho-physiological modifications (Krasensky and Jonak 2012; Anjum et al. 2017).

The range of mechanisms by which plants respond to drought are the result of an integration of the diverse effects of stress and thus, responses are generated at all levels of organization over space and time (Farooq et al. 2009a; Zlatev and Lidon 2012; Krasensky and Jonak 2012). These responses are broadly classified into stress avoidance and stress tolerance strategies. Plants are able to avoid stress by delaying its negative impact through certain adaptive mechanisms. On the other hand, stress tolerance is the ability of a plant to acclimatize to stressful conditions (Touchette et al. 2009). These include modifications to the membrane system, cell wall architecture and cell cycle (Tenhaken 2015). At the molecular level, several stress- inducible/ responsive genes are modulated which encode crucial proteins involved in the synthesis of osmo-protectants, detoxifying enzymes, protein kinases and phosphatases, thereby altering the metabolism of several stress-specific metabolites, ultimately contributing to stress protection (Lawlor 2011; Chelli-Chaabouni 2014). Osmotic adjustment, one of the major mechanisms to maintain cell turgor in many plants, involves the net accumulation of compatible solutes in a cell in response to a reduction in the water potential. As a consequence of this accumulation, the osmotic potential of the cell is lowered. Thereby, water is attracted into the cell and turgor pressure is maintained. This further enables water uptake into the cell thereby maintaining plant metabolic activity, growth and productivity (Lidon 2012; Krasensky and Jonak 2012). These compatible solutes include amino acids (e.g. proline, GABA), amines (polyamines, glycine betaine), carbohydrates and sugar alcohols (raffinose, trehalose, fructans, sorbitol) etc. that accumulate in plants on sensing low water potential. These compounds are able to stabilize proteins and cellular structures and/or maintain turgor pressure in cells by osmotic adjustment. Some of these metabolites also contribute to redox metabolism through the modulation of their own metabolic pathways, thereby maintaining the redox equilibrium in plant cells (Bartels and Sunkar 2005; Valliyodan and Nguyen 2006; Munns and Tester 2008; Janská et al. 2010). The modulation/ accumulation of these metabolites are also substantially influenced or regulated by important phytohormones and transcription factors. The stress hormone, abscissic acid (ABA) is an integral regulator of abiotic stress signaling (Tuteja 2007; Rock et al. 2009; Zhu 2016). ABA promotes stomatal closure, inhibits stomatal opening to reduce water loss by transpiration and induces the

expression of numerous stress-related genes. Recent studies have also indicated the role of ABA in regulation of stress-induced metabolic adjustments. Modulation of some stress-related transcription factors have been shown to induce changes in stress-associated metabolite levels through modulated transcriptional responses to stress (Saddhe et al. 2017; Vishwakarma et al. 2017).

1.4 Polyamines and their involvement in plant stress response

Polyamines (PAs) are ubiquitous, low molecular weight, aliphatic, positively charged amines which are implicated in a variety of cellular processes. The major PAs, putrescine (Put), spermidine (Spd) and spermine (Spm) are present in all cellular compartments in actively proliferating cells of most organisms. The tetra-amine, spermine was first discovered in human spermatozoa by Leeuwenhoek in 1678 (Fariduddin et al. 2013). Polyamines are known to exist in free as well as conjugated forms. These molecules are extremely dynamic in terms of their concentrations in different species, organs and tissues and are influenced by the developmental stage of the organism. Polyamine homeostasis in cells is crucial and is tightly regulated through its biosynthesis, catabolism and conjugation with other compounds in the cells. Since PAs are poly-cationic at physiological pH, they are capable of electrostatically interacting with negatively charged macromolecules such as DNA, RNA, proteins and phospholipids and thus, reversibly stabilize them (Kaur-Sawhney et al. 2003; Miller-Fleming et al. 2015). A large spectrum of studies have thoroughly investigated and demonstrated the role of PAs in a variety of regulatory and cellular processes such as cell division and elongation, replication, transcription, translation, membrane and cell wall stabilization, chromatin organization, ribosome biogenesis, and programmed cell death (Takahashi and Kakehi 2010; Masson et al. 2017). In addition to their role in developmental processes, PAs are among the most effective compatible solutes implicated against abiotic stresses. The role of PAs in biotic and abiotic stress tolerance in plants has been reviewed by premier workers in this field. Richards and Coleman, (1952), the pioneers of PA research were the first to report Put accumulation under potassium deficiency. Thereafter, various plant species exposed to a gamut of abiotic stresses such as drought, salinity, low temperature, oxidative stress and metal toxicity have been studied universally (Fariduddin et al. 2013). These studies have elucidated the involvement of PAs as stress markers/ stress ameliorators under severe environmental stresses. The findings from these studies further implicate and reinforce the role of PA accumulation in promoting stress tolerance. Besides, the patterns and concentrations of PAs accumulated under different kinds of stresses in a variety of plant species have been found to be extensively diverse (as reviewed by Fariduddin et al. 2013, Liu et al., 2015 etc.). In several cases, PA accumulation has been associated with plant tolerance to water stress in various plant species (Yang et al. 2007; Groppa and Benavides 2008; Takahashi and Kakehi 2010; Alcázar et al. 2010; Minocha et al. 2014; Shi and Chan 2014 etc.). As reviewed by premier workers (Atkinson and Urwin 2012; Arora and Pande 2017; Jeandroz and Lamotte 2017; Kumar et al. 2018), during the last decade, different approaches have been undertaken by

plant scientists all over the world to generate plants tolerant to a multitude of abiotic stresses. In the recent years, PA research has been found to focus on a few important strategies for stress response and amelioration (Alcázar et al. 2006; Marco et al. 2011; Bitrián et al. 2012; Sequera-Mutiozabal et al. 2016). These can be enumerated as follows: (i) transgenic manipulation of PA biosynthetic pathway up regulating candidate gene expression has led to an increase in tolerance to a variety of stresses (ii) increased expression of PA biosynthetic genes and activities of their corresponding enzymes is accompanied by an increase in the PA accumulation in plants promoting stress tolerance; (iii) mutant studies have revealed function of PAs in stress tolerance such that mutants of PA biosynthesis have compromised tolerance to stress (iv) while exogenous supply of PAs to plants increase tolerance to stress, plants with inhibited PA biosynthesis are more prone to stress damage. A few examples of PA function in stress tolerance and the implemented strategies are illustrated in **Table 1.** Polyamines not only act as indicators of stress, but also play major roles in stress amelioration. As stress indicator molecules, the concentration of PAs is usually reported to elevate under environmental stresses. As stress ameliorators, PAs often accumulate in cells to serve as compatible solutes, scavenge ROS and promote antioxidant activity, act as signaling molecules in the ABA regulated H_2O_2 and NO production in stress response pathways, regulate ion channels and participate in programmed cell death. Overall, PAs balance the carbon: nitrogen ratio in cells. At both the national and international levels, premier workers have targeted the PA metabolic pathway as a potential tool in agricultural biotechnology to troubleshoot various challenges encountered by the plants during their lifecycle.

Table 1. Strategies and scientific tools implemented to characterize the function of PAs in response to drought stress.

Strategy 1: **Genetic manipulation of candidate genes → Accumulation of one or more PAs → Stress Recovery/ tolerance**		
Genes manipulated	**Hosts**	**References**
ADC	*Oryza sativa* *Solanum meloangena,* *Triticum aestivum,* *Arabidopsis thaliana,* *Nicotiana tabacum,* *Solanum lycopersicum*	Capell et al. 2004, Prabhavathi and Rajam 2007, Bassie et al. 2008, Alcázar et al. 2010, Wang and Liu 2009; Wang et al. 2011b, a
SAMDC	*Nicotiana tabacum,* *Oryza sativa,* *Solanum lycopersicum*	Waie and Rajam 2003, Wi et al. 2006, Peremarti et al. 2009 Hazarika and Rajam 2011

SPDS	*Arabidopsis thaliana, Ipomoea batatas*	Kasukabe et al. 2004, 2006
Strategy 2: **Knockouts to generate single gene mutants to study involvement of PAs in stress tolerance**		
ADC, SPDS, SPMS	*Arabidopsis*	Alcázar et al. 2011
SPMS	*Arabidopsis*	Yamaguchi et al. 2007
CuAO1	*Arabidopsis*	Wimalasekera et al. 2011
Strategy 3: **Exogenous application of PAs to facilitate stress response and tolerance**		
Type of PAs	**Hosts**	**References**
Put, Spd, and Spm	Bermuda grass	Shi et al. 2013
Spm	*Arabidopsis*, White clover	Yamaguchi et al. 2007; Li et al. 2015

1.5 Plant growth promoting rhizobacteria

The soil shelters and supports the existence of a huge and diverse microbial community. The terrestrial photosynthetic plants have evolved as engineers to create and establish specific microbial niches in order to facilitate their own sustainability with respect to growth, development and protection from unfavorable conditions. The layer of soil attached or surrounding the roots is called rhizosphere (Walker et al. 2003). A group of bacteria that are capable of colonizing the rhizosphere/root environment are termed as 'rhizobacteria' (Kloepper and Scroth. 1978; Zablotowicz et al. 1991).. Plant roots synthesize, accumulate, and secrete chemical compounds (root exudates) (Walker et al. 2003) that attract vast number of heterogeneous bacterial populations thereby promoting plant-beneficial/pathogenic interactions. The plant growth promoting rhizobacteria (PGPR), are a class of rhizospheric bacteria that are characterized by the following inherent properties: (i) they colonize the root either endogenously or on the surface (ii) they are able to survive, multiply and compete with other organisms in the soil ecosystem and (iii) they promote plant growth under normal and adverse conditions (Zablotowicz et al. 1991; Antoun and Prévost 2005; Fuentes-Ramirez and Caballero-Mellado 2005; Ngumbi and Kloepper 2016 etc.) Gray and Smith (2005) have classified PGPR into extracellular (ePGPR) and intracellular (iPGPR) based on the degree of bacterial proximity to the root and intimacy of the plant- microbial association. While ePGPR exist in the rhizosphere, on the rhizoplane, or between cells of the root cortex, the iPGPR colonize inside root cells, forming nodules. Some examples of ePGPR include *Azotobacter, Azospirillum, Bacillus, Burkholderia, Pseudomonas* etc. (Figueiredo et al. 2010; Bhattacharyya and Jha 2012; Vacheron et al. 2013; Ahemad and Kibret 2014). These bacteria are free- living and associate with the plant through facultative symbiosis. However, the iPGPR are obligate symbionts and include the genera *Allorhizobium, Azorhizobium,*

Rhizobium etc. Based on their functionality, Somers et al (2004) have broadly classified the PGPR as (i) biofertilizers (promoting nutrient solubilization), (ii) phytostimulators (plant growth promotion, generally through phytohormones), (iii) rhizoremediators (degrading organic pollutants) and (iv) biopesticides (as biocontrol agents, through the production of antifungals, antibiotics, lytic enzymes etc). Several authors have explored the mechanisms of action manifested by PGPR in promoting plant growth and they include a host of direct and indirect mechanisms. Direct mechanisms of PGPR action mainly involve facilitating resource acquisition and hormonal modulation in plant, while indirect mechanisms mostly include bio-control activities of PGPR by counteracting pathogenic invasion and adversities on plant growth and development (Glick 2012). A detailed description of each of these mechanisms as reported by several premier workers is followed.

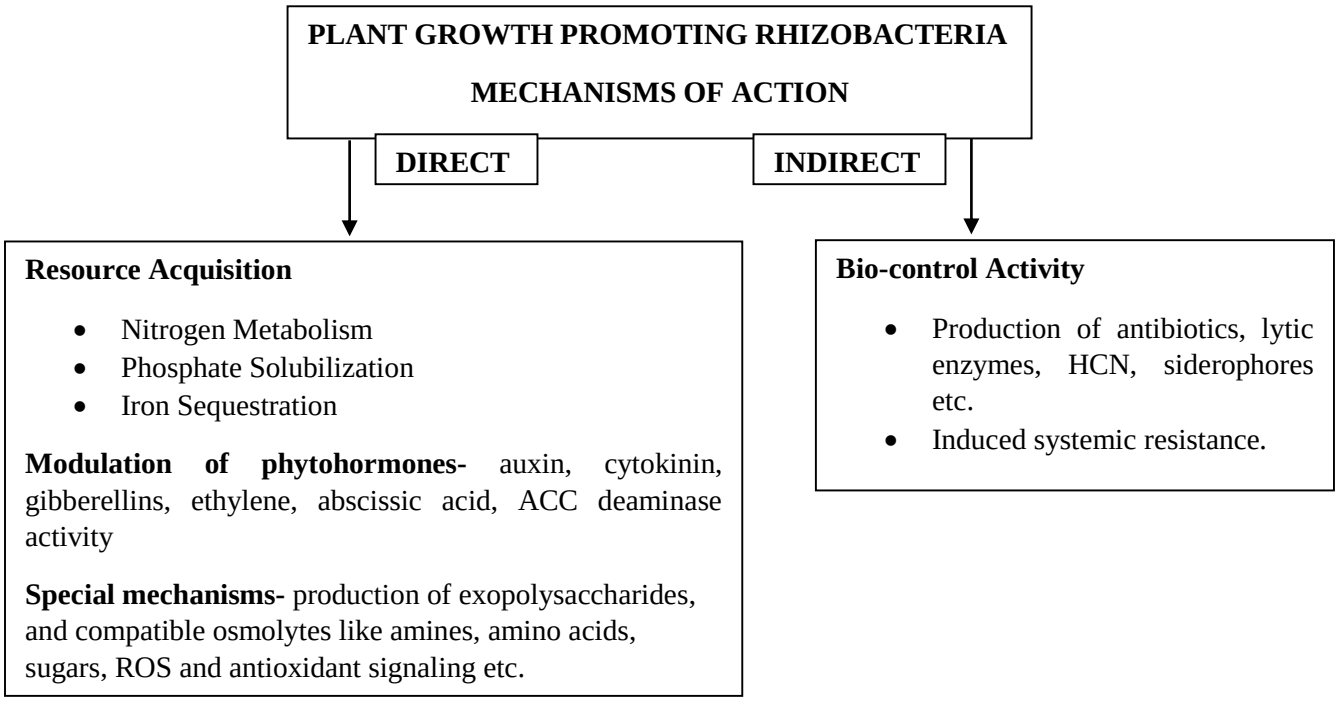

Fig. 1.2 Diverse mechanisms of action of PGPR-mediated plant growth promotion

(Bhattacharyya and Jha 2012; Glick 2012; Ahemad and Kibret 2014 etc.)

1.5.1 Direct mechanisms of plant growth promotion by PGPR

(1) Nitrogen fixation

Nitrogen fixation is a crucial process by which atmospheric N_2 is converted into usable form for plant growth and productivity. About two-third of the atmospheric nitrogen is fixed biologically by symbiotic (eg. *Rhizobium*) and non- symbiotic (eg. *Azotobacter*, *Azospirillum*) bacteria. The biological N_2 fixation is accomplished by the aid of the nitrogenase enzyme complex that reduces N_2 to NH_3 which can be taken up

by the plants. While the non- symbiotic fixers provide only a small amount of the fixed nitrogen to the host plants, the symbiotic N_2 fixers undergo intracellular colonization in the plant roots by nodule formation and establishes a more sustainable mode of N_2 fixation in leguminous plants. The key genes, called 'nif' genes, involved in the process of nitrogen fixation, are found in symbiotic as well as free living organisms. The biological nitrogen fixation is an economically viable and environment-friendly alternative for the use of harmful chemical fertilizers.

(2) Phosphate solubilization

The second most important macronutrient after nitrogen which is required for plant growth and development is phosphorus. However, despite its abundant availability in soil, plants are unable to utilize it directly thereby leading to decline in their growth and productivity. This low availability of phosphorous to plants is because of the fact that majority of phosphorus in soil exists in insoluble forms, while the plants can absorb only the monobasic/ dibasic ions (Bhattacharyya and Jha 2012). A class of phosphate solubilizing microorganisms (PSM) have the ability to synthesize organic acids by the aid of which they are able to solubilize the inorganic phosphorus in soil, thus making it available to the plants. Bacteria in the genera *Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Pseudomonas, Rhizobium* and *Serratia* are reported to be the best phosphate solubilizers. They act as biofertilizers and augment plant growth.

(3) Exopolysaccharide production

Exopolysaccharides are a class of bacterial polysaccharides that are synthesized as extracellular material by cell- wall anchored enzymes and are secreted into the external environment. These are essentially carbohydrate polymers which play a vital role in the formation of biofilms (Bhaskar and Bhosle)and provide protection to the bacteria during desiccation. Exopolysaccharide production is reported to occur under stressed conditions by several PGPR such as *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Streptococcus mutans.* They remain either associated with the cell wall to form a bound capsule layer or they can be released in the vicinity of cells as extracellular slime (Glick et al. 1999). Exopolysaccharides have been reported to improve the moisture-holding capacity. Sandhya and Ali (2015) reported that the inoculation of EPS producing *Bacillus* spp. strains in soil resulted in improved soil permeability through increased aggregation under drought stress conditions. The acquisition of drought tolerance in *Bacillus s*pp. has been reported to be associated with various structural and metabolic changes including a decrease in metabolism and an increase in carbohydrates such as exopolysaccharides (Vardharajula and Sk Z 2014)The presence of EPS in soil bacteria has been described primarily in *Rhizobium* spp. that led to an increased water retention through the mechanism of soil aggregation (Burkert 2016).

(4) Production of compatible solutes

As mentioned earlier, osmotic adjustment is one of the crucial responses of plants to combat stressed conditions. The accumulation of several compatible organic solutes like sugars, polyamines, betaines, quaternary ammonium compounds, polyhydric alcohols, proline and other amino acids (Yancey et al. 1982; Yancey 2001, 2005) and water stress proteins like dehydrins are reported in plants. As mentioned earlier, osmotic adjustment refers to the net accumulation of solutes in a cell due to a lowering in water potential in the cell. Consequently, the osmotic potential of the cell is lowered by the accumulation of solutes, thereby attracting water into the cell and making up for the turgor loss. Few studies have reported the enhanced accumulation of compatible solutes like proline and polyamines in PGPR under stressed conditions (Kohler et al. 2008; Cass et al. 2009; Xie et al. 2014; Zhou et al. 2016). There are scant reports suggesting production of these osmolytes in response to stress by PGPR like *Bacillus, Pseudomonas, Azosprillum* etc. when inoculated with plants. Constitutive accumulation (by overexpression of the responsible gene) of cellular osmolytes is rapidly emerging as a popular approach to enhancing drought tolerance in crops (Bohnert and Shen 1999; Wani et al. 2013). The involvement of PGPR in the production of compatible solutes can thus have promising applications in terms of improving plant growth productivity under water stress.

(5) Production of phytohormones

Plant hormones are crucial in terms of their involvement in growth, development and response to the environment. Whenever plants encounter unfavorable conditions, they modulate the endogenous hormonal levels to mitigate the adverse effects of stress. Several lines of evidences suggest the role of PGPR in modulation of hormonal levels of plants by their ability to synthesize and release hormones such as IAA, GA, cytokinin, ABA etc. as secondary metabolites under environmental stresses. Few examples of bacteria reported to produce IAA include *Agrobacterium, Pseudomonas, Bradyrhizobium, Rhizobium, Azospirillum, Klebsiella, Enterobacter* etc. The drought mitigating *Pseudomonas putida* GAP-P45 has been reported to alleviate water stress by altering endogenous phytohormone accumulation and re- distribution in the roots and shoots of *A. thaliana* under water deficit conditions (Ghosh et al. 2018). Production of auxin by *Bacillus* has been reported in association with improved growth effect on Solanum sp. (Ahmed and Hasnain 2010). It has been reported earlier that the amount of IAA production in plants influences root morphology and functioning thereby modulating nutrient accessibility in soil (Spaepen and Vanderleyden 2011). Cytokinins are known to play a major role in cell division, vascular differentiation and induce the proliferation of root hairs. Cytokinins are known to be actively produced by strains of *Azotobacter, Rhizobium, Rhodospirillum, Pseudomonas, Bacillus* etc. The oriental Thuja seedlings inoculated with cytokinin-producing *Bacillus subtilis* strains were found to be more resistant to drought stress (Liu et al. 2013). The involvement of gibberellin in seed germination, floral induction, flower and fruit development etc. is known for many years.

Gibberellin- producing *Sphingomonas* sp. LK11 promoted growth in tomato seedlings (Khan et al. 2014). Environmental factors that stimulate the hormone production in PGPR include carbon limitation, oxidative stress etc. The plant hormone, ABA, is well known for its involvement during drought stress. During water stress, an increased ABA-mediated stomata closure limits water loss (Bauer et al. 2013). Cohen et al. , (2008) reported that inoculation with *Azospirillum brasilense* Sp245 led to an increase of ABA content in *Arabidopsis*, especially when grown under osmotic stress. ABA is also known to affect the accumulation of osmotic solutes under different stress conditions. Therefore, the PGPR mediated modulation of endogenous hormonal levels in plants is an important aspect that can be explored to develop novel management strategies for sustainable agriculture.

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

Apart from being a plant growth regulator, ethylene has also been established as a stress marker (Saleem et al. 2007). The endogenous level of ethylene is significantly increased under stress conditions like those generated by salinity, drought, water logging, heavy metals and pathogenicity, thereby negatively affecting the overall plant growth. The negative impact of high ethylene concentration is characterized by defoliation, absciscion, premature senescence and other cellular processes ultimately leading to reduced crop productivity (Saleem et al. 2007; Bhattacharyya and Jha 2012). Plant growth promoting rhizobacteria such as *Acinetobacter, Achromobacter, Agrobacterium, Alcaligenes, Azospirillum, Bacillus, Burkholderia, Enterobacter, Pseudomonas, Ralstonia, Serratia and Rhizobium* etc. (Shaharoona et al. 2006; Zahir and Arshad 2007; Saleem et al. 2007; Nadeem et al. 2009) are capable of producing the enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Such rhizobacteria take up the ethylene precursor ACC and by the aid of ACC deaminase, convert it into 2-oxobutanoate and NH_3 thereby lowering ethylene production. This attribute of PGPR is known to promote plant growth and protect under high salt and drought stress (Zahir and Arshad 2007; Nadeem et al. 2009). Other forms of stress which are known to be relieved by ACC deaminase producers include that of pathogen attack, stress from polyaromatic hydrocarbons, heavy metals, radiation, wounding, insect predation etc. (Glick 2005; Viterbo et al. 2010). ACC- deaminase producing bacteria are known to promote plant root elongation, shoot growth, nodulation, nutrient soluilization, mycorrhizal colonization in various crops etc. (Glick 2005; Shaharoona et al. 2006; Zahir and Arshad 2007; Nadeem et al. 2009).

(7) Iron sequestration

Iron is an important nutrient required by all forms of life in varying concentrations. However, in the aerobic environment, iron occurs principally as Fe^{3+} thereby forming insoluble hydroxides and oxyhydroxides, making it generally inaccessible to both plants and microorganisms (Rajkumar et al. 2010). Bacteria are

commonly known to secrete siderophores which are low molecular mass iron chelators. Under conditions of iron limitation, siderophores help in sequestration of iron from minerals and organic compounds (Indiragandhi et al. 2008). Siderophores can also complex with other metals like Al, Cd, Cu, Ga, In, Pb and Zn, as well as with radionuclides including U and Np (Kiss and Farkas 1999; Neubauer et al. 2000) thereby increasing soluble metal concentration in soil and alleviating heavy metal stress in plants (Rajkumar et al. 2010) Plants can assimilate iron from bacterial siderophores by chelation, direct uptake of siderophore-Fe complexes, or by a ligand exchange reaction (Ellermann and Arthur 2017). Improved plant growth due to an increase of iron uptake inside plant tissues was reported in *Arabidopsis thaliana plants* when inoculated with siderophore producing *Pseudomonas fluorescens C7* (Vansuyt et al. 2007). Similar reports suggest improved iron nutrition in plants through microbial synthesis of siderophores leading to crop improvement have been reported in oat (Crowley and Kraemer 2007). maize (Sharma and Johri 2003) etc.

1.5.2 Indirect Mechanisms

The indirect mechanisms by which PGPR promote plant growth and productivity involve their role as biocontrol agents. Their biocontrol activity is mediated by the production of antibiotics, lytic enzymes, HCN etc. (Glick 2005). Many rhizobacteria have been reported to produce antifungal metabolites like, HCN, phenazines etc. (Saharan and Nehra 2011; Bhattacharyya and Jha 2012; Bishnoi 2015) thereby protecting the plants from pathogenic invasion and nutrient limitation. Certain plants are known to develop resistance against some pathogenic bacteria, fungi, and viruses through the biocontrol that is imposed by plant- microbial interactions, a phenomenon called induced systemic resistance (ISR) (Lugtenberg and Kamilova 2009). Moreover, ISR stimulate the host plant's defense responses through jasmonate and ethylene signaling within the plant thereby protecting the plants against a variety of plant pathogens (Glick 2015). Many individual bacterial components, such as lipopolysaccharides (LPS), flagella, siderophores, and volatiles like, acetoin and 2, 3-butanediol (Lugtenberg and Kamilova 2009) are also known to promote ISR in crop plants.

1.5.3. Role of PGPR in drought stress tolerance

There are several direct and indirect mechanisms by which PGPR impact plant growth and productivity under normal as well as stressed conditions. The role of PGPRs on abiotic stress tolerance/mitigation in plants has been investigated thoroughly in the recent years. A wealth of information has emerged out of the work conducted by national and international groups. As reviewed by premier workers, PGPR mediated drought stress tolerance in plants include mechanisms like phytohormonal activity, generation of volatile compounds, alteration in root morphology, ACC deaminase activity, accumulation of osmolytes, EPS production, antioxidant defense and co-inoculations with other organisms etc. When inoculated with plants,

the beneficial plant- microbial interaction induces physical and chemical changes in plants, which result in enhanced tolerance to abiotic stresses, commonly known as induced systemic tolerance (Yang et al. 2009). Some examples of free- living PGPR- mediated drought stress tolerance in various plant species are enlisted below in Table 2. These are classified based on the different mechanisms employed by each rhizobacteria in mediating the drought stress amelioration (Vurukonda et al. 2016).

Table 2: PGPR mediated drought stress amelioration and improved plant growth through direct and indirect mechanisms.

Name of the PGPR strain	Host Plant	Mechanism of action	Reference
Azospirillum. Brasilense	Tomato, Wheat, *Phaseolus vulgaris, Arabidopsis*	Phyto-hormonal modulation, increase in IAA, ABA etc.	Creus et al. 2005, Arzanesh et al. 2011, German et al. 2000, Cohen et al. 2008
	Maize, Rice	Production of compatible solutes like trehalose, proline, cadaverine etc.	Rodrigues et al. 2009, Casanovas et al. 2002, Cass et al. 2009
	Wheat	Alteration of root morphology Induction of drought responsive gene expression	Bashan et al. 1992 Kasim et al. 2013
Azospirillum lipoferum	Maize	Production of compatible solutes, phytohormone modulation	Bano et al. 2013, Cohen et al. 2009
Bacillus subtilis	*Platycladus orientalis,*	Phyto- hormonal modulation through synthesis of ABA, cytokinin,	Liu et al. 2013
	Arabidopsis	Induction of drought responsive genes, production of compatible solutes	Zhang et al. 2010
Bacillus polymyxa, Bacillus megaterium BOFC15	Tomato, *Arabidopsis*	Production of compatible solutes like proline, spermidine etc.	Shintu and Jayaram 2015, Zhou et al. 2016

Bacillus thuringiensis	Wheat	Reduction of volatile emissions	Timmusk et al. 2014
Bacillus sp.	*Cicer arietinum*, pepper	ACC- deaminase activity	Sharma and Khanna 2013, Hui and Kim 2013
Bacillus thuringiensis AZP2, Bacillus licheniformis	Wheat, Pepper	Induction of specific genes	Timmusk and Wagner 1999, Lim and Kim 2013.
P. putida H-2–3	Soyabean	Phytohormonal modulation	Kang et al. 2014
Pseudomonas putida GAP-P45 and P. fluorescens	Maize, tomato	Production of compatible solutes	Sandhya et al. 2010a, Gou et al. 2015, Ansary et al. 2012
P. fluorescense			Mayak et al. 2004
Pseudomonas putida GAP-P45,	Tomato and pepper	ACC- deaminase activity	Sandhya et al. 2010
Proteus penneri (Pp1), Pseudomonas aeruginosa (Pa2)	Sunflower, maize	EPS production	
Pseudomonas chlororaphis O6	*Arabidopsis*	Volatile emissions	Cho et al. 2008

1.6 Gaps in existing research

1. As evident from the detailed review, the unique properties of PGPRs present excellent opportunities to explore and develop sustainable technologies for crop improvement under drought stress. Our knowledge of the mechanisms governing PGPR- mediated plant responses to abiotic stress from a physiological perspective has considerably increased during the last few years. However, the molecular mechanisms governing PGPR function for stress amelioration have never been thoroughly investigated. Therefore the lack of knowledge with respect to the molecular basis of plant- PGPR interactions is the most predominant gap in this research.
2. As mentioned earlier, studies on the role of PAs as stress markers/ stress ameliorators have emerged to be truly promising in the last few decades. However, we are still far from understanding the intricate

regulation and coordination of these complex pathways. Although PA biosynthesis has been explored in response to stress exposure by several authors, research on PA catabolism at the molecular level is still in its infancy. Therefore, comprehensive investigation of the PA metabolic pathway at the molecular level is crucial to understand its regulation during stress responses.

3. Based on our review of literature on plant-PGPR interactions, few instances suggest a possible link between PA metabolism and PGPR mediated stress tolerance. For example, the accumulation of compatible osmolytes such as proline, GABA and ethylene are known to be affected by PGPR inoculation under water stress. The PA metabolic pathway lies in the intersection of these important metabolic pathways and is connected to each of these metabolites in one or another way. Besides, there are scant reports on the accumulation of PAs in plants by symbiotic (Cass et al. 2009; Zahedi and Abbasi 2015) and free- living (Xie et al. 2014; Zhou et al. 2016) PGPRs, with respect to biotic/abiotic stress tolerance. These examples indeed point towards the possibility of an overlap/connection between PGPR mediated stress responses and modulation of PA metabolism in plants under stress. However, a thorough and comprehensive understanding of their relationship/correlation/regulation at the molecular level is scarce. Enumeration of this link and downstream effects thereof is thus the purpose of this research.

1.7 Scope and objectives of the Study

In this study, we have intended to explore the molecular basis of plant-PGPR interactions with special emphasis on PA metabolism in response to drought stress. The major objective of our study is to investigate the role of drought mitigating soil bacteria on PA metabolism under dehydration conditions.

For our analysis, we have selected the model plant *Arabidopsis thaliana* to conduct our experiments. *Arabidopsis* has several advantages over other plant species due to its completely sequenced genome and availability of mutants for several genes involved in various metabolic pathways, including the PA metabolic pathway. Based on available literature we selected *Pseudomonas putida* GAP-P45 to conduct our research. This strain was originally isolated by Sandhya et al., (2009) from the rhizospheric region of sunflower plants grown in semi-arid zones of Hyderabad, India and characterized for its drought ameliorating activity. In both sunflower and maize, inoculation with this particular strain under drought stress led to an improved root and shoot biomass, root and shoot length, relative water content, leaf water potential and minimized electrolyte leakage (Sandhya et al. 2009, 2010a). Furthermore, the strain GAP-P45 had the ability to produce exopolysaccharide, HCN, ammonia, siderophores and phytohormones such as, IAA, GA and cytokinin, therefore serving as a potential PGPR (Sandhya et al. 2010c). The authors (Sandhya et al., 2010a) also observed an

improvement in the physiological and biochemical profile of maize plants under drought stress characterized by elevated accumulation of cellular proteins, amino acids, proline, total soluble sugar and starch when inoculated with *P. putida* GAP-P45. Moreover, significant lowering in the activities of antioxidant enzymes (APX, CAT, GPX) in GAP-P45 inoculated plants as compared to non-inoculated plants was reported under drought stress.

Specific objectives-

Based on the literature review and gaps identified, the specific objectives of this research aimed at understanding the molecular intricacies of plant-PGPR interactions with respect to plant- polyamine metabolism can be enlisted as follows.

1. To test and confirm the efficacy and cross- compatibility of the selected drought- tolerant PGPR strain, *Pseudomonas putida* GAP-P45 on growth promotion and water stress mitigation in *Arabidopsis thaliana* through characterization of certain plant growth specific morpho-physiological parameters in a soil- free experimental set- up.
2. To delineate the impact of *P. putida* GAP-P45 on polyamine metabolism in *A. thaliana* under drought stress; specifically to study/analyze the
    (i) expressions of target genes involved in the PA biosynthetic pathway in response to GAP-P45 inoculation under normal and water-stressed conditions.
    (ii) expressions of target genes and enzymes involved in the PA catabolic pathway in response to GAP-P45 inoculation under normal and water-stressed conditions.
    (iii) accumulation pattern of major PAs Put, Spd ,Spm and the Put catabolic product GABA in response to GAP-P45 inoculation under normal and water-stressed conditions and correlate them with the overall PA metabolism under water stress to understand its role in PGPR-mediated stress tolerance (if any).
3. To study the impact of *P. putida* GAP-P45 on redox state of *A. thaliana* under water stress through analysis of ROS levels and activities of antioxidant enzymes.

Chapter 2

Impact of *Pseudomonas putida* GAP-P45 on the morpho-physiological status of *Arabidopsis thaliana* under water stress.

2.1 Introduction

The impact of drought in plants has been thoroughly discussed and reviewed in the previous chapter. Prior to proceeding with the molecular studies, it was crucial to test the efficacy and the morpho-physiological impact of the selected PGPR strain, *Pseudomonas putida* GAP-P45 on our model plant *Arabidopsis thaliana* under normal and water-stressed conditions. Hence, our laboratory analyzed the overall health of *A. thaliana* when inoculated with *P. putida* GAP-P45 with or without water stress through several parameters such as visual observation of plant growth, fresh weight, dry weight, plant water content, root length, chlorophyll content etc. [Data published in Ghosh et al. (2017); part of Ghosh, D., Ph.D. thesis (2018)].

2.2 Materials and Methods

2.2.1 Plant growth, maintenance and treatments

Wild type (Columbia-0) *A. thaliana* seeds were surface sterilized, stratified in the dark at 4 ^{o}C to break dormancy and sown on square pieces of autoclaved, stainless-steel mesh (0.01 inch wire diameter, 0.015 inch clear opening) in Petri plates containing half strength, sterile Murashige and Skoog (MS) medium (Murashige and Skoog 1962), supplemented with 0.8 % agar and 1 % sucrose. The seeds were then incubated in a controlled environment at 22 (±1) ^{o}C and a 16/8 h light/dark cycle with 150 µmol m^{-2} s^{-1} light intensity at 50-70 % relative humidity. A week after germination, the individual meshes containing seedlings at the 4-leaved stage were transferred to Magenta boxes containing agar-supplemented MS medium for various experiments. Each mesh contained 5-7 seedlings and each Magenta box contained 4 of these meshes. Water-stress was induced 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). The PEG- infusion method for preparation of media was adapted from van der Weele (2000) and has been described in Ghosh et al. (2017) and Sen et al. (2018). Before starting an experiment, *P. putida* GAP-P45 was grown overnight in Luria Bertani (LB) broth (Bertani 1951) in a shaking incubator at 28 ^{o}C 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 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, at least

three replicate Magenta boxes were used, each Magenta box containing 4 meshes, each with 5-7 seedlings.

Throughout the study, bacterial inoculum was collected from MS medium (stressed, non- stressed, inoculated and non- inoculated conditions) and streaked on LB plates to monitor colony formation and growth of *P. putida* GAP-P45. The growth of *P. putida* GAP-P45 was monitored throughout the study by collecting inoculum from MS medium (both inoculated with GAP-P45 and non- inoculated) and checked for colony formation on LB plates. Alongside the use of GAP-P45, the common laboratory strain *E. coli* was used as a negative control in plant growth experiments and inoculated with *A. thaliana* in order to rule out any possible drought mitigation by mere inoculation of bacteria in the medium. Water potential measurements were conducted to detect any plausible change in water potential due to the bacterial growth on MS-agar media. As described in Ghosh et al. (2017), adding 200 µL water caused no significant change to the water potential of the medium.

2.2.2 Morpho-physiological studies on *A. thaliana* under treatments

Physiological studies were performed to assess the impact of *P. putida* GAP-P45 on water-stress alleviation in *A. thaliana* at different time-points (day 2, day 4 and day 7) post treatments. The experimental parameters included visual observations on plant growth, measurement of fresh weight (FW), dry weight (DW), plant water content (PWC) of whole seedlings and primary root length (Ghosh et al., 2017).

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 ºC for 48 h for measurement of DW. Plant water content was measured both on FW and DW basis, by using the formulae:

PWC (DW basis) = [(FW-DW)/DW] X 100 and PWC (FW basis) = [(FW-DW)/FW] X 100 (Turner 1981).

Whole seedlings with intact roots were placed on a glass slide, in order to measure primary root length. The tap root was straightened and the secondary roots were separated using a fine needle. Length of the primary roots was measured using a centimeter scale.

Estimation of chlorophyll content in *A. thaliana* leaves

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 ºC 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 analyzed in the filtrate by a spectrophotometer at 663 nm and 646 nm wavelengths for chl a and chl b respectively. Total chlorophyll content was assessed using the formula:

Chl (a+b) = 7.49*A663 + 18.21*A646 (Barnes et al. 1992).

2.3 Results

2.3.1 Impact of *P. putida* GAP-P45 on plant growth under water-stressed conditions

Plants with similar growth and phenotypes were exposed to GAP-P45 and water stress treatments. Interestingly, GAP-P45 inoculation under non- stressed conditions did not cause any visible change in the overall growth of the plants throughout the study **(Fig. 2.1 E vs. F, I vs. J, M vs. N)**. Water stress, induced by PEG supplementation, led to significant growth stunting as opposed to the all other treatments **(Fig. 2.1 G, K, O)** right from day-2. With GAP-P45 inoculation under water stress, the *A. thaliana* seedlings exhibited improved phenotype as opposed to the non-inoculated ones **(Fig. 2.1 H vs. G, L vs. K, P vs. O)**. While the non- inoculated water-stressed plants showed a progressive decline, those inoculated with GAP-P45 exhibited much better tolerance to dehydrating conditions.

The LB agar plates used for monitoring growth of GAP-P45 exhibited colony formation only in the positive controls, no contamination was observed on the negative control plates **(Fig. 2.2)**. The plant based experiments did not show any growth promoting activity of *E. coli*, rather a decline in growth of *A. thaliana* was observed with *E. coli* inoculation contrary to the beneficial impact of GAP-P45 inoculation **(Fig. 2.3)**.

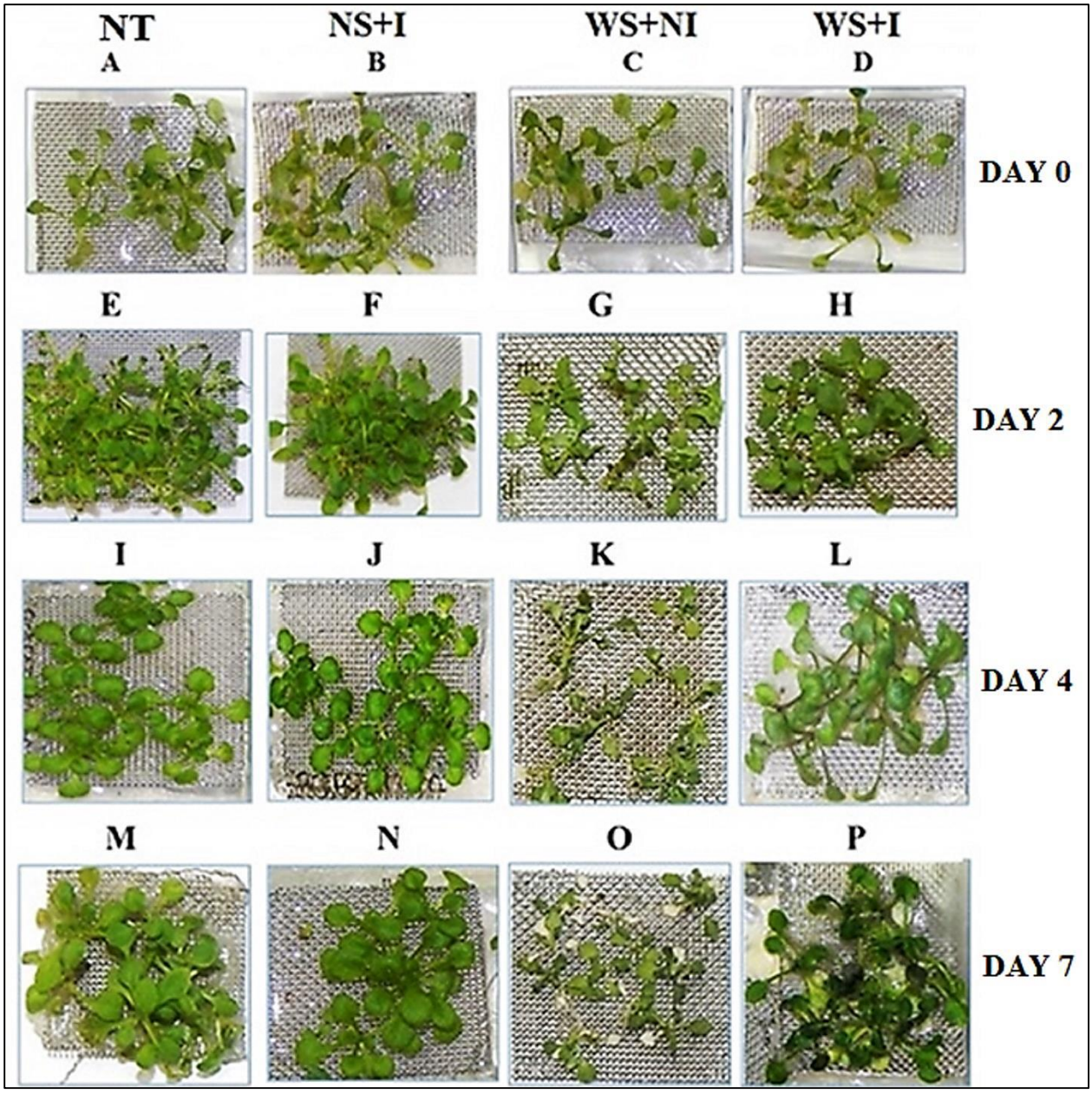

Fig. 2.1 Plant growth and development following *Pseudomonas putida* GAP-P45 inoculation in *Arabidopsis thaliana* under water-stressed conditions (25% PEG). While figures in A-D represent a period, just before treatments, E-H represents day 2; I-L, day 4 and M-P, day 7 post treatments. **NT**-no treatment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

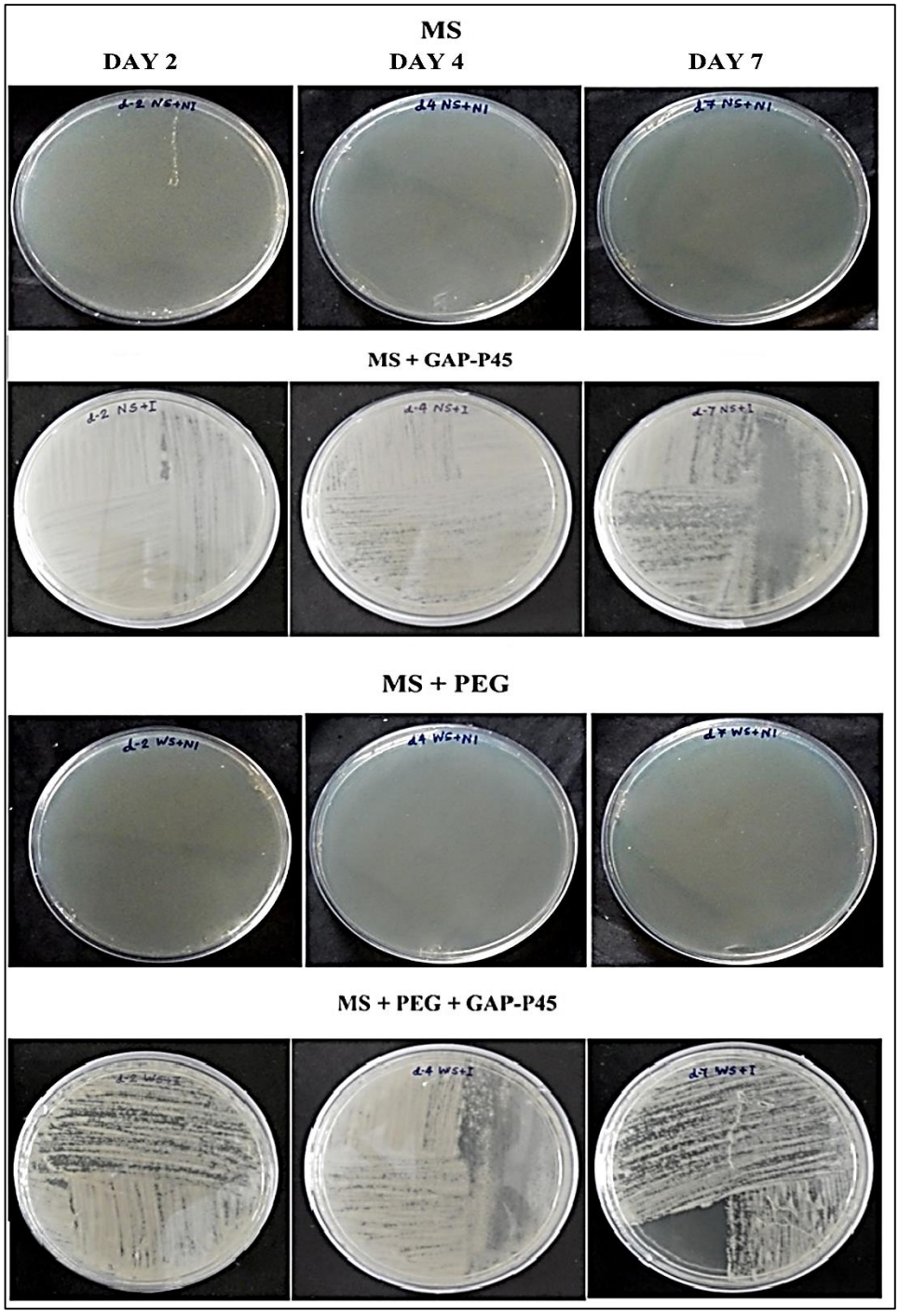

Fig 2.2 Growth monitoring and colony formation of *Pseudomonas putida* GAP-P45 throughout the study (day 2, day 4 and day 7) in all treatments compared to the control (MS).

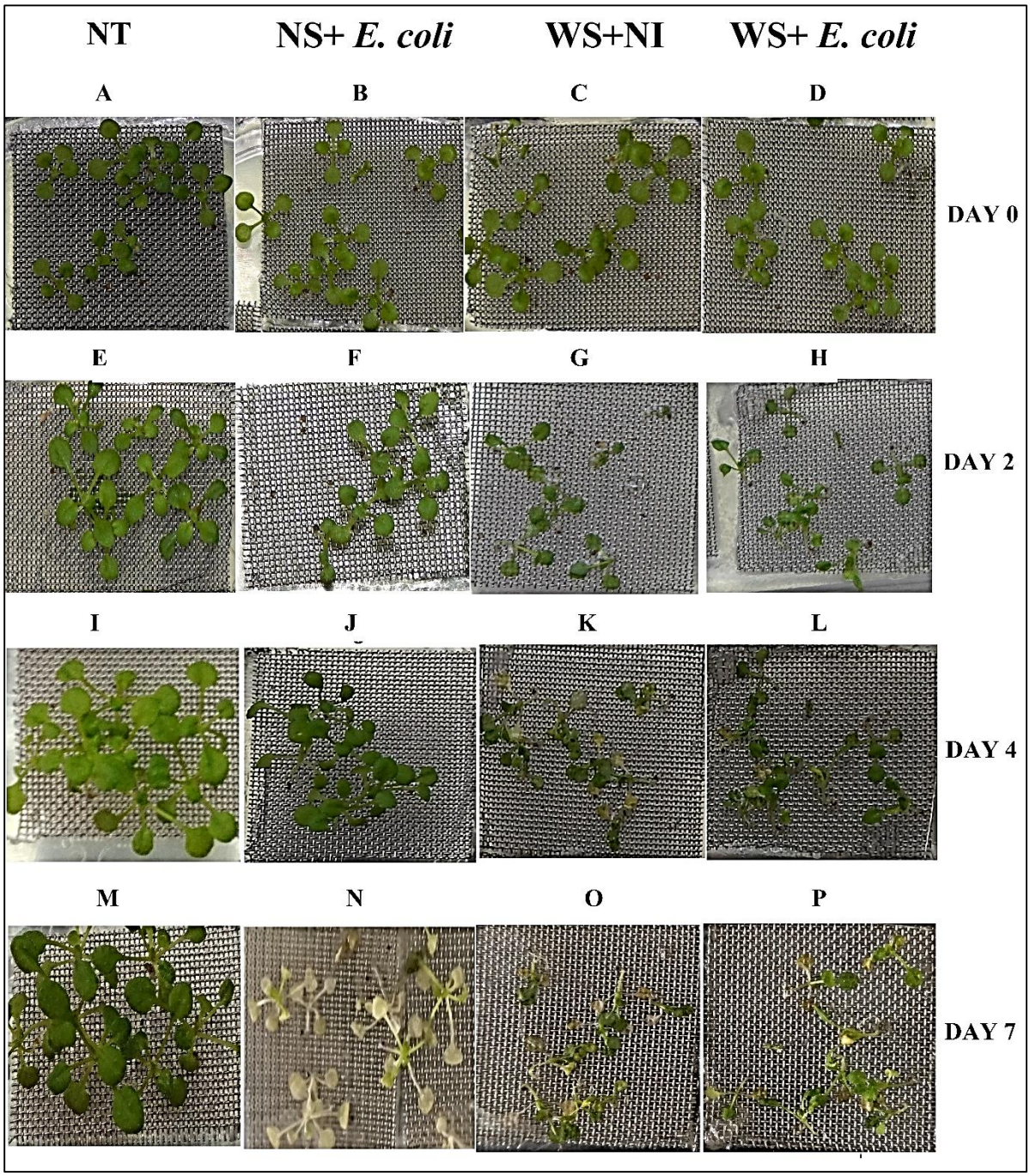

Fig. 2.3 Impact of inoculation of *E. coli* on *Arabidopsis thaliana* under water stress. *E coli* is not a PGPR strain and therefore has been used as a negative control strain in this study in order to prove the essentiality of *P. putida* GAP-P45 as a PGPR. A-D represent the initial period before treatments, 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 Impact of *P. putida* GAP-P45 on physiological parameters in *A. thaliana* under water stress.

2.3.2a Fresh weight, dry weight and plant water content

The results obtained from the physiological experiments have been cumulatively illustrated **(Fig. 2.4)**. Overall, inoculation of *P. putida* resulted in significant improvement in FW, DW and PWC (calculated both on FW and DW, Turner, 1981) in *A. thaliana* under water stress, while the water-stressed plants without inoculation recorded drastic reduction in PWC, FW and DW.

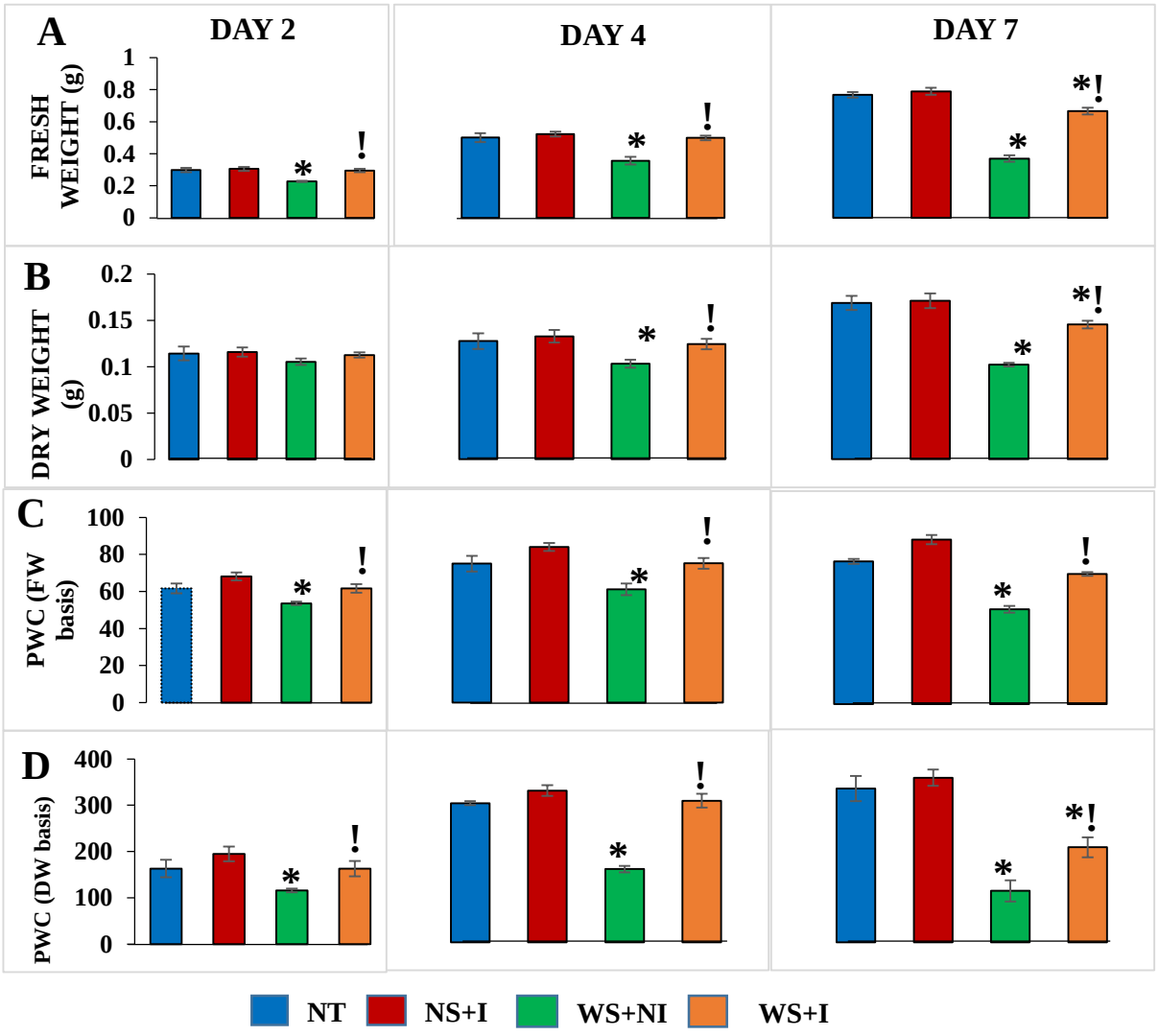

Fig. 2.4 Impact of *Pseudomonas putida* GAP-P45 on water-stress amelioration in *Arabidopsis thaliana* studied by quantifying fresh weight 'A', dry weight 'B' and plant water content 'C'- fresh weight basis and 'D' – dry weight basis of *A. thaliana* whole seedlings day 2, day 4 and day 7 post treatments. 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.

2.3.2b Primary root length and root morphology

Previous reports suggest that primary root length of plants tends to increase under drought conditions in many plants (Pace et al. 1999; Jacobs et al. 2004; Grossnickle 2005). In our study as well, water-stressed, non-inoculated plants exhibited the highest primary root length **(Fig. 2.5, 2.6)** and enhanced branching at day-2, day-4 and day-7 **(Fig 2.6)** while in case of water-stressed, GAP-P45 inoculated plants, primary root length **(Fig 2.5)**.and branching pattern **(Fig. 2.6)** were similar to control plants at most time periods of the study as evident from the data obtained.

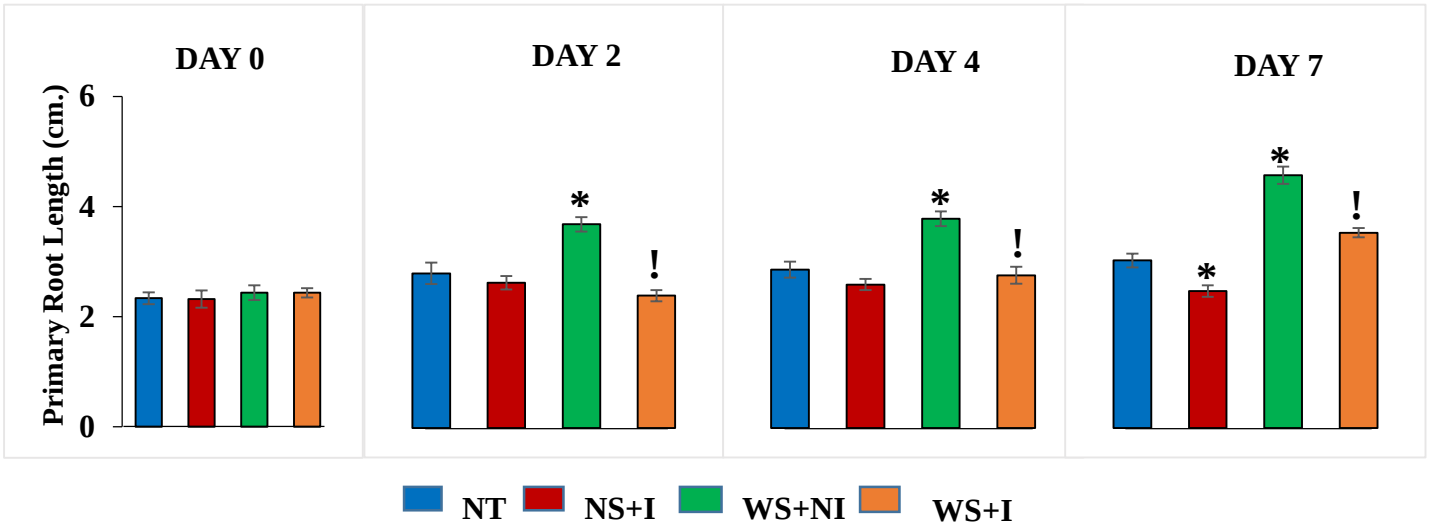

Fig. 2.5 Measurement of primary root length of *Arabidopsis thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 inoculation with or without water stress, day 2, day 4 and day 7 post treatments. Each bar represents mean ± SE of 10 replicate plants, taken from 3 Magenta boxes. '*' indicates 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.

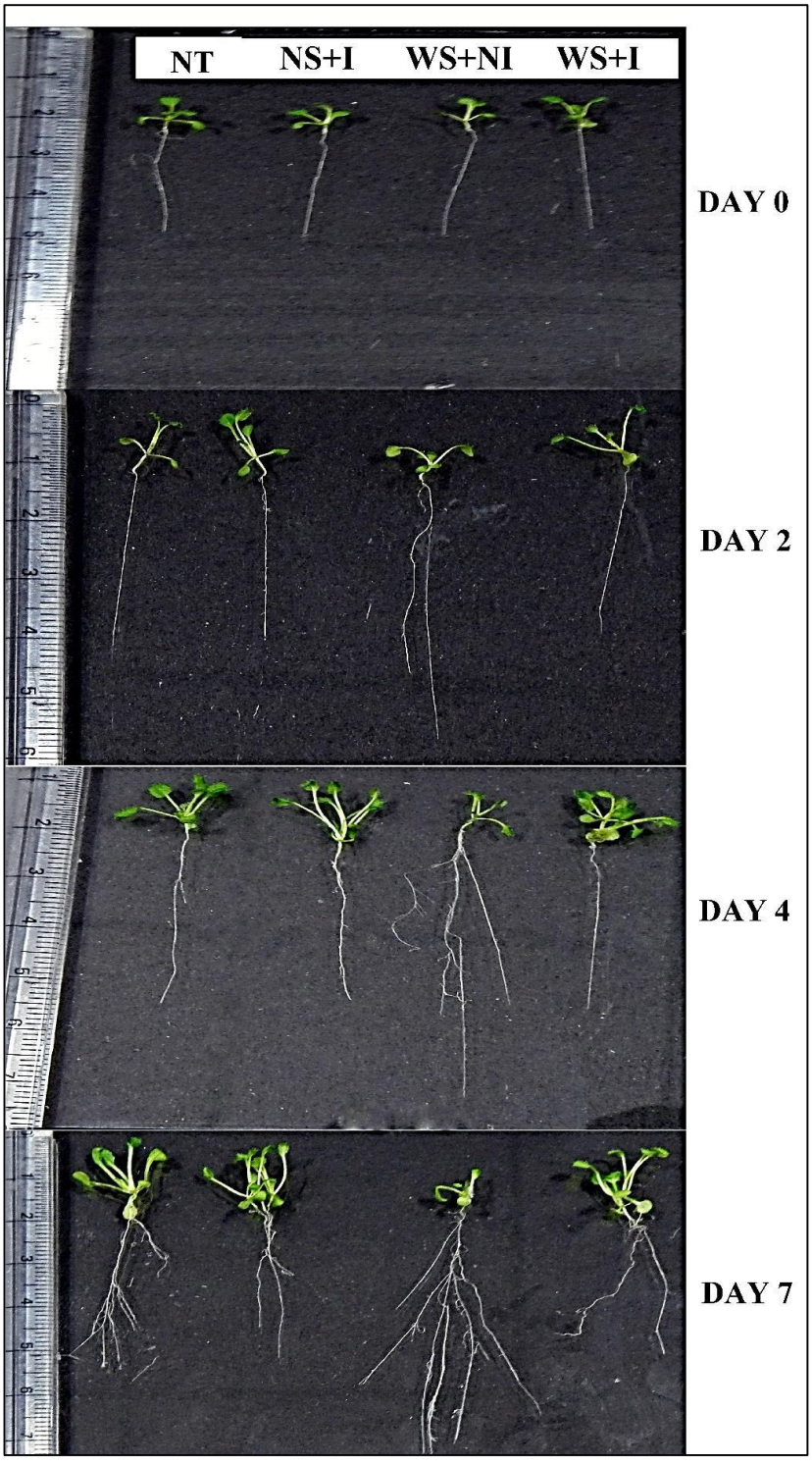

Fig. 2.6 Illustrations of root architecture as observed in *Arabidopsis thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 treatment with or without water stress obtained from each magenta box after day 2, day 4 and day 7 of each treatment. Day 0 represents seedlings collected prior to subjecting them to any treatments.

2.3.2c Chlorophyll content

Chlorophyll content followed a similar trend as PWC **(Fig. 2.4).** 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 conditions, significantly elevated the chlorophyll content in leaves **(Fig. 2.7).**

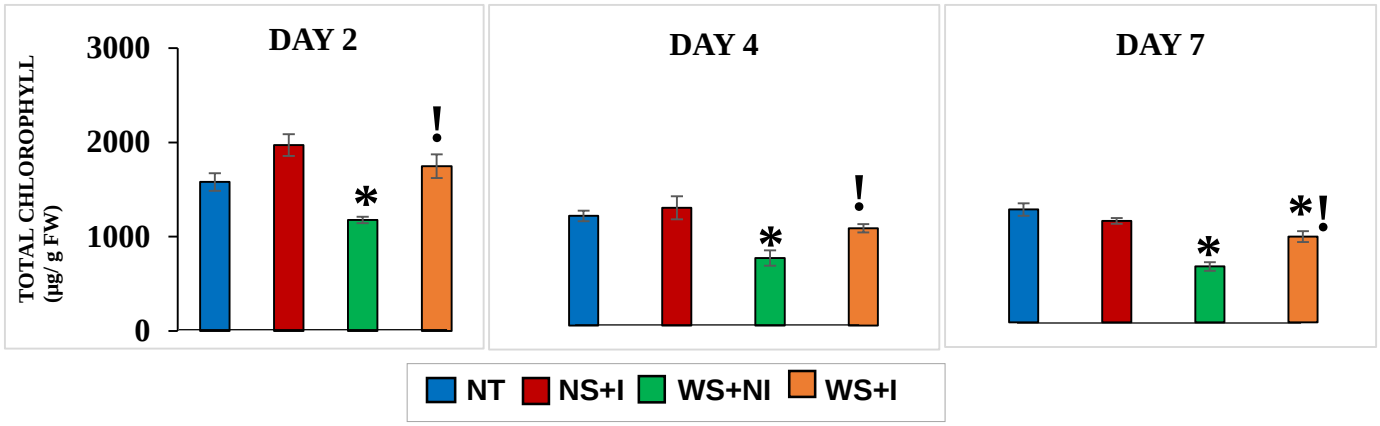

Fig. 2.7 Quantification of chlorophyll content in *Arabidopsis thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 inoculation with or without water stress, day 2, day 4 and day 7 post treatments. Each bar represents mean ± SE of 6 replicate sets, each with 40 mg leaf sample. **'*'** indicates significant difference ($p \leq 0.05$) 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

The bacterial strain *Pseudomonas putida* GAP-P45 used in this study, had been previously characterized as a drought- mitigating PGPR strain in maize and sunflower (Sandhya et al. 2009, 2010a, b). Our objective in this study was to assess the broad spectrum efficacy of this strain in mitigating water stress in *A. thaliana* seedlings thus proving cross- compatibility of this strain under laboratory conditions. Hence, we conducted some visual and physiological experiments to establish the suitability of this strain for drought- mitigation in *A. thaliana* so that it could be further used for conducting molecular studies. To simulate water stress conditions and to induce osmotic stress, PEG- 6000 was supplemented to the MS medium such that it would lower the water potential of the medium. Quite expectedly, under water stress, the health of the *A. thaliana* seedlings declined drastically, as evident from **Fig. 2.1**. However, when GAP-P45 was inoculated in the PEG supplemented medium, the health of the seedlings was not only restored but also displayed significant improvement in the plant phenotype than

the non- inoculated ones. Following this, the data obtained from physiological studies pertaining to FW, DW, PWC **(Fig 2.4)**, chlorophyll content **(Fig. 2.7)** etc. further corroborated our observations that GAP-P45 indeed improved the water status and ultimately the overall health of the *A. thaliana* seedlings under water stress. Under non- stressed conditions however, there were no significant improvement in plant phenotype over the no treatment controls, reinstating the ability of *P. putida* GAP-P45 to primarily mitigate drought conditions in *A. thaliana*. Several studies in the past have emphasized the ability of certain PGPRs to improve plant growth only under stressed conditions and not otherwise (Chanway and Holl 1994; Timmusk and Wagner 1999; Sandhya et al. 2010a; Rubin et al. 2017). Surprisingly, GAP-P45 did not change the water potential of the medium and yet was capable of improving the shoot growth and overall water status of the *A. thaliana* seedlings.

As discussed and reviewed earlier, PGPRs manifest their plant growth promoting and stress mitigating activities through diverse mechanisms (Cohen et al. 2009; Sandhya et al. 2010a, b; Liu et al. 2013b; Khan et al. 2014; Kang et al. 2014; Selvakumar et al. 2018). In addition to the production of phytohormones, the strain *P. putida* GAP-P45 was previously reported to secrete exopolysaccharides, siderophores and ammonia (Sandhya et al. 2010a), which are important plant growth promoting traits that can mitigate water stress in *A. thaliana* seedlings. Several authors have reported the occurrence of elongated primary root in plants under water stress to minimize stress injury and water consumption (Boyer 1985; Tardieu et al. 2011; Bresson et al. 2013). Reduction in shoot growth also conserves energy under water stress by lessening the consumption of water. In accordance to this theory, we observed similar trends of enhanced elongation and branching of roots in non-inoculated water-stressed plants in our study. Inoculation of seedlings with GAP-P45 significantly lowered root elongation and branching under water-stress **(Fig. 2.5, 2.6).** Another important aspect of plant growth that gets compromised under stressed conditions is the photosynthetic efficiency of plants. As reviewed in the previous chapter, ABA-mediated stomatal closure is among the foremost responses of plants to stressed conditions. This causes lesser CO_2 uptake, reduced ATP generation and subsequent ROS aggravation. Furthermore, this leads to photo-damage and disruption of the photosynthetic machinery and photosynthetic pigments, like chlorophyll (Munné-Bosch and Alegre 2004; Santos 2004; Anjum et al. 2017). This phenomenon was observed in our study as well such that the chlorophyll content of the water-stressed non-inoculated plants drastically reduced at all the time points of analysis, corroborating the observed decline in plant growth. Contrarily, the chlorophyll content in *A. thaliana* seedlings inoculated with GAP-P45 was significantly higher compared to the non- inoculated water-stressed plants and were much closer to the chlorophyll levels of the non- stressed plants. Our results positively correlated with previous studies reporting enhanced chlorophyll production due to beneficial plant- PGPR interactions leading to crop improvement as reviewed by (Adesemoye and Kloepper 2009).

2.5 Key Findings

Our experiments on morpho-physiological parameters of *A. thaliana* under water stress and PGPR inoculation established that at the particular concentration of the PGPR, *P. putida* GAP-P45 used in our studies, there was significant improvement in plant health and physiology under water-stress. However, non-stressed plants inoculated with the same concentration of the bacterium exhibited no significant morpho-physiological changes. The preliminary data obtained from this study supported the use of *P. putida* GAP-P45 for conducting mechanistic studies through molecular techniques in *A. thaliana* under water stress. Hence, we used this particular strain for further experimentation in order to accomplish the remaining objectives of our study.

Chapter 3

Modulation of polyamine metabolism in *Arabidopsis thaliana* by drought mitigating PGPR, *Pseudomonas putida* GAP-P45

<u>Section 1</u>: Modulation of polyamine biosynthetic gene expression in *A. thaliana* by *P. putida* GAP-P45 with or without water stress

3.1.1 Introduction

We have described the intricate mechanisms of drought adaptation in plants in the previous chapters. As mentioned previously, since several years, polyamines (PAs) have been implicated in stress response as stress markers and stress ameliorators. The well regulated concentration of PAs in plant cells serve a multitude of important functions related to survival and growth. As mentioned previously, the three major PAs in plants are spermidine (Spd), spermine (Spm) and their diamine precursor, putrescine (Put). As illustrated in **Fig. 3.1**, PA biosynthesis in *A. thaliana* is initiated by the conversion of arginine into the diamine Put through three enzymes. The rate limiting enzyme arginine decarboxylase (ADC) encoded by paralogs *ADC1* and *ADC2* (Urano et al. 2003; Urano et al. 2004; Alcázar et al. 2006; Cuevas et al. 2008) converts arginine into agmatine and subsequently to N-carbamoyl putrescine and Put mediated by agmatine iminohydrolase encoded by *AIH* (Janowitz et al. 2003) and N- carbamoyl putrescine amidohydrolase encoded by *CPA* (Piotrowski et al. 2003) respectively. A third route to Put biosynthesis has also been recently discovered that states the contribution of arginase in converting agmatine to Put (Patel et al. 2017). The conversion of Put to Spd and subsequently Spm requires successive addition of aminopropyl moieties to Spd synthase,(encoded by paralogs *SPDS1* and *SPDS2*), and Spm synthase, (encoded by *SPMS*) respectively (Panicot et al. 2002). The aminiopropyl moieties are donated by decarboxylated S-adenosylmethionine (dcSAM) via SAM decarboxylase, encoded by *SAMDC1-4* (Urano et al. 2003).

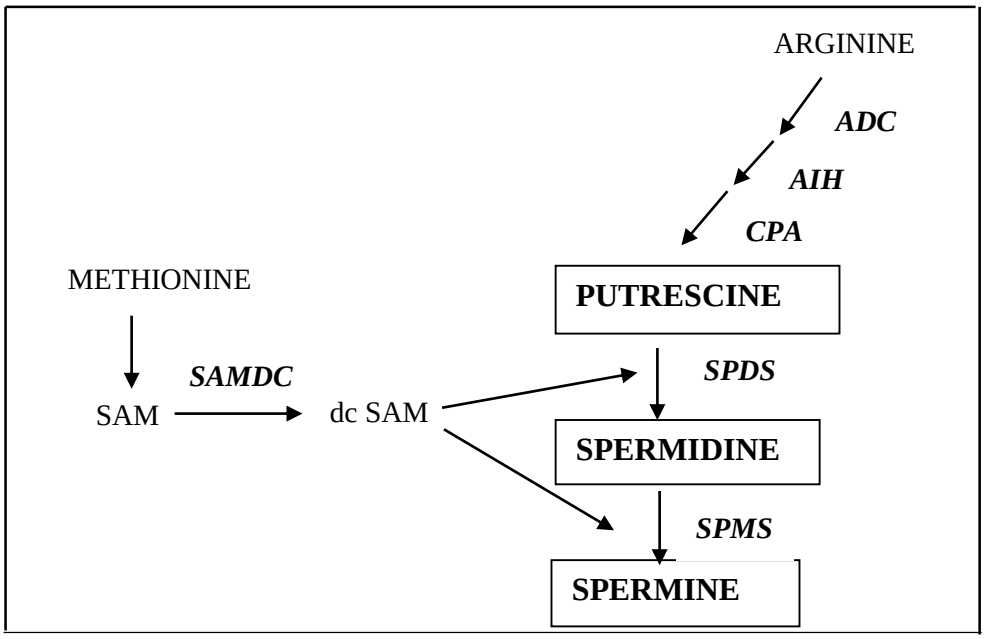

Fig. 3.1: PA biosynthetic pathway in *A. thaliana* [Alcázar et al. 2006]

The importance of PAs in biotic and abiotic stress response in plants has been well-documented in the past decade through molecular and genetic studies by Takahashi and Kakehi (2010); Alcázar et al. (2010); Hussain et al. (2011); Marco et al. (2011); Bitrián et al. 2012; Minocha et al. (2014) and others.

In this work we focused on understanding the modulation of PA biosynthesis, at the transcriptional level in *A. thaliana*, under water-stressed conditions when inoculated with the drought-mitigating, free living rhizobacteria, *Pseudomonas putida* GAP-P45 (Sandhya et al. 2009, 2010a; Sandhya and Ali 2015, Ghosh et al. 2017). As mentioned earlier, this strain was originally isolated, characterized and tested for drought amelioration in maize and sunflower by Sandhya et al. (2010a, 2009). Thereafter, we have established and reported (Ghosh et al., 2017) the beneficial impact of *P. putida* GAP-P45 on water-stress alleviation in *A. thaliana*. In the present study, we examined the expression patterns of key genes involved in the biosynthesis of Put, Spd and Spm in *A. thaliana* seedlings in response to GAP-P45 inoculation with or without water stress. [Data published in Sen et al., (2018)].

3.1.2 Materials and Methods

3.1.2a Growth of *A. thaliana*, water-stress induction and *P. putida* GAP-P45 inoculation

The procedures involved in germination, growth, water-stress induction and GAP-P45 inoculation have been thoroughly described in Chapter 2.

3.1.2b Measurement of the transcript levels of polyamine biosynthetic genes in *A. thaliana* seedlings

Expression studies for the genes involved in the PA biosynthetic pathway in *A. thaliana* seedlings exposed to water stress and GAP-P45 inoculation were carried out with untreated seedlings used as the reference controls. Whole seedlings were collected at three different time points, namely day 2, day 4 and day 7 post treatments. Roots were rinsed with distilled water to get rid of media and surface bacteria, if any. The seedlings were then subjected to RNA extraction and cDNA synthesis. For total RNA extraction, 200 mg of whole seedlings were homogenized and treated with TRI reagent (Ambion, Life Technologies) and the extraction process was performed according to the manufacturer's protocol. Genomic DNA was removed using an RNAse free DNAse kit (NEB). cDNA synthesis was carried out using Superscript III 1st- strand synthesis kit (Life Technologies). Total RNA (2 µg) was reverse transcribed into cDNA in a 20 µl total volume of reaction mix containing the Superscript III (Invitrogen) reverse transcriptase enzyme, buffer, dNTPs, oligo-dT primer and DTT, based on the manufacturer's instructions. Semi-quantitative PCR was performed using appropriate primers **(Table 3)**, for the following genes – *ADC1, ADC2, AIH, CPA, SPDS1, SPDS2, SPMS, SAMDC1, SAMDC2, SAMDC3* and *SAMDC4.* The gene-specific primer sequences (Jumtee

et al. 2008; Jubault et al. 2008) used for the analyses were manufactured by Oligos, Sigma. The obtained qualitative expression pattern of these genes (analyzed through agarose gel electrophoresis of amplicons as illustrated in **Fig 3.2**) were corroborated using quantitative real-time PCR (Step One Plus, Applied Biosystems, USA) using a SYBR green PCR master mix (Invitrogen). Prior to relative quantification of the cDNA samples from different treatments, all the primers were tested at different concentrations (ranging from 50-200 nM per reaction) with the serially diluted cDNA (ranging from 0.24-150 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. The cDNA samples used for standardization were synthesized from the RNA of no treatment control seedlings. Amplification of the desired cDNA segment was ensured by analyzing the melt-curves generated in real-time PCR. Melt-curve is the determination of melting temperature (Tm) of the amplicon following PCR. Formation of primer dimers or any non-specific amplifications usually exhibit a lower Tm value than the desired amplicon. Presence or absence of primer-dimers and any other non-specific products/ contamination were confirmed by the amplification of "no template control". The standard curves and typical melt-curves (representative examples) of all the genes of interest are illustrated in **Fig. 3.3** and **Fig. 3.4** respectively. Thereafter, for relative quantification of cDNA samples in a total volume of 10 µl PCR solution, 1.5 µl of cDNA template was mixed with 100-200 nM each of forward and reverse primers and 2x SYBR Green PCR Master Mix. The reaction was first incubated at 95ºC for 10 min., followed by 40 cycles of incubation at 95 ºC for 15 s and 60 ºC for 1 min. (Jumtee et al. 2008). The qRT PCR was performed in three replicate cDNA samples for each treatment. Expression of all the genes were normalized to the endogenous control gene, *β-actin-2* and the fold change in expression of the treated vs untreated samples was computed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The formulae used to calculate the ΔΔCT and relative quantification (fold change) values are shown below:

Step 1: ΔCT = CT of the target genes - CT of the endogenous control (within same treatment).

Step 2: ΔΔCT = ΔCT of the target gene in treatment - ΔCT of the target gene in reference sample.

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

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

3.1.2c Statistical analysis:

Statistical analysis was performed by two-way Anova with Tukey's HSD test (level of significance, $p \leq 0.05$). 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.

Table 3: Oligonucleotide sequences of the PA biosynthetic genes used for semi-quantitative and quantitative PCR reactions (Jumtee et al. 2008; Jubault et al. 2008)**.**

Genes	Forward primer	Reverse primer
β-ACTIN2	5'-AGATTCAGATGCCCAGAAGTCTTGT-3'	5'-TGGATTCCAGCAGCTTCCAT-3'
ADC1	5'-CAGACTCTTAAACACCGAGCCG -3'	5'-GACTTAGCAAGACACGATGCGA-3'
ADC2	5'-TCTCTGTTCGTCCTCATGGCTC-3'	5'-AGCTGCAATCCTAAACCACCG-3'
AIH	5'-TCGAGAATGCAAGAGAGATCGTT-3'	5'-CATTTTCGGCGACGGAAGTA-3'
CPA	5'-GATCAAGTCGAAAAGGCAAAGCT-3'	5'-CCATCCATAGTAAGAAGCACCTTGT-3'
SPDS1	5'AATCACCACCTCTCACAAACCC-3'	5'TCGGTGGCAGAGGTTTCTTTA-3'
SPDS2	5'TGGTGGTTGATGTGGCTAAGC-3'	5'GGTTCCTTCAGCAGCGTTCTT-3'
SPMS	5'-GTGGAGGTGATGGTGGTGTTCT-3'	5'-AACACGAGGATCGTCAAACCC-3'
SAMDC1	5'-TCGAGCCCAAGCAATTCTCT-3'	5'-CAAATGTCCTCTCTCTGCACCC-3'
SAMDC2	5'-ACCATTCCCTCACCGCAACTT-3'	5'-GGTTTCATCGTCATTGCCCAT-3'
SAMDC3	5'-ACCCCGGAAGATGGTTTTAGC-3'	5'-GCCAATACCTCGGTTCCAAGA-3'
SAMDC4	5'-ACAACCACGAGGTGACTAAGCG-3'	5'-GGCGTGAAGGACTGATAAACGA-3'

The possibility of contamination from genomic DNA was also ruled out by using *P5CS1*, *SPDS2* and *SPMS* primers through semi- quantitative PCR followed by analysis of products on agarose gel. These primers amplify intron-spanning regions from genomic DNA template generating larger products (550 bp, 311 bp and 234 bp respectively) as opposed to the amplicons obtained from amplification of cDNA specifically (135 bp, 119 bp and 146 bp).

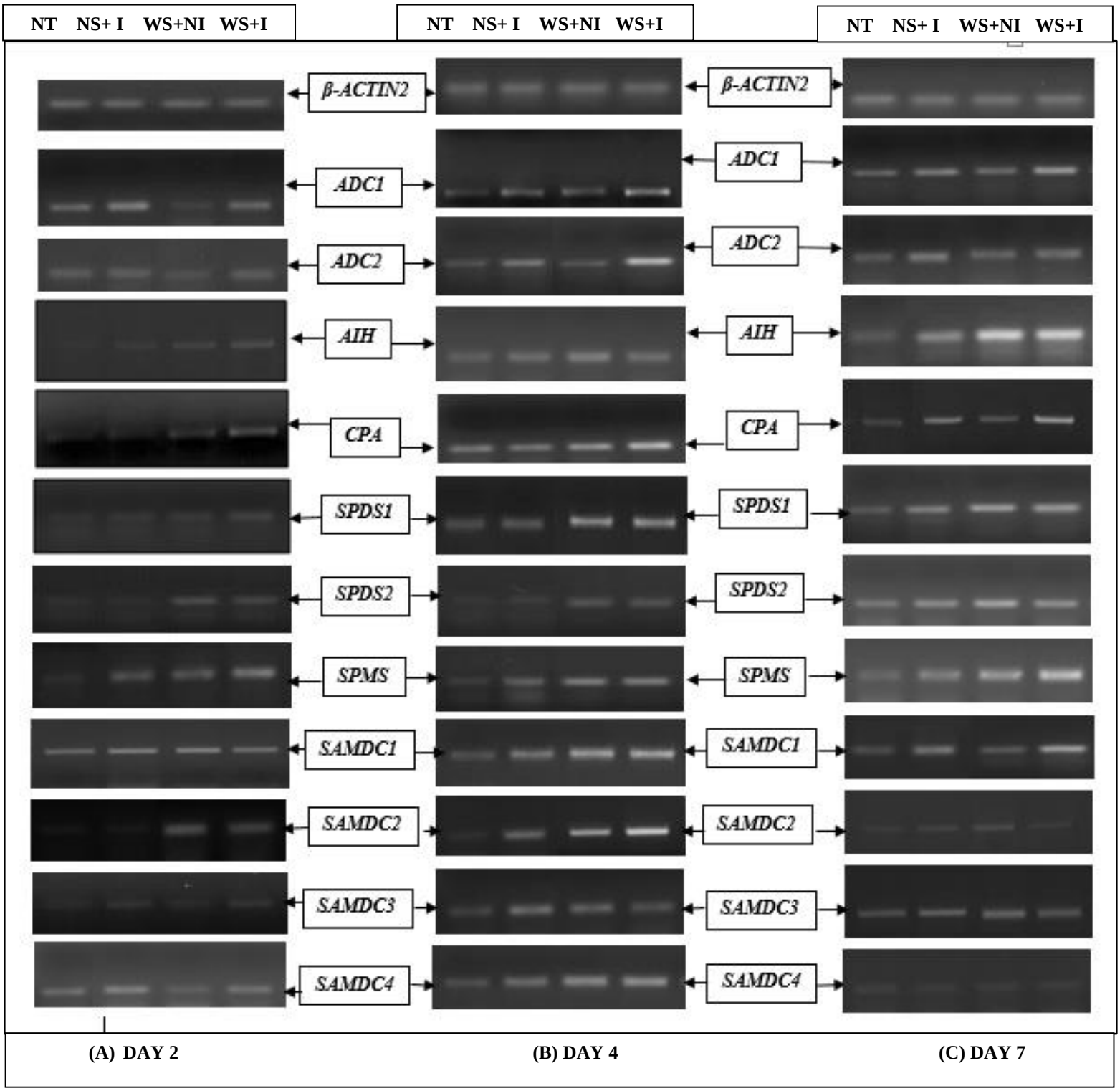

Fig. 3.2 Semi quantitative PCR analysis of expression pattern of the polyamine biosynthetic (*ADC1, ADC2, AIH, CPA, SPDS1, SPDS2, SPMS, SAMDC1, SAMDC2, SAMDC3 and SAMDC4*) genes post water-stress induction and GAP-P45 inoculation (A) day 2 (B) day 4 (C) day 7 after treatments.

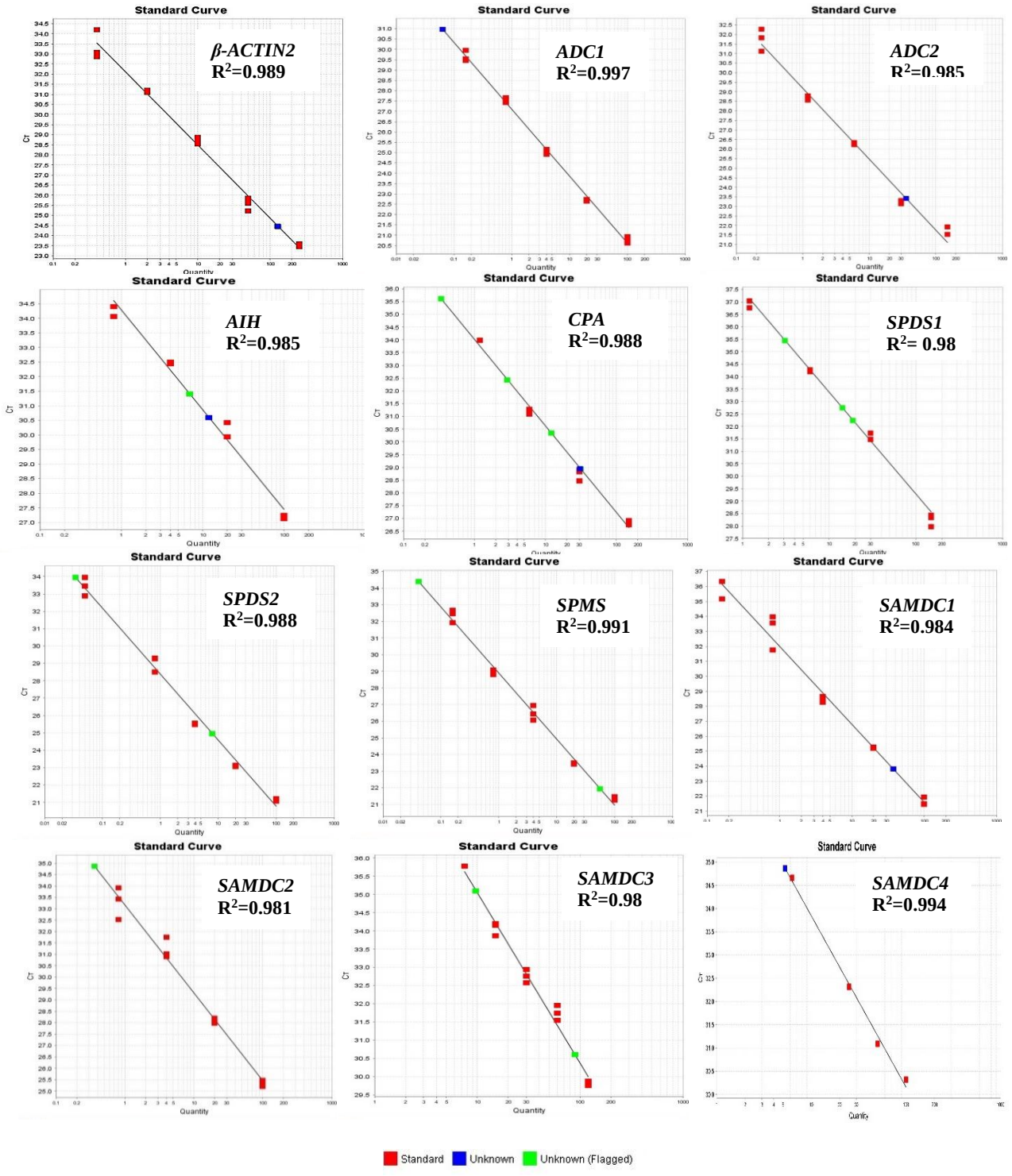

Fig. 3.3 Standard curves of all the gene primers to optimize the primer and template concentration for the relative quantification of expression of the genes of interest in real-time PCR analysis. The genes include *β-Actin2*, *ADC1*, *ADC2*, *AIH*, *CPA*, *SPDS1*, *SPDS2*, *SPMS*, *SAMDC1*, *SAMDC2*, *SAMDC3* and *SAMDC4*. Standard curves were generated using cDNA template from no treatment controls.

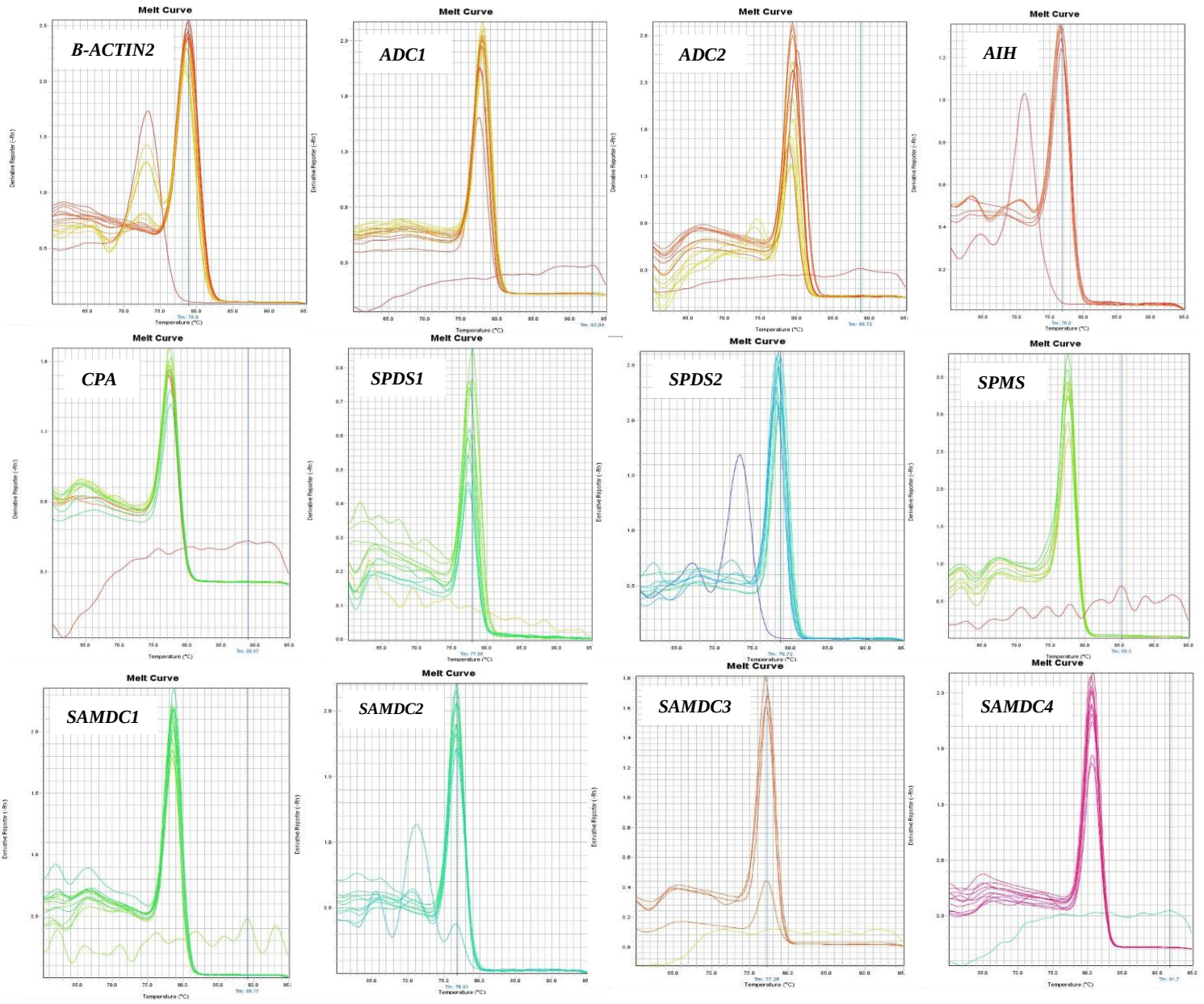

Fig. 3.4 Representative examples of melt curves of all the gene products to determine presence/absence of any non-specific amplification of template DNA in quantitative real-time PCR. The genes analyzed were *β-Actin2*, *ADC1*, *ADC2*, *AIH*, *CPA*, *SPDS1*, *SPDS2*, *SPMS*, *SAMDC1*, *SAMDC2*, *SAMDC3* and *SAMDC4*. Primer- dimers were observed in case of *β-Actin2*, *AIH*, *SPDS2 and SAMDC2* with T_m values lower than the actual amplicons.

3.1.3 Results

P. putida GAP-P45 modulates the expression of PA biosynthetic genes in _A. thaliana_ with or without water stress:

The Put biosynthetic genes:

The expression of all the Put biosynthetic genes (*ADC1*, *ADC2*, *AIH* and *CPA*) were found to be modulated by all treatments and at almost all time points of the study, as evident from **Fig. 3.5**. On day 2 **(Fig. 3.5 A)**, both *ADC1* and *AIH* displayed a slight (although statistically insignificant) induction (~ 1.5 fold) in expression in the non-stressed seedlings inoculated with GAP-P45, as opposed to the no-treatment controls.. *ADC2* and *CPA* remained unaltered under these conditions. Water-stress, by itself, caused either a decrease (*ADC2*), a slight increase (*AIH*) or no change (*ADC1*, *CPA*) in expression as opposed to the no-treatment controls. A ~ 2-2.5-fold increase in the transcript levels of all these genes was observed in the water-stressed samples, inoculated with GAP-P45, as compared to their non- inoculated counterparts.

On day 4 **(Fig. 3.5 B)**, all four genes exhibited an upregulation in response to all treatments. The expression of *ADC1* continued to be >1.5-fold in response to GAP-P45 inoculation without water stress as opposed to the untreated controls. Water stress itself (without GAP-P45 inoculation) caused a 1.5-fold increase in *ADC1* expression, while GAP-P45 inoculation of water-stressed plants resulted in a >2-fold increase in *ADC1* expression. *ADC2* also exhibited almost similar patterns of regulation by the various treatments on day 4 of analysis. In case of *AIH*, inductions were seen in all treatments; however, its highest induction was recorded in water-stressed conditions, without GAP-P45 treatment. The expression of *CPA* showed a gradual, treatment-dependent upregulation from day 2 to day 4.

Subsequently, on day 7, **(Fig. 3.5 C)**, *ADC1* displayed a similar pattern of expression as on day4, but the transcript abundance was marginally higher in some treatments as opposed to day 2 and day 4. On day 7, *ADC2* displayed a different trend in its expression pattern as opposed to *ADC1*. *ADC2* saw a significant surge (~ 3-fold) in expression on inoculation with GAP-P45 without water-stress. Water stress by itself caused no change in the expression of *ADC2* (as opposed to the no-treatment controls), while treatment with GAP-P45 under water-stress, caused a slight increase in the expression of *ADC2* **(Fig. 3.5 C)**. In case of *AIH*, the day 7 trends were comparable to day4, although, the transcript abundance was slightly higher in all treatments on day 7 as opposed to day 4. The expression of *AIH*, as shown in **Fig. 3.5 (A-C)**, exhibits a gradual, time-dependent up-regulation in almost all treatments from day 2 to day7. As for *CPA*, much like day 4, there was up-regulation of its expression in all treatments on day 7 of analysis as well. The highest up-regulation was seen in the water-stressed + GAP-P45 inoculated plants.

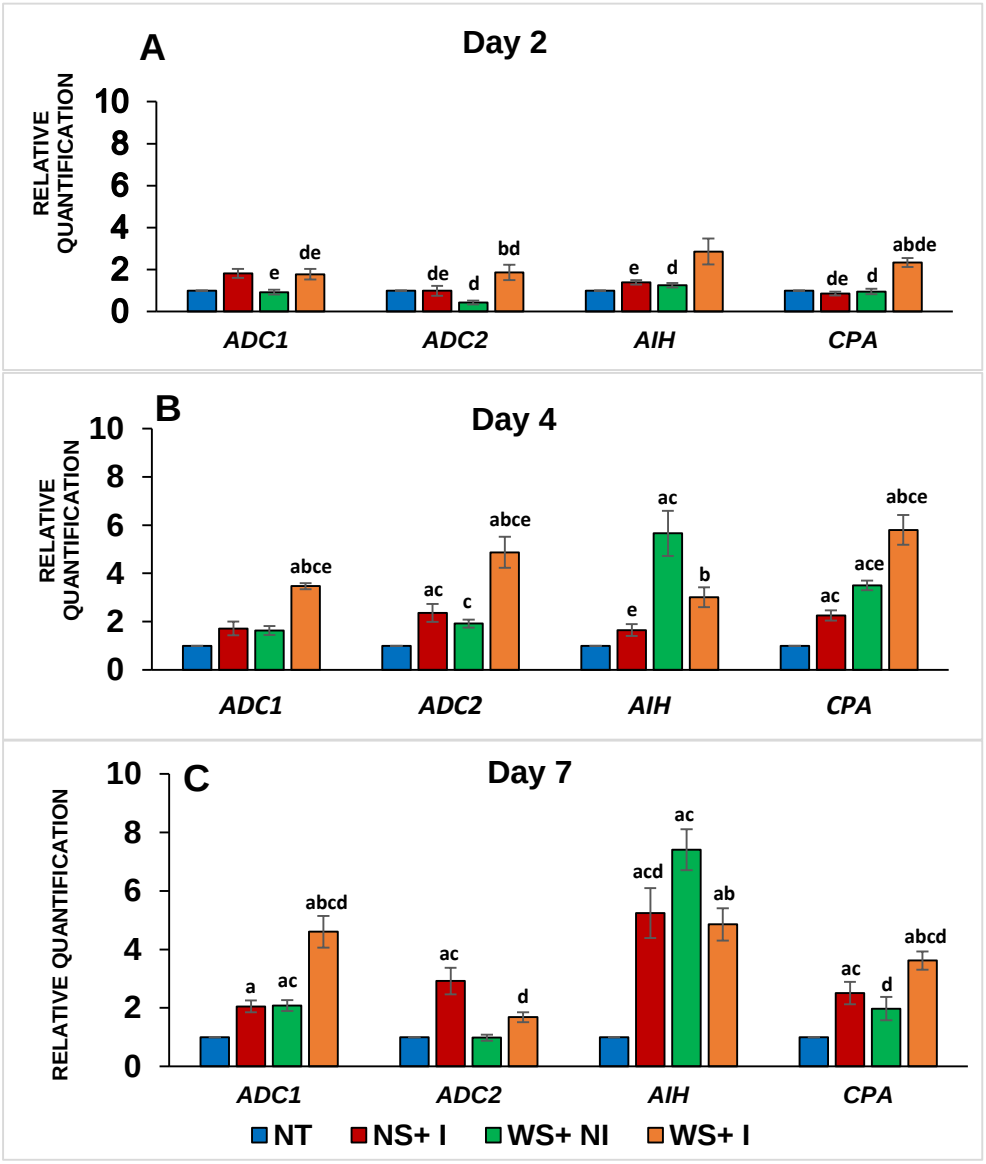

Fig. 3.5 Relative expression of putrescine biosynthetic genes (*ADC1*, *ADC2*, *AIH* and *CPA*) in *A. thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 inoculation with or without water stress, day 2 **(A)**, day 4 **(B)** and day 7 **(C)** post treatments (**NT**: no treatment; **NS+ I**: non-stressed, inoculated; **WS+NI**: water-stressed, non-inoculated; **WS+I**: water-stressed, inoculated). Each bar represents mean (±) S.E. of 6 replicates. 'a' represent significant difference ($p \leq 0.05$) of all treatments from **NT**. 'b' represent significant difference ($p \leq 0.05$) between **WS+NI** and **WS+I** seedlings. 'c', 'd' and 'e' denote significant differences of a gene within a treatment at a particular day from day 2, 4 and 7 respectively.

The Spd and Spm biosynthetic genes

The Spd biosynthetic gene *SPDS1* exhibited an initial surge (day 2) in expression in response to water stress and a greater increase in expression with GAP-P45 inoculation under water-stressed conditions **(Fig. 3.6 A)**, but no change in expression was observed in the non-stressed inoculated seedlings. Similarly, *SPDS2* did not show any significant change in expression on inoculation with GAP-P45 under normal conditions. However, it exhibited a water stress- induced increase in expression, and a downregulation in the stressed + GAP-P45 treated seedlings (as opposed to the water-stressed seedlings).

On day 4 **(Fig. 3.6 B)**, gradual, treatment-dependent elevation in inductions were seen in the expression of *SPDS1*. The expression of *SPDS2* was also impacted under various treatments with a significant upregulation seen in case of water-stressed seedlings on day 4. However, the magnitudes of upregulation were several-fold higher in *SPDS1* as compared to *SPDS2*, for the individual treatments. In case of *SPDS1*, the difference between day 4 and day 7 data is that, on day 7, the water-stressed plants showed a decrease in expression on inoculation with GAP-P45. Thus, the expression of *SPDS1* in this treatment, exhibits a 4-fold surge from day2 to day 4, followed by a down-regulation of the almost same magnitude on day7. Trends on day 7 were almost identical between *SPDS1* and *SPDS2* **(Fig. 3.6 C)**.

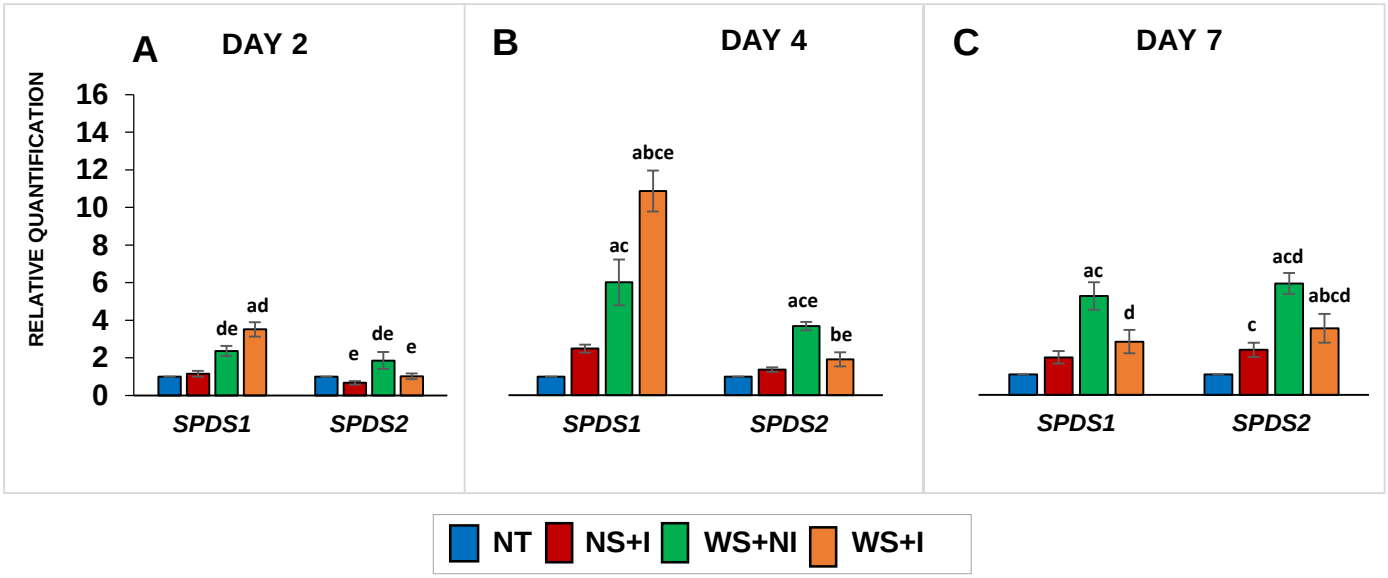

Fig. 3.6 Relative expression of spermidine biosynthetic genes (*SPDS1 and SPDS2*) in *A. thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 inoculation with or without water stress, day 2 **(A)**, day 4 **(B)** and day 7 **(C)** post treatments. Each bar represents mean (±) S.E. of 6 replicates. 'a' represent significant difference ($p \leq 0.05$) of all treatments from **NT**. 'b' represent significant difference ($p \leq 0.05$) between **WS+NI** and **WS+I** seedlings. 'c', 'd' and 'e' denote significant differences of a gene within a treatment at a particular day from day 2, 4 and 7 respectively.

All the treatments caused an elevation in the *SPMS* expression at all the time points with respect to the no-treatment controls **(Fig. 3.7)**. On day 2 and day 7 water-stressed + GAP-P45 treated plants showed the highest expression. On day 4 while all treatments showed similar levels of upregulation, on day7, there was a >3-fold elevation in the expression of *SPMS* in GAP-P45 inoculated seedlings under water stress, compared to the non-inoculated, water-stressed seedlings.

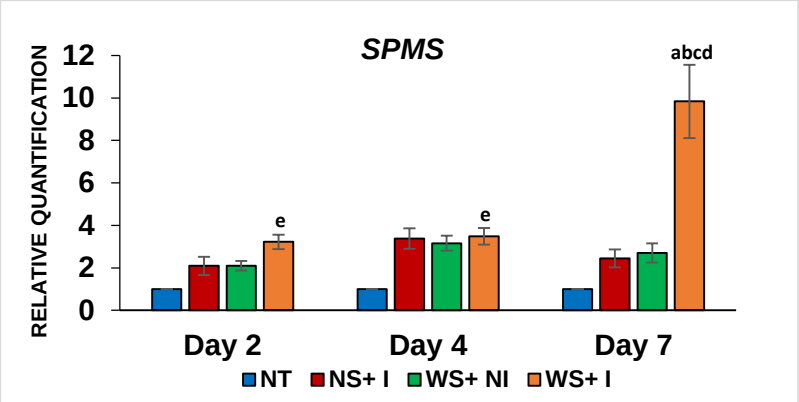

Fig. 3.7 Relative expression of spermine biosynthetic gene (*SPMS*) in *A. thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 inoculation with or without water stress, day 2, day 4 and day 7 post treatments. Each bar represents mean (±) S.E. of 6 replicates. 'a' represent significant difference ($p \leq 0.05$) of all treatments from **NT**. 'b' represent significant difference ($p \leq 0.05$) between **WS+NI** and **WS+I** seedlings. 'c', 'd' and 'e' denote significant differences of a gene within a treatment at a particular day from day 2, 4 and 7 respectively.

As illustrated in **Fig. 3.8**, the expression of almost all *SAMDC* paralogs was induced in response to most treatments and at most time points compared to the no treatment control. An overall elevation in expression was noted for all the four genes on inoculation with GAP-P45 under non-stressed conditions. Exceptions to this were shown by *SAMDC2* which displayed a decrease in expression on day 2 **(Fig. 3.8 A)**, *SAMDC3* and *4*, displaying negligible change at day 2 and day 7 respectively **(Fig. 3.8 A, C)**. On day 2, water stress caused an upregulation in *SAMDC1* and *SAMDC2*, while *SAMDC3* exhibited negligible change and *SAMDC4* exhibited a slight downregulation, as opposed to the no-treatment controls. On day 4, all the paralogs exhibited significantly elevated expression levels in response to water stress, *SAMDC2* being the most induced **(Fig. 3.8 B)**. On day 7, all except *SAMDC4* showed upregulation under water-stressed conditions **(Fig. 3.8 C)**. With respect to GAP-P45 inoculation under water stress, the expression of different paralogs seemed to be impacted differently.

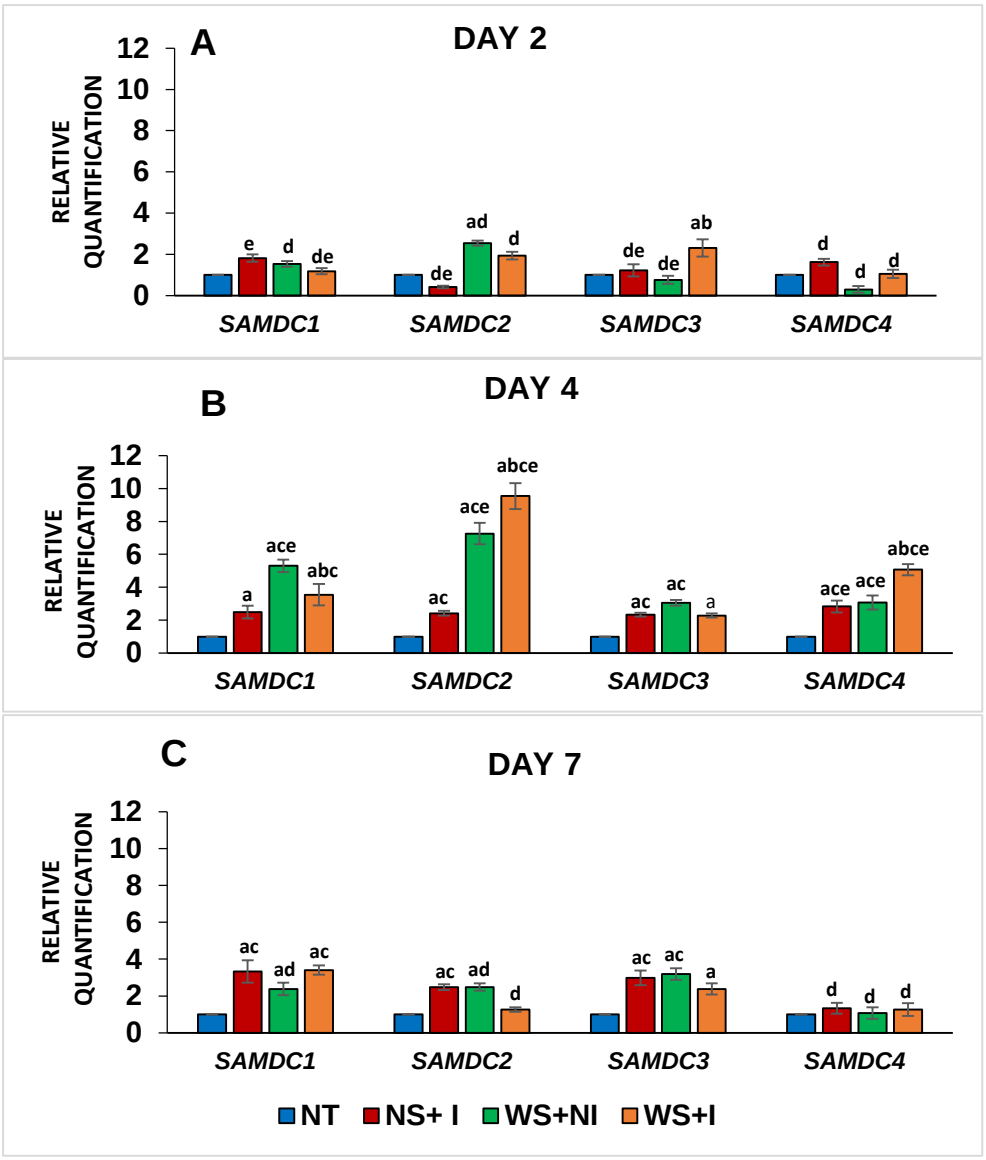

Fig. 3.8 Relative expression of *SAMDC* genes (*SAMDC1*, *SAMDC2*, *SAMDC3* and *SAMDC4*) in *A. thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 inoculation with or without water stress, day 2 **(A)**, day 4 **(B)** and day 7 **(C)** post treatments. Each bar represents mean (±) S.E. of 6 replicates. 'a' represent significant difference ($p \leq 0.05$) of all treatments from **NT**. 'b' represent significant difference ($p \leq 0.05$) between **WS+NI** and **WS+I** seedlings. 'c', 'd' and 'e' denote significant differences of a gene within a treatment at a particular day from day 2, 4 and 7 respectively.

While *SAMDC1* showed a down-regulation (day 2 and day 4) followed by a marginal upregulation (day 7), as opposed to water-stressed (non-inoculated) plants, *SAMDC2*, in presence of GAP-P45 under water-stressed conditions, showed a down-regulation on day 2 and day 7 and an upregulation on day 4 as opposed to water-stressed (non-inoculated) plants. In case of *SAMDC3*, inoculation with GAP-P45 under

water stress resulted in a ~ 2.5-fold increase in expression on day 2 while remaining down-regulated on day 4 and day 7 when compared to the water-stressed non-inoculated seedlings. The expression of *SAMDC4* remained upregulated on day 2 and day 4 on inoculation with GAP-P45 under water-stress as compared to the water-stressed seedlings without inoculation, while exhibiting no change on day 7 under any treatment.

3.1.4 Discussion

P. putida GAP-P45 modulates PA biosynthetic gene expression under normal (non-stressed) as well as water-stressed conditions.

As mentioned previously, PAs are important molecules that are obligate requirements for cell survival and sustenance. They are known to play crucial roles in the process of plant development and stress tolerance. Yet, the modulation of PAs and their metabolism has seldom been studied in response to plant inoculation with beneficial soil bacteria. When the expression of PA biosynthetic genes was analyzed under inoculated, non-stressed conditions, we observed that, interestingly, GAP-P45 affected (either induced or downregulated) the expression of all PA biosynthetic genes at almost all time points of analysis even without water-stress **(Fig. 3.5- 3.8)**. Overall, all the PA biosynthetic genes were mostly upregulated on all three days of analysis when inoculated with GAP-P45 under normal conditions. Prior to our study, transcriptional profiles of PA metabolic genes in response to PGPR inoculation have never been reported. Our data presents interesting and novel observations suggesting the ability of soil bacteria to trigger PA biosynthetic gene expression as an early response in plants, within two to four days of bacterial inoculation under non-stressed conditions.

The PA metabolic enzymes are under complex regulation due to their crucial balance necessary for efficient regulation of cellular responses. The genes encoding these enzymes are reported to be differentially regulated under various cellular conditions. In *Arabidopsis*, while *ADC2* is reported to be strongly induced by dehydration/ high salinity etc. (Urano et al. 2003, 2004; Alcázar et al. 2006; Takahashi and Tong 2015), *ADC1* is either reported to be constitutively expressed (Takahashi and Tong 2015) or mainly induced by cold (Cuevas et al. 2008, 2009), and seldom reported to be induced under drought (Alcázar et al. 2010, 2011, 2012), implicating a differential expression pattern for both these genes. The other two genes involved in Put biosynthesis in *Arabidopsis*, *AIH* and *CPA* are not reported to be affected under any stresses (Alcázar et al. 2006, 2011, 2012). In our study, we found that, under water-stressed conditions, most of the Put biosynthetic genes exhibited an overall upregulation/induction in their expression as opposed to the no-treatment controls at almost all time-periods of analysis **(Fig. 3.5).** The exceptions to this observation are: (1) *ADC2* showed a downregulation on day 2 and no induction on day 7 **(Fig. 3.5 A, C)**, (2) *CPA* exhibited a delayed induction i.e. not observed until day 4 of analysis **(Fig. 3.5 B)**. While our observations mostly

corroborated previous reports in literature, there were a few deviations, i.e. *AIH* and *CPA* were also upregulated in response to water stress at most time points during the study.

The SPDS enzyme encoding genes *SPDS1* and *SPDS2* are also among thoroughly studied stress responsive genes. While, *SPDS1* expression is reported to increase under stress, *SPDS2* is mostly reported to follow a constitutive expression pattern under various stresses (Alcázar et al. 2006), including drought (Alcázar et al. 2011). As far as our results are concerned, both *SPDS1* and *SPDS2* exhibited significant induction (day 2 onwards) in response to water-stress **(Fig. 3.6)**. The gene *SPMS,* encoding the enzyme spermine synthase, as previously reported to be induced under most stresses (Urano et al. 2004; Alcázar et al. 2010), followed the same pattern in our study **(Fig. 3.7)**. Although the enzyme SAMDC is encoded by at least 4 genes (*SAMDC1-4*), only *SAMDC1* and *SAMDC2* have been reported earlier as being differentially responsive to abiotic stresses such as drought (Alcázar et al. 2011), cold and to some extent salinity (Gill and Tuteja 2010; Marco et al. 2011). In our study as well, the *SAMDC* genes mostly showed an upregulation in response to water stress, except for *SAMDC3* on day 2 and *SAMDC4* on day 2 and day 7 **(Fig. 3.8)**.

Under water-stressed, GAP-P45 inoculated conditions, the Put biosynthetic genes were induced **(Fig. 3.5)** as compared to water-stressed, non-inoculated plants on all days of analysis (exceptions being *AIH* on day 4 and day 7). *SPDS1* followed the same pattern as the Put biosynthetic genes on day 2 and day 4, while exhibiting a downregulation on day 7 as opposed to the water-stressed, non-inoculated plants. Such a down-regulation was also exhibited by *SPDS*2 at all time-periods of analysis **(Fig. 3.6).** Therefore, under water-stress, while GAP-P45 mostly induced the expression of the Put biosynthetic genes, it mostly downregulated the Spd biosynthetic genes. *SPMS* also showed an overall induction by GAP-P45 inoculation under water-stressed conditions **(Fig. 3.7)**.

In most genes directly involved in PA biosynthesis, by day 2 **(Fig. 3.5 A, 3.6A, 3.7)**, even when other treatments had not caused any induction in expression (except *ADC1* showing an upregulation in non-stressed inoculated seedlings), GAP-P45 inoculated plants under water-stressed conditions were already exhibiting significantly elevated expression levels. This induction, as compared to the water-stressed, non-inoculated plants was mostly sustained on day 4, except *AIH*, *SPDS2* and *SPMS*. Day 7 showed a more complicated dynamics. While in *ADC1*, *ADC2* and *CPA*, this induction was sustained, *SPDS1* actually showed a down-regulation on day 7 as opposed to the water-stressed, non-inoculated plants. In case of *AIH* **(Fig. 3.5 B, C)**, the sudden and sharp surge in its expression in the water-stressed, non-inoculated plants on day 4 and day 7, actually superseded the induction due to GAP-P45 inoculation under water-stress on both these days. With respect to *SPMS* expression **(Fig. 3.7)**, while on day 4, the water-stressed, non-inoculated samples caught-up with the water-stressed inoculated plants, a sudden and sharp surge in its expression was

seen in the water-stressed, inoculated plants on day 7. As far as the *SAMDC* genes are concerned, such an induction was also seen in *SAMDC3* on day 2 **(Fig. 3.8 A)**, *SAMDC2* on day 4, *SAMDC4* on day 2 and day 4 **(Fig. 3.8 A, B)** and *SAMDC1* on day 7 **(Fig. 3.8 C)**. Hence, it can be seen that, while an overall induction was observed in the expression of all genes under all treatments, on most time points, in many cases, especially with the genes involved directly in PA biosynthesis, the highest induction was exhibited by the water-stressed, GAP-P45 inoculated plants. The substantially higher expression of these genes in the water-stressed + GAP-P45 inoculated plants could be attributed to either or both of the following:

1. Gradual and sustained increase in their expression following the initial surge on day 2.
2. A combined/synergistic effect of water-stress and GAP-P45 inoculation, since these treatments also, individually, led to the up-regulation of their expression on day 4 (*ADC1*, *ADC2*, *CPA* and *SPDS1*) and/or day 7 (*ADC*, *CPA* and *SPMS*).

The fluctuations observed in the PA transcript levels could possibly be a result of the pluralistic response generated due to an intensive molecular reprogramming of genes that could be PGPR responsive as well as PA-associated.

3.1.5 Key Findings

1. Most of the PA biosynthetic genes were induced under non-stressed, GAP-P45 inoculated conditions, even though no morpho-physiological alterations were manifested in *A. thaliana* plants by this treatment, when compared to no-treatment controls.
2. While water-stress caused a change in expression of most PA biosynthetic genes, GAP-P45 inoculation further modulated their expression. In fact, GAP-P45 inoculation under water- stress caused the most statistically significant fluctuations in the gene expressions.

<u>Section 2</u>: Modulation of polyamine catabolic gene expression and enzyme activity in *A. thaliana* by *P. putida* GAP-P45 with or without water stress.

3.2.1 Introduction

In plants, PA homeostasis is a resultant of both its biosynthesis as well as catabolism. The role of PA catabolism in optimally maintaining the PA pool in plant cells is known to be as crucial as its biosynthesis for the plant's survival, development and response to external stimuli like biotic/ abiotic stresses (Cona et al., 2006; Moschou et al., 2012, 2008b; Planas-Portell et al., 2013). As mentioned earlier, the involvement of PAs in stress response has been thoroughly studied by premier workers in the field (Urano et al. 2004; Page et al. 2007; Cuevas et al. 2008, 2009, Mohapatra et al. 2009, 2010a; Takahashi and Kakehi 2010; Alcázar et al. 2011, 2012; Minocha et al. 2014). However, the focus of most of these studies have been to elucidate the role of PA biosynthesis and accumulation in response to various stresses in plants (Urano et al. 2004; Alcázar et al. 2006, 2012, Cuevas et al. 2008, 2009; Sen et al. 2018).

Polyamine catabolism, in plants is mediated by amine oxidases (AOs) **(Fig. 3.9)**. Copper containing amine oxidases (CuAOs) or diamine oxidases (DAOs) terminally catabolize the diamines, Put and cadaverine through oxidative deamination to generate catabolic products H_2O_2 and aminoaldehydes which are also precursors of γ-aminobutyric acid (GABA). The triamine, Spd has also been reported to be a substrate for this class of enzymes (Moschou et al. 2008b, 2012; Ghuge et al. 2015). In recent years, several genes encoding these enzymes have been characterized in *A. thaliana*. These genes include *AO1, CuAO1, CuAO2, CuAO3* and the most recent *CuAO8* which exhibit typical CuAO activity i.e. PA oxidation (Moller and McPherson 1998; Wimalasekera et al. 2011; Naconsie et al. 2014; Qu et al. 2014; Ghuge et al. 2015; Groß et al. 2017). The other class of amine oxidases are the flavin containing PA oxidases (PAOs) which mostly catabolize Spm, Spd and their conjugated or acetylated forms with varying substrate specificity. In dicots, such as *A. thaliana*, PAOs, namely *AtPAO1, AtPAO2, AtPAO3, AtPAO4 and AtPAO5* oxidize the carbon at the exo-side of the N^4-nitrogen of Spd and Spm, giving rise to a PA back-conversion pathway (as opposed to the conventional terminal catabolism of PAs in monocots), with the production of Spd from Spm and Put from Spd, in addition to H_2O_2 and aminoaldehydes (Moschou et al. 2008a, b, 2012, Fincato et al. 2011, 2012; Planas-Portell et al. 2013) **(Fig. 3.9)**. While *AtPAO1* and *AtPAO5* preferentially back-convert thermospermine to Spd, *AtPAO4* is involved in back- conversion of Spm to Spd and *AtPAO2* and *AtPAO3* mainly convert Spd to Put (Angelini et al. 2010; Sagor et al. 2016; Tavladoraki et al. 2016).

Most of the genes encoding plant CuAOs and PAOs are known for their involvement in developmental processes like cell wall maturation, vascular development etc. (Cona et al., 2006; Moschou et al., 2012). Besides, their expression is reported to be highly stimulated by external stimuli such as wounding, pathogen attack, methyl jasmonate treatment, salicyclic acid, ACC, ABA, drought, salinity,

light, heat etc. in various plants like barley, chickpea, tobacco and others (Jimenez-Bremont et al., 2014; Planas-Portell et al., 2013; Quinet et al., 2010; Tisi et al., 2011; Toumi et al., 2010; Yoda et al., 2006).

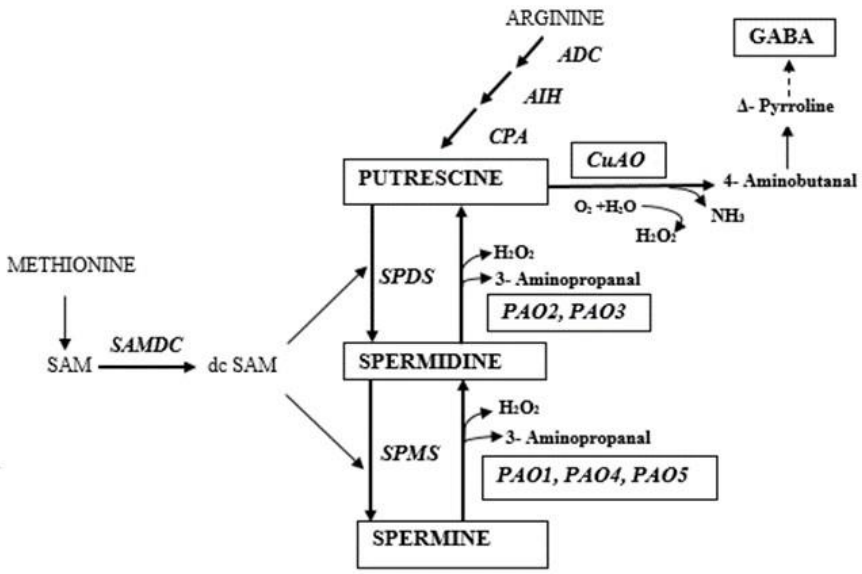

Fig. 3.9 Polyamine metabolic pathway in *Arabidopsis thaliana* (Alcázar et al., 2006; Fincato et al., 2011; Sequera-Mutiozabal et al., 2016).

The role of PA catabolism has been studied with respect to drought and salinity stresses (Berberich et al., 2015; Bouchereau et al., 1985; Cona et al., 2006; Kusano et al., 2015; Sequera-Mutiozabal et al., 2016). In some cases, the PA biosynthesis and catabolism was found to exhibit a reciprocal relationship, thereby impacting PA accumulation under stress (Aziz et al. 1998; Sagor et al. 2016). There are also many instances wherein the possible integration of both, increased biosynthesis and catabolism with increased PA accumulation/ turnover, manifested in stress responses (Roy and Ghosh 1996; Waie and Rajam 2003; Prabhavathi and Rajam 2007; Toumi et al. 2010; Hatmi et al. 2015). However, despite several years of research, the precise role of PA catabolism in the plant response to environmental stress has remained elusive.

An association of PGPR and PAs in mitigating abiotic stress tolerance in plants has been explored in the recent years by a few workers (Cass et al. 2009; Zhang et al. 2010; Xie et al. 2014; Zhou et al. 2016; Sen et al. 2018). While a link between PA catabolism and abiotic and biotic stress responses has been described, there are no reports on the modulation of PA catabolism or PA turnover in response to beneficial soil microorganisms under abiotic stress conditions.

In the previous section (section 1), we had described the impact of a free living, drought-mitigating rhizobacterial strain, *P. putida* GAP-P45 on the expression of key genes in the polyamine biosynthetic

pathway in water-stressed *A. thaliana*. We observed and reported (Sen et al. 2018) that inoculation with GAP-P45 under water stress led to a significant increase in the expression of most of the PA biosynthetic genes. In continuation of the previous work, in this section, we describe the impact of this particular rhizobacterial strain on the expression of PA catabolic genes (*CuAO1-3, PAO1-5*) and activities of the corresponding catabolic enzymes copper amine oxidase (CuAO) and polyamine oxidase (PAO) in water-stressed *A. thaliana* [Manuscript under review].

3.2.2 Materials and Methods

3.2.2a Measurement of the transcript levels of PA catabolic genes in *A. thaliana* seedlings

The procedures involved in germination, growth, water-stress induction and GAP-P45 inoculation have been thoroughly described in chapter 2 (Ghosh et al., 2017, Sen et al., 2018). The transcript levels of all PA catabolic genes were analyzed through similar procedures followed for the biosynthetic genes as mentioned in Section 1 of this chapter. Briefly, total RNA (2 µg) isolated from treated and untreated *A. thaliana* seedlings was reverse transcribed into cDNA in a 20 µl total volume of reaction mix containing the MuLV reverse transcriptase enzyme (NEB), buffer, dNTPs, oligo dT primer and DTT, based on the manufacturer's instructions. Semi-quantitative as well as quantitative real time PCR for analysis of gene expression was performed using appropriate primers **(Table 4)**, for the following genes- *CuAO1, CuAO2, CuAO3, PAO1, PAO2, PAO3, PAO4* and *PAO5*. The gene-specific primer sequences (Hou et al., 2013; Marina et al., 2013; Planas-Portell et al., 2013; Wimalasekera et al., 2011) used for the analyses were manufactured by Oligos, Sigma. All the primers were tested at different concentrations (ranging from 50-500 nM per reaction) with the serially diluted cDNA (ranging from 6.25ng - 200ng, using 1:2 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. The cDNA samples used for standardization were synthesized from the RNA of no treatment control seedlings. Quantitative real time PCR (qRT PCR) was performed using an Applied Biosystems detection system, using SYBR Green PCR master mix (2X) kit (GeneSure, Genetix). For relative quantification of cDNA samples in a total volume of 10 µl PCR solution, 0.5µl of cDNA template was mixed with 200-500 nM each of forward and reverse primers and 2x SYBR Green PCR Master Mix. The reaction was first incubated at 95^{o}C for 10 min., followed by 45 cycles of incubation at 95 ^{o}C for 15 s and 60 ^{o}C for 1 min. (Jumtee et al. 2008). The qRT PCR was performed in three biological replicate cDNA samples for each treatment from at least two independent experiments. Expression of all the genes were normalized to the endogenous control gene, *β-actin-2* and the fold change in expression of the treated vs untreated samples was computed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) as described earlier. The semi- quantitative PCR analysis of

amplicons through agarose gel electrophoresis, standard curves and melt curves (representative examples) for the genes generated through qRT PCR are illustrated in **Fig. 3.10, Fig. 3.11** and **Fig. 3.12** respectively.

Table 4: Oligonucleotide sequences of the PA catabolic genes used for semi-quantitative and quantitative PCR reactions.

(Hou et al., 2013; Marina et al., 2013; Planas-Portell et al., 2013; Wimalasekera et al., 2011)

Genes	Forward primer	Reverse primer
CuAO1	5'-TTGCCACCTACTCTTGGGTCTTTG-3'	5'-ACTTGACGATTCGAGCCGAGAGTT-3'
CuAO2	5'-GTCAAGATGGAACTCCCGC-3'	5'-TCGCCACATGATATCTCCAG-3'
CuAO3	5'-GTAAGTTTGTGCCACTCCCCC -3'	5'-GCCACTCGACAAAGTAACCCC-3'
PAO1	5'-ACAGAGGATGGTTCCGTATACGA-3'	5'-AAGATCAGATTGGAGAACACCGATA-3'
PAO2	5'-TCGTCAAATGCGTAGAGC-3'	5'-AAATCCCACCGAAACCAC-3'
PAO3	5'-ACAAACCTCACGACCTCTATG-3'	5'-TCAAGCACACGCATCCTG-3'
PAO4	5'-ATCCAGAACTAAGGCAAG-3'	5'-ATGACCACCTGAAAGACA-3'
PAO5	5'-TGATCAAGCCAAGGTTCATGAG-3'	5'-GGCACCATGAGTTGTGGAGTAA-3'

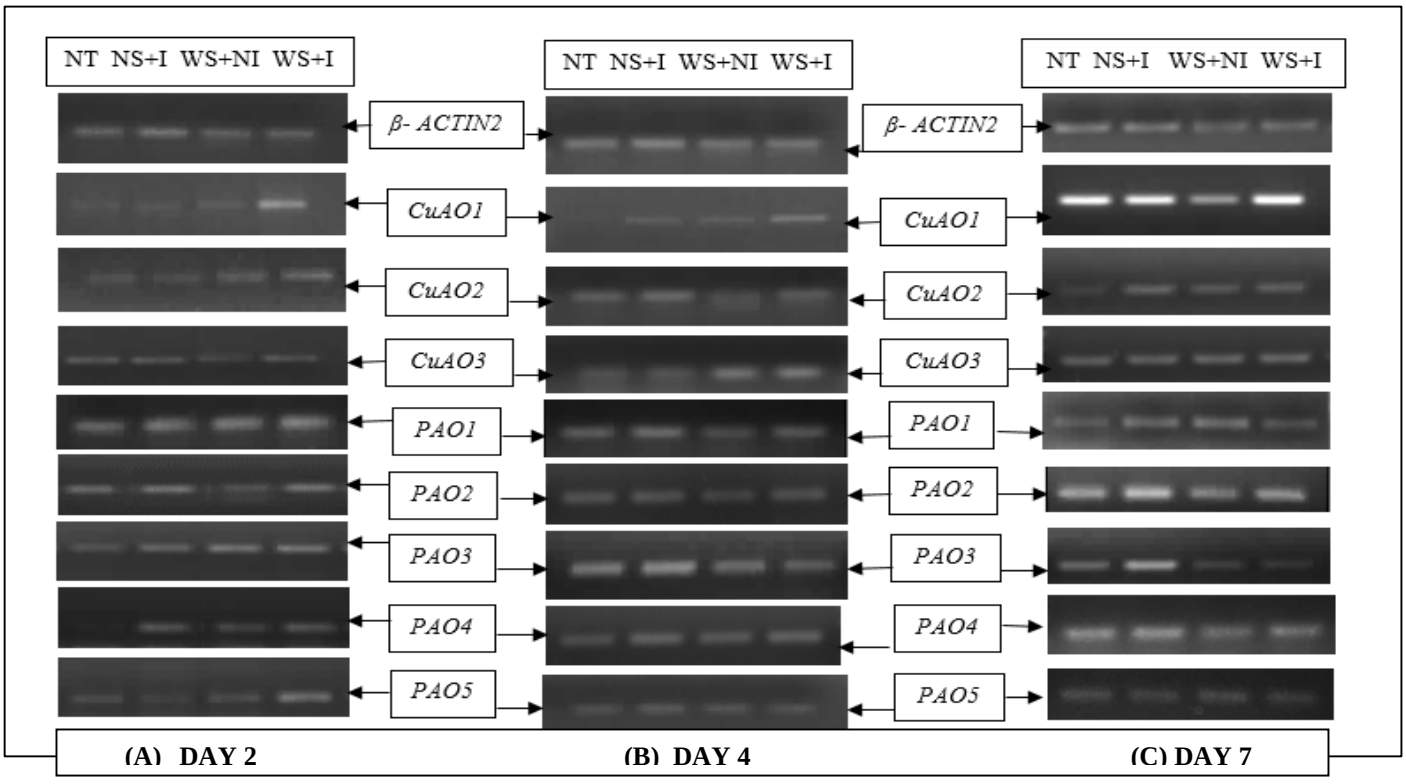

Fig. 3.10 Semi quantitative PCR analysis of expression patterns of the polyamine catabolic genes (*CuAO1, CuAO2, CuAO3, PAO1, PAO2, PAO3, PAO4 and PAO5*) post water-stress induction and GAP-P45 inoculation (A) day 2 (B) day 4 (C) day 7 after treatments.

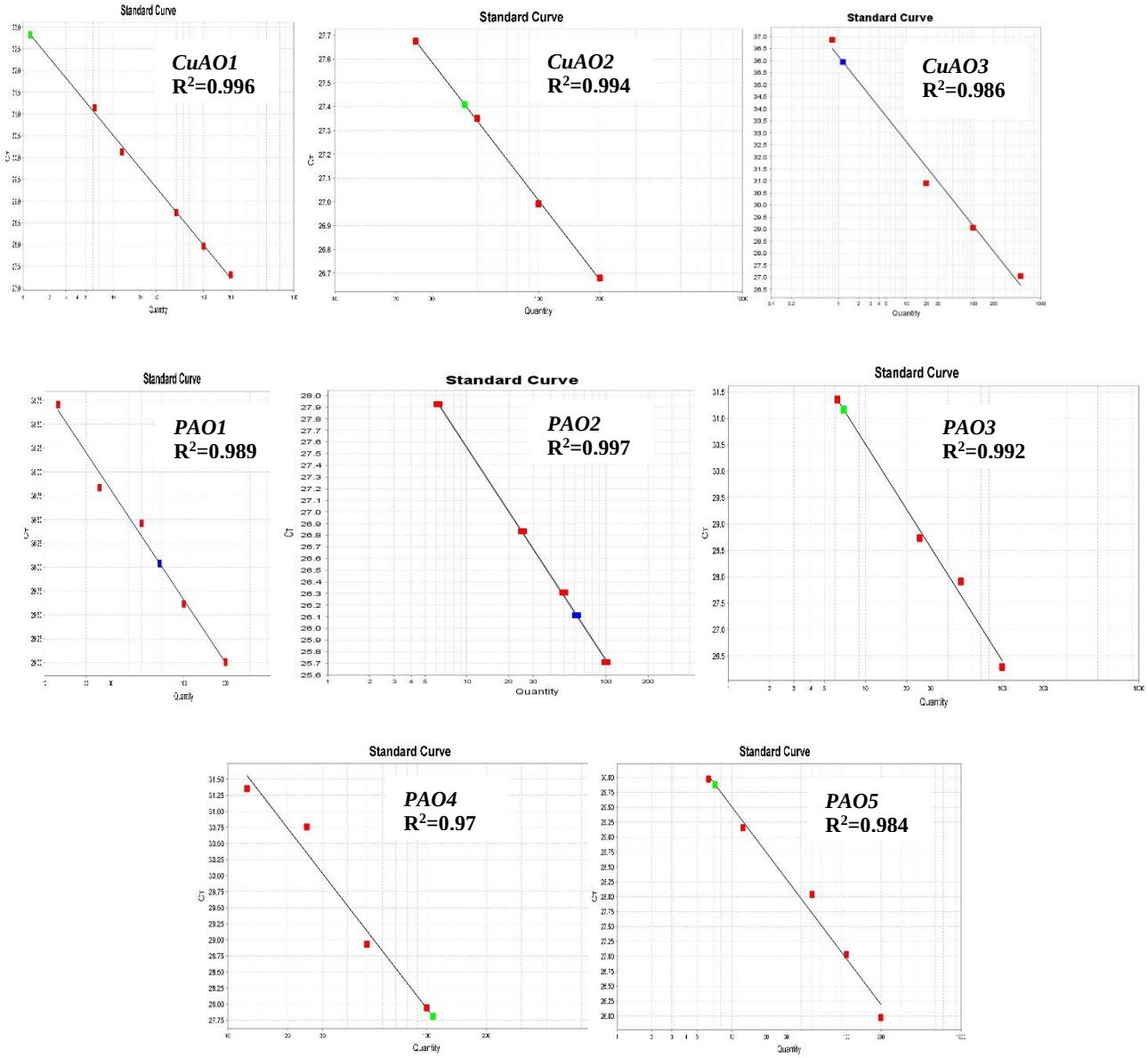

Fig. 3.11 Standard curves of all the gene primers to optimize the primer and template concentration for the relative quantification of expression of the genes of interest in real-time PCR analysis. The genes include *CuAO1, CuAO2, CuAO3, PAO1, PAO2, PAO3, PAO4* and *PAO5*. Standard curves were generated using cDNA template from no treatment controls.

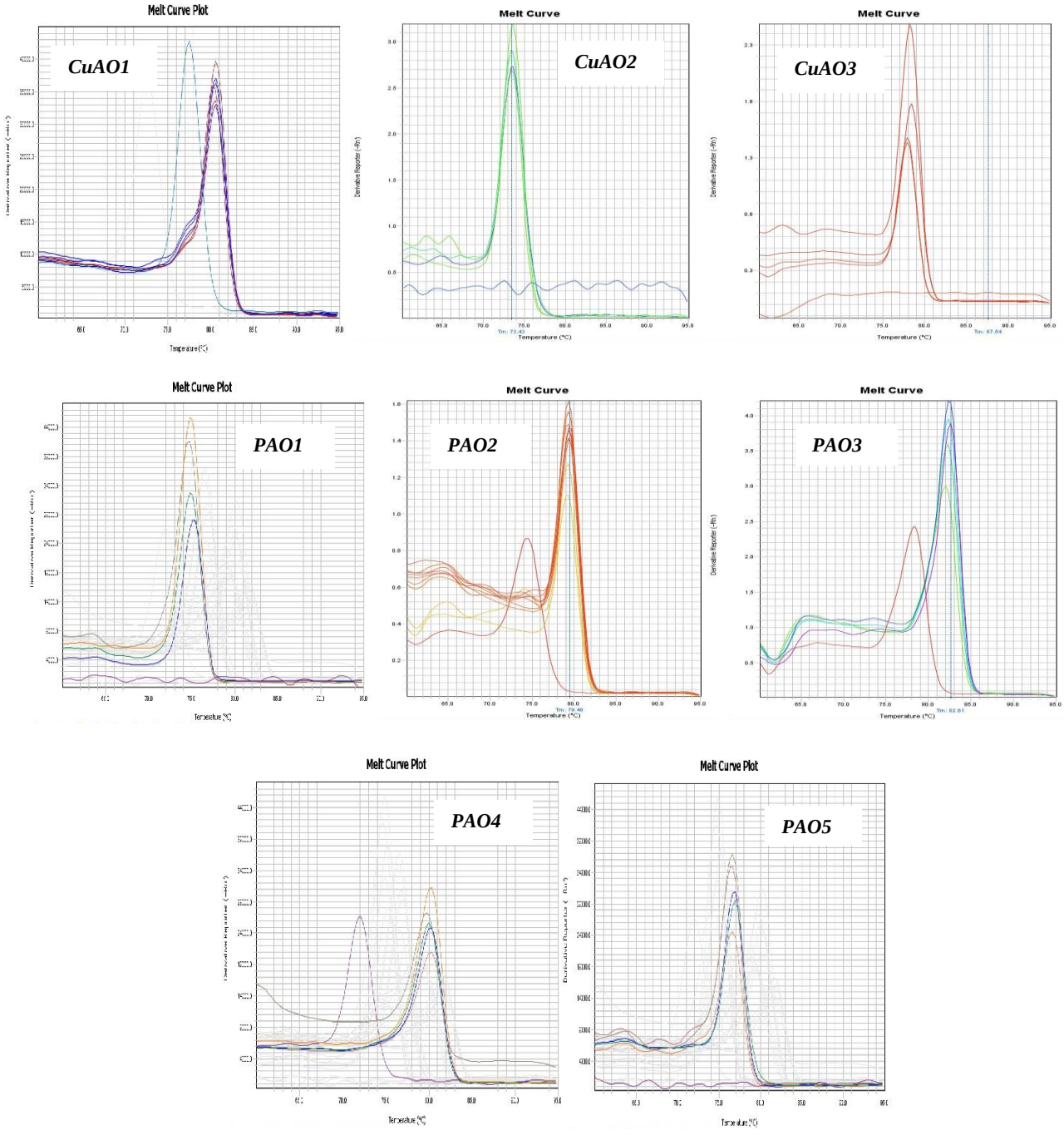

Fig. 3.12 Representative examples of melt curves of all the gene products to determine presence/absence of any non-specific amplification of template DNA in quantitative real-time PCR. The genes analyzed were *CuAO1*, *CuAO2*, *CuAO3*, *PAO1*, *PAO2*, *PAO3*, *PAO4* and *PAO5*. Primer- dimers were observed in case of *CuAO1*, *PAO2*, *PAO3 and PAO4* with T_m values lower than the actual amplicons.

3.2.2b Measurement of PA catabolic enzyme activity in *A. thaliana* seedlings

The CuAO and PAO enzyme activities were estimated spectrophotometrically by a modified method based on the colorimetric assay of Δ^1-pyrroline using Put (CuAO), Spd and Spm (PAO) as substrates as originally described by Holmstedt et al. (1961) and Asthir et al. (2002). Briefly, 200 mg of *A. thaliana* seedlings subjected to different treatments were collected on day 2, day 4 and day 7 post treatments. Thereafter, the seedlings were homogenized (triplicate samples) at 4 °C in 100 mM K-phosphate buffer (pH 7.0) containing 5 mM dithiothreitol and the extract centrifuged at 16,000 *g* for 20 min at 4 °C. The residue was then sequentially extracted twice for 10 min with 100 mM K-phosphate buffer (pH 7.0) containing 20 mM ethylene diamine tetraacetic acid. In a 2.0 ml final reaction volume, 0.2 ml of extract was combined with 50 units of catalase, 0.1% 2-aminobenzaldehyde and the reaction started by adding 10mM of Put/ Spd/ Spm as substrates for analyzing CuAO/ PAO activity respectively. The reaction was carried out at 37 °C for 2 h, and then stopped with 2.0 ml of 10% (v/v) perchloric acid. The tubes were then centrifuged at 5000 rpm for 15 min. Formation of the Δ-pyrroline product was determined by reading the absorbance at 430 nm. Control reactions were carried out with inactivated enzyme prepared by heating for 20 min in a boiling water bath. Activities are shown as the means of six determinations and are expressed in μmol Δ-pyrroline min^{-1} g^{-1} fresh weight.

3.2.2c Statistical analysis

Statistical analysis was performed by two-way Anova with Tukey's HSD test (level of significance, $p \leq 0.05$). 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.

3.2.3 Results

P. putida GAP-P45 modulates the expression of PA catabolic genes in _A. thaliana_ with or without water stress

The Put catabolic genes:

In this study, all the treatments were found to affect the expressions of the Put catabolic genes (*CuAO1*, *CuAO2*, and *CuAO3*) at most time points (**Fig. 3.13)**. On day 2 **(Fig. 3.13 A)**, neither of these genes showed any modulation under non-stressed conditions when inoculated with GAP-P45. Water-stress, on its own, caused a ~ 2- fold increase in the expression of only the *CuAO1* gene while *CuAO2* remained unchanged and *CuAO3 was* slightly down-regulated as opposed to the no-treatment controls. When water-stressed samples were inoculated with GAP-P45, a ~ 2-2.5-fold increase in the transcript levels of all these genes was observed, as compared to their non- inoculated counterparts **(Fig. 3.13 A).** In both, *CuAO1* and *CuAO2*,

the highest and statistically significant (*CuAO1*) up-regulation was seen in the water-stressed, GAP-P45 inoculated samples.

On day 4 **(Fig. 3.13 B),** at least two out of the three genes (*CuAO1 and CuAO3*) exhibited an up-regulation in response to all treatments. The expression of *CuAO1* surged by > 2.5 fold in response to GAP-P45 inoculation without water stress as opposed to the untreated controls. Water stress itself (without GAP-P45 inoculation) caused a ~ 2.5 fold induction in the *CuAO1* expression. Also, this observed induction was 1.5 times greater than that observed on day 2. GAP-P45 inoculation of water-stressed plants further resulted in a >1.5-fold increase in *CuAO1* expression as compared to the non- inoculated seedlings. The gene *CuAO2* exhibited highest expression (> 3.5 fold) in the GAP-P45 inoculated, water-stressed samples as compared to all other treatments on day 4 which either remained down-regulated (non-stressed inoculated seedlings) or unchanged (water-stressed seedlings) as compared to the untreated control **(Fig. 3.13 B)**. The expression of *CuAO3* showed a gradual, treatment-dependent up-regulation from day 2 to day 4. While the non-stressed inoculated plants were induced by 1.5 fold than the untreated controls, the highest and most significant up-regulation was seen in the GAP-P45 inoculated water-stressed samples which was 2 fold greater than the non- inoculated, water-stressed plants.

Interestingly, on day 7, **(Fig. 3.13 C),** all the three genes exhibited similar patterns of expression in that, both the non-stressed, inoculated and water-stressed samples displayed almost equal levels of significant up-regulation as compared to the untreated controls. Further elevation in gene expression for all these genes was observed with GAP-P45 inoculation under water stress. *CuAO1* displayed a similar pattern of expression as on day 4, but the transcript abundance was significantly higher (~3- 4 fold) in all treatments. On day 7, *CuAO2* displayed a gradual treatment-dependent up-regulation. *CuAO2* saw a significant surge (~ 3.5 -fold) on inoculation with GAP-P45 without water-stress. Water stress by itself also caused a similar increase of > 3-fold in the expression of *CuAO2* (as opposed to the no-treatment controls), while treatment with GAP-P45 under water-stress, caused further induction of *CuAO2* by ~ 2 fold over the water-stressed, non- inoculated plants **(Fig. 3.13 C)**. As for *CuAO3*, all the treatments displayed a significantly high up-regulation (> 6 fold) from the untreated controls on day 7. Besides, when compared to day 4, this gene exhibited a significant surge (~ 4 fold) in the non-stressed, inoculated seedlings and > 1.5 fold higher induction in the water-stressed ones with or without inoculation **Fig. 3.13 (A-C)**. However, on day 7, although the GAP-P45 inoculated seedlings displayed highest expression of *CuAO3* under water stress as compared to the other two treatments, this increase was found to be statistically insignificant.

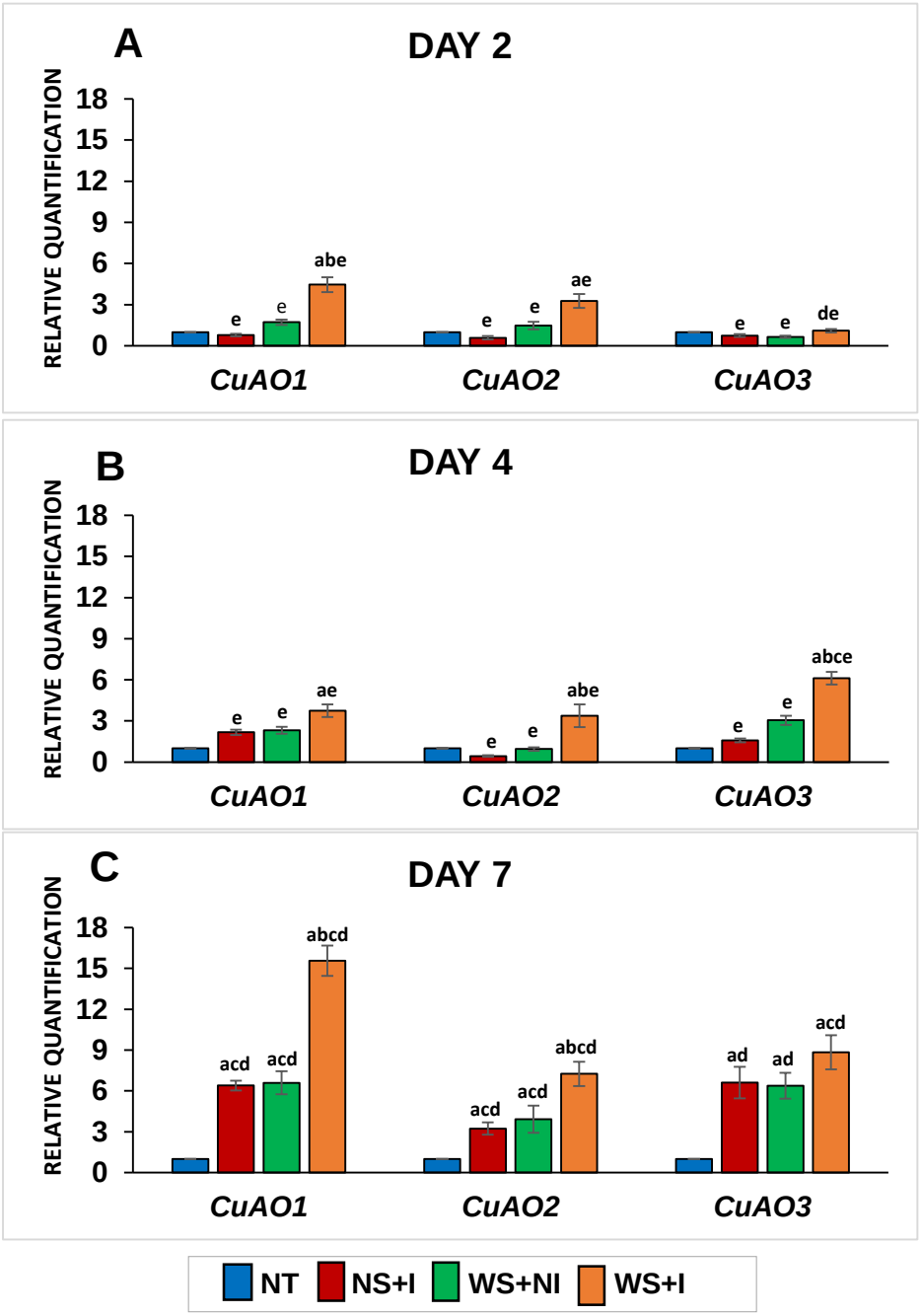

Fig. 3.13 Relative expression of putrescine catabolic genes (*CuAO1*, *CuAO2* and *CuAO3*) in *A. thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 inoculation with or without water stress, day 2 **(A)**, day 4 **(B)** and day 7 **(C)** post treatments (**NT**: no treatment; **NS+ I**: non-stressed, inoculated; **WS+NI**: water-stressed, non-inoculated; **WS+I**: water-stressed, inoculated). Each bar represents mean (±) S.E. of 6 replicates. 'a' represent significant difference ($p \leq 0.05$) of all treatments from NT. 'b' represent significant difference ($p \leq 0.05$) between WS+NI and WS+I seedlings within a gene and time-point. 'c', 'd' and 'e' denote significant differences of a gene within a treatment on a particular day from day 2, 4 and 7 respectively.

The Spd and Spm back- conversion genes

The expression patterns of PAO encoding genes *PAO1-PAO5*, known to be involved in the back-conversion of Spm and Spd to Put displayed an overall similarity to the expression patterns of the Put catabolic genes, as described above. However, interestingly, there were some variations too. On day 2 **(Fig. 3.14 A)**, GAP-P45 inoculation under non-stressed conditions caused slight increase in two of these genes namely *PAO1* and *PAO4* while the expression of the other three genes remained unchanged *(PAO2, PAO3 and PAO5*) in these samples. At the same time-point, water stress by itself caused up-regulations of > 2.5-7 fold in all these genes, with statistically significant increases in *PAO1*, *PAO3* and *PAO4*. When the expression of *PAO1-PAO5* was analyzed in the water-stressed samples post GAP-P45 inoculation, the expression of all these genes was further induced by ~1.5 (*PAO3*, *PAO4*) >3 (*PAO1, PAO2*) or >5 fold (*PAO5*).

Subsequently on day 4 **(Fig. 3.14 B)**, under non-stressed conditions, except *PAO1* (which was marginally up-regulated), the expression of all others remained similar to the untreated controls. Under water stress, all the genes were slightly up-regulated except *PAO3*. The water-stressed GAP-P45 inoculated seedlings displayed ~1.5- 3 fold significantly higher up-regulation for *PAO1* and *PAO2* genes as compared to the non- inoculated ones. However, interestingly, on day 4, there was a significant dip in the expression levels of most of these genes in the water-stressed inoculated samples when compared to their expression on day 2, albeit, still being higher than their non-inoculated counterparts.

On day 7, the *PAO* genes showed differential patterns of expression **(Fig. 3.14 C)**. The non- stressed GAP-P45 inoculated samples showed significantly high expression levels of all these genes (except *PAO5*) as compared to the untreated controls. Water stress, itself, up-regulated some of them (*PAO-1, 2, 4*) and left the others unchanged *(PAO-3, 5)* as compared to the untreated controls. Inoculation with GAP-P45 under water-stressed conditions, on one hand, significantly down-regulated *PAO1*, while on the other, led to a further increase in the expression of both *PAO2* and *PAO4* as compared to all other treatments. Marginal increases (statistically insignificant) with inoculation were also seen in case of *PAO5* and *PAO3* under water-stressed conditions **(Fig. 3.14 C)**.

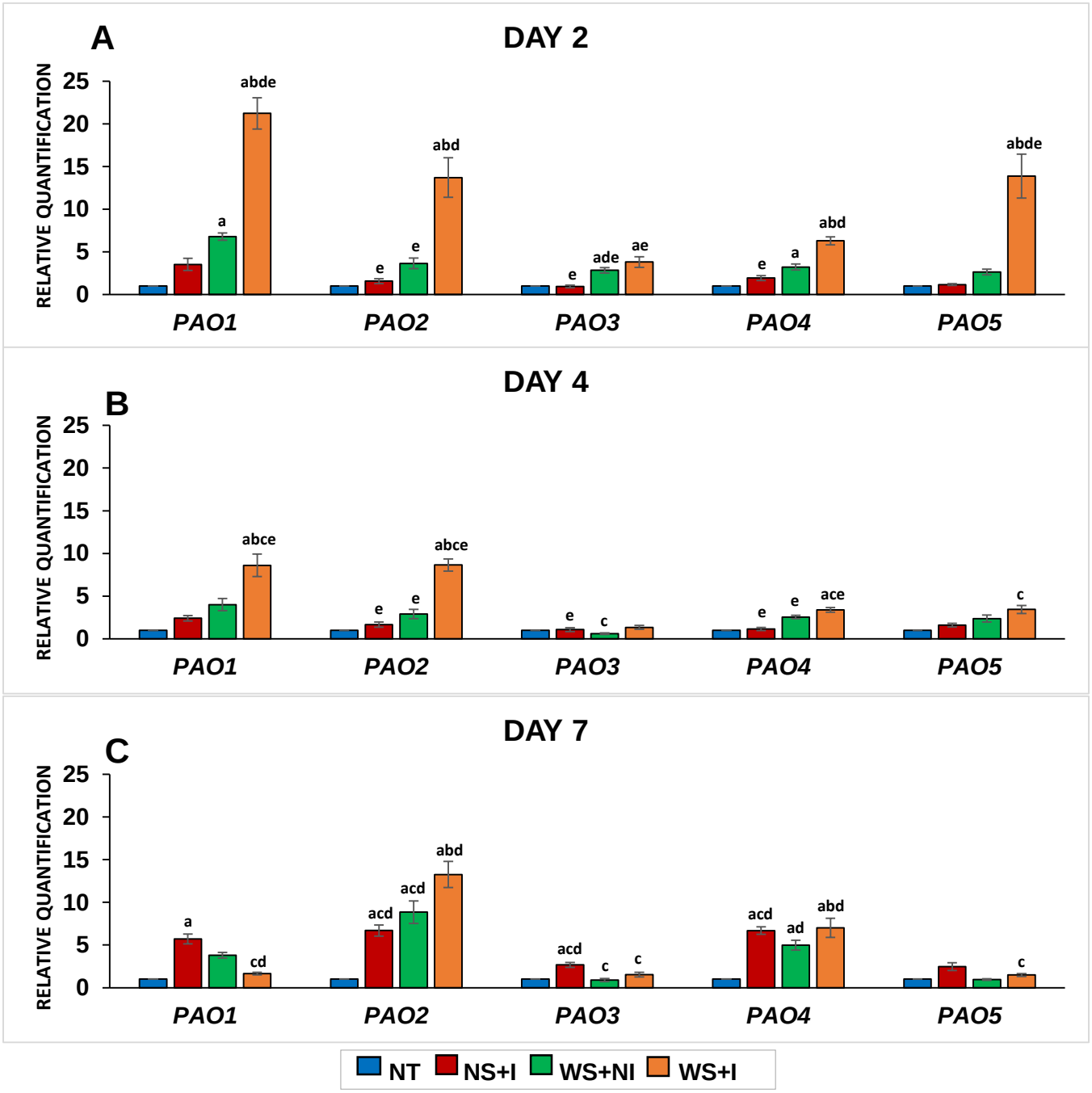

Fig. 3.14 Relative expression of spermidine and/or spermine catabolic genes (*PAO1*, *PAO2*, *PAO3*, *PAO4* and *PAO5*) in *A. thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 inoculation with or without water stress, day 2 **(A)**, day 4 **(B)** and day 7 **(C)** post treatments. Each bar represents mean (±) S.E. of 6 replicates. 'a' represent significant difference ($p \leq 0.05$) of all treatments from NT. 'b' represent significant difference ($p \leq 0.05$) between WS+NI and WS+I seedlings within a gene and time-point. 'c', 'd' and 'e' denote significant differences of a gene within a treatment on a particular day from day 2, 4 and 7 respectively.

***P. putida* GAP-P45 differentially modulates the expression of PA catabolic enzymes in *A. thaliana* with or without water stress**

Copper- amine oxidase

On day 2, the activity of the CuAO enzyme exhibited similar patterns **(Fig. 3.15 A)** with the observed transcriptional profile of the *CuAO* genes **(Fig. 3.13).** As with gene expression data, while the non- stressed inoculated seedlings showed almost similar activity of this enzyme as the untreated controls, elevations were observed in the activities of the water-stressed plants with the highest and most significant elevation found in the water-stressed, inoculated seedlings. On day 4, all treatments caused remarkable enhancement in enzyme activity as opposed to untreated controls. Interestingly, the up-regulation was similar in all treatments, except a significantly higher increase in water-stressed, GAP-P45 inoculated plants. Trends on day 7 were similar to day 4. It is interesting to note that, the activity of this enzyme, in all treatments (i.e. minus the no-treatment controls), was elevated on day 4 as opposed to day 2, coming down on day 7 to almost day 2 levels. Also, on day 7, there was no difference in the activity observed in water-stressed non-inoculated seedlings as compared to the water-stressed seedlings with GAP-P45 inoculation, which is contrary to gene expression data **(Fig. 3.13 C).** The CuAO specific activity was almost identical to the activity observed at all time-points **(Fig. 3.15 B).**

Polyamine oxidase

The PAO enzyme activity with Spd as a substrate displayed some interesting dynamics. All treatments displayed significant elevation in PAO activity on day 2 (except non-stressed, inoculated ones) **(Fig. 3.15 C)**. However, contrary to the gene expression data **(Fig. 3.14 A),** the activity observed in the GAP-P45 inoculated water-stressed samples on day 2 did not surpass the activity seen in the water-stressed plants without inoculation **(Fig. 3.14 C).** On day 4, all treatments showed further elevation in PAO activity, with somewhat higher activity in the water-stressed GAP-P45 inoculated seedlings and thus correlated well with the transcriptional profile of the Spd catabolic genes **(Fig. 3.14 B).** On day 7, while all the treatments showed significantly higher PAO activity compared to the untreated controls there was hardly any difference in activities observed between the water-stressed inoculated vs. water-stressed, non- inoculated seedlings **(Fig. 3.15 C).** As with CuAO activity, the PAO activities were also highest on day 4 as opposed to day 2 and day 7 in all treatments**.** The specific activity displayed almost identical patterns to the activity observed at all the time points of analysis **(Fig. 3.15 D).** Modulations in PAO activity and specific activity with Spm as substrate were mostly similar to that of PAO activity and specific activity with Spd as a substrate **(Fig. 3.15 E, F).**

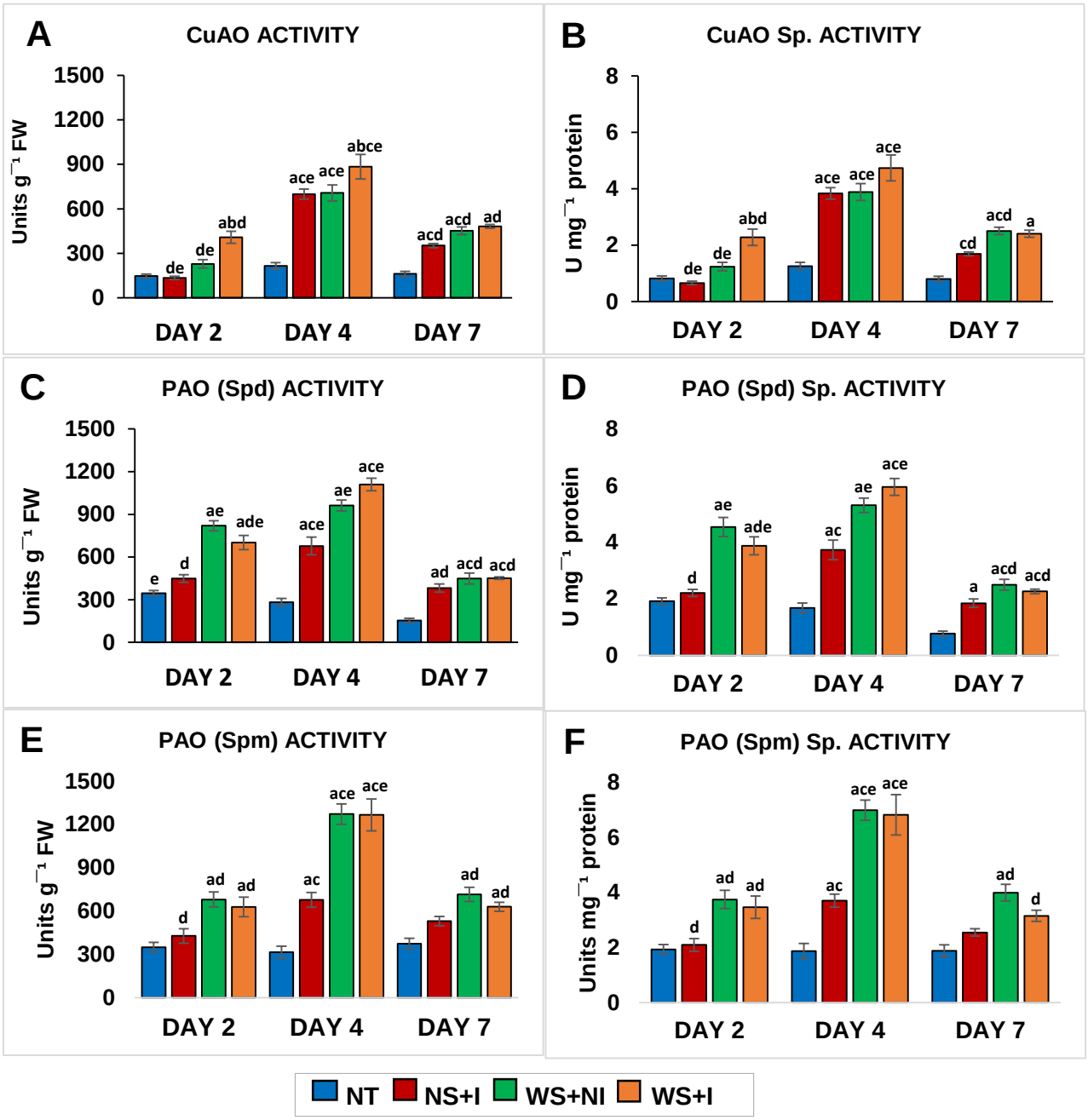

Fig. 3.15 Activity of CuAO and PAO in *A. thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 inoculation with or without water stress, day 2, day 4 and day 7 post treatments. Activities and specific activities of CuAO with Put as substrate (**3.15 A, B**), PAO with Spd **(3.15 C, D)** and Spm **(3.15 E, F)** as substrates expressed as U g^{-1} FW **(3.15 A, C and E)**, U= µ mole Δ pyrroline synthesized; U mg^{-1} protein (specific activity, **3.15 B, D and F**) respectively. Each bar represents mean of U of enzyme activity (±) S.E of 6 independent replicates of each treatment. 'a' represent significant difference ($p \leq 0.05$) of all treatments from NT. 'b' represent significant difference ($p \leq 0.05$) between WS+NI and WS+I seedlings within an

enzyme and time-point. ‘c’, ‘d’ and ‘e’ denote significant differences of enzyme activity within a treatment on a particular day from day 2, 4 and 7 respectively.

3.2.4 Discussion

Although there are a few reports on the role of PA oxidation in stress response, these have mostly focused on the PA oxidation mediated ROS (H_2O_2, $O_2^{\cdot-}$) and NO generation and PA oxidative deamination products like DAP, GABA etc. (Xing et al. 2007; Moschou et al. 2008a, b, 2012; Palavan-Unsal and Arisan 2009; Rodríguez et al. 2009; Toumi et al. 2010). The direct impact of drought stress on the regulation of PA catabolic gene expression has rarely been reported (Toumi et al. 2010; Alcázar et al. 2011; Hatmi et al. 2015; Sagor et al. 2016). The impact of plant – microbial interactions in modulating plant PA metabolism have been investigated earlier but mostly focused on biotic interactions (Berberich et al., 2015; Cona et al., 2006; Jimenez-Bremont et al., 2014). Reports on the influence of beneficial plant- microbial associations on PA metabolism in plants are still relatively scarce (Cass et al. 2009; Zhang et al. 2010; Xie et al. 2014; Zhou et al. 2016; Sen et al. 2018) in comparison with that obtained from studies on pathogenic associations.

***P. putida* GAP-P45 modulates PA catabolic gene expression under normal (non-stressed) as well as water-stressed conditions.**

It is now well- known that PA homeostasis in plants is critically regulated by both its biosynthesis as well as catabolism and/or back-conversion (Hatmi et al., 2015; Planas-Portell et al., 2013; Prabhavathi and Rajam, 2007; Sequera-Mutiozabal et al., 2016; Waie and Rajam, 2003 etc.). So far, the impact of any PGPR on the transcriptional profiles of PA catabolic genes in plants has not been reported. When we analyzed the expression of PA catabolic/ back- conversion genes with GAP-P45 inoculation under non-stressed conditions, we observed that, interestingly, GAP-P45 mostly induced the expression of almost all PA catabolic genes at almost all time points of analysis **(Fig. 3.13, 3.14)**. On day 2 **(Fig. 3.13 A, 3.14 A),** with GAP-P45 inoculation under non-stressed conditions, most of these genes remained unchanged (or marginally down-regulated in few) compared to the untreated controls, except for *PAO1* and *PAO4* where an increase was observed. On day 4, *CuAO1, CuAO3, PAO1* and *PAO5* were induced with GAP-P45 inoculation under non- stressed conditions **(Fig. 3.13 B, 3.14 B)**. On day 7, GAP-P45 inoculation caused significant elevation in the expression of all these genes **(Fig. 3.13 C, 3.14 C)** so as to even surpass all other treatments in some cases (*PAO1, PAO3,* and *PAO5*). Taken together, these observations suggest an ability of this bacterium to modulate the expression of these genes even under non- stressed conditions, albeit, without any significant phenotypic alterations as compared to the untreated control seedlings (Ghosh et al. 2017; Sen et al. 2018).

Interestingly, in our study we observed that, almost all these genes were induced under water stress except *CuAO2* (day 2, day 4), *CuAO3* (day 2), *PAO3* (day 4, day 7) and *PAO5* (day 7) as opposed to the untreated controls **(Fig. 3.13, 3.14)**. Inductions (although statistically insignificant) were observed in *CuAO1* (day 2, day4), *CuAO3* (day 4), *PAO2, PAO5* (day 2, day 4) and *PAO4* (day 4) in response to water stress while the rest were significantly induced. All the three Put catabolic *CuAO* genes displayed a gradual time- dependent up-regulation under water stress as compared to the untreated controls **(Fig. 3.13 A-C)**. Interestingly, in case of the *PAO* transcripts (involved in Spd and Spm back- conversion), while the expression of *PAO1*, *PAO3* and *PAO5*, gradually reduced from day 2 to day 7 and came closer to that of the untreated controls, an opposite trend of time- dependent increase was observed in case of *PAO2* and *PAO4* as opposed to the untreated seedlings **(Fig. 3.14 A-C)**. The water stress induced up-regulation observed in most of the *CuAO* and *PAO* transcripts in our study correlates well with earlier studies with drought and/ or salt and cold stress treatments (Hatmi et al., 2015; Shelp et al., 2012; Tanou et al., 2014). The *AtCuAO* genes have been earlier reported to be responsive to external stimuli such as MeJA treatment, involved in exogenous PA and ABA mediated ROS/ NO generation, stomatal closure, response to wounding etc. (Wimalasekera et al. 2011; Planas-Portell et al. 2013). This is the first report on the observed induction of the *CuAO 1-3* genes in response to water stress in *A. thaliana*.

Through mutant studies the roles of *AtPAOs* have been elucidated under abiotic stresses such as salt and drought. Mostly, *AtPAO1, AtPAO2 and AtPAO3* are reported to be salt stress responsive (Sagor et al. 2016). The expression of *PAO/PAO2* in citrus/grapevine and *Arabidopsis* was observed to be induced under salt and/or drought stress respectively (Alcázar et al., 2011; Hatmi et al., 2015; Tanou et al., 2014). While *AtPAO3* is known to be involved in ROS modulation and maintenance of balanced respiration through Spd oxidation (Andronis et al. 2014), its direct role in drought stress has never been explored earlier. Similar to *AtCuAOs*, the role of *AtPAOs* have never been comprehensively studied under water stress conditions. Thus, our study contributes significantly to the understanding of the impact of water stress on PA oxidation genes.

Under water-stressed, GAP-P45 inoculated conditions **(Figs. 3.13, 3.14)**, almost all these genes were significantly up-regulated as compared to the water-stressed, non- inoculated plants at almost all time points of the study except *CuAO3* (day 7), *PAO1* (day 7), *PAO3* (all time points) and *PAO5* (day 4, day 7). In fact, in all genes studied, for the most part, the highest up-regulation of expression was seen under inoculated, water-stressed conditions. Similar to their non- inoculated counterparts, the expression of all *CuAO* genes displayed a time- dependent increase in up-regulation even under water-stressed, inoculated conditions **(Fig. 3.13 A-C)**. This up-regulation surpassed that of all other treatments at almost all time points of study except *CuAO3* (day 2). The *PAO* transcripts, at most time points, displayed highest up-regulation

(as seen in case of *CuAO* genes) in response to GAP-P45 inoculation under water stress **(Fig. 3.14 A-C)**. The exceptions to this were *PAO1* with a down-regulation on day 7, *PAO3* with similar expression throughout the study and *PAO5* with mostly marginal (statistically insignificant) up-regulation (except day 2) with GAP-P45 inoculation under water stress as compared to the non- inoculated water-stressed seedlings. Also, similar to their non-inoculated counterparts, *PAO1* and *PAO5* displayed a decreased transcript abundance from day 2 to day 7 with GAP-P45 inoculation under water stress **(Fig. 3.14 C).** This could be correlated with earlier studies suggesting a link between lowering of cytoplasmic *PAOs (PAO1, PAO5)* with improved stress tolerance in *A. thaliana* (Sagor et al. 2016), grapevine (Hatmi et al. 2015) etc.

Overall, GAP-P45 significantly induced the expression of all these genes under water stress at most time points of analysis. Similar to the PA biosynthetic genes reported earlier (Sen et al. 2018), this observed induction was mostly highest in the GAP-P45 inoculated water-stressed seedlings at most time points of analysis. This is consistent with previous reports suggesting a correlation of higher expression of PA oxidation genes with that of stress tolerant phenotypes as observed in other plants such as grapevine, citrus etc. (Alcázar et al., 2011; Hatmi et al., 2015; Tanou et al., 2014).

Correlation between PA catabolic gene expression and enzyme activity.

As described earlier, our results with respect to the Put catabolizing CuAO enzyme activity **(Fig. 3.15 A, B)** correlated well with the gene expression data of the corresponding genes **(Fig. 3.13 A, B)** especially on day 2 and day 4 in case of all the treatments. On day 7, while the non-stressed, inoculated and the water-stressed plants showed significant elevation in CuAO activity as expected from the gene expression data, the GAP-P45 inoculated water-stressed plants which were expected to show further elevation in enzyme activity (based on gene expression data), did not show any further up-regulation. This could be attributed to possible post- transcriptional/ post- translational silencing of this protein mediated by GAP-P45 under inoculated conditions. This can be confirmed through further experiments involving post-transcriptional silencing and/or protein modifications.

In case of PAO activity with both Spd and Spm as substrates, on day 2, while the other treatments exhibited similar patterns to that of the gene expression data, surprisingly, in the GAP-P45 inoculated water-stressed seedlings, as opposed to the *PAO* transcript profiles, there was no further elevation of PAO activity suggesting a transcriptional/ post- translational silencing **(Fig. 3.15 C-F),** similar to CuAO**.**

On day 4, all the treatments showed positive correlation with most of the *PAO* transcript patterns **(Fig. 3.14 B)** with respect to PAO (Spd) activity. While all treatments displayed elevated enzyme activity as compared to the untreated controls, highest activity was observed in GAP-P45 inoculated water-stressed seedlings indicating active back- conversion of Spd into Put **(Fig. 3.15 C, D).** The genes *PAO1, PAO4* and

PAO5 are reported to have higher substrate specificity for Spm (Moschou et al. 2008b, 2012; Planas-Portell et al. 2013)). As far as a correlation is concerned, the gene expression data **(Fig. 3.14 A-C)** differed from Spm catabolic PAO activity mostly in case of the GAP-P45 inoculated water-stressed seedlings, indicating a possible post- transcriptional silencing in these genes as well **(Fig. 3.15 E, F).**

On day 7, for both substrates (Spd and Spm), the enzyme activities drastically lowered in all treatments though they were still significantly higher than the untreated controls. The pattern of expression in all treatments was similar to that observed on day 4 except that the PAO activity in water-stressed GAP-P45 inoculated seedlings was not significantly higher from that observed in the non- inoculated seedlings **(Fig. 3.15 C-F).**

3.2.5 Key Findings

1. Inoculation with GAP-P45 induced most of the PA oxidation/ catabolic genes under normal growth conditions in *A. thaliana.*
2. While water-stress itself altered the expression of most PA oxidation/catabolic genes, GAP-P45 inoculation further induced the expression of most of these genes. To be precise, GAP-P45 inoculation under water- stress conditions led to the most statistically significant inductions in gene expression/ enzyme activities.
3. While under water stress, the transcript levels of almost all genes and enzyme activities correlated positively, in case of GAP-P45 inoculation under water-stressed conditions, an overall positive correlation between gene expression and enzyme activity was found only in case of Put catabolism. In case of Spd and Spm catabolism, possible post transcriptional silencing in response to GAP-P45 inoculation under water stress can be hypothesized.

<u>Section 3</u>: Impact of *Pseudomonas putida* GAP-P45 on the accumulation of major polyamines (putrescine, spermidine, and spermine) and their oxidative deamination product γ-aminobutyric acid (GABA) in *Arabidopsis thaliana* under normal and water-stressed conditions.

3.3.1 Introduction

The present scenario of research regarding the involvement of PAs in abiotic stress tolerance has been thoroughly reviewed in Chapter 1. The elevation of PA concentrations in *A. thaliana* under abiotic stress has been investigated by several workers (Alcázar et al. 2006, 2011; Groppa and Benavides 2008). Among the three major PAs, Put accumulation under abiotic stresses like drought/cold/salinity, and elevation in its levels is a well-studied phenomenon (Liu et al. 2006, 2015; Cuevas et al. 2008). The overall accumulation of PAs under abiotic stress conditions is largely a result of multiple events. The *de novo* biosynthesis, catabolism, conjugation, inter-conversion etc. determine the ultimate PA concentration in the cells. Moreover, the influence of several factors, such as the plant species, tolerance capacity, stress intensity, types and conditions, and the overall physiological status at the whole plant level is known to determine PA accumulation over space and time (Bitrian et al 2012, Liu et al., 2007). The PA metabolic pathway has been illustrated in the previous section of this chapter **(Section 2, Fig. 3.2.1)**.

Polyamines play a very important role in coordinating the carbon: nitrogen balance due to their connection with other metabolites that contribute to carbon metabolism in plant cells. Interestingly, these metabolites, such as, proline (Pro), and GABA are all synthesized from a common substrate glutamate (Glu), which forms the hub of nitrogen metabolism in plant cells (Mattoo et al. 2010). GABA is generally derived from glutamate, although a contribution from PAs is also possible (Shelp et al., 2012) through PA catabolism. As evident from the PA metabolic pathway **(Section 2, Fig. 3.2.1)**, Put oxidation (oxidative deamination) in plants produces 4-aminobutanal, which spontaneously cyclizes to $^{\Delta 1}$-pyrroline and further converts to γ-aminobutyric acid (GABA) (Petřivalský et al. 2007). Fincato et al., (2011) also suggested the formation of GABA from Spd and Spm oxidation by PAOs in addition to Put oxidation by CuAO. The transamination and oxidation of GABA further yields succinic acid, which is a crucial component of the Krebs cycle (Rea et al. 2004). GABA is an important metabolite which usually displays altered levels during biotic/ abiotic stress exposure in various plant species (Kim et al. 2013, Shelp et al. 2012, and Xing et. al 2007), although the molecular basis of its function is currently unknown (Shelp et al. 2012).

We have previously described the transcript profiles PA biosynthetic (Sen et al., 2018) and catabolic genes and activities of PA catabolic enzymes (manuscript under review) in *A. thaliana*, with or without water stress when inoculated with the drought-mitigating, free living rhizobacteria, *P. putida* GAP-P45. In this section, we describe the accumulation patterns of PA- related metabolites under similar experimental conditions. The metabolites included in this study are the three major PAs, Put, Spd and Spm

[data published in Sen et al., (2018)] and their oxidative deamination product GABA (manuscript under review).

3.3.2 Materials and Methods

3.3.2a Measurement of cellular polyamine content:

The procedures involved in germination, growth, water-stress induction and GAP-P45 inoculation have been previously described in Chapter 2 (Ghosh et al., 2017, Sen et al., 2018). Whole seedlings were collected at the three different time points, as mentioned earlier and subjected to cellular PA analysis. Cellular, free PAs were analyzed by high-performance liquid chromatography (HPLC) using a relative fluorescence (RF) detection system (Shimadzu, RF-20A) for the detection of dansyl chloride-derivatized PAs. The procedure followed for the extraction and determination of dansyl-PAs through RF-HPLC is a modification of the methods described by Minocha et al. (1990, 1994) and Mohapatra et al. (2010). The analyses were performed in triplicates in at least two independent experiments. Briefly, 100 (±20) mg of whole seedlings were collected at three different time points, namely day 2, day 4 and day 7 post treatments and mixed with 400 (±20) µL of 5% (v/v) perchloric acid (PCA). For extraction of PAs, the samples were exposed to freeze/thaw cycles at -20 ºC/ room temperature respectively, homogenized sonicated and vortexed until they were well mixed. Following centrifugation and collection of the supernatant, extracted PAs were derivatized using 20 mg ml^{-1} dansyl chloride (freshly prepared in acetone) in a highly alkaline solution of Na_2CO_3. Following an hour of heat incubation at 60 ºC, excess dansyl chloride was removed by adding 20 mg ml^{-1} of L-asparagine to the reaction mix and incubated at 60 ºC for 30 min. The dansylated PAs were then partitioned into a toluene fraction and vacuum evaporated. The vacuum dried PAs were dissolved in methanol and used for PA titer analysis. Commercially available diaminoheptane (HD) was used as an internal standard. Polyamine content was quantified by extrapolating from standard curves **(Fig. 3.16 A-C)** generated through derivatization of known concentrations of commercially available PAs and expressed as µmol PA content g^{-1} FW of *A. thaliana* seedlings. The identity specificity of PAs in samples were determined through peaks generated from commercial standards for each of the PAs **(Fig. 3.17)**.

3.3.2b Measurement of cellular GABA content

The estimation of GABA accumulation in *A. thaliana* seedling in response to various treatments was performed by a modified method based on the Berthelot reaction for detection of ω-amino acids as described originally by Kitaoka and Nakano (1969) and Karladee and Suriyong (2012). Briefly, 100 mg of seedlings were homogenized and extracted in 80% methanol. The crude extract was then sequentially treated with Berthelot reagents (phenol and NaOCl) in the presence of borate buffer. The reaction mixture was then exposed to a few cycles of boiling and subsequent cooling to generate a bluish colored reaction product.

Optical density of the bluish colored solution generated in the samples were determined against blanks by spectrophotometry at a wavelength of 630 nm. GABA content was quantified by extrapolation from a standard curve **(Fig. 3.16 D)** generated from known concentrations of commercially available GABA solution and expressed as µg GABA content g^{-1} FW of *A. thaliana* seedlings.

3.3.2c Statistical analysis

Statistical analysis was performed by two-way Anova with Tukey's HSD test (level of significance, $p \leq 0.05$). 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.

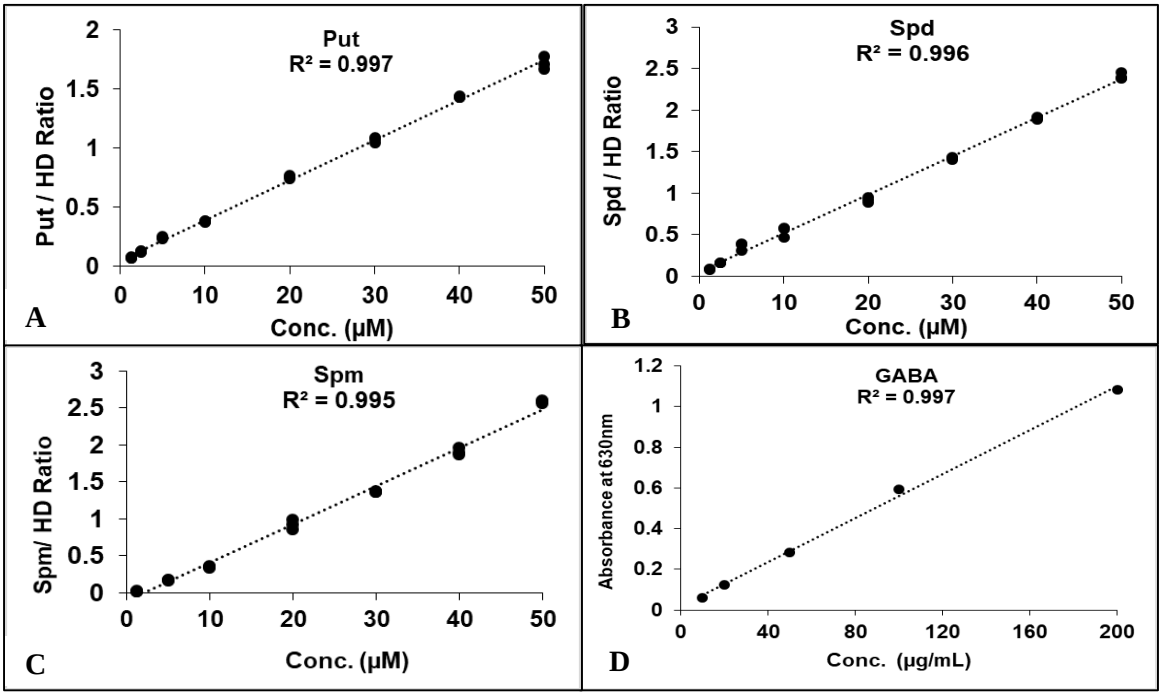

Fig. 3.16 Standard curves of major PAs, **(A)** Put **(B)** Spd **(C)** Spm and the Put catabolic product **(D)** GABA. The standard curves of PAs were generated through derivatization of a range of known concentrations of commercially available PAs by using RF-HPLC while for GABA spectrophotometry technique was used.

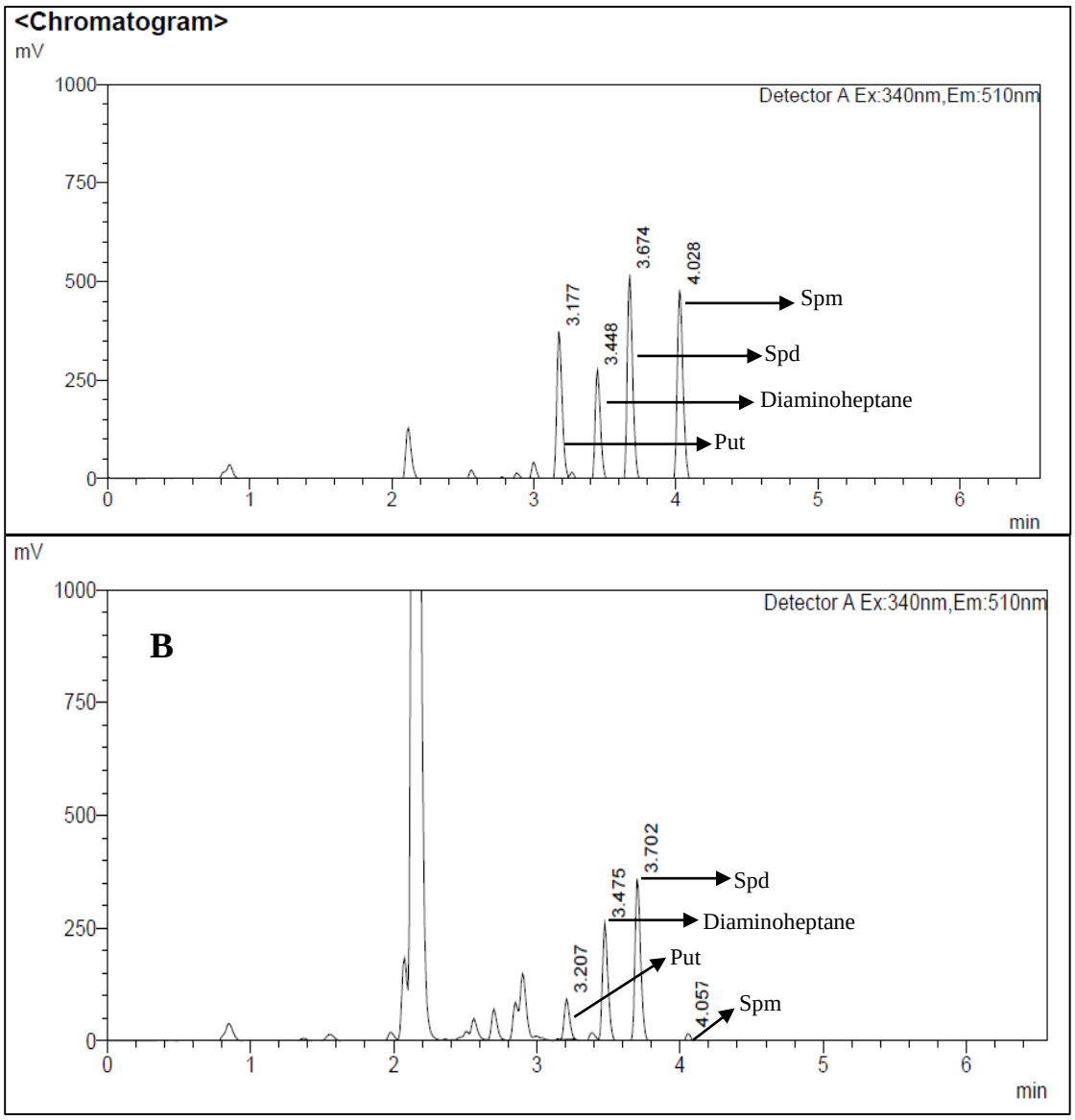

Fig. 3.17 Representative peaks of PAs obtained by chromatographic separation (RF-HPLC) from **(A)** commercial standards and **(B)** experimental samples for Put, Spd, Spm and the internal standard, Diaminoheptane.

3.3.3 Results

Cellular accumulation of free PAs under PGPR inoculation with or without water stress

The cellular content of Put fluctuated on all days of analysis under the various treatments, as evident from **Fig. 3.18 A**. On day 2, water-stress induced accumulation of Put, which was induced further by GAP-P45 inoculation. Similar trends were seen on day 4, except that, GAP-45 inoculation even without water-stress

caused an induction in Put levels. A gradual surge was seen in Put levels from day2 to day7 in the no-treatment controls, while the non-stressed, inoculated and water-stressed plants did not show any change in Put levels from day 4 to day 7. A substantial elevation in Put levels was observed in the water-stressed, inoculated samples on day 7, making it the highest Put concentration among all treatments and time-periods.

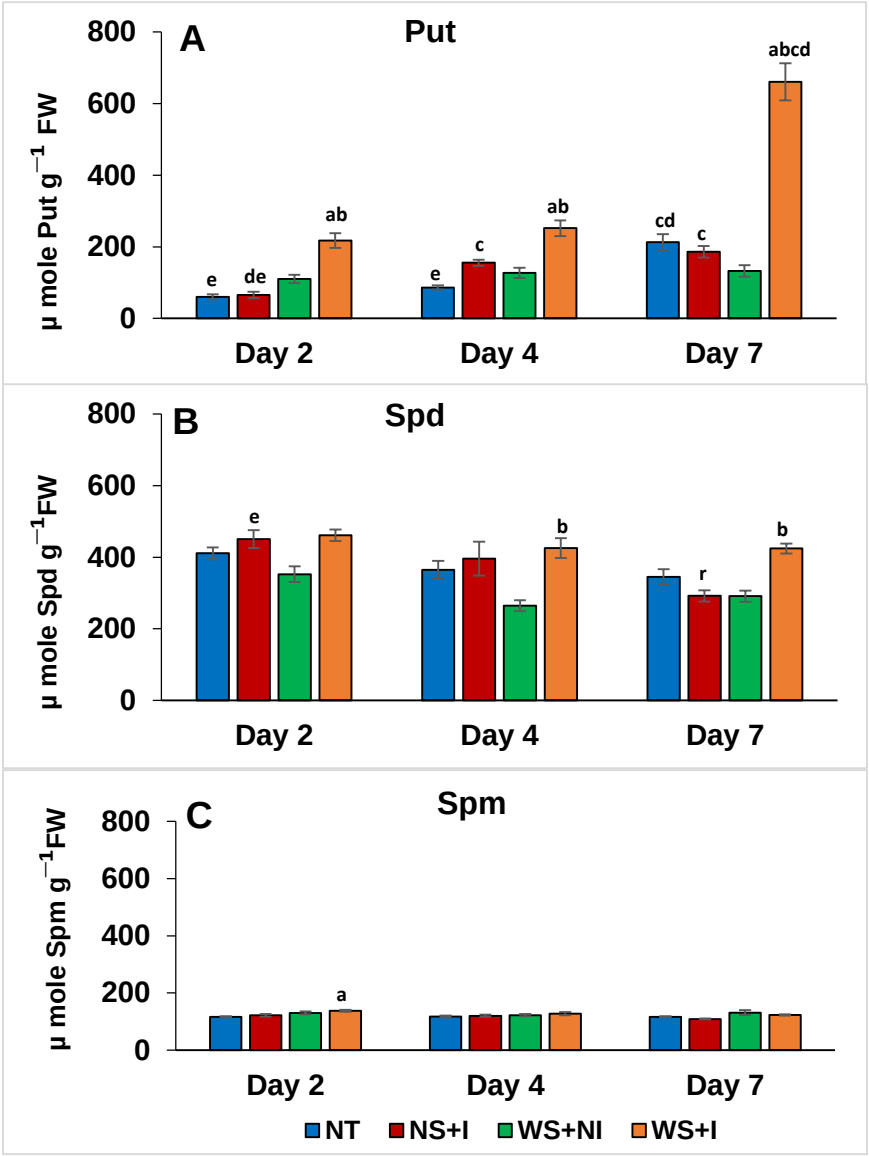

Fig. 3.18 Accumulation of polyamines (**A)** Put, (**B)** Spd, (**C)** Spm in *A. thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 inoculation with or without water stress, day 2, day 4 and day 7 post treatments (**NT**: no treatment; **NS+ I**: non-stressed, inoculated; **WS+NI**: water-stressed, non-inoculated; **WS+I**: water-stressed, inoculated). Each bar represents mean (±) S.E. of 6 replicates. 'a' represent significant difference ($p \leq 0.05$) of all treatments from **NT**. 'b' represent significant difference ($p \leq 0.05$) between **WS+NI** and **WS+I** seedlings. 'c', 'd' and 'e' denote significant differences of a gene within a treatment at a particular day from day 2, 4 and 7 respectively.

Cellular levels of Spd were about 1.5-4-fold higher than Put at all time periods of analysis and in all treatments (except day 7, GAP-P45 + water-stressed) as illustrated in **Fig. 3.18 B**. While the Put content in the no-treatment controls increased from day 2 to day 7 (as expected), no major change was seen in the cellular content of Spd from day 2 to day 7. GAP-P45 inoculation without water-stress did not cause any change in Spd levels on day 2 and day 4, but did cause a slight decrease on day 7. Water-stress caused a decrease in Spd levels at all time-points, while GAP-P45 inoculation under water-stress increased the Spd titers to either the same level as the no-treatment controls (day 2 and day 4) or higher (day 7). Spermine levels exhibited little (day 2) or no change (day 4 and day 7) on any given day under the various treatments **(Fig. 3.18 C)**.

Cellular accumulation of GABA with PGPR inoculation with or without stress.

As evident from **Fig. 3.19**, the accumulation of GABA in *A. thaliana* shows interesting modulations in response to GAP-P45 inoculation under non- stressed and stressed conditions. On day 2, the GABA content in the non- stressed inoculated seedlings was the highest among all other treatments. GABA content in the other treatments were similar. On day 4, however, the water-stressed, non-inoculated samples showed highest levels of GABA, while other treatments showed similar GABA levels. On day 7, a gradual, treatment dependent increase was seen in GABA levels as compared to untreated controls with significant elevations in the water-stressed samples (both inoculated and non-inoculated).

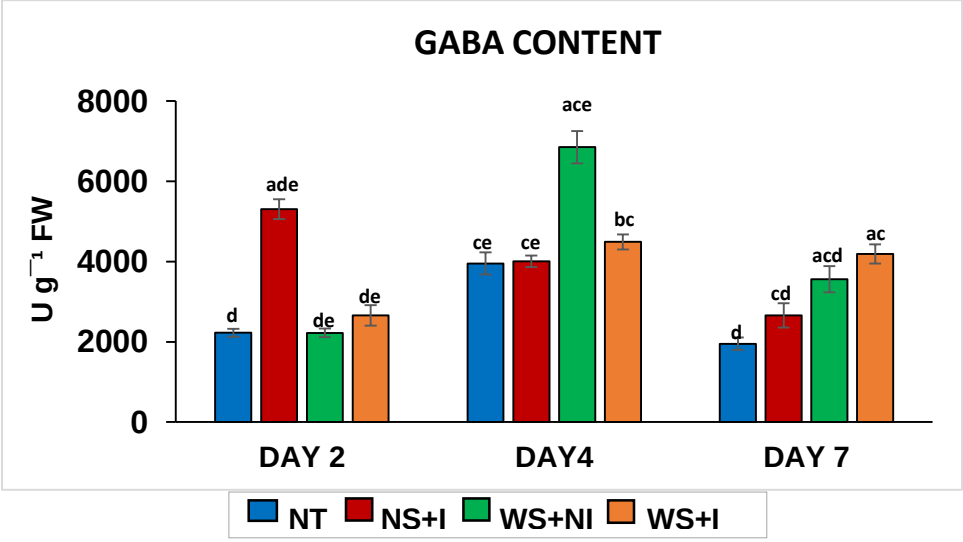

Fig 3.19. Accumulation of GABA in *A. thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 inoculation with or without water stress, day 2, day 4 and day 7 post treatments. Each bar represents mean of U; U= µg GABA synthesized (±) S.E of 6 independent replicates of each treatment. 'a' represent significant difference ($p \leq 0.05$) of all treatments from NT. 'b' represent significant difference ($p \leq 0.05$) between WS+NI and WS+I seedlings within a treatment and time-points. 'c', 'd' and 'e' denote significant differences within a treatment on a particular day from day 2, 4 and 7 respectively.

3.3.4 Discussion

***P. putida* GAP-P45 modulates PA cellular titers under water stress.**

The expression of PA biosynthetic as well as catabolic genes were found to be significantly induced with GAP-P45 inoculation under water stress (as described in the previous sections of this chapter). In continuation, when we analyzed the accumulation of PAs under similar experimental conditions, we observed that GAP-P45 inoculation significantly elevated the levels of Put and Spd under water stress as compared to the other treatments at all the time points of analysis **(Fig 3.18)**. The complicated dynamics of the PA pool in plant cells under abiotic stresses has been explored by many authors. While in some cases, the three most abundant PAs, Put, Spd and Spm, have displayed substantial elevations in response to abiotic stresses (Yang et al. 2007), there are indeed many examples (Liu et al. 2006; Wang and Liu 2009; Do et al. 2014; Ikbal et al. 2014) which describe significant increases in only one of the three PAs, in most cases, Put. Since Put is the precursor for the biosynthesis of higher molecular weight PAs (Spd and Spm), in this context, Capell et al. (2004) suggested that Put levels must exceed certain threshold to enhance the synthesis of Spd and Spm under stressed conditions.

Correlation between PA biosynthetic gene expression and PA accumulation under *P. putida* GAP-P45 inoculated, water-stressed *A. thaliana*

The accumulation of PAs under abiotic stress conditions is largely due to the increased *de novo* synthesis of free PAs. There are several reports suggesting both transcriptional (Liu et al., 2015) as well as post-transcriptional (Alcazar et al., 2006) and/ or post- translational (Majumdar et al., 2016) regulation of PA biosynthesis under various stresses. Therefore, an understanding of the expression patterns of the biosynthetic genes is crucial to be able to understand the regulation of PA levels.

Clearly, in our study, as described in section 1, under normal (non-stressed) conditions, while GAP-P45 modulated the expression of several PA biosynthetic genes at all time periods of study **(Section 1, Fig. 3.5- 3.8)**, the cellular levels of PA titers did not exhibit much fluctuations with GAP-P45 inoculation, except for a slight increase in Put content on day 4 and a decrease in Spd content on day 7 **(Fig. 3.18 A, B)**. The former was the only instance of a positive correlation between an induction in gene expression and concomitant induction in PA concentration under non-stressed, inoculated conditions.

In our study, as far as the modulation of PA titers under water-stressed conditions (both, inoculated and non-inoculated) are concerned, a straight-forward, positive correlation is not seen between the cellular PA content and the gene expression data. While in some cases, there is a positive correlation, in others, there is not.

An overall induction in Put levels in water-stressed + GAP-P45 samples positively correlates with the observed induction of *ADC1*, *ADC2*, *AIH*, and *CPA* in some or all time-periods of analysis. Such a

correlation was not seen between the *SPDS* gene expression levels vs. Spd accumulation. From the gene expression data **(Fig. 3.6)** of spermidine synthase paralogs *(SPDS1* and *SPDS2)*, one could hypothesize that Spd is expected to accumulate under water-stressed conditions, at all time periods of analysis. However, a reduction of Spd levels was observed under water-stressed conditions at all time periods of analysis **(Fig 3.18 B)**. Again, from the gene expression data, it can be expected that Spd levels will decrease when water-stressed plants are treated with GAP-P45 (down-regulation is seen at all the time points in *SPDS2* and day 7 in *SPDS1*) **(Fig. 3.6)**. However, the Spd accumulation data indicates an induction in Spd levels on GAP-P45 inoculation of water-stressed plants, as opposed to water-stressed plants without inoculation **(Fig. 3.18 B)**. The *SPMS* gene was found to be upregulated under all treatments throughout the study **(Fig. 3.7)**. However, the cellular Spm levels only slightly increased on day 2 under water-stressed GAP-P45 inoculated conditions, while no further increase was seen in their levels on inoculation with GAP-P45 **(Fig. 3.18 C)**. Our data point towards (a) transcriptional modulation of polyamine biosynthetic genes and (b) complex post transcriptional regulation and/ or inter-conversion/ canalization of polyamines, by *P. putida* GAP-P45 under normal and water-stressed conditions. The lack of correlation between PA metabolic gene expression and the accumulation of cellular PA content under abiotic stresses has been reported and reviewed earlier by several workers (Liu et al. 2006, 2015, Alcázar et al. 2006, 2010, Cuevas et al. 2008, 2009; Krasensky and Jonak 2012; Minocha et al. 2014). It has been suggested that PA accumulation is ultimately governed more by post-translational regulation of PA biosynthetic enzymes (Majumdar et al. 2016) as opposed to post-transcriptional regulation. The availability of dcSAM is also known to limit the biosynthesis of Spd and Spm (Ge et al. 2006; Bitrián et al. 2012). In our study, the fluctuations observed in the transcript levels of the *SAMDC* paralogs indicate a complex modulation involved in the biosynthesis and accumulation of the higher PAs, Spd and Spm.

Correlation of PA catabolism with PA accumulation under *P. putida* GAP-P45 inoculated, water-stressed *A. thaliana*.

As with PA catabolism, inoculation with GAP-P45 under water stress led to significantly increase the expression of PA catabolic genes in *A. thaliana* **(Section 2, Fig. 3.13, and Fig. 3.14)**. We also observed that among the PAs, only Put oxidation (characterized by *CuAO* gene expression and CuAO enzyme activity) was mostly elevated to highest levels in response to GAP-P45 inoculation under water stress. This suggests that inoculation with GAP-P45 under water stress promotes high Put turnover (especially on day 2 and day 4) through enhanced Put biosynthesis and catabolism in *A. thaliana* seedlings. The net accumulation of Put is a consequence of both its biosynthesis and catabolism. As stated previously, several authors have discussed the involvement of *AtPAOs* in the direct/ sequential back- conversion of higher PAs (Spm and Spd) into Put (Moschou et al. 2008b, 2012; Toumi et al. 2010; Planas-Portell et al. 2013). In light

of this information, since Spd and Spm can convert back into Put, therefore, Put levels can be influenced by such back- conversion reactions as well.

Interestingly, as described earlier in Section 2 of this chapter, our study reflects a disparity between PAO gene expression and enzyme activity indicating post- transcriptional silencing of PAOs with GAP-P45 inoculation under water stress. At most time –points of analysis, the PAO back- conversion activity was found to be inhibited, possibly restricting any further accumulation of Put from back- conversion of Spm and Spd. This inhibition can be speculated to be a feedback inhibition due to enhanced Put accumulation on all days of analysis **(Fig. 3.18 a)**. Further studies will have to be conducted to confirm this hypothesis. It will be interesting to see if this inhibition is occurring at the post-transcriptional or post-translational level. Also, the lack of increased Spd oxidation (i.e. back- conversion) in response to GAP-P45 under water stress, as evidenced by the stagnating PAO activity described earlier in section 2 **(Fig 3.15 C, D)**, in most cases, supports the slightly increased Spd accumulation in these seedlings **(Fig. 3.18 B).** Our results suggest a tight coordination between PA inter-conversion and accumulation in response to GAP-P45 inoculation under water stress. Overall, our study on PA metabolism presents interesting and novel observations suggesting the ability of *P. putida* GAP-P45 to trigger enhanced PA accumulation through integrated modulation of PA biosynthesis and catabolism in *A. thaliana* under water stress.

Correlation between PA metabolism and plant health under GAP-P45 inoculated conditions, with or without water-stressed conditions

As mentioned before in Chapter 2, *A. thaliana* plants exhibited improved health and sustenance, and, therefore, enhanced tolerance to water stress at the particular concentration of GAP-P45 used in this study (Ghosh et al. 2017). Although, at the same concentration, under non- stressed conditions, GAP-P45 did not seem to enhance plant growth and development (Ghosh et al. 2017). One of the most interesting aspects about our data is that, even though, no phenotypic manifestation is observed, GAP-P45 under non-stressed conditions, did cause significant modulations in PA metabolism in almost all time-periods of study. Not only were the expression of PA metabolic genes affected, but, PA levels also changed in response to GAP-P45 inoculation under non-stressed conditions. Inoculation under water-stressed conditions caused an overall increase in Put and Spd levels. Our results suggest a tight coordination between PA inter-conversion and accumulation in response to GAP-P45 inoculation under water stress. These PA concentrations are also found to positively correlate with the enhanced water-stress tolerance under GAP-P45 inoculation that we have reported earlier (Ghosh et al., 2017). Also, as mentioned earlier, there are reports on drought tolerant rhizobacteria, themselves secreting PAs (Zhou et al. 2016). If this is true for GAP-P45 as well, then, the uptake of bacterial PAs could be contributing towards ultimate PA levels at a given point of time within the plants. Further experiments will be required to confirm such hypotheses.

***P. putida* GAP-P45 modulates GABA accumulation under normal (non-stressed) as well as water-stressed conditions.**

As evident from **Fig. 3.19**, the accumulation of GABA was differentially impacted with different treatments in *A. thaliana*. Under non-stressed conditions, GAP-P45 inoculation spiked GABA levels most significantly on day 2, decreasing drastically thereafter as the study progressed. Water stress itself significantly elevated GABA levels on day 4 with a subsequent decrease on day 7. With GAP-P45 inoculation under water stress, the GABA levels never increased significantly than the water-stressed non- inoculated seedlings, rather, on day 4, they displayed significant down-regulation. On day 7, there was somewhat a positive correlation between GABA accumulation patterns as observed with the Put and/ or Spd catabolism.

GABA production from CuAO mediated Put/ Spd degradation has been reported to be affected by abiotic stresses (Bouché and Fromm 2004; Xing et al. 2007; Shelp et al. 2012; Berberich et al. 2015; Majumdar et al. 2016). However, as mentioned earlier, a direct correlation between the two has been seldom observed. GABA in plants is believed to be majorly derived from glutamate although the contribution of PAs is also marked (Berberich et al., 2015). However, as opposed to PA accumulation in response to various stresses, the accumulation of GABA is governed by processes other than Put availability such as reported in various studies (Xing et al. 2007; Shelp et al. 2012). The regulation of glutamate decarboxylases as well as AOs have a major influence on GABA accumulation in addition to the O_2 availability and cellular redox balance due to stress (Bitrián et al. 2012; Berberich et al. 2015; Hatmi et al. 2015; Sequera-Mutiozabal et al. 2016; Masson et al. 2017).

3.3.5 Key findings

P. putida GAP-P45 impacts the accumulation of key metabolites of the PA pathway with significant elevations in Put and Spd levels under water stress as compared to non- inoculated water-stressed plants.

Chapter 4

Modulation of redox state in *Arabidopsis thaliana* by a drought mitigating *Pseudomonas putida* GAP-P45

4.1 Introduction

As stated previously, plants often generate aggravated levels of ROS when exposed to environmental stresses. Reactive oxygen species are known to exert a wide range of physiological responses in plants. These responses lead to modifications of enzymes, proteins, nucleic acids, lipids and other cellular components (Choudhury et al. 2013, 2017; You and Chan 2015). If kept unchecked, ROS concentrations can substantially increase in cells leading to oxidative stress characterized by cell death (Mittler, 2002).

In plant cells, under optimal growth conditions, ROS are constantly produced at a basal level in mitochondria, chloroplast, peroxisomes (Ozden et al. 2009), apoplast, cytosol, vacuoles and nuclei (Gautam et al. 2017). However, during abiotic stress conditions, ROS production occurs as a consequence of disruptions in metabolic activity (metabolic ROS) or to trigger various signaling cascades/ signal transduction network involved in abiotic stress response (signaling ROS) (Choudhury et al. 2013, 2017). Thus, the extent of their accumulation at a given time is the major determinant of their positive or negative effect in the biological systems. (Choudhury et al. 2013, 2017; Baxter et al. 2014a).

The predominant ROS include superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O_2$). One of the predominant ways by which $O_2^{\cdot -}$ is generated is from reduced CO_2 fixation and a subsequent loss of ATP during photosynthesis. This further triggers chain reactions to produce more aggressive and potentially toxic oxygen radicals (Apel and Hirt 2004; Suzuki 2015). Under water deficit conditions, ABA- induced stomatal closure results in the decreased availability of $NADP^+$. Hence, ferredoxin is over-reduced during photosynthetic electron transfer in the chloroplast and leads to the formation of $O_2^{\cdot -}$ due to transfer of electrons from PS-I to oxygen. Peroxisomes and mitochondria too host several key metabolic processes like photorespiration and apoplastic electron transport aided by enzymes NADPH oxidase, class III peroxidases, amine oxidases, oxalate oxidases etc. These actively contribute to the cellular pool of $O_2^{\cdot -}$ and H_2O_2 (Choudhury et al. 2013, 2017; Baxter et al. 2014b; Suzuki 2015). In this context, an interesting aspect of this study is also the fact that H_2O_2, besides being produced by several other enzymes as discussed above, is also generated as a by- product of PA catabolism by the activities of amine oxidases [as illustrated in the PA metabolic pathway **(chapter 3, section 2, Fig. 3.9)** (Moschou et al. 2008b; Andronis et al. 2014). Few studies in the past decade have also elucidated the role of PA-mediated H_2O_2 as a signaling molecule in generating stress responses (Pottosin et al. 2014).

Several workers have thoroughly investigated and reviewed the deleterious effects of ROS, whose production is stimulated under water stress (Blokhina et al., 2003). Higher plants have thus acquired dedicated pathways to protect themselves from ROS toxicity, as well as to use ROS as downstream signaling molecules (Foyer and Noctor, 2013; Considine et al., 2015; Dietz, 2015; Mignolet- Spruyt et al., 2016). Plants, as with other organisms are armed with an antioxidant machinery to combat the toxic effects

of high ROS levels. These mechanisms stringently control the homeostasis between the continuous process of ROS generation and scavenging at a whole plant level as an integrated system in all cellular compartments (Apel and Hirt 2004; Kar 2011; Suzuki et al. 2011; Choudhury et al. 2013, 2017; Suzuki 2015; Gautam et al. 2017; Mehla et al. 2017). Among the antioxidant enzymes, superoxide dismutases (SODs) that can dismutate $O_2^{\cdot-}$ into the more stable H_2O_2 constitute the first line of defense against ROS (Suzuki 2015; Choudhury et al. 2017). The H_2O_2 thus generated is subsequently detoxified by other enzymes such as catalases (CAT), peroxidases (POD), ascorbate peroxidases (APX) etc. (Ahmad et al. 2010; Mehla et al. 2017). Catalase can directly decompose H_2O_2 in peroxisomes though its affinity to H_2O_2 is much lower than APX. Ascorbate peroxidases are localized in chloroplasts, mitochondria, peroxisomes and cytosol. The enzyme has a very high affinity to H_2O_2 and requires ascorbate (AsA) as an electron donor to manifest its activity (Pandey et al. 2017b). Ascorbate and reduced glutathione (GSH), the two most important non- enzymatic antioxidants with redox activity which are known to ameliorate oxidative stress by activating the AsA- GSH cycle (Apel and Hirt 2004; Ahmad et al. 2010). The regeneration of GSH from oxidized glutathione (GSSG) is catalyzed by glutathione reductase (GR). High reductive potential of GSH allows it to scavenge cytotoxic H_2O_2, thus serving as one of the key controllers of cellular ROS in plants (Foyer and Noctor 2005; Mohapatra et al. 2009; Ahmad et al. 2010; Gautam et al. 2017). Several authors have particularly reviewed the activities of different antioxidant enzymes under drought stress (Ahmad et al. 2010; Mehla et al. 2017). The orchestration of plant responses to drought through modulation of antioxidant enzyme activity depends on the cellular compartment, physiological stages etc. resulting in differential ROS signature in plants. While generally an increased antioxidant activity is noted under water-deficit stress (Pastori et al. 2000; Yang et al. 2008), a decrease in antioxidant enzyme activity has also been reported under water deficit stress (Sharma and Dubey 2005; Pan et al. 2006). Under stressed conditions, it is crucial for the plant to maintain a tight regulation of the steady state concentrations of ROS thereby channeling its performance towards efficient signaling and minimizing oxidative damage (Petrov et al. 2015; Nachimuthu et al. 2017).

As described previously, plant growth promoting rhizobacteria (PGPR) constitute a group of soil bacteria that positively benefit plants by secreting phytohormones, exopolysaccharides, antibiotics, siderophores, HCN etc. (Timmusk and Wagner 1999; Cho et al. 2008; Hayat et al. 2010; Zhang et al. 2010; Bishnoi 2015; Ngumbi and Kloepper 2016). In the recent times, many workers have also demonstrated the role of PGPR in modulating antioxidant machinery in addition to other components in plant systems leading to altered metabolic fluxes and tolerance to stress. While in many cases, increased antioxidant activity was noted as a response to PGPR under different stresses (Kohler et al. 2008, 2009; Baltruschat et al. 2008; Ghorbanpour et al. 2013; Bharti et al. 2016), on the contrary, lowering of antioxidant enzyme activity in response to PGPR inoculation under stressed conditions have also been reported (Porcel and Ruiz-Lozano

2004; Sandhya et al. 2009; Upadhyay et al. 2012; Gururani et al. 2013; Naseem and Bano 2014). Few bacteria which are reported to modulate the antioxidant activity under drought stress include *P. entomophila*, *P. stutzeri*, *P. putida*, *P. syringae*, *P. montelli*, *Bacillus lentus*, *A. brasilense*, *B. thuringiensis* etc. All these PGPRs were known to ameliorate drought stress through either elevation or reduction of antioxidant enzyme activity in maize, wheat, rice, *Lavandula, Ocimum* etc. as reviewed by Vurukonda et al. (2016). These are relevant reports suggesting that PGPR impacts the abiotic stress related defense pathways to a certain extent and modulates the concentrations of antioxidants to mediate plant tolerance to a variety of stresses (Bharti et al. 2016).

Since *P. putida* GAP-P45 is known to down-regulate activities of some antioxidant enzymes under drought in maize (Sandhya et al. 2010a), we wanted to test the impact of this strain on the anti-oxidant machinery of *Arabidopsis* under water stress, since the oxidative status of a plant is the determinant of its overall health. [Data published in Ghosh, Sen et al., (2018)].

4.2 Materials and methods

4.2.1 Plant growth, maintenance and treatments

The procedures involved in germination, growth, water-stress induction and GAP-P45 inoculation of *A. thaliana* seedlings has been previously described in chapter 2 (Ghosh et al. 2017).

4.2.2 Estimation of ROS

Hydrogen peroxide (H_2O_2)

H_2O_2 was extracted and its levels quantified by modified method of Slesak et al., 2008 as reported by Mohapatra et al., 2009. About 25-50 mg (FW) of frozen seedlings were thawed, homogenised using mortar and pestle and were mixed with 250- 500 µL acetone, vortexed, and centrifuged (10,000 xg, 5 min.). To 0.25 mL of the supernatant, 25 µL titanium reagent (20 % titanium tetrachloride in concentrated HCl, v/v) was added. The peroxide-titanium complex was precipitated by adding 500 µL NH_4OH to the above mixture. Following centrifugation (10 min, 10, 000 × g), the supernatant was discarded and the precipitate was repeatedly washed with acetone. The precipitate was then dissolved in 1 mL of 2N H_2SO_4 and absorbance of the resultant solution was measured at 415 nm using a spectrophotometer. The absorbance was compared against a standard curve generated from known concentrations of H_2O_2.

Superoxide anion radical ($O_2^{\cdot-}$)

The detection and quantitative determination of superoxide radicals in *A. thaliana* seedlings was performed as a function of reduction of nitroblue tetrazolium (NBT) into formazan as described by Grellet Bournonville and Díaz-Ricci, 2011. A modified version of the previously described assay for superoxide

quantification was used. Briefly, *A. thaliana* seedlings subjected to different treatments as described previously were treated with 200 µL of 1 mg ml^{-1} NBT solution which was added in the vicinity of roots on the MS agar contained in the Magenta boxes. Further, the NBT-stained plantlets were ground in liquid nitrogen, the formazan content was extracted and solubilized in 2 M potassium hydroxide : dimethylsulfoxide (KOH : DMSO; 1/1.16) (v/v), and then centrifuged for 10 min at 12,000 x g at 4°C under light- protected conditions. Similar experimental sets without addition of NBT were used as controls. The absorbance at 630 nm was immediately measured, and compared with a standard curve obtained from known amounts of NBT in the KOH-DMSO mix.

4.2.3 Activities of ROS metabolizing enzymes

Superoxide dismutase (SOD)

The activity of SOD was assayed and quantified using a modified protocol of Giannopolitis and Ries, 1977. Briefly, 25 – 50 mg (FW) of frozen seedlings were thawed, homogenised and mixed with about 0.5 mL of 0.1 M potassium phosphate (K-Pi) buffer and 0.1 mM ethylenediamine tetraacetic acid (EDTA) (pH 7.8) followed by centrifugation of the homogenate at 10,000 g for 10 min at 4 °C. The supernatant was collected and subjected to the assay. The reaction mixture was composed of 1.3 µM riboflavin, 13 mM methionine, 63 µM nitroblue tetrazolium (NBT), 0.05M Na_2CO_3 (pH 10.2), and 40 µL of enzyme extract. Distilled H_2O was added to bring to the final volume of 3 ml. The mixtures were illuminated in glass test tubes for 30 min. Two test tubes (one illuminated while the other kept in dark) containing all components except the enzyme extract served as the blanks. The reaction was initiated and terminated by turning the light on and off respectively. There was no detectable amount of the reaction occurring under room light during preparation of the solutions and spectrophotometric measurements. The absorbance at the end of 30 min was read at 560 nm in a UV/Vis spectrophotometer. The enzyme activity is expressed as 50 % inhibition of NBT reduction min^{-1} g^{-1} FW of *A. thaliana* seedlings.

Peroxidase (POD)

The activity of POD was quantified in seedlings by modified method of Hamilton et al., 1999. About 25 – 50 mg (FW) of frozen seedlings were thawed, homogenised and mixed with about 0.5 mL of 0.1 M K-Pi buffer and 0.1 mM EDTA (pH 7.8) followed by centrifugation of the homogenate at 10,000 xg for 10 min at 4 °C. The supernatant was collected and subjected to the assay as the enzyme extract. The assay mixture composed of 3 mL of 0.1 M K-Pi buffer (pH 7), 100 µL of 0.5% o-phenylenediamine and 25 µL of the enzyme extract. The reaction was initiated by adding 100 µL of H_2O_2 and the absorbance was read at 440 nm in a UV/Vis spectrophotometer just after starting the reaction as well as at the end of 5 min. The enzyme

activity was expressed as the change in Abs min^{-1} g^{-1} FW as units of POD activity g^{-1} FW of *A. thaliana* seedlings.

Catalase (CAT)

Activity of CAT was determined according to Aebi, 1984 as reported by Elavarthi and Martin, 2010. Followed by collection of the enzyme extract as described above, the decomposition of H_2O_2 was studied as a decrease in absorbance at 240 nm in a UV/Vis spectrophotometer. The 3 mL assay mixture contained the enzyme extract diluted in 0.1 M K-Pi buffer and 0.1 mM EDTA (pH 7.8) and H_2O_2 was added to a final concentration of 10 mM in order to initiate the reaction. The enzyme activity that was expressed in terms of mmoles of H_2O_2 decomposed $min^{-1}g^{-1}$ FW of *A. thaliana* seedlings.

Glutathione reductase (GR)

For extraction and estimation of GR enzyme, as described by Mohapatra et al., 2009, (modifications of the method of Schaedle and Bassham, 1977 and Jahnke et al., 1991) 100 mg (FW) of frozen seedlings were thawed, homogenised, sonicated and collected in 200 µL of 50 mM K-Pi buffer (pH 7.0) containing 0.2 mM diethylenetriamine pentaacetic acid (DTPA). After a freeze-thaw cycle, the mixture was vortexed for 5 min and centrifuged (16,000 × g, 10 min). To 50 µL of the supernatant, 850 µL of K-Pi buffer (25 mM, pH 7.8 with 0.2 mM DTPA) was added. Then, 50 µL of 3 mM NADPH made in 3 mM NaOH were added. Change in absorbance (340 nm) was monitored for up to minimum of 30 s using UV/Vis spectrophotometer. This was followed by addition of 50 µL of 10 mM GSSG and the rate of its reduction (to GSH) was monitored again by measuring the change in absorbance for 30 s. This rate of change of absorbance was subtracted from the one determined in the absence of GSSG. The enzyme activity was expressed as mmol NADPH oxidized/min g^{-1} FW of *A. thaliana* seedlings (Jahnke et al., 1991).

Ascorbate peroxidase (APX)

Following similar homogenisation methods applied to the seedlings as explained above, APX was assayed and quantified, such that 50 µL of the supernatant (100 mg seedlings in 400 µL of 50 mM K-Pi buffer) was mixed with 850 mL K-Pi buffer, 25 µL of 10 mM ascorbic acid and 50 µL of 10 mM H_2O_2 and the decrease in absorbance (290 nm) was monitored for 30 s. Enzyme activity is expressed as mmol AsA oxidized/min g^{-1} FW of *A. thaliana* seedlings (Jahnke et al., 1991). The method of Nakano and Asada, 1981 as described by Jahnke et al., 1991 was modified as reported by Mohapatra et al., 2009) to assay this enzyme.

4.2.4 Total protein estimation

Seedlings (500 mg) were collected from different treatments and homogenized in liquid nitrogen. Total protein content was determined according to Bradford method (Bradford 1976) with bovine serum albumin as the standard. Specific enzyme activity was expressed as unit mg^{-1} protein.

4.2.5 Statistical analysis

Statistical analysis was performed by student's t-test (level of significance, $p \leq 0.05$). 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 ROS levels

Superoxide radical ($O_2^{\cdot-}$)

Under non-stressed conditions, the inoculated seedlings recorded lowest $O_2^{\cdot-}$ content at all the time points as opposed to the non-inoculated counterparts **(Fig. 4.1 A)**. The seedlings under water stress without GAP-P45 inoculation recorded highest content of $O_2^{\cdot-}$ across all time points.

Hydrogen peroxide (H_2O_2)

H_2O_2 levels exhibited some interesting dynamics under water-stressed and GAP-P45 treatments. As evident in **Fig. 4.1 B**, the H_2O_2 levels in the GAP-P45 treated seedlings at the end of day 2 were significantly elevated as opposed to the untreated seedlings. Interestingly, in the later time periods, the H_2O_2 levels decreased in the GAP-P45 inoculated plants, both under water-stress and normal conditions. Water-stressed plants exhibited an increase in H_2O_2 content from day 2 to day 4, followed by a decline on day 7. On both day 4 and day 7, the H_2O_2 concentration in the water-stressed, non-inoculated plants recorded higher levels than the water-stressed, inoculated plants. Overall, in all groups except the no-treatment controls, a reduction of H_2O_2 levels was seen from day 2 to day 7.

4.3.2 Antioxidant enzymes

Superoxide dismutase (SOD)

Superoxide dismutase exhibited the highest activity in water-stressed plants without GAP-P45 inoculation for the most part **(Fig. 4.2 A)**. On day 2, the activity of SOD was comparable in the other three treatments. On day 4, the water-stressed, GAP-P45 inoculated plants exhibited a marked decrease in the activity of SOD, while a similar decrease was seen in the non-stressed, inoculated plants on day 7. An overall decreasing trend was seen in SOD activity under all treatments from day 2 to day 7. Trends in specific activity (U mg^{-1} protein) of SOD were almost identical to its activity (U mg^{-1} FW) on all days in all treatments **(Fig. 4.2 B).**

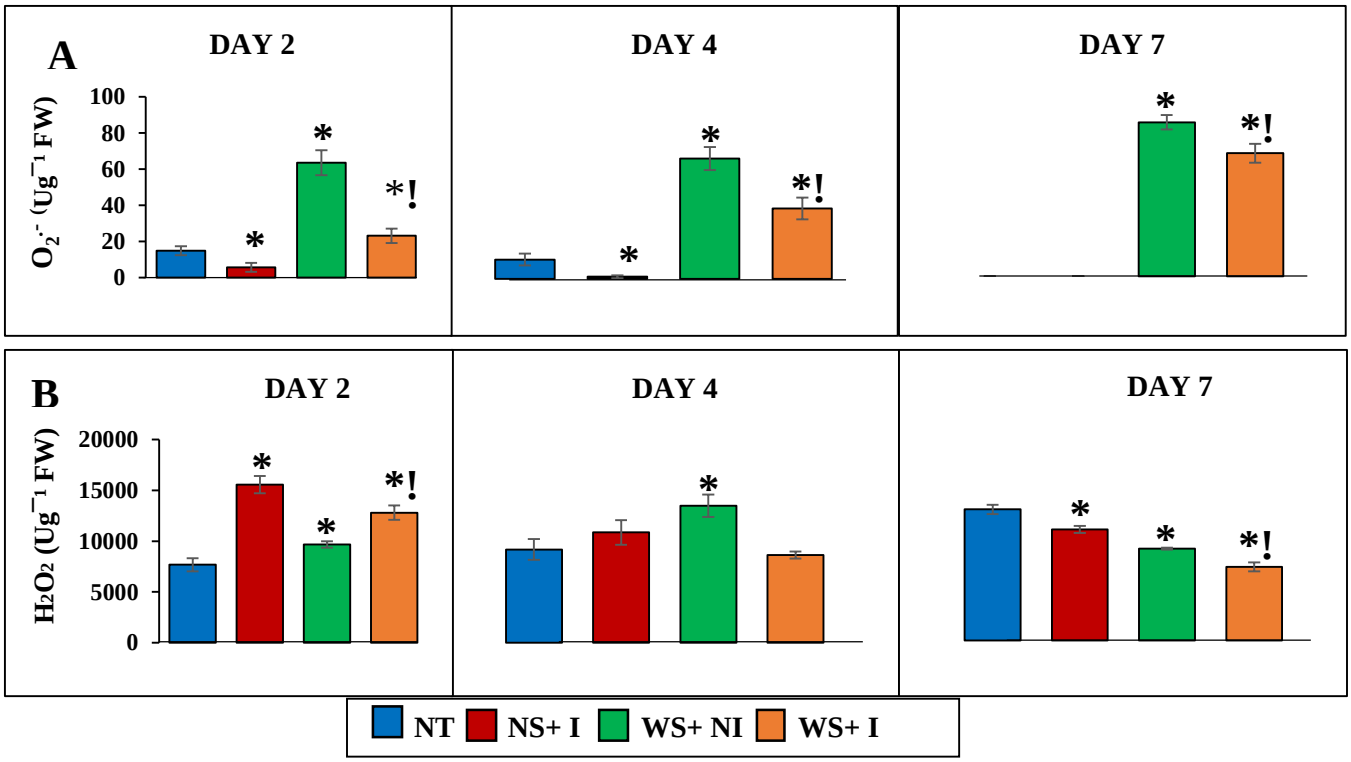

Fig. 4.1 Quantitation of accumulated ROS levels in *A. thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 inoculation with or without water stress, day 2, day 4 and day 7 post treatments (**NT**: no treatment; **NS+ I**: non-stressed, inoculated; **WS+NI**: water-stressed, non-inoculated; **WS+I**: water-stressed, inoculated). **(A)** Accumulation of superoxide anion radical ($O_2^{\cdot-}$), U= µmole of formazan content in seedlings; **(B)** Accumulation of hydrogen peroxide (H_2O_2), U= µmole H_2O_2 content in seedlings. The bars represent mean (±) S.E of 6 independent replicates of each treatment. "*" represent significant difference ($p \leq 0.05$) of all treatments from the untreated control, NT. "!" represent significant difference ($p \leq 0.05$) between WS+NI and WS+I within a component on a particular day.

Peroxidase (POD)

Among all four treatments, POD activity was highest in the water-stressed, non-inoculated samples, followed by the water-stressed, inoculated samples on all days of analyses **(Fig. 4.3 A)**. The no-treatment controls and GAP-P45 inoculated plants under normal conditions exhibited similar POD activity on all days of analyses, both being significantly lower than the water-stressed plants (with or without GAP-P45 inoculation). Trends in specific activity (U mg^{-1} protein) of POD were almost identical to its activity (U mg^{-1} FW) on all days in all treatments **(Fig. 4.3 B)**.

Catalase (CAT)

The activity of the enzyme CAT recorded significantly high levels in seedlings with GAP-P45 treatments under normal growth conditions across all time points **(Fig. 4.3 C)** as opposed to the no treatment controls. In seedlings treated with water stress without GAP-P45 inoculation, CAT activity was found to be elevated after day 2 and day 4 post water stress induction than in all other treatments. However, a time- dependent decrease in the same was seen from day 2 to day 7. The inoculated plants under water stress exhibited significantly lower levels than the non-inoculated seedlings on day 2 and day 4. On day 7, the CAT activity was similar in these two treatments. Trends in specific activity (U mg^{-1} protein) of CAT were almost identical to its activity (U mg^{-1} FW) on all days in all treatments **(Fig. 4.3 D).**

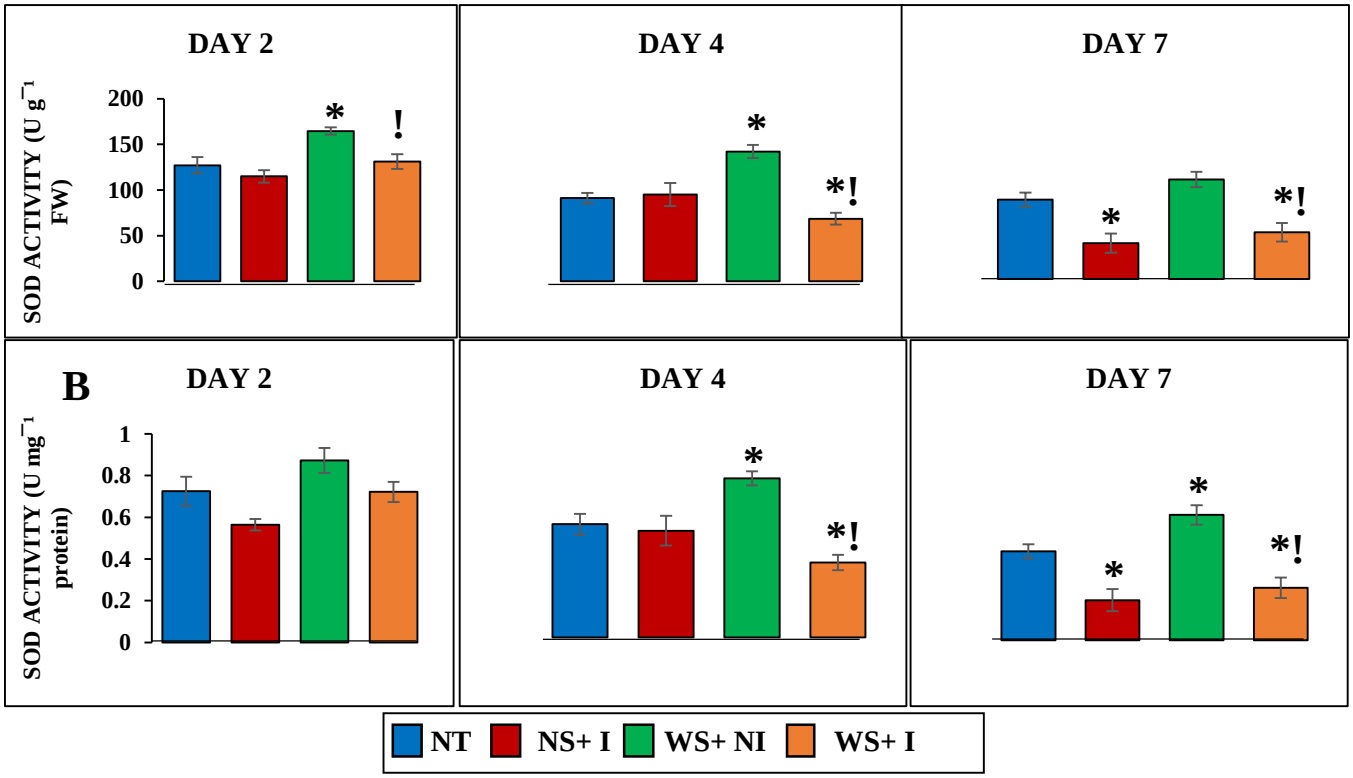

Fig. 4.2 Activity of antioxidant enzymes in *A. thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 inoculation with or without water stress, day 2, day 4 and day 7 post treatments. Activity of superoxide dismutase (SOD) expressed as **(A)** units (U) g^{-1} FW, U= 50 % inhibition of NBT reduction, **(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, NT. "!" represent significant difference ($p \leq 0.05$) between WS+NI and WS+I on a particular day.

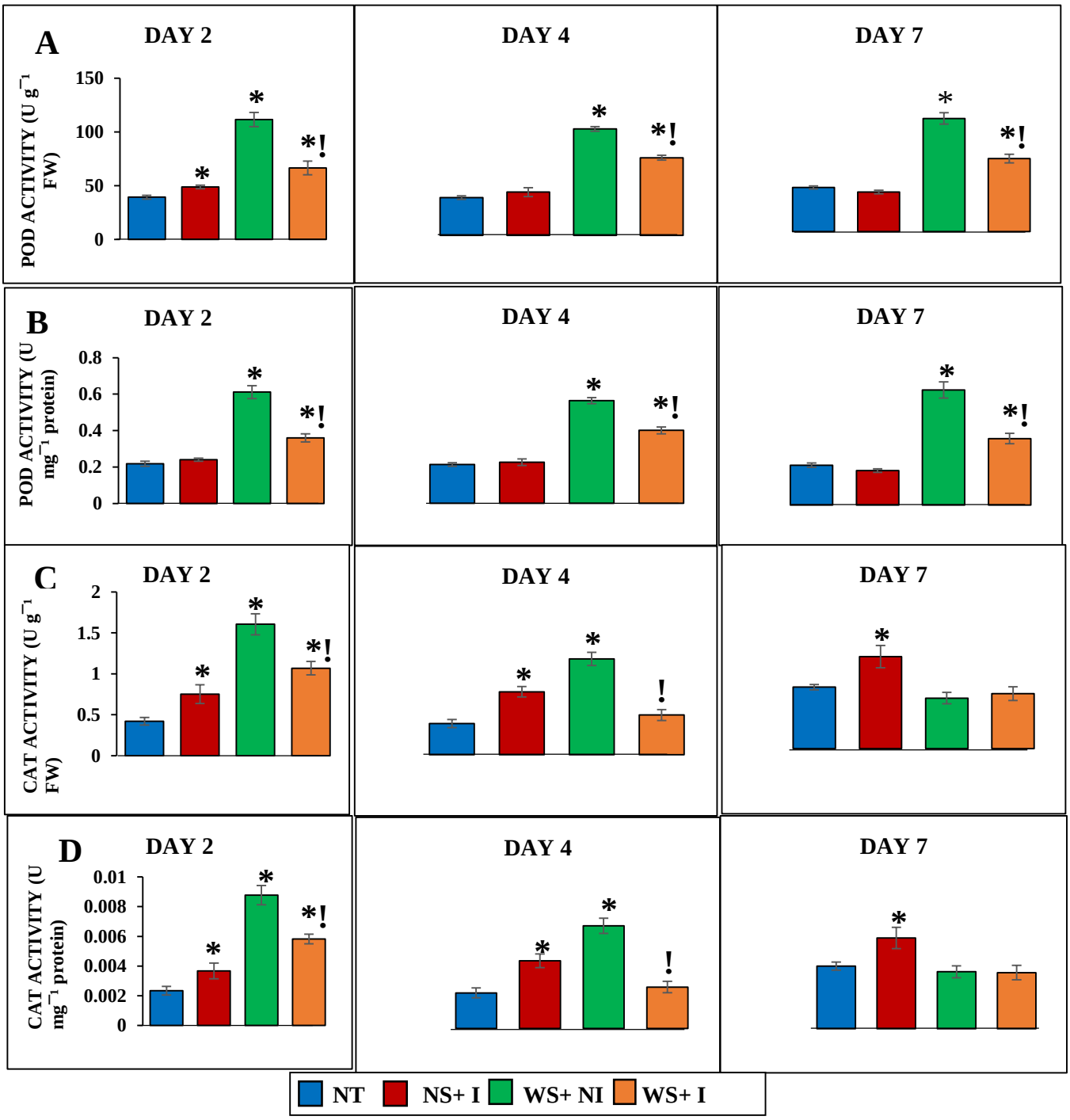

Fig. 4.3 Activity of antioxidant enzymes in *A. thaliana* seedlings in response to *Pseudomonas putida* GAP-P45 inoculation with or without water stress, day 2, day 4 and day 7 post treatments. Activity of peroxidase (POD) expressed as **(A)** U g^{-1} FW, U=Change in Abs min^{-1}; **(B)** U mg^{-1} protein (specific activity); activity of catalase (CAT) expressed as **(C)** U g^{-1} FW, U= m moles of H_2O_2 decomposed min^{-1}; **(D)** 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≤0.05) of all treatments from the untreated control, NT. "!" represent significant difference (p≤0.05) between WS+NI and WS+I within an enzyme on a particular day.

Glutathione reductase (GR)

The GR activity was found to be highest in the water- stressed seedlings without GAP-P45 treatment across all time points **(Fig. 4.4 A).** The enzyme activity in the GAP-P45 treated seedlings under both normal as well as water-stressed conditions displayed a gradually decreasing trend from day 2 to day 7. Only on day 2 was the enzyme activity of non-stressed inoculated seedlings higher than the untreated controls. Specific activity (U mg^{-1} protein) of GR was almost identical to its activity (U mg^{-1} FW) on all days in all treatments **(Fig. 4.4 B).**

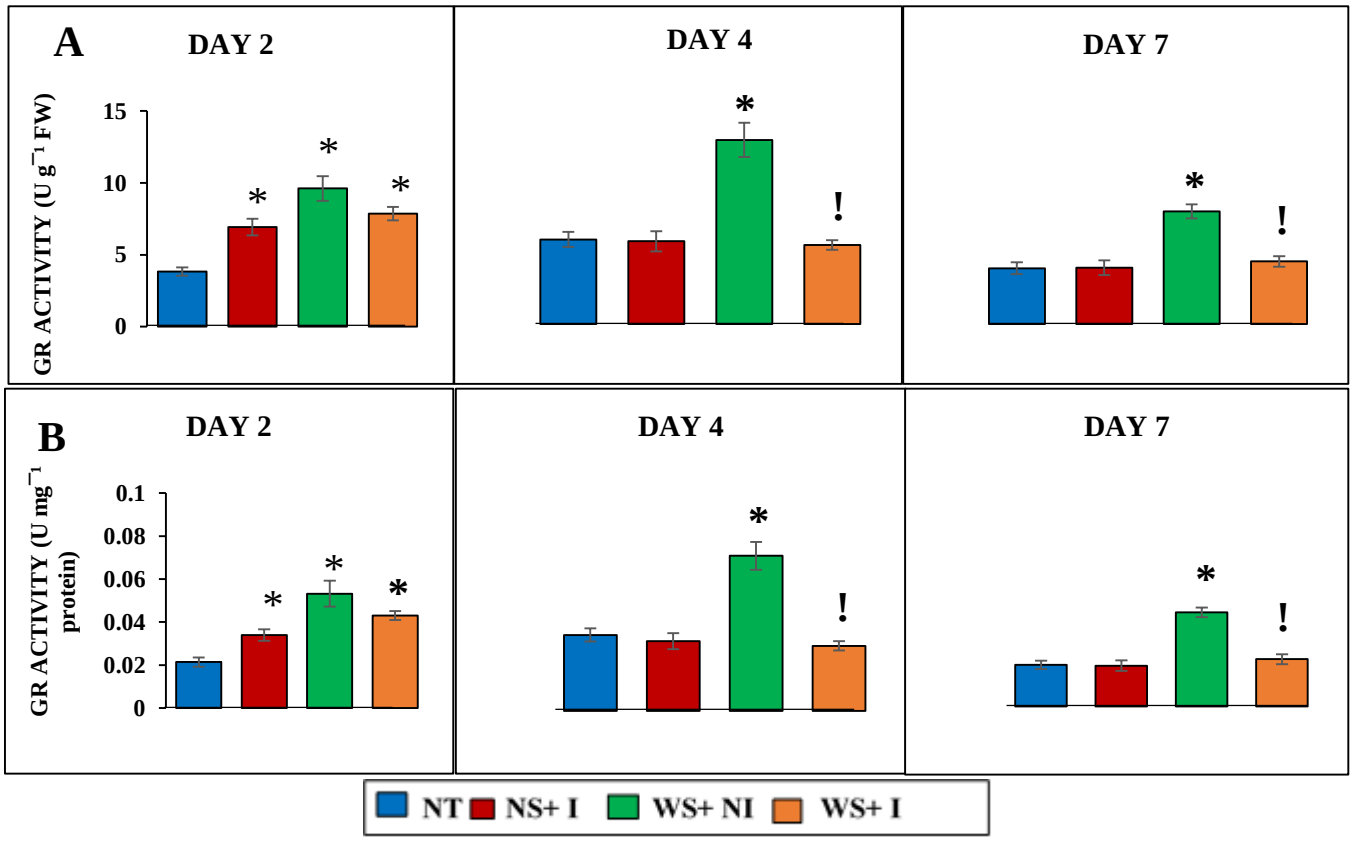

Fig. 4.4 Activity of antioxidant enzyme **(A, B)** glutathione reductase (GR) in *A. thaliana* seedlings in response to *P. putida* GAP-P45 inoculation with or without water stress, day 2, day 4 and day 7 post treatments, expressed as **(A)** U g^{-1} FW, U= m mol NADPH oxidized min^{-1}; **(B)** U mg^{-1} 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≤0.05) of all treatments from the untreated control, NT. "!" represent significant difference (p≤0.05) between WS+NI and WS+I on a particular day.

Ascorbate peroxidase (APX)

The activity of enzyme APX, in the seedlings with GAP-P45 treatments under normal growth conditions recorded high APX activity on days 4 and 7 **(Fig 4.5 A)**. However, GAP-P45 treatment under water-stressed conditions, caused a reduction in APX activity on day 2 and 4. The activity of APX decreased in water-stressed non- inoculated plants from day 2 to day 7. By day 7, it was lower than the water-stressed inoculated seedlings. Specific activity (U mg^{-1} protein) of APX was almost identical to its activity (U mg^{-1} FW) on all days in all treatments **(Fig. 4.5 B).**

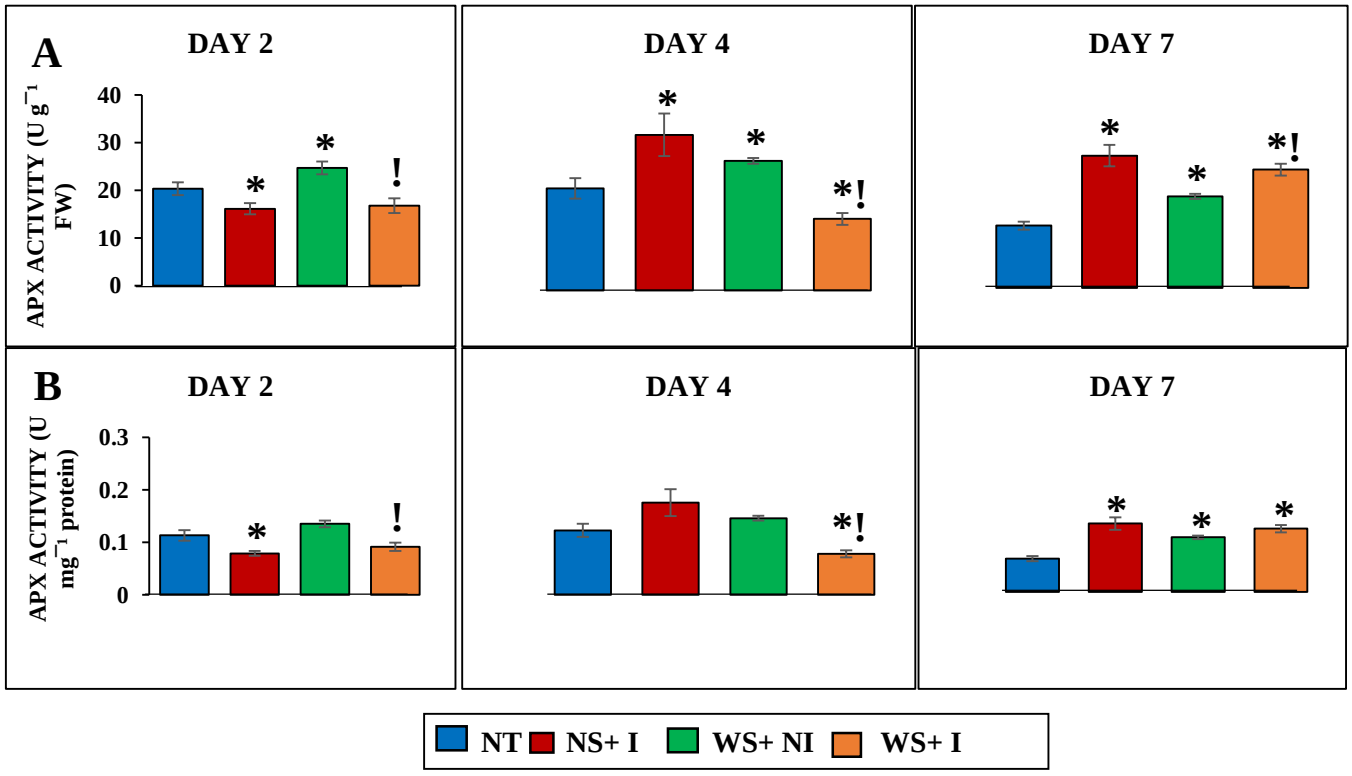

Fig. 4.5 Activity of antioxidant enzyme **(A, B)** ascorbate peroxidase (APX) in *A. thaliana* seedlings in response to *P. putida* GAP-P45 treatment under normal and water stress induced conditions compared to untreated controls, expressed as **(A)** U g^{-1} FW, U= m mol AsA oxidized min^{-1}; **(B)** 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, NT. "!" represent significant difference ($p \leq 0.05$) between WS+NI and WS+I on a particular day.

4.4 Discussion

GAP-P45 mostly decreases the levels of ROS and ROS scavenging enzymes under water-stress conditions

It had been previously reported Sandhya et al., (2010) that the PGPR strain *P. putida* GAP-P45, when inoculated with maize under drought stress conditions, significantly lowered the activities of antioxidant enzymes APX, CAT and glutathione peroxidases as compared to the un-inoculated water- stressed controls. Hence, we wanted to comprehensively investigate the oxidative status of water- stressed *A. thaliana* when inoculated with *P. putida* GAP-P45.

Out of the two ROS whose endogenous concentrations we tested in response to our treatments, $O_2^{\cdot-}$ was several-fold lower in the non-stressed plants as opposed to the water-stressed plants, on all days of analysis **(Fig. 4.1 A)**. In stressed as well as non-stressed plants, GAP-P45 inoculation significantly lowered the concentration of $O_2^{\cdot-}$. Hence, a GAP-P45 mediated decrease was seen in $O_2^{\cdot-}$ levels, both under stressed and non-stressed conditions. However, interestingly, from day 2 to day 7, the levels of $O_2^{\cdot-}$ kept decreasing in the non-stressed plants, while, it increased in the stressed plants (in both cases, with or without GAP-P45 inoculation) **(Fig. 4.1 A)**. $O_2^{\cdot-}$ is a substrate for SOD, which converts it to H_2O_2. The relative activity of SOD in the different treatments correlates positively with the levels of accumulated $O_2^{\cdot-}$, especially in the stressed plants **(Fig 4.2)**. Both, $O_2^{\cdot-}$ and SOD activity are up-regulated under water-stress, with GAP-P45 inoculation lowering the levels of both **(Fig 4.1 A & Fig 4.2)**. Hence, it can be argued that in all samples, the scavenging of $O_2^{\cdot-}$ by SOD is able to keep pace with its generation. Since, SOD converts $O_2^{\cdot-}$ to H_2O_2, one can expect a positive correlation between $O_2^{\cdot-}$ and H_2O_2 levels at a given point of time, especially with the positive correlation seen between $O_2^{\cdot-}$ and SOD activity. However, H_2O_2, exhibited a different pattern of accumulation **(Fig. 4.1 B)**. To begin with, on day 2, an inverse correlation was seen between $O_2^{\cdot-}$ and H_2O_2 levels. It appears that, on day 2, all treatments induced H_2O_2 accumulation as compared to the no-treatment controls. Because SOD activity negatively correlates with H_2O_2 accumulation data on day 2 **(Fig 4.1 B & Fig 4.2)**, hence, it appears that the relatively higher H_2O_2 in the inoculated samples vs. their non-inoculated counterparts could be a consequence of other H_2O_2 generating pathways and/or their reduced scavenging by H_2O_2 scavenging enzymes. This could be attributed to the fact that besides being the most stable and non- radical ROS, H_2O_2 can also be generated via several enzymatic reactions in various cellular compartments of the plant cells. These include the chloroplastic photosynthesis, mitochondrial and apoplastic electron transport chains, photorespiration, oxidative deamination of amines, activity of peroxidases, amine oxidases, β- oxidation of fatty acids, catabolism of sugars and metabolites like proline, polyamines etc. From the pattern of accumulation of H_2O_2 on day 2, it can be assumed that, on day 2, higher scavenging of H_2O_2 is going on in the non-inoculated samples vs. the inoculated samples **(Fig 4.1 B)**. This seems to be the case in all H_2O_2 scavenging enzymes (POD, CAT, GR, APX) when the water-stressed

inoculated samples are compared with the water-stressed non-inoculated samples **(Fig. 4.3 and Fig. 4.5)**. All these enzymes exhibit lower activity and specific activity in the water-stressed inoculated samples as opposed to the water-stressed non-inoculated samples. In the non-stressed samples, however, only a partial correlation is seen. While POD, CAT **(Fig 4.3 A-D)** and GR **(Fig 4.4)** are higher in the non-stressed, inoculated samples (vs. the inoculated ones), APX was observed to be lower in them **(Fig 4.5)**.

From day 2 onwards, a gradual decrease was seen in the H_2O_2 levels of the inoculated plants, both under non-stressed as well as stressed conditions. The water-stressed plants exhibited an increase in H_2O_2 content from day 2 to day 4, followed by a decline on day 7. The no-treatment control seedlings did not show any significant fluctuations, rather an overall increase was observed in their H_2O_2 content from day 2 to day 7 **(Fig 4.1 B)**. Hence, one would assume that, in the inoculated plants, a gradual scavenging of H_2O_2 would have occurred, leading to their decrease on day 4 and day 7. However, as can be seen from the activity of the H_2O_2 scavenging enzymes, such a clear correlation was not seen. In the non-stressed plants, catalase **(Fig 4.3 C** and **D)** and APX **(Fig 4.5)** showed enhanced activity (and specific activity) in the inoculated plants (vs. the non-inoculated plants), which could be responsible for the gradual decrease in H_2O_2 levels in the non-stressed, inoculated plants from day 2- day 4/ day 7. However, as far as the water-stressed plants are concerned, the inoculated plants exhibited lower activities and specific activities of all H_2O_2 scavenging enzymes studied on day 4 and day 7 **(Fig. 4.2-4.5)**. This points towards a different mode of scavenging and/or utilization of the higher H_2O_2 in these samples. In other words, the gradual decline in H_2O_2 levels, especially in the water-stressed, inoculated plants, despite there being no surge in the activities of the H_2O_2 scavenging enzymes could mean that the H_2O_2 produced is being degraded by some other means or utilized by some other reactions. The utilization of H_2O_2 in regulating a multitude of physiological responses in plants such as photosynthesis, stomatal movement, strengthening of cell wall etc. has been well- reviewed in the past decade (Kar 2011; Petrov and Van Breusegem 2012; You and Chan 2015) including its dynamic nature under stress as elucidated by several workers (Hung et al. 2005; Cheeseman 2007; Quan et al. 2008; Kar 2011; Mittler et al. 2012). The small size of the molecule coupled with its long half-life as compared to other ROS and the fact that H_2O_2 can diffuse across cell membranes and reside in different cellular compartments makes it a more easily accessible, interactive and highly utilized molecule supporting its contribution in signaling processes as a response to different environmental cues (Neill et al. 2002; Kar 2011; Petrov and Van Breusegem 2012). Under drought stress, in *Arabidopsis*, ROS, in particular, H_2O_2, mediates ABA induced stomatal closure (Bindschedler et al. 2006; Baxter et al. 2014) demonstrating its role as a secondary messenger in phyto-hormone mediated signaling in plants (Golldack et al. 2014). H_2O_2- induced protein modifications, oxidation of amino acid residues, interactions with various transcription factors serve as the fundamental step in redox- driven regulation of signal transduction cascades (Choudhury et al. 2017). It is interesting to note that, in low concentrations, H_2O_2 induces stress

tolerance in plant (He et al. 2009; Terzi et al. 2014). Besides being utilized for a range of cellular modifications and activities, H_2O_2 levels are also regulated by a host of other non-enzymatic antioxidants such as peroxiredoxins/ thioredoxins, ascorbate, glutathione, tocopherols, flavonoids, carotenoids etc. (Fratelli et al. 2004; Ahmad et al. 2010; Choudhury et al. 2017). Therefore, it is likely that the decline in the H_2O_2 levels on account of its plausible utilization in other cellular processes as argued above, could be subsequently leading to lower enzyme activities in water-stressed, inoculated plants as opposed to water-stressed, non-inoculated plants.

4.5 Key Findings

Our data point towards the observation that GAP-P45 under water-stressed conditions leads to improved phenotype (Ghosh et al. 2017) of *A. thaliana* which is correlated with;

1. Reduced production of $O_2^{\cdot -}$
2. Enhanced utilization of H_2O_2
3. Consequently, reduced activities of ROS scavenging enzymes.

Data from ROS accumulation and ROS scavenging enzyme activities suggest an overall up-regulation of redox metabolism under water-stressed conditions, and a GAP-P45 mediated down-regulation of the same.

Chapter 5

Summary and Conclusions

Based on the results obtained with respect to plant morpho-physiological characteristics, PA metabolic gene expression, enzymatic activities, accumulation of PA- related metabolites, ROS and antioxidant profiles etc. in water-stressed *A. thaliana* under *P. putida* GAP-P45 inoculated conditions, the following conclusions could be summarized as follows.

5.1 Drought- tolerant *Pseudomonas putida* GAP-P45 alleviates water- stress in *Arabidopsis thaliana.*

- We observed improved morpho-physiological status, and enhanced physiological parameters in *Arabidopsis thaliana* seedlings under water-stress when inoculated with *Pseudomonas putida* GAP-P45 as compared to non- inoculated plants. This is evident from our data on plant growth, fresh weight, dry weight, plant water content, chlorophyll content etc.
- In conclusion, *P. putida* GAP-P45 ameliorated water stress in *A. thaliana.*

5.2 Drought- tolerant *Pseudomonas putida* GAP-P45 modulates PA metabolism in *Arabidopsis thaliana.*

- The drought mitigating rhizobacterial strain *P. putida* GAP-P45 impacted PA metabolism in *A. thaliana* under water stress.
- At the transcriptional level, GAP-P45 significantly induced PA biosynthetic as well as catabolic genes in *A. thaliana* under water stress. Even when other treatments had not shown an induction, GAP-P45 inoculated water-stressed *A. thaliana* seedlings were already upregulated in most of the PA candidate genes. This particular treatment led to highest upregulation of PA metabolic genes for most part of the study.
- The overall increase in Put catabolism in addition to increased Put biosynthesis point towards enhanced Put turnover resulting in net Put accumulation in GAP-P45 inoculated water- stressed *A. thaliana* as compared to the non-inoculated, water-stressed plants. An overall positive correlation in the transcript levels of PA biosynthetic and catabolic genes and the accumulation of PAs was also found mostly in case of Put.
- The lack of increase in Spd/Spm catabolism in response to GAP-P45 inoculation under water stress as compared to water-stressed non- inoculated plants could be possibly a Put-mediated feedback inhibition of Spd/Spm catabolic enzyme activity to prevent further back- conversion of Spd/ Spm to Put.
- The overall increase in Put and Spd levels in the water-stressed, GAP-P45 inoculated plants as compared to the non-inoculated, water-stressed plants could also be correlated with an improvement in the morpho-physiology of water-stressed plants on inoculation with GAP-P45

5.3 Drought- tolerant *Pseudomonas putida* GAP-P45 modulates redox state in *Arabidopsis thaliana*

- Inoculation of water-stressed *A. thaliana* with *P. putida* GAP-P45 led to a decline in ROS levels and decreased activity of antioxidant enzymes while water-stressed plants without inoculation mostly recorded highest ROS levels and antioxidant enzyme activity. While water-stressed plants showed an upregulation of redox metabolism, GAP-P45 inoculation downregulated the same in water-stressed *A. thaliana*
- These observations corroborated the GAP-P45 mediated stress tolerant phenotype of *A. thaliana* under water stress.

Chapter 6

Specific contributions and

Future perspectives

6.1 SPECIFIC CONTRIBUTIONS

- First report on the modulation of plant polyamine biosynthetic genes at the transcriptional level by a free-living, drought-mitigating rhizobacterial strain.
- First report on the impact of a free-living, drought-mitigating rhizobacterium on plant polyamine catabolic gene expression.
- First report on the dynamic and complex transcriptional and/ or post transcriptional regulation, inter-conversion/ canalization of polyamines, by *P. putida* GAP-P45 under water-stress.
- Modulation of polyamine metabolism in response to *P. putida* GAP-P45 corroborated the water-stress tolerant phenotype observed in *A. thaliana* seedlings thereby indicating that PGPR mediated water stress tolerance and PGPR- mediated modulation of PA metabolism are indeed interconnected. Future studies are required to further validate the essentiality of such an interconnection for plant stress tolerance.

6.2 FUTURE PERSPECTIVES

The knowledge obtained from our study opens up several opportunities to extensively investigate PA metabolism with respect to plant –PGPR interactions under stress to be able to translate into field studies and serve agriculture from a larger perspective. A few examples describing possible future prospects of this research can be enumerated as follows,

- Studies on PA biosynthetic mutants and/ or PA inhibitors can be employed to elucidate the role of plant- PGPR interactions with respect to the modulation of PA metabolism in ameliorating stress.
- Our results clearly indicate the possible involvement of post- transcriptional/ post- translational modifications/ mechanisms to regulate PGPR- mediated PA homeostasis under stress. This area needs to be comprehensively studied in order to understand PA regulation during plant- PGPR interactions.
- The employment of transport/ uptake inhibitors can be utilized to understand whether the increase in PAs in response to PGPR inoculation is a result of an uptake of bacteria-secreted PAs (if any) or a consequence of PGPR-mediated endogenous modulation of PA accumulation through signal transduction processes.

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List of Publications

1. **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
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, **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
4. 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 review

5. "Drought- mitigating *Pseudomonas putida* strain modulates polyamine catabolism in *Arabidopsis thaliana"*- **Sunetra Sen**, Sridev Mohapatra.

List of Conferences Attended

- Presented a poster on "*Modulation of polyamine biosynthesis and redox state in Arabidopsis thaliana by a drought-mitigating Pseudomonas putida strain*" at Life Science Research & its Interface with Engineering and Allied Sciences (LSRIEAS) 2018, organized by Birla Institute of Technology and Sciences, Pilani.

- Presented a poster on "*Modulation of polyamine biosynthesis by a drought-mitigating Pseudomonas putida strain in Arabidopsis thaliana*" at International Conference on Plant Developmental Biology (ICPDB) and 3rd National *Arabidopsis* Meeting, 2017, organized by NISER, Jatni.

- Presented a poster on "*An auxin secreting Pseudomonas putida rhizobacterial strain that negatively impacts water-stress tolerance in Arabidopsis thaliana*" at 3rd International Plant Physiology Congress, 2015, held at JNU, New- Delhi.

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 on plant molecular 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 2002, Dr. Sridev Mohapatra has almost 16 years of academic experience in the field of Plant Molecular Biotechnology. He has published over 10 research papers with good citations in reputed international journals and conferences. He has served as a reviewer 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

Ms. Sunetra Sen 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. She has obtained her Master's degree (M. Sc.) in Microbiology from Kristu Jayanti College, Bangalore University, India in 2010 and started her research as a research fellow in 2013. She is well versed in various microbial, plant, molecular and analytical techniques. She has good number of publications and awards to her credit and has presented her work in several national and international conferences. Currently, her career interests are focused on the development of stress-tolerant crop varieties by transgenic technology based on the understanding of plant-microbial interactions.

Appendices