Differential expression analysis of Calmodulin-binding (CaM) gene in Pea plants under drought stress and the effects of Plant Growth Promoting Rhizobacteria and Plant Growth Regulators

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QUAID-I-AZAM UNIVERSITY
ISLAMABAD, PAKISTAN
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AUTHOR'S DECLARATION

By submitting this dissertation entitle **"Differential expression analysis of Calmodulin-binding (CaM) gene in Pea plants under drought stress and the effects of Plant Growth Promoting Rhizobacteria and Plant Growth Regulators"** I **Tasmia Bashir** hereby declare that this thesis is my own novel work and effort that it has not been submitted anywhere for any award/Degree. Where other source of information has been used, they have been acknowledged. Furthermore, the research work presented in the dissertation was carried out in the Plant Physiology Laboratory, Department of Plant Sciences, Quaid-I-Azam University, Islamabad and Crop Physiology Laboratory, Martin Luther University Halle-Wittenberg, Germany.

TASMIA BASHIR

Dated: 28th August, 2020

PLAGRISIM UNDERTAKING

I solemnly declare that the research work presented in this dissertation entitled **"Differential expression analysis of Calmodulin-binding (CaM) gene in Pea plants under drought stress and the effects of Plant Growth Promoting Rhizobacteria and Plant Growth Regulators"** is purely my original research work with no substantial involvement from any other person/resources. Minor input or assistance taken from anywhere has been appropriately acknowledged.

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APPROVAL CERTIFICATE

This is to certify that the dissertation entitled **"Differential expression analysis of Calmodulin-binding (CaM) gene in Pea plants under drought stress and the effects of Plant Growth Promoting Rhizobacteria and Plant Growth Regulators"** submitted by Ms. Tasmia Bashir is acceptedin its present form by the Department of Plant Sciences, Quaid-i-Azam University Islamabad, Pakistan, as satisfying the dissertation requirement for the degree of Ph.D. (Doctor of Philosophy) in Plant Sciences.

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DEDICATION

"Put Allah first and everything will work out, may be not the way you planned but, just how it's meant to be".

(Anonymous)

*This dissertation is dedicated to the sustainer and the best planner of the Universe "**The Almighty Allah**" who always has a greater plans for me. I always pray for the directions to follow it, patience to wait on it and knowledge to know when it comes. Without the hardships I would not have valued ease.*

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TASMIA BASHIR

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LIST OF ABBREVIATIONS

	Abbreviations
%	Percent
~	Approximation
°C	Degree Celsius
µl	Micro litre
µM	Micro Mole
µmol $m^{-2}s^{-1}$	Micromole per meter square per second
ABI1	ABA-insensitive1 gene
ABREs	ABA-responsive elements
ACC-deaminase	1-aminocyclopropane-1-carboxylate deaminase
a.m	Ante meridian
AMS	Arbuscular mycorrhizal association
ANOVA	Analysis of Variance
AtCML8	*Arabidopsis thaliana* CaM-like protein 8
B.C	Before Christ
BNF	Biological nitrogen fixation
Ca^{2+}	Calcium
CaM	Calmodulins
CaMBD	CaM-binding domain
CAMTA	CAM binding transcription factor
CAT	Catalase
CBD	CaM-binding domain
CBL	calcineurin B-like proteins
CBL-CIPK	CBL-interacting protein kinase
CBNAC	Calmodulin-binding NAC protain
CBP	CaM-binding protein
CC	Chlorophyll content
CCaMK	Ca^{2+}/calmodulin-dependent protein kinase
cDNA	Complementary deoxyribonucleic acid
CDPK	Calcium Dependent Protein Kinase
CDPKs	Calcium-dependent protein kinases
CF	Chlorophyll fluorescence
cfu/ml	Colony forming unit per micro litre
CIPK	Calcineurin-interacting protein kinase
CK	CaM-kinase
CML	CAM-like proteins
CRKs	CDPK related protein kinases
CRT	Calreticulin
d	Days
DREB	dehydration-responsive element binding
DREB/CBF	DRE-binding factor/C-repeat binding factor

DW	Dry weight
EPS	Exopolysaccharide
ETI	Effector triggered immunity
Fe	Iron
FLIR	Forward looking infrared camera
FV/FM	Variable fluorescence by maximum fluorescence
FW	Fresh weight
g	Gram
GA	Gibberellic acid
GmCaM4	*Glycine max* Calmodulin 4
h	Hour
H_2O_2	Hydrogen per oxide
$HClO_4$	Per chloric acid
HNO_3	Nitric acid
IAA	Indole acetic acid
ISR	Induce-systemic resistance
IST	Induced-systemic tolerance
K	Potassium
$kgha^{-1}$	Kilogram per hectare
KPK	Khyber Pakhtunkhwa
LSD	Least significant difference
MAPK	Mitogen activated protein kinase
MC	Moisture content
mg	Milligram
mg/L	Milligram per litre
miRNAs	micro Ribonucleic acids
ml	Millilitre
Mn	Manganese
mRNA	Messenger ribonucleic acid
MYB	DNA-binding activity of transcription factor
N_2	Nitrogen
Na^+	Sodium
NCBI	National Center for Biotechnology Information
ND	Nanodrop
ng	Nano gram
NPR1	non-expressor of pathogenesis-related
NUP85	Nucleoporin 85
O.D	Optimum density
P	Phosphorus
p	Probability
PC	Princiopal component
PCA	Principal component analysis
PGPR	Plant growth promoting rhizobacteria

PGRs	Plant growth regulators
PH	Plant height
PR	Pathogenesis-related
PS II	Photosystem II
PSB	Phosphate solubilizing bacteria
PsCaM1	*Pisum sativum* Calmodulin
PsDREB2	*Pisum sativum* dehydration-responsive element binding
PsNIN	*Pisum sativum* nodule inception
qRT-PCR	Quantitative real time polymerase chain reaction
RIDER	Rhizobacterial induced drought endurance and resilience
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPK1	Receptor like protein kinase 1
RWC	Relative water content
SA	Salicylic acid
SC	Stomatal conductance
SI	Stomatal index
SID	salicylic acid induction-deficient
SlCaM	*Solanum lycopersicum*
SOS	salt-overly-sensitive
SPAD	Soil plant analysis development meter
SRK2C	Stress responsive protein kinase
SYMRK/DMI2	Symbiosis receptor like kinase/does not make infection2
TFs	Transcription factors
TGA	TGACG motif-binding factor
TP_1	Time point 1
TP_2	Time point 2
v/v	Volume by volume
VFs	Varimax loading factors
YEM	Yeast extract mannitol
Zn	Zinc

Summary of Dissertation

Abstract

In the proceeding climate change, drought stress has been identified as the major stress factor. To survive under such conditions, plants respond these changes by manipulating key physiological processes and modulation of expression of various Calmodulin (CaM) genes.

The present study was aimed to investigate the effect of plant growth hormones, salicylic acid (SA), abscisic acid (ABA) and *Rhizobium pisi* (strain DSM 30132) applied singly and in combination, on pea (*Pisum sativum* L.) cv. Florida plants under control and drought stressed conditions. Prior to the sowing, seeds were soaked for 5h in broth culture (10^8 cfu/ml) of *Rhizobium pisi* and SA /ABA. The seeds soaked for 5h in distilled water served as control. Three weeks old (21d) seedling were subjected to drought stress by discontinuing water supply and the effects were examined at two different time points of drought i.e., 4d (TP_1) and 8d (TP_2) of induction of drought stress. The salient physiological parameters studied were; moisture content of rhizosphere soil, plant biomass, and relative water content (RWC), canopy temperature, stomatal index, stomatal conductance, chlorophyll content, chlorophyll fluorescence and nutrient content. The inoculation effects of *Rhizobium pisi* and priming of SA and ABA on the expression analysis of PsCaM1 was also evaluated. The expression level of PsCaM1 (*Pisum sativum* Calmodulin) gene was identified by qRT-PCR among the treatments. The actin gene was used as a reference gene.

Results revealed a higher retention of soil moisture content in rhizosphere soil of abscisic acid treated plants at TP_1 and TP_2. Abscisic acid decreased the fresh and dry weight of plants under unstressed condition but increased the fresh weight as well as relative water content under drought stress. *Rhizobium* and SA ameliorated the adverse effects of drought stress more effectively than ABA alone. The *Rhizobium* inoculation significantly increased stomatal conductance under drought stress at TP_2. Under drought stress, at TP_1 all the treatments alone and in combination increased the RWC significantly over drought stressed plants. The FV/FM ratio was higher in SA treatment followed by combined treatment with SA, *Rhizobium* and ABA. Under drought stress, both Na and K uptake was significantly increased in *Rhizobium* + SA + ABA and *Rhizobium* inoculation. ABA, *Rhizobium* + SA treatments enhanced the Mg uptake under drought stress. Whereas, ABA, *Rhizobium* + SA + ABA increased accumulation of Ca content. Fe was significantly higher in *Rhizobium* and combined treatment of *Rhizobium* + SA + ABA. Similarly, Zn and Mn accumulation was also improved in *Rhizobium* treatment. Expression analysis demonstrated a significant upregulation of PsCaM1 gene under drought stress. ABA showed significantly higher (~1.5 folds) expression under drought stress at TP_2

followed by *Rhizobium* + ABA, Rhizobium + SA that divulged an increased expression of 0.8, 0.5 and 0.4 folds respectively at long term drought stress (TP_2). PsDREB2 gene is positively induced in *Rhizobium*, ABA and combined treatment of *Rhizobium* + ABA under long term drought stress (TP_2).

It is deduced from the data that *Rhizobium* alone or in association with SA may be used to mitigate drought induced inhibition on plant growth. *Rhizobium*, ABA and SA treatments exhibited better growth effect on pea plants at short term drought stress. Whereas, *Rhizobium* assisted SA and ABA to alleviate drought induced adverse effects over long term drought. The PsCaM and PsDREB2 gene is induced under long term drought stress. It is inferred that ABA, *Rhizobium* and consortium of **ABA, SA and Rhizobium** can be ideal candidate to enhance drought tolerance in pea plants by the upregulation of PsCaM gene.

Keyword: *Pisum sativum*, Drought stress, ABA, SA, CaM, PsCaM, PsDREB

Chapter No.1

Introduction and Review of Literature

INTRODUCTION

Abiotic stresses include salinity, low and high temperatures, and drought. They are considered as the major abiotic stresses that occurred globally. Plants have established different mechanisms to withstand these extreme conditions (Salehi-Lisar and Bakhshayeshan-Agdam, 2016). A better understanding of these mechanisms can aid in the improvement of stress tolerant crops.

The world population is expanding rapidly and estimated to reach 9.4 billion by the end of year 2050 (Béné et al., 2015). To fulfil food demands of increased population significant increase in crop production is necessary, while keeping the renewable and ecological resources of our plant preserved (Hertel, 2015). Different abiotic (i.e., salinity, cold, drought, frost and waterlogging) and biotic (i.e., insects, weeds and pathogens) factors are restricting the agricultural production by reducing the quality and quantity of crop yield that results into limited plant growth (Waraich et al., 2011). Drought, a major stress contributor influences crop yield worldwide (Singh and Laxmi, 2015). Expected temperature rise will bring noteworthy change in annual global rainfall that will subsequently increase the drought frequency (OECD, 2012). According to the European Union (EU), from 1991-2006 drought area has been doubled with a 25% estimated yield loss. In fact, the United Nations estimates two-thirds of the world population possibly will be under conditions of drought in 2025 and 1.8 billion people will be an inhabitant in countries/states with absolute water scarcity (FAO 2007; Chartres and Varma, 2010).

1.1. Role of secondary messenger

Through signal transduction networks plants react to different environmental fluctuations and developmental cues that comprises non-protein or multiple protein elements. Environmental fluctuations mostly includes different transcription factors, receptors and enzymes, while the developmental cues includes some secondary messengers, such as active oxygen species, cyclic nucleotides, lipids, calcium (Ca^{2+}) and hydrogen ions. Among them, Ca^{2+} is the significant secondary messenger (Liese and Romeis, 2013; Valmonte et al., 2014; Simeunovic et al., 2016). The Ca^{2+} is also essential in maintenance of cell wall and membrane stability, root hair elongation, stomatal guard cell movement, pollen tube growth, and as an important plant nutrient (White and Broadley, 2003; Kim et al., 2009). Plants evoke specific spatiotemporal calcium signals in the form of transient changes in Ca^{2+} concentration in cells due to environmental and developmental stimuli. External factors (e.g., temperature salt, osmotic stress, light) can establish diverse Ca^{2+} changes which are recognized by specific calcium sensors/receptors to instigate further outcome of transcriptional and metabolic reactions (Batistič, and Kudla, 2012; Shi et al., 2018). The transient intensification of the cytosolic Ca^{+2} concentration is generated by salt, cold, drought, mechanical, oxidative and osmotic stress (Matthus et al., 2019). Sensor proteins perceive specific Ca^{+2} spike signatures in signalling cascade; namely, Ca^{2+} CDPK (Calcium Dependent Protein Kinase), CML (CAM-like proteins), CAMs (Calmodulins), CCaMK (Ca^{2+} or Ca^{2+}/calmodulin dependent protein kinase), CBL (calcineurin B-like proteins), and their CIPK (Calcium interacting protein kinases), and CRT (calreticulin), that are controlled directly or indirectly by signature spike of Ca^{2+} (Ray et al., 2007; Magnan et al., 2008; Weinl and Kudla, 2009; Galon et al., 2010, 2008; Takahashi et al., 2011; Xu et al., 2011). Transcriptional factors transcribed transduced signal in differential gene expressions and regulate it by Ca^{2+} binding proteins, named as CAM binding transcription factor, CAMTA. Characterization of sensor protein include "sensor responders" and "sensor relays" (Hashimoto and Kudla, 2011). Calmodulins (CaM), calcineurin B-like proteins (CBL) and calmodulin-like protein (CML) are "sensor relays". Due to lack of catalytic domain they transmit Ca^{2+} through protein-protein interactions. The CBLs link with a family of protein identified as CIPKs (i.e., CBL interacting protein kinases) and the "CBL-CIPK complex" is designated as bimolecular "sensor-responders". Sensor-responders are CDPKs because protein is a combination of kinase domain and Ca^{+2} sensing calmodulin-like domain (Monshausen, 2012). Structurally CDPKs and CCaMKs are alike they also have regulated kinase, which have a Ca^{2+}-CaM binding domain overlapping with the auto inhibitory domain and visinin-like domain for Ca^{2+} binding (DeFalco et al., 2012).

1.2. Plant responses to drought stress

Plant roots have the ability to sense soil moisture deficiency rapidly, this environmental stress influence crop yield more severely (Peleg et al., 2011). Drought stress of plant has described in Figure (1.1), which represents modified molecular, morphological and physiological plants attributes under drought stress. The photosynthetic activity is badly affected under drought stress, modification in carbon and nitrogen metabolism as well as plant water relation have been reported (Mejri et al., 2016). Plant growth inhibition minimizes water loss from leaves because of stomatal closure that is first response in plant. To derive water from the deep down surfaces of soil, augmented root length is an adaptive strategy. Cell hydration maintenance by osmotic adjustment and minimized water loss via transpiration may compose in another adaptive mechanism (Boughalleb et al., 2016). To cope with drought stress though plants have their own mechanisms, but different soil microorganisms role in drought tolerance induction is worth mentioning (Glick, 2012).

The phenological stages of plants, length of exposure to stress and the severity are significant determinant for damage caused by stress (Figure 1.2). Salt/drought induce secondary stress which occurs as osmotic stress. Root growth attribute is extremely sensitive to osmotic stress as compared to leaf growth such as mild osmotic stress can inhibit leaves and stem growth (Ahanger et al., 2014). Under water deficit conditions, alleviated leaf size is evaluated more beneficial due to concomitant reduced transpiration rate, although photosynthetic rate is adversely affected. During moderately dry climates, the direct evaporation from soil persists relatively wet. This strategy is not as efficacious as anticipated (Tardieu, 2005; Forni et al., 2017).

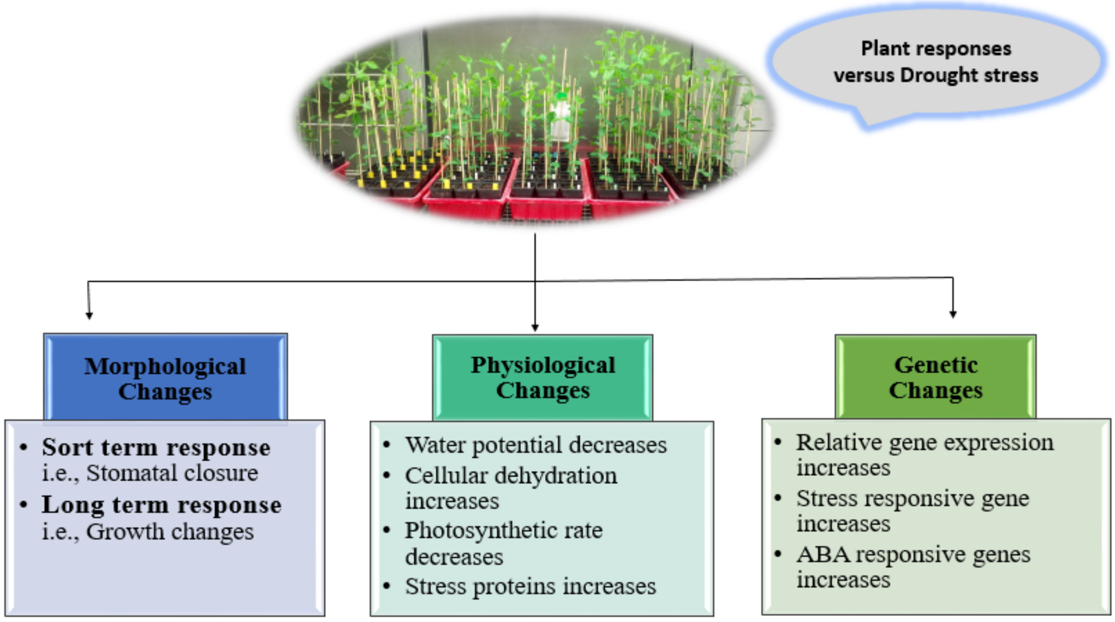


Figure 1.1: A diagram presenting plant responses against drought stress.
Source: Akinci, (2013).

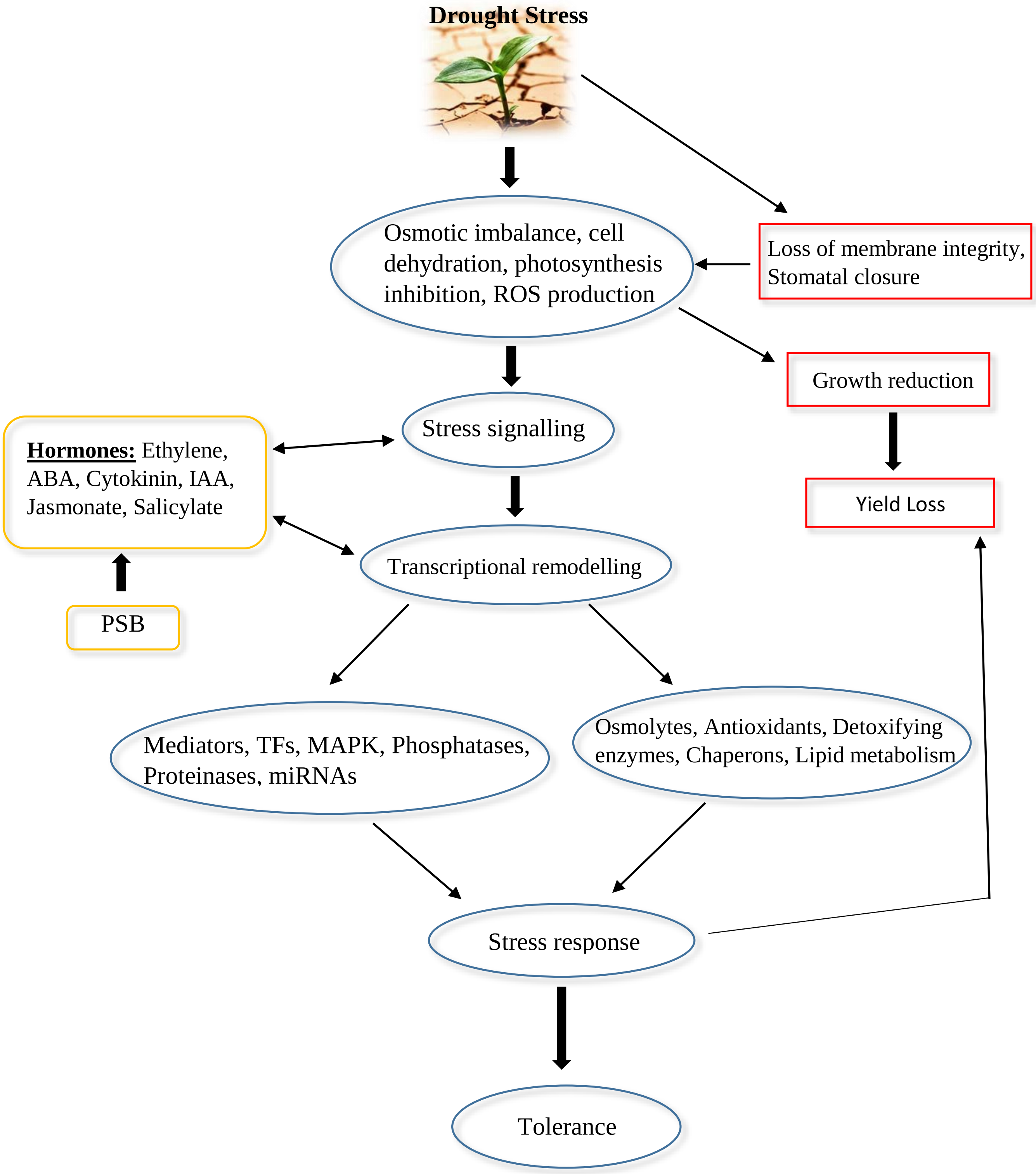


Figure 1.2: A schematic overview of drought stress responses in plants

Source: Forni et al., 2017

1.3. Leguminous

Legumes are thought to be the most important staple crops worldwide. They are the primary source of oil, fibre, minerals, micronutrients and vegetable proteins that are pertinent for human consumption and livestock feed (Xiao et al., 2017; Hummel et al., 2018). Because of symbiotic consortium with rhizobia, they have the specific to fix nitrogen as well. Due to frequent exposure to drought, salinity, pH and/or temperature stress climate specifically to drought their biological nitrogen fixation ability is impaired (BNF) (Furlan et al., 2019). Considering the expansion of semi-arid regions and projected expansion of global population (IPCC, 2014), the investigation prone to drought stress condition is of significant interest (Naya et al., 2007; Larrainzar et al., 2014; Larrainzar and Wienkoop, 2017).

1.3.1. Pea (*Pisum sativum* L.)

Pea is one of the leading legume used as a staple food in temperate cultivating systems all over Europe, North America and Asia, in East African highlands it is also known as a traditional protein crop (Zohary et al., 2012) which is cultivated as a source of forage, green seeds for processing and vegetable crop in addition e.g., snap pea (Stone et al., 2015). The concentration of genetic variation is much greater in the cultivated pea (*P.sativum*) species than the wild species *P. fulvum* (Zong et al., 2009). The word 'pea' has its origin to the Italian word: pisello (derived by the Latin: pisselo) that traced to ancient Greek word 'pison' ('πίσον'). In Afghanistan and Abyssinia peas had probably originated, with areas in the Mediterranean area colonised later. Afterwards pea spread to other regions of Asia and Europe (Karkanis et al., 2016). In Middle East with barley and wheat, pea was also domesticated simultaneously, not later than the sixth millennium B.C (Karkanis et al., 2016).

Pisum sativum L. belongs to the Fabaceae (Leguminosae) family with a small genus. Pea is one of the well-recognized rabi season vegetable. It is an annual herbaceous self-pollinated vegetable with a trailing, climbing or dwarf growth habit. However, it a cool season crop, but frost can influence its pod devotement and flowering (Rahman et al., 2020). The rise in temperature confer a great loss in seed yield and poor pod setting. In Pakistan, an area of 56,200 hectares was under pea cultivation during 2011-12. The estimated total production was of 36,900 tonnes. Whereas, Khyber Pakhtunkhwa (KPK province) shared production of 800 tons with an average of 667 kgha^{-1}. It was considered as the least production of pea in comparison to other provinces (Khan et al., 2013). Pea crop has inclusive adaptability under agro-ecological regimes of Pakistan. Therefore, it is being cultured in plains in winter, whereas the cultivation of pea on highlands in summer. There are various reasons for pea yield loss in Pakistan than the number of advanced countries.

1.3.2. Adaptability of pea to abiotic stress

Pea is a well acclimatized and improved crop to a broad range of regimes/environments from temperate maritime to semiarid conditions. The optimum temperature for the base germination of seeds is around 20 ± 1.1 °C (Raveneau et al., 2011; Karkanis et al., 2016). Though, the chances of damage due to frost depends on the developmental phase of the plant. The chilling temperature of about -4.5 °C can kill 50% of seedlings (Sallam et al., 2015). Generally, legumes can easily be affected by the freezing temperatures, specifically at the formation of a pod, seed filling, and flowering stage (Maqbool et al., 2010). By the process of “cold acclimatization” pea can tolerate frost because during the process they are exposed to low temperatures (Balwin et al., 2014). The production of cysteine and methionine has been interrelated with tolerance to low temperatures in pea (Legrand et al., 2013). It has been observed that autumn sowing led to a greater yield of pea +56% than spring sowing in Italy (Annicchiarico and Filippi, 2007). The compact nature of soil with increased temperature or drought influences the growth and yield of pea crop during flowering and filling of grain. In addition, pea flower earlier in winter. Therefore, they are less susceptible to drought stress by the end of the cycle (Vocanson and Jeuffroy, 2008; Neugschwandtner et al., 2015; Neugschwandtner et al., 2019). Drought stress impact can be avoided in the semi-arid region through crop management practices such as early sowing of pea (Khan et al., 2010). Pea is very sensitive to salinity and a high rate of waterlogging that is why they should not be cultivated in soil having a low infiltration rate (Duhan et al., 2018). Lately, some more salt-tolerant interesting landraces have been derived from China and Greece. These salt-tolerant cultivars ameliorate the tolerance in field pea crop under salinity. Thus, they are considered to be used in the breeding programmes (Leonforte et al., 2013).

The prime importance is to manage water resources through enhanced water use efficiency. The traditional breeding practices comprise the selection of stress tolerant varieties with appropriate agronomic attributes. By genetic engineering, the drought tolerant crops can be developed. The method involves the recognition of fundamental genetic components significant for drought/stress tolerance in plants which enables them to introduce these stress-responsive genes into crops. A number of physiological events in plants are triggered by drought that in turn influence the expression level of genes (Sahi et al., 2006). Several genes entailed in stress tolerance has initially been derived from *Arabidopsis thaliana*. With the introducing stress inducible genes an upsurge tolerance to cold, drought and salinity stresses in plants has been achieved by the process of genetic engineering (Shinozaki and Yamaguchi-Shinozaki, 2007).

The phytohormones including; cytokinins (CK), abscisic acid (ABA), salicylic acid (SA), and gibberellic acid (GA), antioxidants (e.g., hydrogen peroxide (H_2O_2)), ascorbic acid, and osmoprotectants have been employed as seed priming and foliar practices (Farooq et al., 2009) in order to mitigate the consequences of drought stress in plants. The adverse impact of stresses can be alleviated as a result of an exogenous utilization of plant growth regulators (PGRs), for instance gibberellins (Afzal et al., 2005), cytokinins (Merewitz et al., 2011), auxins (Fahad et al., 2015). They can also improve seed germination, seed yields, and yield quality, development, and growth (Egamberdieva, 2017). Furthermore, the application of cytokinins under abiotic stress environments by scavenging free radicals can deferral leaf senescence directly (Sarafraz- Ali et al., 2011; Ardakani et al., 2014).

1.4. Plant Growth Promoting Rhizobacteria (PGPRs)

Soil is defined as the upper layer of the earth’s crust that is made up of air, water, minerals, and other living organisms, and supports numerous substantial functions. The rhizosphere is demarcated as a thin layer of soil adjoining to the plant roots augmented in beneficial bacteria. These bacteria perform an imperative role in the plant growth preferment. Rhizospheric bacteria are characterized as plant growth promoting rhizobacteria (PGPRs) which remarkably improved growth parameters along with the suppression of various crop plant diseases (Mehmood et al., 2018). PGPR triggers the growth of associated host plants (Bhattacharyya and Jha, 2012). Further, PGPR are categorized as biocontrol agents, biopesticides, and biofertilizers, subjected to their mode of activities. These PGPRs are potentially capable of establishing their community community in the soil as they have the tendancy to adapt diverse conditions. The rapid growth rate of these PGPRs along with their biochemical adaptability helps them to digest an extensive variety of xenobiotic and natural compounds that played a significant role in their foundation (Narasimhan et al., 2003).

PGPR triggers a number of processes in an ecosystem such as nutrient cycling, nutrient uptake, the establishment of seeds and stress tolerance in biological control (Bisen et al., 2015). They have the ability to resist the environmental stresses, for instance, low and high temperatures, extreme soil salinities, heavy metals, heat and drought (Liddycoat et al., 2009; Chakraborty et al., 2015; Hidri et al., 2016). The diverse and wide range of microorganisms have been reported so far in ameliorating plant growth and mitigating the detrimental influence of drought stress.

1.4.1. Rhizobacterial response to drought stress

Bacteria are directly coupled with the accessibility of water through cellular functioning because it confirms the functionality of essential proteins and the integrity of macromolecules (Ngumbi and Kloepper, 2016). Usually, bacteria cope with the unfavourable environmental condition by modulating morphological modifications in the form of cysts and spores. The abundance and colonization of bacterial communities present in rhizospheric soil are affected by the environmental traits such as physicochemical attributes accompanied by species and age of the plant (Verma et al., 2019; Saharan and Nehra, 2011).

Several strategies have been used by bacteria to adjust to limited water conditions. The role of exopolysaccharides (EPS) in drought tolerance has been well demonstrated in bacterial cells (Tamaru et al., 2005). It acts as a binding agent in the soil which sequentially amended soil quality. A number of studies verified that EPS remarkably increases the resistance of both prokaryotes and eukaryotes dehydrated cells in terrestrial habitats. It also provides assistance to reclaim growth after desiccation (Naseem et al., 2018).

The production of glutamate, trehalose, proline, glycine betaine and osmo-protectants through K assist bacteria to acclimatize to drought conditions (Glick, 2012). Secondary metabolites and volatile compounds stimulate bacteria to subsist with the drought stress besides influence the root colonization (Cho et al., 2008). Bacteria involves a multistage up regulation gene for stabilization in the gene expression of stress-responsive genes under drought stress (Valentine et al., 2018). Drought stress can be prevented by an augmented the intensity of H_2O_2 resistance proteins to combat oxidative stress (Gulez et al., 2012).

1.4.2. Mechanism of PGPRs under drought stress

At present, it has established that PGPR strains are just as effective for ameliorating legumes growth, vegetables and cereals cultivated under stress environments (Khan et al., 2019a; Dubey et al., 2019; Lin et al., 2019; Debnath et al., 2019; Khan et al., 2020). Several researches have identified the strong influence of rhizobacteria in mitigating the antagonistic impact of salinity on crop growth *in vivo* along with *in vitro* conditions (Ansari and Ahmad, 2019; Waghmode et al., 2019; Kaushal, 2019).

PGPR demonstrated an alternative approach to alleviate the influence of drought stress in crop plants. A wide range of direct and indirect mechanisms can be utilized to escalate the WUE (water use efficiency) of plants that includes; production of PGRs, secondary metabolites and upregulation of stress-responsive genes in plants. They have the likelihood to modify plant health status which led to an increase in maize crop yield to a maximum level. A remarkable role of rhizobacterial communities has been discerned in the development and growth of maize plants i.e., with the inoculation of PGPR the proline, and sugar content in the leaves of maize plants increased to overcome the unfavourable conditions of drough stress (Sandhya et al., 2010).

Various mechanisms of action proposed that PGPR arbitrated tolerance to plants under drought stress condition. It comprises of modification in root morphology, the production of phytohormones, ACC deaminase activity, antioxidant defense and co-inoculations, volatile compounds, and accumulation of osmolytes, exopolysaccharide (EPS) production. In induced systemic tolerance (IST) the microbial communities instigate modifications in plants that led to an enhanced tolerance under biotic stresses (Yang et al., 2009; Atkinson and Urwin, 2012). Drought and salt stress tolerance is being occurred via elicitation of alleged ITS (induced systemic tolerance) mechanism induced by PGPR in plants (Figure 1.3) the process comprises of number of biochemical and physiological alterations (Yang et al., 2009). The rhizobacterial induced drought endurance and resilience (RIDER) mechanisms has been reviewed by number of researchers and demonstrated that PGPR enables plants to improve their growth under stress condition (Kaushal and Wani, 2016), it consist of inflection of phytohormonal levels (Kang et al., 2014a; Belimov et al., 2014; Glick, 2015; Cohen et al., 2015; Liu et al., 2016; Egamberdieva, 2017) (Figure 1.3), antioxidant defence (Wang et al., 2012; Armada et al., 2016), osmotic modification (Sarma and Saikia, 2014), stress-responsive genes(Kim et al., 2014), bacterial exopolysaccharides (Vardharajula et al., 2011; Naylor and Coleman-Derr, 2018) and volatile organic compounds (Zhang et al., 2008). PGPR not only stick to the root surface (rhizoplane) but also inhabit the rhizosphere along with root cortex as endophyte (Singh, 2018). Figure 1.3 recapitulates the events of PGPR on plants.

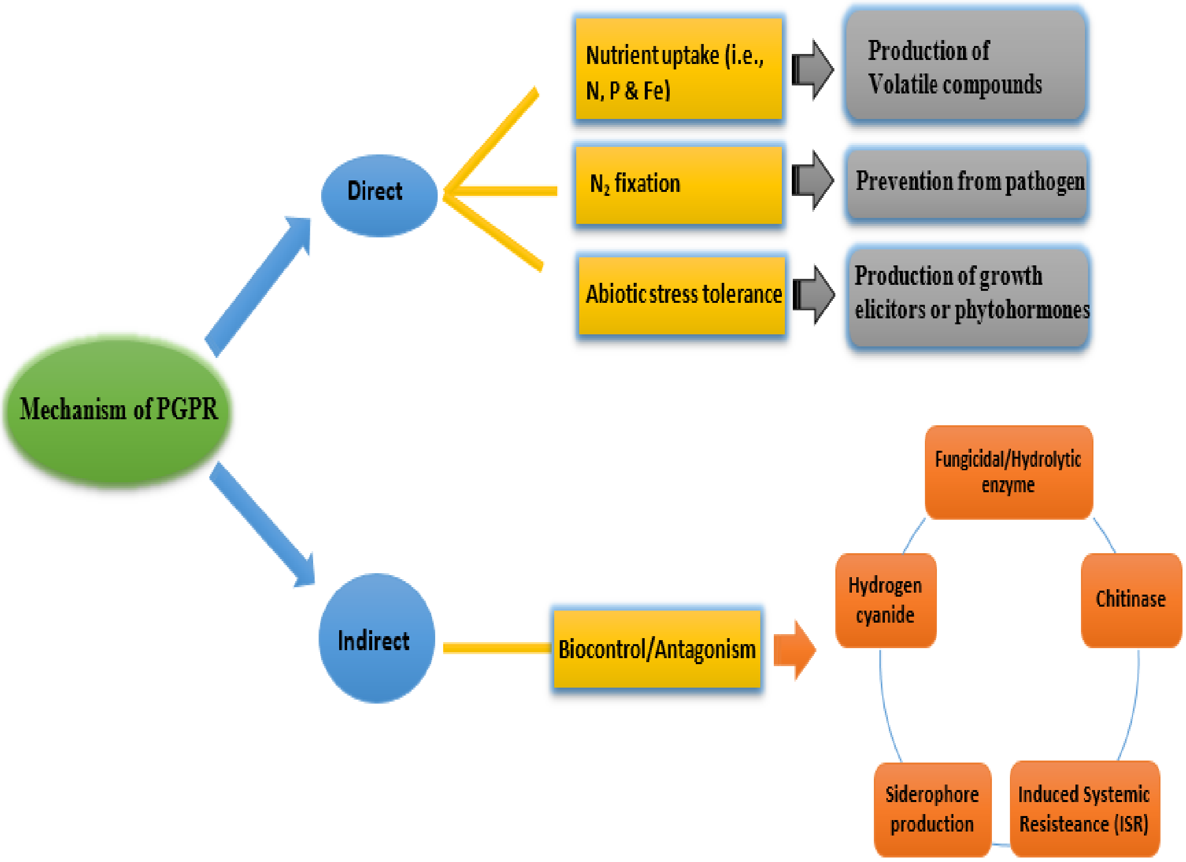


Figure 1.3: Illustrative representation of mechanism of PGPR in enhancing plant growth against environmental stresses. Source: (Singh, 2018).

1.4.3. Role of PGPRs in relation to macro nutrients

Studies on PGPR strains delineated to be effectual when used in combination with other microbial populations. The repercussion of co-inoculation with *Rhizobium tropici* and *Paenibacillus polymyxa* on nodulation of common beans (*Phaseolus vulgaris L.)*, nitrogen content, plant growth has been evaluated under drought environment in a greenhouse (Ferreira et al., 2018; Naseer et al., 2019; Khaitov et al., 2020). Previous studies were orchestrated at three levels of drought with two strains of *P. polymyxa* alone or in combination (Puri et al., 2016).

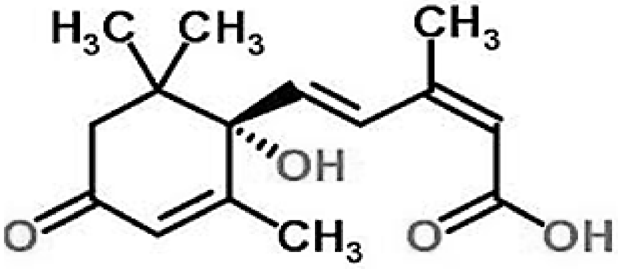
Pseudomonas fluorescens increased the antihypertension alkaloid (ajmalicine) content in *Catharranthus roseus* plants (Vimal et al., 2016; Pandey, 2017; Arivalagan and Somasundaram, 2017). In the same way, the inimical impacts of drought stress on pea plant growth can be alleviated by PGPR comprising ACC deaminase (Belimov et al., 2019; Sapre et al., 2019). The rhizobacteria have the potential to construct exopolysaccharides that can further be used efficaciously against drought stress for augmenting plant growth in sunflower plants (Naseem et al., 2018; Ojuederie et al., 2019; Meena et al., 2019). PGPRs have established various mechanisms for the growth promotion of plants in contaminated soils, the lowering of ethylene concentration is one of the mechanisms (Sarwar et al., 2017; Grobelak et al., 2018). The plausible reason for the enhancement of plant growth under heavy metal stress is attributed to the role of PGPR because they accumulate metals in their cells and mitigate their availability to plants. Another salient feature of PGPR is to enhance resistance against pathogens. They impart indemnity to plants against diseases. They have been shown as effective biocontrol agents against various plant pathogens (Ramadan et al., 2016; Liu et al., 2017; Sahu et al., 2018; Altinok and Yildiz, 2019).

Klebsiella oxytoca inoculated plants comprise of ACC-deaminase that improves the absorption of major nutrients for instance; N, P, K and Ca (Bhise and dandge, 2019; Syyed et al., 2019; Verma et al., 2019). *Klebsiella* stimulates plant growth by alleviating the negative impacts of stresses. However, *Pseudomonas spp*. inoculated seedlings intensify the growth of eggplant by depleting the uptake of Na^+ and amplifying the activities of antioxidant enzymes under salinity stress (Etesami and Alikhani, 2019; Kaymak, 2019). Moreover, under drought stress, PGPRs are intricate in regulating plant nutrition by augmenting the K^+ uptake over Na^+ in plants (Singh et al., 2019a; Shinwari et al., 2019; Rezakhani et al., 2019). The uptake of other major nutrients in plants also improves with the inoculation of PGPR besides the enhancement of water content in stressed plants (Bakhshandeh et al., 2019; Chiappero et al., 2019; He et al., 2019). The PGPR strains are considered not only for mitigating plant growth under salinity stress but are also beneficial for ameliorating plant development under flooding, drought stress and heavy metals (Vivas-Peris et al., 2018; Kerchev et al., 2019; Singh et al., 2019b; Manoj et al., 2020).

1.5. Plant growth regulators (PGRs)

1.5.1. Abscisic acid (ABA)

Abscisic acid is a phytohormone which triggers a series of key processes intricately implied in plant adaptation and development to abiotic and biotic stress responses. ABA is an inhibitory phytohormone that aid plants to acclimate stresses. Plants synthesize ABA in a number of organs that instigate defense mechanisms under stress conditions. The fundamental mechanisms comprise of defense-related gene expression and regulation of stomatal aperture discussing resistance to the environmental stress conditions (Lim et al., 2015; Sah et al., 2016). The striking attribute to the pathogen defence and the control of water loss through the process of transpiration is the pronouncement of stomatal opening and closure. Likewise, it plays a significant role in bud development, seed dormancy, leaf senescence, closure of stomata, resistance and abscission (Daszkowska-Golec, 2016; Kuromori and Shinozaki, 2018). ABA is also recognised as a stress hormone for the reason that the production of the hormone is stimulated through waterlogging, drought and other severe environmental conditions (Vishwakarma et al., 2017; Islam et al., 2018). Abscisic acid is documented as dormin as well because it gives rise to dormancy in stems, seeds, and buds (Wang et al., 2016a). Other names to ABA is inhibitor-B and abscission II (Toungos, 2018).



ABA (Abscisic acid)

1.5.1.1. Role of ABA in Plants

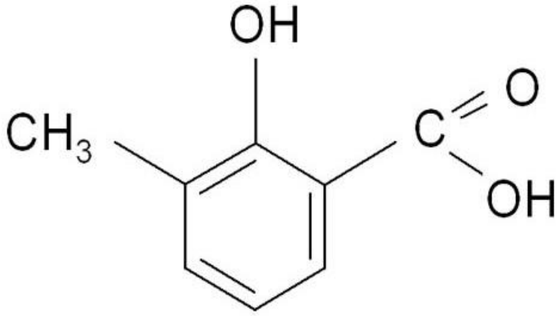
Throughout plant cycles, the phyto-hormone ABA activates several physiological mechanisms. ABA stimulates stomatal closure and the expression of various stress-responsive genes in regard to drought or water deficient conditions (Nambara et al., 2010; Fujita et al., 2011; Upadhyay et al., 2017). Plants persistently come across varied biotic and abiotic climatic stresses, together with high salinity, drought, and numerous pathogen because plants are sessile organisms. The effect of these environmental stresses on plant growth and development can adversely impede crop production (Sah et al., 2016). Under water stress, the elongation and growth of the roots is a consequence of low water prospect that is predominantly initiated by the accumulation of ABA (Kuromori et al., 2018). The accretion of ABA, biosynthesis, and stomatal closure as well have documented in plants under the water stress condition (Valluru et al., 2016; Manzi et al., 2017). A considerably significant concentration of ABA is a prerequisite to sustain lateral root development under osmotic stress (Zhang et al., 2018).

Abscisic acid as a phytohormone plays its role as a chemical signal transducer in feedback to the ecological stresses. Plants sense these indicators and transform them to ABA. The instigation of series of plant physiological, and development mechanisms triggered through ABA thereby inducing acclimatization to the stress conditions (Raghavendra et al., 2010; Lee and Luan, 2012; Huang et al., 2012; de Zelicourt et al., 2016). These stresses adversely influence plant growth and instigate drastic diminution in agricultural crop production. Primarily, plant drop water through stomata on their leaves by gaseous exchange. Regulation of water status and stomatal movement occurred through the key hormone ABA. Plants accumulate and produce an increased amount of ABA in guard cell that led to the stomatal closure to conserve water under drought stress condition. The cellular and molecular mechanism have been comprehensively studied that is underlying ABA induced stomatal closure (Hubbard et al., 2010; Lim and Luan, 2014). The catabolism and biosynthesis of ABA are familiar to be the leading determining factor of endogenous ABA levels in plant cells (Cutler et al., 2010; Seo and Koshiba, 2011; Yamaguchi et al., 2018).

Abiotic and biotic stress have been comprehensively scrutinized in defense responses (Chinnusamy et al., 2008; Popko et al., 2010; Wilkinson and Davies, 2010; Sirichandra et al., 2010). During seed development, abscisic acid triggers the agglomeration of seed storage compounds. In addition, it is necessary for the preservation and induction of seed dormancy. Furthermore, it is intricated in plant pathogen responses (Kuromori et al., 2018).

1.5.2. Salicylic acid (SA)

Salicylic acid (SA) is a phenolic compound that is synthesized through the phenyl propanoid pathway in every plant kingdom. It has an imperative role in abiotic stress tolerance. SA has the potential to induce a protective effect under stresses (Martel and Qaderi, 2016).



SA (Salicylic acid)

1.5.2.1. Role of SA in plants

Plant growth regulators (PGRs) are chemical phytohormones phenolic in nature. They remarkably influence the growth and demarcation of tissues and cells (Gadzovska et al., 2013; Khanna et al., 2016). SA considerably alleviated growth inhibition by drought. It is further manifested through less decreased fresh and dry biomass, root length, plant height and many other physiological roles (Kang et al., 2014b). In addition to this, SA act as an intercellular communication messenger (Arteca, 2013). They have been linked with the upholding water conservation status in plants along with control of biotic and abiotic stresses (Sharma et al., 2019). It is primarily concerned with the modulation of developemental mechanisms and growth of plants in feedback to drought stress (Miura and Tada, 2014). It is apparent that SA provide protection to plants contrary to abiotic stresses by stimulating important physiological mechanisms such as; proline metabolism, antioxidant defence approach, photosynthesis, and water associations (Khan et al., 2010; Nazar et al., 2011; Miura and Tada, 2014).

The earlier studies have explained the role of SA in morphological, biochemical and physiological mechanisms of chickpea (War et al., 2011). A number of researches have validated the contribution of SA in the regulation of drought response in various other species

(Bijanzadeh, et al., 2019; Kareem et al., 2019; Gupta et al., 2020; Sohaq et al., 2020). SA has beneficial effects on growth, production of flavonoids in ornamental and crop plants, and flowering (Pacheco et al., 2013). Under stress condition, SA functions as a signalling compound which induces gene as chaperones, heat shock proteins, antioxidant enzymes, in addition to the production of secondary metabolites (Jumali et al., 2011). The inducible pathogenesis-related (PR) gene are instigated by means of drought stress in plants such as PR1 and PR2 (Miura and Tada, 2014). The generation of ROS induced by SA probably caused an escalation in endogenous hormone level which promotes closure of stomata (Lee et al., 2019). Likewise, it can occur due to the exogenous application of SA tends to H_2O_2, Ca^{2+} accumulation and ROS (Patni and Ansari, 2019; Chavoushi et al., 2019; Abbas et al., 2019). The naturally eventuating diamines and putrescine assumed to be the latent plant growth regulators in water conservation subsequently encourage root development (Khan et al., 2019; Irfan et al., 2019; Sujatha-Edupuganti and Anuradha, 2019). The major role of PGRs is in mediating plant defense responses against abiotic stress and pathogen attacks. Under stress, they intricate metabolic expression in plants. They are also intricate in the mechanisms of water conserving balance, stomatal closure and regulating stress-responsive genes. In addition to this, a number of other processes are involved like flowering, fruiting, ripening, senescence, plant development, and expression of secondary metabolites linked with the drought tolerance (Damalas, 2019; Canalis et al., 2019).

1.6. Calmodulin binding proteins (CaM)

Calmodulin (Calcium modulated protein) is a small calcium binding protein expressed in all eukaryotic cells that acts as secondary messenger in a variety of cellular responses. There is a structural and functional homology between plants, animals and yeast calmodulin, but multiple isoforms of the protein appear to be the distinguishing feature of higher plants (Villalobo et al., 2019). In response to extracellular calcium concentration calmodulin binds to the short peptide sequence of the target proteins and initiates the calcium dependent signalling pathways (Edel and Kudla, 2015). Calmodulin is the most eminent calcium transducer, regulating the activity of different proteins with wide range of cellular functions. Most of the functions of calmodulin and its downstream effectors are alike in eukaryotes and plants. On the other hand, the plants have a unique series of calmodulin linked and downstream target proteins (Bouch´e et al., 2005).

Calcium is a second messenger; it plays a key role in various cellular mechanisms like; development and growth in plants. Plant hormones, sunlight, abiotic stresses, mechanical disturbances, and pathogen elicitors acts as modulator for intracellular calcium level (Chakraborty et al., 2015; Chakraborty and Acharya, 2017; Sindhu and Sharma, 2019). There are different calcium sensors which alter the calcium signals into an inclusive range of cellular responses (McCormack et al., 2005; Pei and Gilroy, 2018). Calcium sensor responders and sensor relays are the types of calcium sensors which are involved in different signalling responses in plants. CDPKs (calcium dependent protein kinases) are the well characterized class of Calcium sensor responders in plants (Kudla et al., 2010; Valmonte et al., 2014). The CDPKs comprise four functional domains among which the C terminal CaM like Ca^{2+} -binding domain and a Calmodulin (CaM) binding domain are present. As calcium regulates the activity of CDPKs, therefore CDPKs are recognised to play key role in Calcium mediated cell signalling (Liese and Romeis, 2013). In addition to Calcium-dependent protein kinases, the CRKs have similarly been delineated in plants. However, on contrary the CDPKs the activity of CRKs (CDPK related protein kinases) is an independent of the Calcium levels (Rigó et al., 2013; Wang et al., 2016; Badmi et al., 2018). The sensor relays and calcineurin-B-like proteins go through the calcium bring about conformational changes and then act together with their downstream target proteins (Reddy and Reddy, 2004; Chen et al., 2012; Boudsocq and Sheen, 2013).

1.6.1. Pathways involved in CaM

There are numerous stimuli for the plant cells in response to which there is a rapid increase in the cytosolic calcium levels. The high calcium level transduces the different cellular signalling pathways via calmodulin and other calcium binding proteins (Steinhorst and Kudla, 2013; Bergey et al., 2014). In the recent past, a massive range of calmodulin related proteins have been recognized which are specific to plant cells. Moreover, calmodulin proteins play novel role in plant cells signalling in response to environment signals (Aldon et al., 2018).

The environmental stimuli include the biotic and abiotic stimuli which modulate the calcium levels in cytosol or other organelles such as nucleus. The rise in free calcium levels leads to the binding of free calcium with calcium modulated proteins, calmodulin and CRPs (Chen et al., 2015). The structural changes in these proteins help them to link up with variety of cellular targets that control vital cellular functions, such as cellular metabolism, cytoskeleton and protein modifications. Moreover, the calcium and calmodulin signalling also modulate the gene expression of certain genes by either directly binding to the different transcription factors or initiating the signalling cascade. The binding of CaM or CRP can result in rapid cellular changes (within seconds or minutes). However, the steady responses need gene transcription and protein synthesis (it may take minutes to days). The calmodulin-mediated signalling, and its interplay with other signalling cascades, comprises the reaction of the plant to the external stimuli.

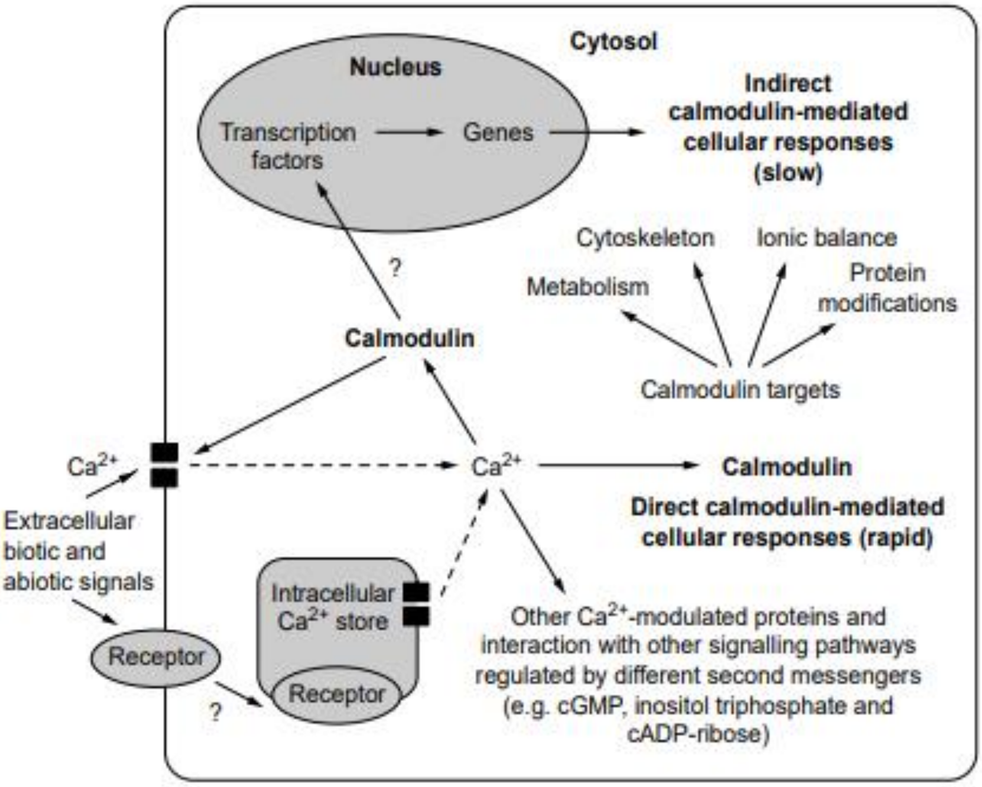


Figure 1.4: Ca^{2+}bound calmodulin arbitrated signal transduction pathways in plants under stress. Stress signals are discerned via receptors, in some cases triggering the transient changes in Ca^{2+} concentrations in organelles and/or cytosol. Dashed arrows indicate Ca^{2+} fluxes from intracellular or extracellular stores, and question mark denotes unidentified signal transduction intermediates.
Source: (Seybold et al., 2014).

1.6.2. CaM and drought stress

The main environmental stresses for plant cells are high salinity and drought which results in osmotic stress. The osmotic stress persuades the cellular and molecular level responses among which the primary response is the transient increase in the calcium level and ultimately calcium signal transduction pathways to alleviate the potential damages (Huang et al., 2012; Tripathy et al., 2019). Furthermore, the SOS (salt overly sensitive) signalling pathway the calmodulin and calmodulin related protein signalling is also known to play role as a response to osmotic stress in plants (Zhu, 2016; Saddhe et al., 2019; Ma et al., 2019). The increased expression of osmotic stress induced GmCaM4 genes in *Arabidopsis*, and calmodulin (CaM) genes from Soybean deliberates the osmotic stress tolerance by increasing the DNA-binding activity of transcription factor MYB. Moreover, it has also been reported that MYB2 interacts with calmodulin in calcium dependent way and thus regulate the osmotic and salt stress-responsive genes (Yoo et al., 2019; Kahraman et al., 2019). Another gene AtCML8, which is the ortholog of GmCaM4, was observed to be stimulated using salt treatment (Zhou et al., 2016). AtCML9, a protein like CML was also originate to be implicated in salt stress tolerance *via* ABA arbitrated signalling pathways (Dai et al., 2018). During the seed germination and seedling growth, the induction of ABA along with the abiotic stress the expression of AtCML9, and the knock-out mutants of actm19 exhibited an oversensitive response to ABA. In addition to this they showed increased tolerance to osmotic as well as salt stresses. Moreover, the expression of ABA-responsive genes comprising; RAB18, RD20 and RD29A and many osmotic stresses was dysregulated in *atcml9*. The CML gene in rice i.e., *OsMSR2* was potentially intricated in ABA arbitrated salt and dehydration tolerance (Xu et al., 2011).

1.7. Association of PGPR to CaM proteins

Numerous studies propose that Calcium levels, Calmodulin and CRPs are key players of plant response to different pathogens and symbionts attack. To supply nitrogen for plant growth, different legumes have a symbiotic relationship with nitrogen fixing bacteria which can convert nitrogen to ammonia (Mus et al., 2016). The signalling molecule, Nod factor produced by *Rhizobium* bacteria establish a symbiotic relationship between legume and *Rhizobium* (Tan et al., 2019). The Nod factor is recognized by the root hair cells of the host and it includes calcium responses which are separated spatiotemporally (de Bruijn, 2020).

Initially the calcium flux occurs at the tip of the root hairs then calcium spearing occurs in the surrounding of the nucleus. Mutation analysis of the root nodules of *M. truncatula* was found to be informative about the role of Calcium in the signalling of Nod factor (Ding et al., 2008).

1.8. Molecular Characterization

The diverse range of calmodulins and CMLs can be well explained by the tissue specific or subcellular localization of these proteins. The CaM is mostly found in cytosol however certain studies have reported the localization of calmodulin in nucleus (Cheval et al., 2013) (Fig. 1.6). The microarray analysis has shown that the most part of calmodulins and *CMLs* genes do not present unnecessary patterns in response to environmental stimuli or during plant development (McCormack et al., 2005).

The key role of DREB (dehydration responsive element binding protein) also known as C-repeat binding factor protein in osmotic, and heat stress have found in *Arabidopsis* (Sakuma et al., 2006; Kim et al., 2012). There are two types of dehydration-responsive element binding (DREB) proteins; i.e., DREB2A and DREB2B. They have been known to perform a major function in ABA independent pathway under heat and osmotic stresses (Yoshida et al., 2014). The expression of DREB2A is independent of ABA water stress. However, it is induced *via* greater salinity, heat, drought, and osmotic stresses. Sakuma et al. (2006) has explained the role of DREB2A under drought stress conditions. The DREB2A modulates the water stress-responsive gene expression, as a result increasing the water stress tolerance.

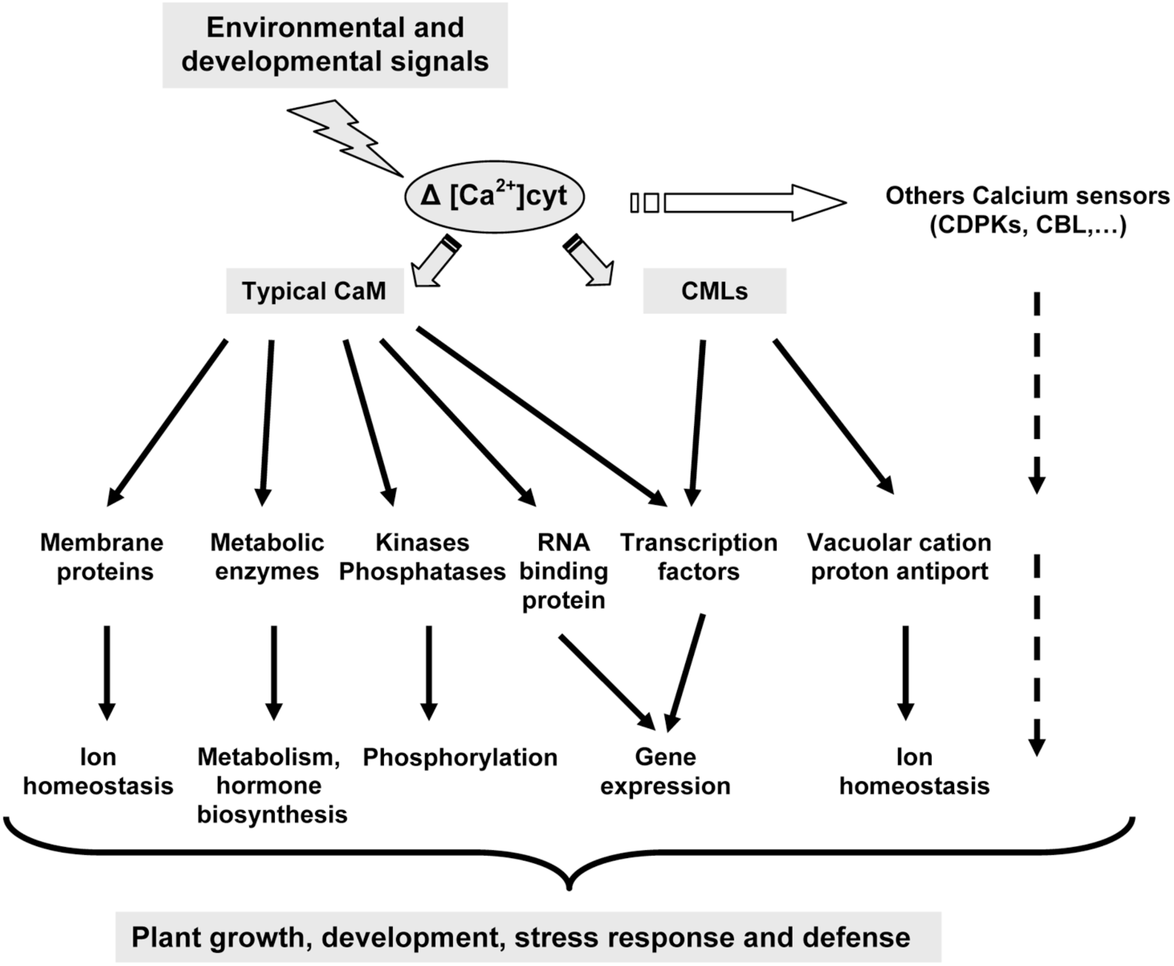


Figure 1.5: Calcium signalling with CaMs and CMLs in response to environmental stress.
Source: Ranty et al., 2006.

1.9. Aims of the study

- Differential expression analysis of calmodulin-binding (CaM) gene in pea plants under drought stress and the effects of PGPR and PGR
- To assess the role of PGPR (*Rhizobium pisi*) and PGRs (ABA and SA) on the growth of pea (*Pisum stivum)* under drought stress.
- Scrutinize the expression level of calmodulin gene in pea (PsCaM1) by inducing different treatments of PGPR and PGRs under the influence of drought stress.

Chapter No. 2

Phenotypic and physiological effects of PGPR and PGRs on Pea plants under drought stress

INTRODUCTION

The drastic change in climate leads to global food security which is being challenged and compromised because of a rapid increase in population (Swinnen, 2018). The utmost important limiting factors to crops productivity and ultimate food security have been demonstrated by earlier climate change outlooks, which are heat and drought stress. The frequent inception of drought around the world is triggering by the reduction in the precipitation and modifications in the rainfall pattern (Hsiang and Burke, 2014; Cook et al., 2014; Lobell and Asseng, 2017). The extreme drought stress is the prime source of a significant decline in crop production by exerting negative impacts on the growth of plant (Fahad et al., 2017; Wojtyla et al., 2020). A number of physiological (e.g., translocation of ions, ions uptake, carbohydrates, nutrient metabolism, hormones, respiration, and photosynthesis) and biochemical processes are inhibited through the mitigation of plant growth (Bita and Gerats, 2013; Ahmad et al., 2019).

Pea (*Pisum sativum* L.), is a food legume that is cultivated during the cool season. It has an extensive diversification of uses. It is cultivated worldwide as an economic source of protein. Pea contains a high concentration of lysine, tryptophan, and grain protein ranging from 19 to 27%. Pea also comprises a high level of carbohydrates. It is low in fiber and contains total digestible nutrients of about 87% (Mevlüt and Albayrak, 2012; Venkidasamy et al., 2019; Senapati et al., 2019; FAOSTAT, http://faostat3.fao.org) and is consumed as green seeds (fresh, canned or frozen), dry seeds, or green pods. Field pea is also used for animal feed (Karkanis et al., 2016).

2.1. Plant growth promoting rhizobacteria (PGPR)

The group of microorganisms which inhabit in the root of many plants and are identified as plant growth promoting rhizobacteria (PGPR). They are generally recognized as rhizobacteria and involves bacteria occupying the rhizosphere. They confer beneficial effects to plants by enabling plant growth either through direct mechanisms (i.e., improved availability of nutrients, production of phytohormones) or by indirect mechanisms (i.e., induced systemic resistance (ISR), subduing the pathogens using antibiosis, and synthesize lytic enzymes) (Goswami et al, 2016; Flores-Gallegos and Nava-Reyna, 2019). Furthermore, these microorganisms are involved in the synthesis of antioxidant enzymes production to preserve plants from ecological stresses that result in the initiation of ROS (reactive-oxygen species). Later it gives rise to cell damage or the use of PGPR to interact with those crops (Olanrewaju et al., 2017). They play a remarkable role in enhanced crop yields under sub-optimal conditions together with drought and high salinity.

2.1.2. Mechanism of PGPR induced drought tolerance

2.1.2.1. Drought tolerance by *Rhizobium*

Legumes have an association with the symbiotic root nodulating bacteria that fix N_2 for them and they are extremely sensitive to the environmental stresses in particular to drought stress (Niste et al., 2013). The accumulation of N_2 can be restricted due to the reduction in soil water content to gaseous exchange in leaves. The potential yield of legumes are subjected to the drying out of soil called drought (Beebe et al., 2014; Ansari et al., 2019; Nadeem et al., 2019). The delimitation of water is a major constraint in world agriculture. In general, the majority of the crop plants are extremely sensitive to even mild dehydration (Benešová et al., 2012; Llorens et al., 2020). Drought lowers the water content in soil and resulting in the inhibition of cell expansion, cell division, and eventually dehydrating cells. As a result, ensuing osmotic stress (Figure 2.1). In addition to this, ROS produced during drought stress in plants gives rise to oxidative stress (Vurukonda et al., 2016). These beneficial microorganisms are the integral constituent of agricultural practices to augment crop yield in an eco-friendly environment and in a sustainable way under severe stress circumstances (Gill et al., 2015; Gillet et al., 2017).

A number of studies have been delineated the activities of PGPRs under drought stress besides salinity stress in tomato (Mayak et al., 2004), maize (Bano and Fatima, 2009; Vardharajula et al., 2011), wheat (Tiwari et al., 2011), cucumber (Wang et al., 2012), *Vigna radiata* (Sarma and Saikia, 2014), pea (Barnawal et al., 2014), white clover (Han et al., 2014) and *Cicer arietinum* L. (Tiwari et al., 2016). Through the process of induced systemic resistance (ISR) (also termed as helicitation), PGPR induces salt and drought stress in plants that consist of a number of biochemical and physiological alterations (Lucas et al., 2014). It take account of regulation of phytohormonal levels (Belimov et al., 2014; Glick, 2015; Cohen et al., 2015; Liu et al., 2016; Egamberdieva et al., 2017; Park et al., 2017), bacterial exopolysaccharides (Vardharajula et al., 2011; Timmusk et al., 2013), osmotic adaptation (Sarma and Saikia, 2014), stress responsive genes (Kim et al., 2014), antioxidant defense (Wang et al., 2012; Armada et al., 2014), and volatile organic compound (Gutiérrez-Luna et al., 2010; Bitas et al., 2013) which can enhance tolerance in plants under stress conditions. However, major constraints to agriculture such as salinity along with drought attributes of drylands can be alleviated through RIDER (rhizobacterial-induced drought endurance and resilience) processes (Kaushal, 2019).

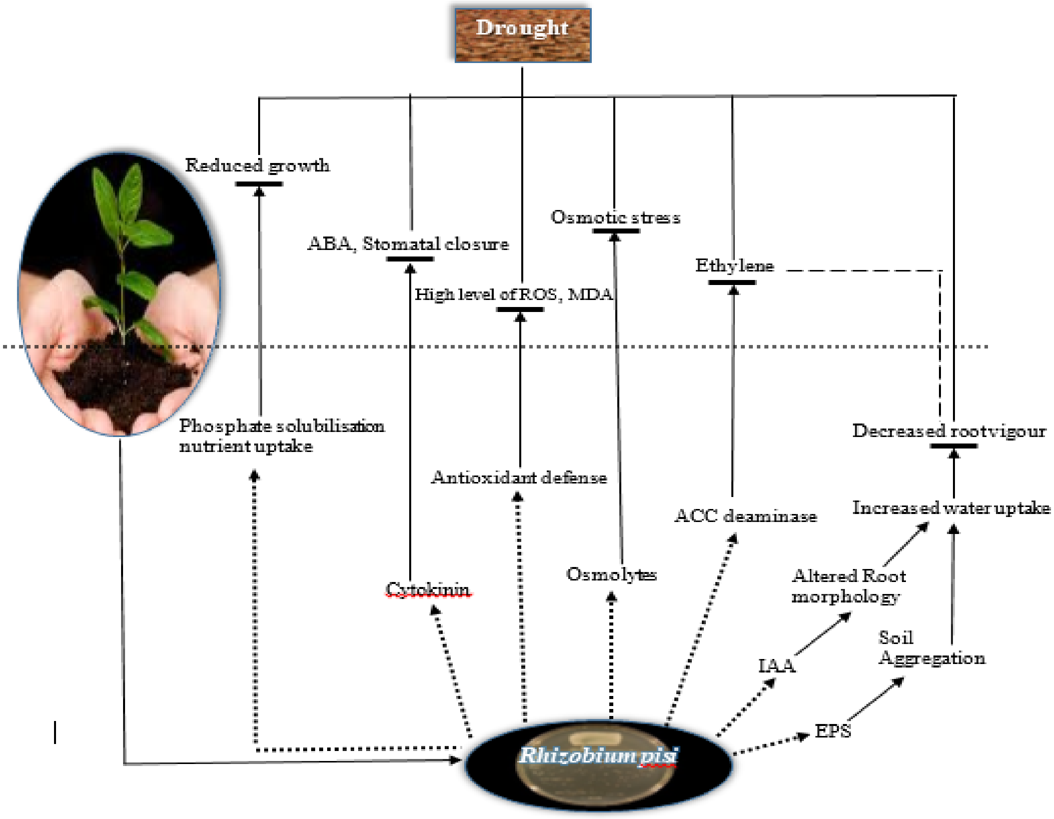


Figure 2.1. The PGPR (Plant Growth Promoting Rhizobacteria) in association to the particular processes in the course of drought stress. Solid arrows specify drought stress instigated effects on plants; the dotted arrows designate rhizobacterial components opposing stress consequences. Acronyms: ABA (abscisic acid); ROS (reactive oxygen species); MDA (malondialdehyde); ACC (1-aminocyclopropane-1-carboxylate); IAA (indole-3-acetic acid); EPS (exopolysaccharides).

2.1.3. Salicylic Acid (SA)

In natural environments, plants have to withstand against a number of abiotic and biotic stresses, generally employing in combination. That is the reason that they have developed distinctive stress signalling pathways that are arbitrated by phytohormones, reactive-oxygen species (ROS) additionally to some other signalling molecules. These signalling pathways concurrently curtail the damage and preserving substantial reservoir for the process of growth and reproduction (Cabello et al., 2014; Jayakannan et al., 2015; Verma et al., 2016). Recent studies have identified the combined effect of biotic and abiotic stresses impacting each other. There are a number of interesting points that occurred between the reactive-oxygen species (ROS) and the stress-responsive hormonal pathways. These pathways substentially could play an essential role in modulating plant response to various stresses (Sewelam et al., 2016; Shukla et al., 2019). Under stresses, salicylic acid (SA) together with the abscisic acid (ABA) accrue in plants and equally related to ROS. They act as a signal to stress responses (Rivas-San and Plasencia, 2011; Mittler and Blumwald, 2015; Suzuki et al., 2016).

The role of SA is very diverse and the most established role is the production of signalling molecule in plants. The production of these signals occurred in both local and systemic plant defense primarily contrary to biotrophic and hemi-biotrophic pathogens (Rivas-San-Vicente and Plasencia, 2011). In the salicylic acid induction deficient (SID) mutants in *Arabidopsis* do not accrue SA. They tend to be more prone to pathogens (Maruri-López et al., 2019). The suggested model of SA mechanism of action is the suppression of catalase (CAT) which is an essential H_2O_2 scavenging enzyme that results in an elevated H_2O_2 level. ROS sequentially can trigger the synthesis of SA regulating the activity of benzoic acid-2-hydroxylase. It transform benzoic acid into SA. Hence, both SA and ROS takes part in the regulatory loop. Where, ROS initiates the synthesis of SA. Whereas, SA promotes their accumulation that was claimed to prompt antioxidants. Ultimately, resulting in the reduction of ROS concentration (Khokon et al., 2011; Herrera-Vásquez et al., 2015; Khan et al., 2015; Silva et al., 2019). SA plays a dual role in plant feedback to abiotic stress. However, by the exogenous application of SA, the negative impacts of drought stress can be mitigated or undermine the plant response to stress. The reduction in plant responses against stresses depends on the interval of the treatment, and the concentration of SA and plant species (Barba-Espín et al., 2011; Khan et al., 2015).

2.1.4. Abscisic acid (ABA)

Abscisic acid (ABA) is a stress phytohormone. It plays an important role in plant response against drought stress, as a cellular signalling in the movement of water from root to leaf (Alves & Setter, 2004). Cellular signalling leads to the adaptation in the entire physiological and morphological mechanism of plants (Yin et al., 2004). Moreover, ABA is originated in root tissues, transported *via* xylem to shoot through the process of transpiration stream that results in the closure of stomata in order to minimize the water loss during drought (Seo & Koshiba, 2011).

ABA has been used with different concentrations ranging from 1 to 1,000 μM depending on the part of plant tissues and to be able to influence protein synthesis and gene expression entailed in anti-oxidative defense (Guan et al., 2000). Drought stress remarkably reduced the concentration of IAA and GA in leaves than that of the control (Xie et al., 2003; Bano and Yasmeen, 2010). However, an exogenous application of ABA caused an intensification of IAA and GA content as compared to untreated control plants under stressed conditions (Farooq & Bano, 2006). ABA acts together with the signalling pathways of SA in an intricate manner. ABA can also stimulate the biosynthesis of SA. SA in response can increase the concentration of ABA (Seo and Park, 2010).

2.1.4.1. Mechanism of action

The mechanism of action initiated with the closure of stomata under a limited supply of water. Specifically, multiple sites of ABA biosynthesis have been suggested on plant water relations such as vascular cells and guard cells (Figure 2.2) (Nambara, et al., 2010; Cao et al., 2011). It is evident from the earlier studies that the mechanism depends on the phenotypes where ABA is required in the signal transduction of defective mutant or the biosynthesis of ABA (Kaushal and Wani, 2016). It might possibly be transported from the areas of biosynthesis to the guard cells. Multiple trans-membrane ABA transporters specify the movement of ABA within a plant. They are actively modulated in an intercellular network. ABA modulates a number of molecular processes in various tissues, organs in addition to guard cells to withstand water stress reliant on the environmental circumstances. Multiple classes of ABA transporters have been established and specifies that plants are equipped with an extremely refined system. This system enables plants to sense and retort to water availability under adversely changing environments (Kuromori et al., 2018).

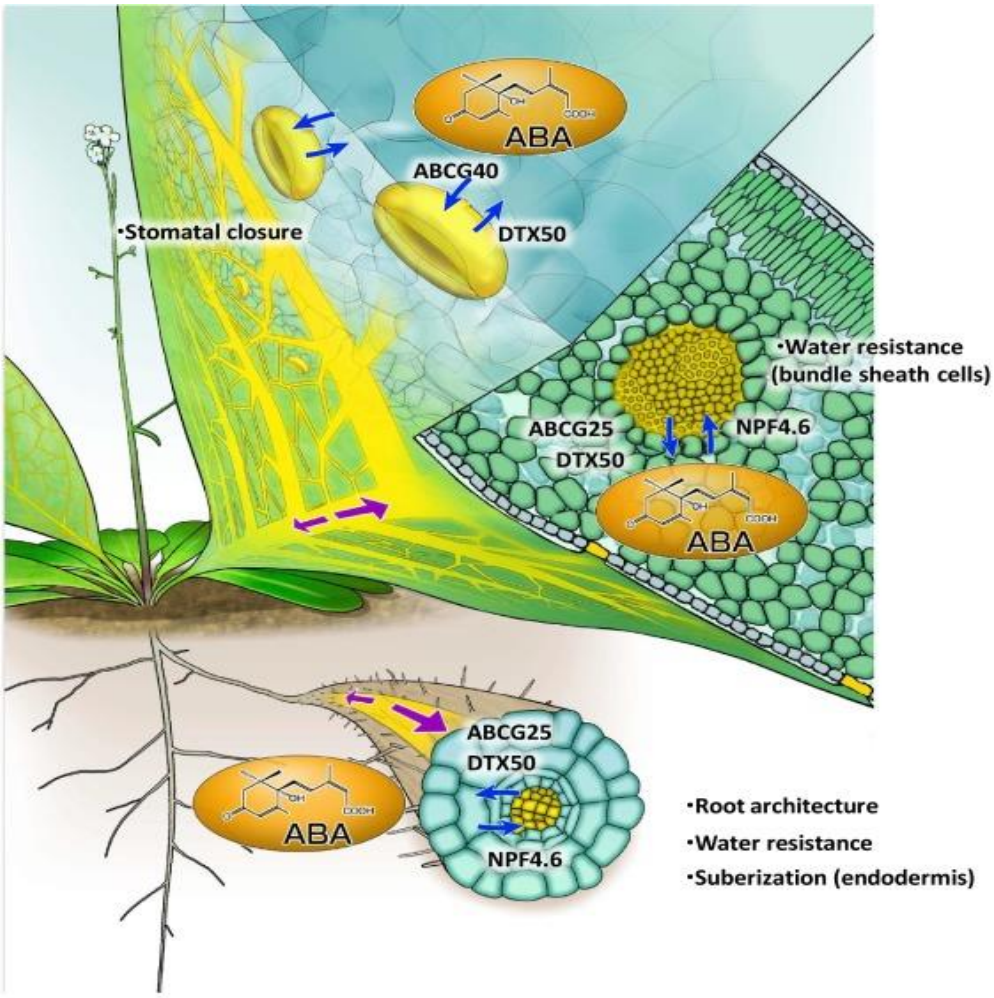


Figure 2.2. Abscisic Acid (ABA) regional functions and transport in plant drought Stress responses. The illustrative representation depicting three potential areas of ABA biosynthesis: root (vascular tissue), guard cells, and leaf vascular tissue. Tissues and cells articulating ABA transporters are shaded yellow. The blue arrows point out trans-membrane ABA transport facilitated through transporters. Purple arrows specifies the probable movement of ABA *via* xylem and phloem.

Source: Kuromori et al., 2018

By the utilization of PGPR, crop yield can be preserved to a specific level (Sandhya et al., 2010; Glick 2012; Glick, 2014). There are many converging points where expression of the stress responsive gene and ROS interacts that led to biotic and abiotic stresses (Glombitza et al., 2004; Sewelam et al., 2016). The PGRs such as SA and ABA are considered as the elicitors which accumulate in plants under drought environments. The role of SA is well demonstrated under stress condition where it aids plants to tolerate against pathogenic attack. However, it is required for the activation of plant growth, flowering, development, ripening of fruits and abiotic stresses respectively (Miura and Tada, 2014). While ABA increases 55 fold of the original under drought stress. ABA interacts with SA signalling pathways in an intricate manner. The use of PGPR has been proven as a solution for the sustainability of the agro-ecosystem under stress. These biological agents (PGPR) and elicitors (PGRs) are in control for alleviating plant growth from abiotic and biotic stress responses.

Globally, preceding the climate change is projected to have a considerable repercussion on rainfall, intensifying the drought stress. There is a dire need to improve drought tolerance in crops so as to improve their growth and yield using a number of PGPRs and PGRs (Khan et al., 2019). Previous studies demonstrate the favourable effects of PGPRs and PGRs on wheat and maize crops to alleviate drought stress (Khan et al., 2018; Mega et al., 2019; Kumar et al., 2019). However, literature is scanty on pea plants. Present study was aimed to assess the role of PGPR (*Rhizobium pisi*) and PGRs (SA and ABA) on the growth of pea under drought stress.

2.2 Materials and methods

2.2.1. Plant material and growing conditions

The seeds of pea (*Pisum sativum* var. Pea-Florida) were sown in pots (14×12 cm^2) filled with sieved and autoclaved ED73 soil under in vitro conditions. Experiment was organized in completely randomize design, conducted in triplicates. Plants were grown in walk-in-chamber maintained at 16h photoperiod with temperature 24 ± 2 °C (day/night), 65% relative humidity and light intensity of 100 µmol m^{-2}s^{-1} (LI-COR LI-250A, serial No. Q 101421). Pea seeds were surface sterilized with 95% (v/v) ethanol followed by shaking in 5% (v/v) sodium hypochlorite with slight modification (addition of 50 µl of Tween 100) and subsequently washed thrice with autoclaved distilled water (Lindsey et al., 2017).

2.2.2. Exogenous application of SA and ABA

SA and ABA were used as PGRs. A stock solution of 10^{-6} M was prepared to conduct the experiment (Hadi et al., 2010). The seeds were soaked in aqueous solution of SA and ABA for 6h prior to sowing (Safari et al., 2018).

2.2.3. Preparation of *Rhizobium* inoculum

Rhizobium pisi DSM 30132 strain was used as PGPR. Broth cultures of *Rhizobium* was prepared by growing the *Rhizobium* in yeast extract mannitol (YEM) media for 3 days (10^8 cfu /ml and O.D ~ 1 at 660 nm).

2.2.4. Induction of drought stress

Drought stress was induced after three weeks of germination through withholding the supply of water followed by constant watering to maintain the moisture content of stressed plants at 40% (Pain et al., 2018). The experiment was performed with six replicates each for control and drought conditions. Treatment were: untreated control (C), inoculated with *Rhizobium pisi* (R), treated with salicylic acid (S), treated with abscisic acid (A), combined treatment of *Rhizobium* combined with salicylic acid (B), combined treatment of *Rhizobium* with abscisic acid (D) treated with both SA and ABA with PGPR (E).

2.2.5. Moisture content

Soil samples were taken at a uniform depth of 6 inches from the soil surface and its moisture content was determined by applying given formula (Valarmathi et al., 2019):

Soil moisture (%) = Weight of wet soil (g)-Weight of dry soil (g)/Weight of dry soil (g) × 100

2.2.6. Plant fresh, dry biomass and plant height

Fresh weight of seedling were measured. The seedlings were dried in an oven at 90 °C till a constant weight was obtained. Plant height was measured from the base of the stem to the apex. Six biological replicates were made.

2.2.7. Stomatal conductance

Stomatal conductance evaluates the rate of gas exchange (carbon dioxide uptake) and transpiration (water loss) though the stomata of leaf. It is ascertained *via* degree of stomatal aperture. Measurements were taken at 11:00 am. Stomatal conductance of three different leaves from each plant with three biological replicates was measured by a Porometer (AP-4, Delta T-Devices, Cambridge UK).

2.2.8. Stomatal Index

Leaves were randomly taken from the upper part of plant to remove the mesophyll. The adaxial surface of leaves were peeled off and stomata were observed under a light microscope (Leica DM1000, Meiji infinity 1, Canada) at 20x. The total number of stomata and other epidermal cells in the area of 1mm^2 were counted. Stomatal Index (SI) was calculated (Ogaya et al., 2011).

SI (%) = (No. of Stomata / No. of Stomata + No. of Epidermal cells) x 100

2.2.9. Canopy temperature

To measure leaf temperature, an infrared thermal camera (calibrated) was used. Pots with plants were moved to the middle of the table, one day prior to the measurements. Infrared thermal snaps were taken such that plants were not moved from their position. Results regarding the change in temperature were calculated by FLIR Tools software, Version 5.2.

2.2.10. Relative water content (RWC) of leaves

Relative water content of leaves was measured at two time points after the periods of induction of water stress, following the method of Garcı´a-Mata and Lamattina (2001). Relative water content was calculated by the formula:

$$\text{Relative Water Content (RWC \%)} = \frac{\text{Fresh weight (FW)} - \text{Dry weight (DW)}}{\text{Turgid weight (TW)} - \text{Dry weight (DW)}} \times 100$$

Fresh weight (FW) was measured for each time point of drought period. The dry weight (DW) was acquired after desiccating the samples at 90 °C for at least 72h. Turgor weight (TW) was find out by subjecting leaves to rehydration for 24h after drought treatments (Garcı´a-Mata and Lamattina, 2001).

2.2.11. Chlorophyll Content

Chlorophyll content of pea leaves were calculated using chlorophyll meter (SPAD, Minolta). The different areas of a single leaf was measured (Koshy et al., 2018), and the biological replicates were used to determine chlorophyll content.

2.2.12. Chlorophyll fluorescence (PS II efficiency)

Chlorophyll fluorescence was estimated using a portable Chlorophyll Fluorimeter (MINI-PAM, Portable Chlorophyll Flourometer, Walz-Germany) after 10 min of dark adaptation. Chlorophyll fluorescence was quantified by the Fv/Fm ratio, which represented the maximum quantum yield of photosystem II. It was calculated as Fv/Fm = (Fm – Fo) / Fm, where Fm and Fo are maximal and minimal fluorescence of dark adopted leaves respectively and Fv is variable fluorescence (Jifon and Syvertsen, 2003).

2.2.13. Plant nutrient analysis

For plant nutrient determination, acid digestion was carried out. For this purpose stock solution of HNO_3:$HClO_4$ in 3:1 ratio was prepared. Plant shoot (1g) material was ground and transferred in flask having 8 ml of digestion mixture which was kept for overnight in acid. Afterwards, the flasks were placed on the hot plat and the plant material was digested until brown fumes turned to white fumes. After few minutes, 40 ml distilled water was added. The samples were filtered through Whatman No. 42 filter paper and collected filtrates were used for the determination of minerals (Na, Mg, Ca, Mn, Fe, Zn and K) using atomic absorption spectrophotometer (AAS, Varian, GTA 120-AA240FS).

2.2.14. Statistical analysis

The data was evaluated statistically using analysis of variance (ANOVA) technique for all performed attributes via completely randomized plots design. The comparison between the mean values of treatments were made by Least Significant Difference (LSD) to test significant differences at $P \leq 0.05$ using Statistix 8.1 (Gomez and Gomez, 1984). The data were graphically represented on Microsoft excel 2013.

2.3. Results

2.3.1. Moisture content

The drought was induced at 59% soil moisture even at this stage, the rhizosphere soil of ABA treated plants retained higher moisture content at short term stress (TP_1), but at long term stress (TP_2) the ABA treatment (A) though having higher percentage of soil moisture than other treatments but the moisture content dropped down to 42%. The indication of drought resulted in significance decrease in the moisture content of rhizosphere soil. The percent decrease was linear with the duration of drought stress (Table 2.1). A significant decrease in moisture content occurred in treatment S (SA), whereas a slight decrease was observed in treatment R (*Rhizobium pisi*) and treatment E (combined *Rhizobium*, ABA and SA) has no significant effects compared to control (C). Noteworthy, the least decrease was observed in treatment A (ABA) over C at TP_1. However, at TP_2 the decrease in moisture was non-significantly higher over C.

Table 2.1. Soil moisture content (%) after sowing

Treatments	0 d	5 d	10 d	15 d	20 d Induction of drought	TP1 d (after 4 days)	T.P2 d (after 8 days)
C	65 ± 0	64.91 ± 0.66	61.5 ± 0.39	64.41 ± 0.47	59 ± 0.79	49.16 ± 1.71	40 ± 0
R	65 ± 0	62.74 ± 0.58	62 ± 0.34	63.16 ± 0.69	59.16 ± 0.48	48.33 ± 1.72	40.1 ± 0.18
S	65 ± 0	63.33 ± 0.63	60.67 ± 0.45	60 ± 0.45	59.5 ± 0.49	46.33 ± 1.87	40 ± 0
A	65 ± 0	62 ± 0.51	61.83 ± 0.41	67.08 ± 0.6	65.16 ± 0.8	54.83 ± 1.24	42 ± 0.36
B	65 ± 0	61.33 ± 0.66	59.5 ± 0.46	61.83 ± 0.56	59.33 ± 0.88	47.5 ± 1.12	40.2 ± 0.17
D	65 ± 0	61.91 ± 0.5	60.5 ± 0.35	60 ± 0.59	59.92 ± 0.99	46.66 ± 1.42	39 ± 0.2
E	65 ± 0	64.83 ± 0.66	60.5 ± 0.49	59.66 ± 0.7	57.16 ± 0.96	49.83 ± 1.19	39.6 ± 0.35

Seedling moisture content under stressed condition. Effect of different treatments on plant moisture content (values are the mean from six biological replicates mean ± SE (n=6) in days (d), Control with stress (C); *Rhizobium pisi* with stress (R); salicylic acid (SA) with stress (S); abscisic acid (ABA) with stress (A); *Rhizobium pisi* along with salicylic acid under stress (B); *Rhizobium pisi* with abscisic acid under stress (D); *Rhizobium pisi* with both PGRs (SA and ABA) under stress (E). Irrigated data is not shown because the moisture content was maintained at 65% for both time points under unstressed condition.

2.3.2. Plant fresh and dry biomass

Under unstressed condition fresh weight of the plant was not affected significantly at TP_1 or TP_2 except treatment B (inoculation of *Rhizobium* with SA), treatment A (ABA) and treatment E (*Rhizobium* combined with SA and ABA) which showed 43% significant increase in fresh biomass at TP_1 and 20% decrease in fresh weight at TP_2 whereas no significant effects were visible in treatments as compared to C (Figure 2.3). Under drought stress at TP_1 except treatments D (*Rhizobium* with ABA) and E (combined treatment with *Rhizobium*, ABA and SA) which differ non-significantly, all the treatments showed increase over the C. The maximum increase was due to R > A > S > at TP_1 and TP_2.

Under unstressed condition the dry weight of the plants at TP_1 was significantly higher in R (*Rhizobium* alone), S (SA alone), B (*Rhizobium* combined with SA) treatments (Figure 2.4). Whereas, treatments A (ABA alone), D (*Rhizobium* combined with ABA) and E *(Rhizobium* combined with SA and ABA) have no significant effect when compared with the C. Drought stress enhanced the dry biomass (15% to 16%) at TP_1 in treatments R (*Rhizobium* alone), S (SA alone) and B (*Rhizobium* with SA). While, treatments A (ABA alone), D (*Rhizobium* combined with ABA) and E (*Rhizobium* combined with SA and ABA) showed significant reduction over C (control). Significant increases of dry biomass were depicted in treatments, R, S and B (*Rhizobium* with SA) over C. Though, significant decreases were observed in A, D and E treatments at TP_2.

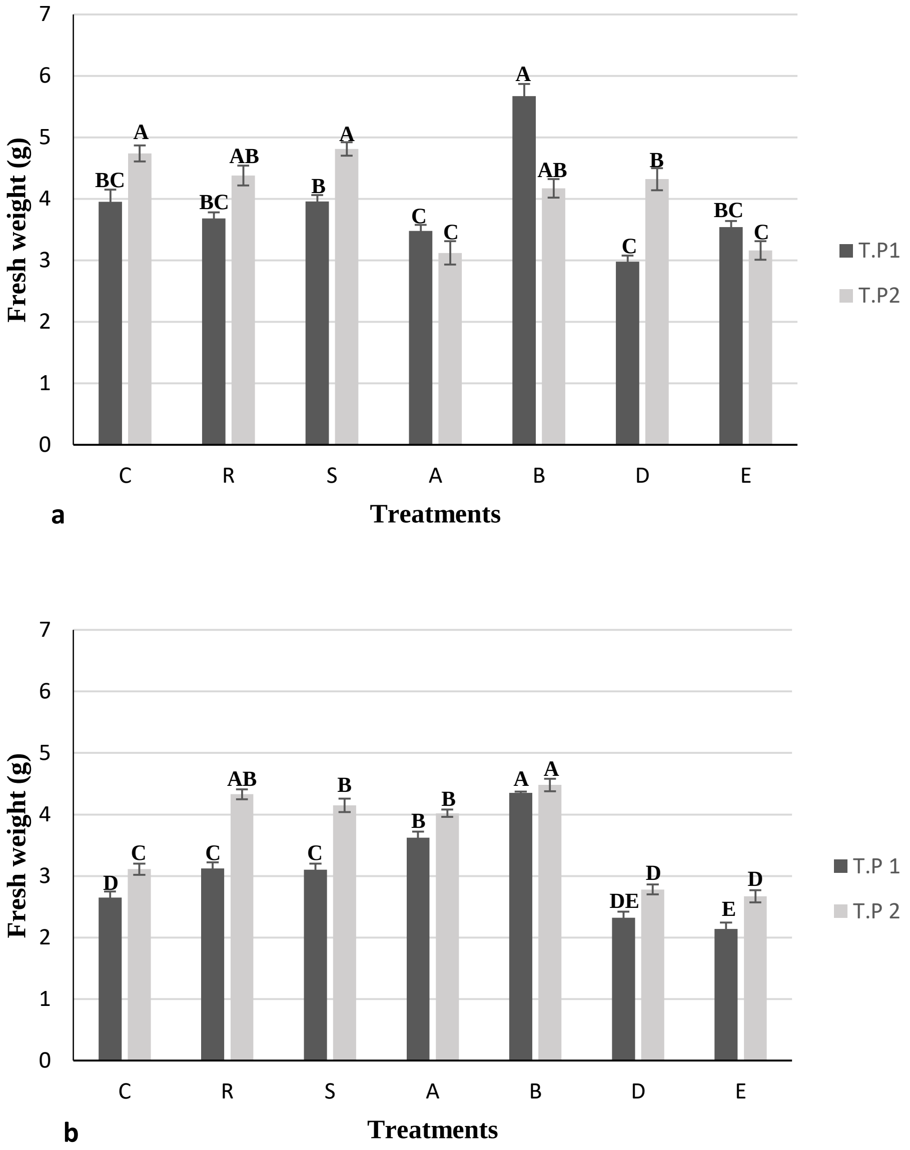


Figure 2.3. Effect of different treatments on seedling fresh biomass (values are the mean from six biological replicates (mean ± SE (n=6), **a:** Seedling fresh biomass under un-stressed condition; **b**: Seedling fresh biomass under drought stressed condition. **Untreated drought stressed Control (C)**; *Rhizobium pisi* (R); salicylic acid (SA) (S); abscisic acid (ABA) (A); *Rhizobium pisi* along with salicylic acid (B); *Rhizobium pisi* with abscisic acid (D); *Rhizobium pisi* with both PGRs (SA and ABA) (E)

Uppercase alphabetic letters heading the bars exhibited significant differences within treatments, LSD significance difference test at $P \leq 0.05$). Time point 1= induction of 4 days of drought (TP1), Time point 2= induction of 8 days of drought stress (T.P$_2$).

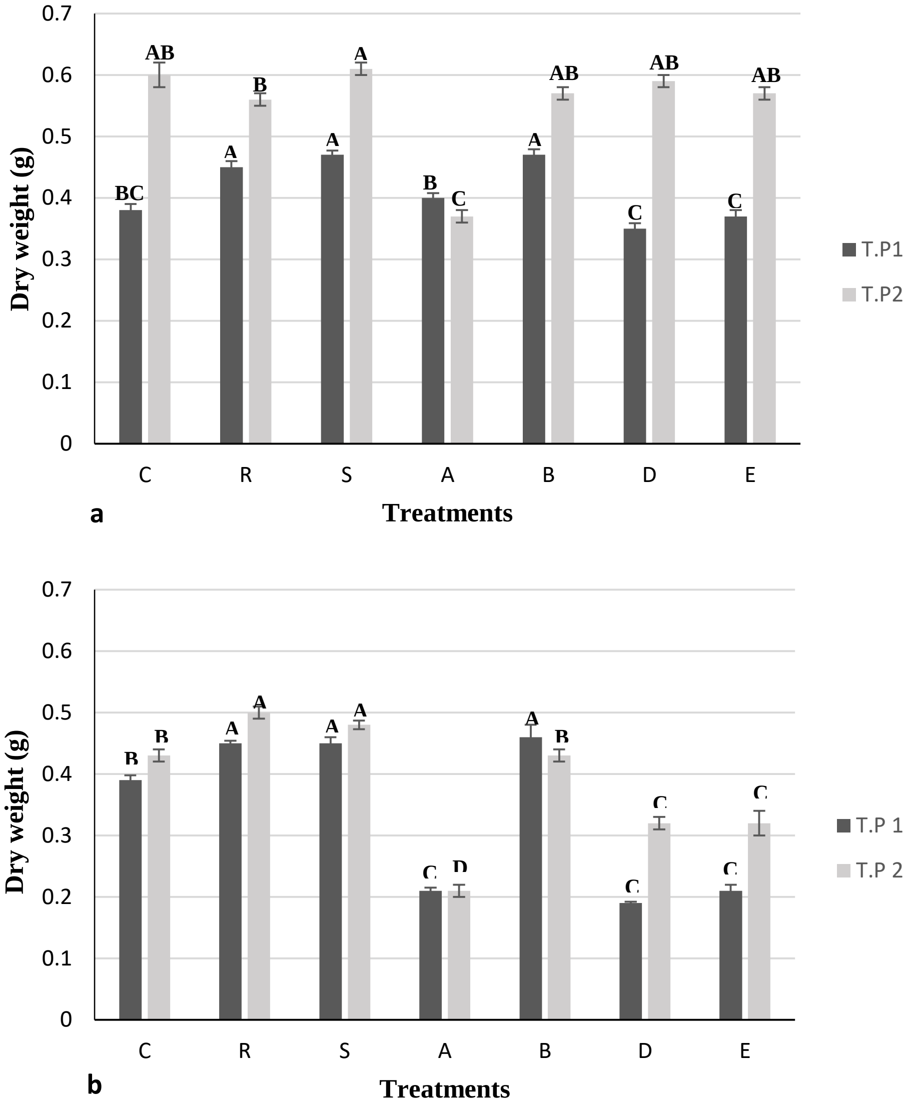


Figure 2.4. Effects of different treatments on seedling dry biomass (values are the mean from six biological replicates (mean ± SE (n=6), **a:** Seedling dry biomass under un-stressed condition; **b**: Seedling dry biomass under drought stressed condition. **Untreated drought stressed Control (C)**; *Rhizobium pisi* (R); salicylic acid (SA) (S); abscisic acid (ABA) (A); *Rhizobium pisi* along with salicylic acid (B); *Rhizobium pisi* with abscisic acid (D); *Rhizobium pisi* with both PGRs (SA and ABA) (E)

Uppercase alphabetic letters heading the bars exhibited significant differences within treatments, LSD significance difference test at *P* ≤ 0.05). Time point 1= induction of 4 days of drought (TP1), Time point 2= induction of 8 days of drought stress ($T.P_2$).

2.3.3. Plant Height

At TP_1 under unstressed condition the height of the plants was not significantly affected in treatments R, S and B, whereas, treatments A and D showed decreases in comparison to C. At TP_2, R showed significant increase whereas A and E showed decreases over C.

Induction of drought stress indicated a significant increase in plant height in R > S treatments over control at TP_1 (Figure 2.5). At TP_2 maximum increase in height was observed in treatment R (*Rhizobium*). But, the treatments S, B, and D displayed no significant difference over control. Though, A, and E treatments showed decreases over C.

2.3.4. Stomatal conductance (SC)

Under unstressed condition the treatments showed significant increases in treatment B, A, S and D over C. Treatment R displayed decrease in stomatal conductance at TP_1 and treatment E has no significant effect (Figure 2.6). At TP_2 the treatments S, A, B and E showed significantly higher SC over C. whereas, treatment R showed decrease and D has no significant effect at TP_2.

Under drought stress R and S have no significant effect whereas, A, B, D and E showed increases over control at TP_1. The maximum increase was due to A> D over C. At TP_2 all the treatments showed significant increases whereas B had no significant effect.

2.3.5. Stomatal Index (SI)

Under unstressed condition at TP_1 treatments showed significant decreases in stomatal index (Figure 2.7). At TP_2 the SI was not effected significantly in treatments A, D and E all other treatments showed significant decreases over C. Under drought stress there was no significant difference in SI in the treatments over C except treatment B but at TP_2 the SI value was similar to C in all the treatments.

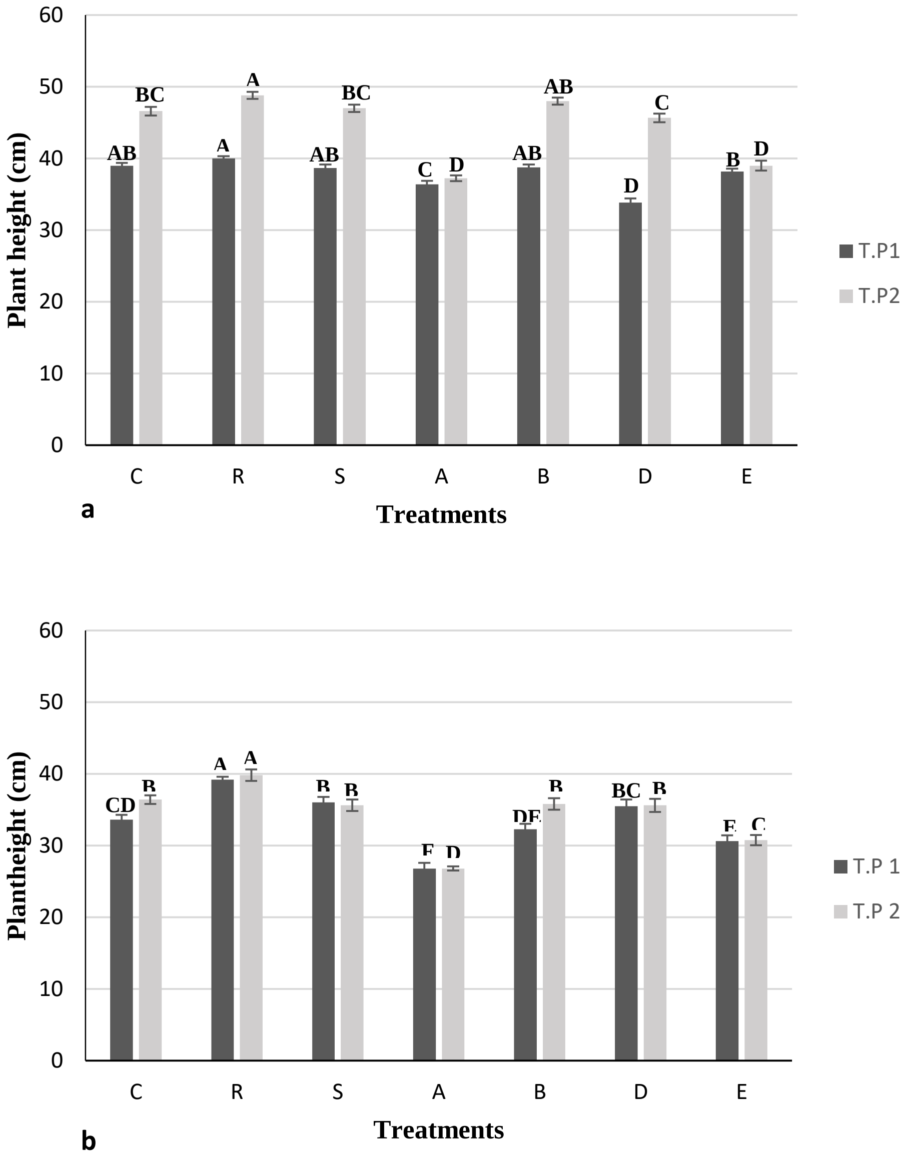
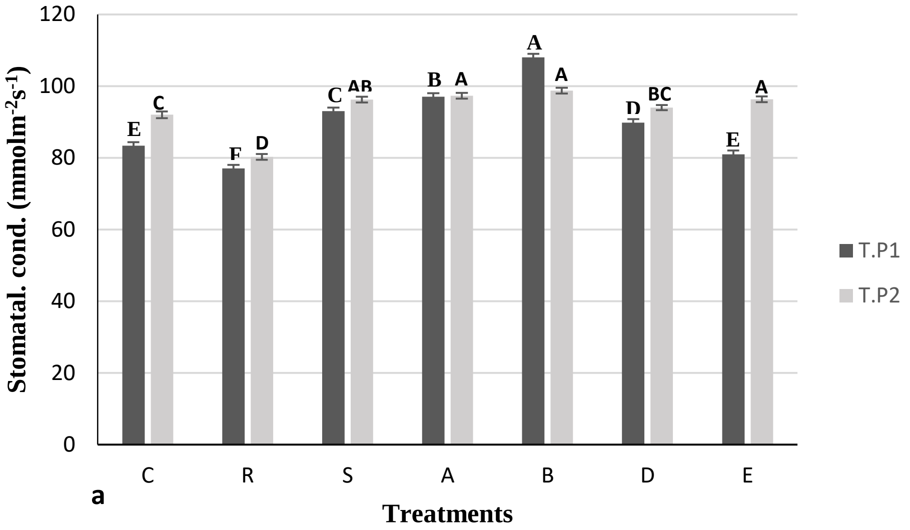


Figure 2.5. Effect of different treatments on Seedling height (values are the mean from six biological replicates (mean ± SE (n=6), **a:** Seedling height under un-stressed condition; **b**: Seedling height under drought stressed condition. **Untreated drought stressed Control (C)**; *Rhizobium pisi* (R); salicylic acid (SA) (S); abscisic acid (ABA) (A); *Rhizobium pisi* along with salicylic acid (B); *Rhizobium pisi* with abscisic acid (D); *Rhizobium pisi* with both PGRs (SA and ABA) (E)

Uppercase alphabetic letters heading the bars exhibited significant differences within treatments, LSD significance difference test at $P \leq 0.05$). Time point 1= induction of 4 days of drought (TP1), Time point 2= induction of 8 days of drought stress (T.P$_2$).



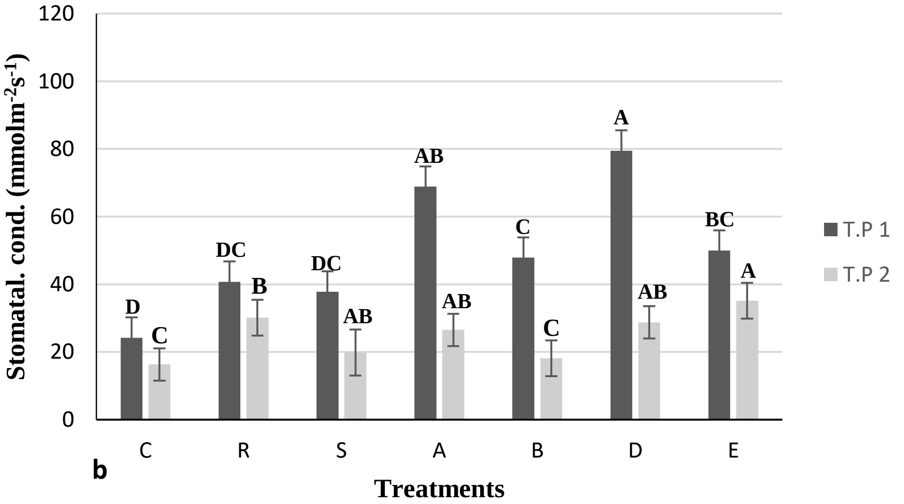


Figure 2.6. Effect of different treatments on stomatal conductance (values are the mean from six biological replicates (mean ± SE (n=6), **a:** Stomatal conductance under un-stressed condition; **b**: Stomatal conductance under drought stressed condition. **Untreated drought stressed Control (C)**; *Rhizobium pisi* (R); salicylic acid (SA) (S); abscisic acid (ABA) (A); *Rhizobium pisi* along with salicylic acid (B); *Rhizobium pisi* with abscisic acid (D); *Rhizobium pisi* with both PGRs (SA and ABA) (E)

Uppercase alphabetic letters heading the bars exhibited significant differences within treatments, LSD significance difference test at $P \leq 0.05$). Time point 1= induction of 4 days of drought (TP1), Time point 2= induction of 8 days of drought stress (T.P$_2$).

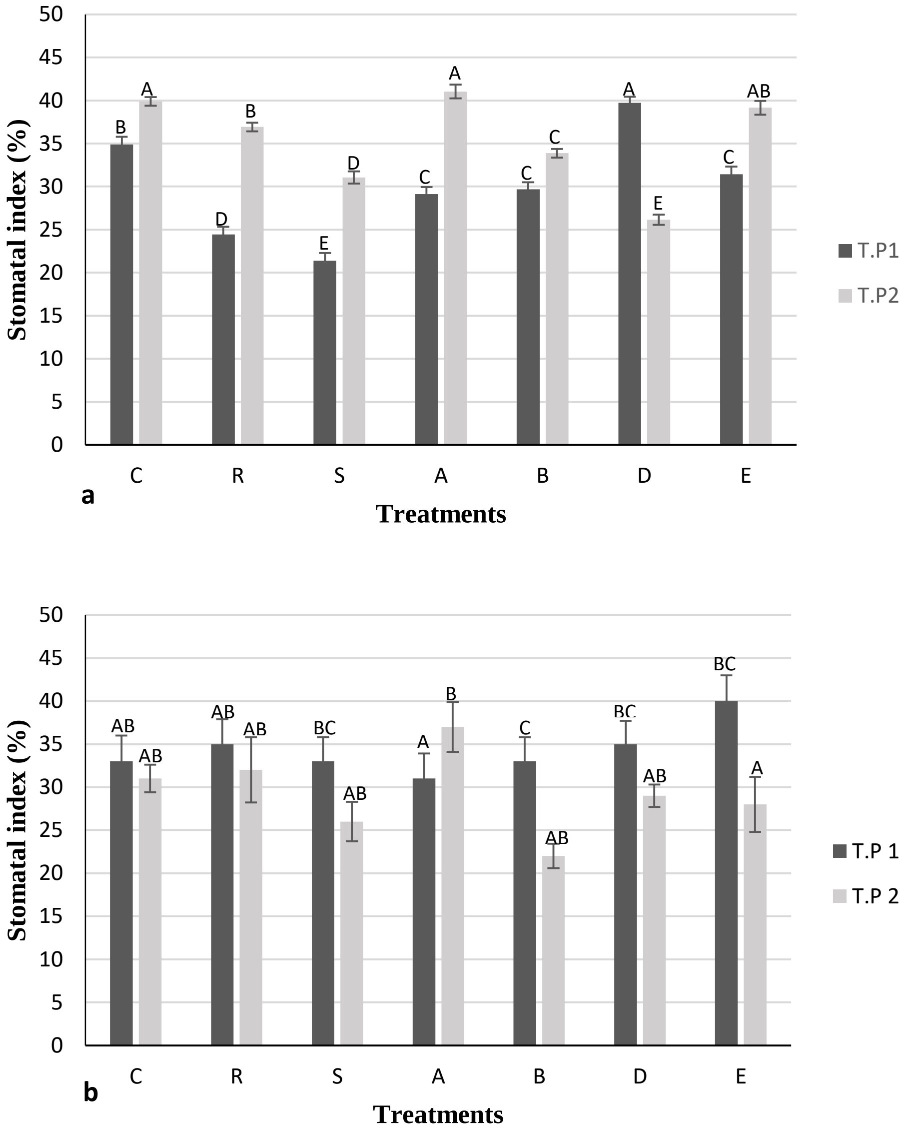


Figure 2.7. Effect of different treatments on stomatal index (SI) (values are the mean from six biological replicates (mean ± SE (n=6), **a:** Stomatal index (SI) under un-stressed condition; **b**: Stomatal index (SI) under drought stressed condition. **Untreated drought stressed Control (C)**; *Rhizobium pisi* (R); salicylic acid (SA) (S); abscisic acid (ABA) (A); *Rhizobium pisi* along with salicylic acid (B); *Rhizobium pisi* with abscisic acid (D); *Rhizobium pisi* with both PGRs (SA and ABA) (E)

Uppercase alphabetic letters heading the bars exhibited significant differences within treatments, LSD significance difference test at $P \leq 0.05$). Time point 1= induction of 4 days of drought (TP1), Time point 2= induction of 8 days of drought stress (T.P$_2$).

2.3.6. Canopy temperature

Under unstressed condition, the results revealed a decrease in canopy temperature in treatments A, B, D and E over C at TP_1 (Figure 2.8). At TP_2 treatments E showed significant increase in canopy temperature over C (control), all other treatments showed no significant decreases over C (control). The maximum decrease in canopy temperature was in treatment A (ABA) at both TP_1 and TP_2 except treatment S which had no significant effect over C.

Under drought stress, at TP_1 all the treatments showed increases over C (Figure 6). The maximum increase 3% over C was due to treatment D. At TP_2, except treatment A and treatment D which showed no significant affects in canopy temperature. There were slight decreases in canopy temperature maximum decrease in canopy temperature was noticed in treatment R.

2.3.7. Relative water content (RWC)

Under unstressed condition, treatments A, D and S showed decrease in RWC, other treatments have no significant effect compared to C at TP_1 (Figure 2.9). At TP_2 reassesses occurred in all the treatments, maximum was due to treatment E.

On induction of drought stress at TP_1, the RWC was decreased in all the treatments S, R, A, B, D compared to C (Figure 7). The maximum decrease 30 % was due to treatment E over C. At $T.P_2$ all the treatments increased the RWC significantly over control, 91 % was in treatments S > B.

2.3.8. Chlorophyll content

The results showed no significant effects of treatments on chlorophyll content either at TP_1 or TP_2 over C (Figure 2.10). Under drought stress also treatments have no significant effect over C at TP_1 and at TP_2 (Figure 8). The chlorophyll content decreased under drought stress.

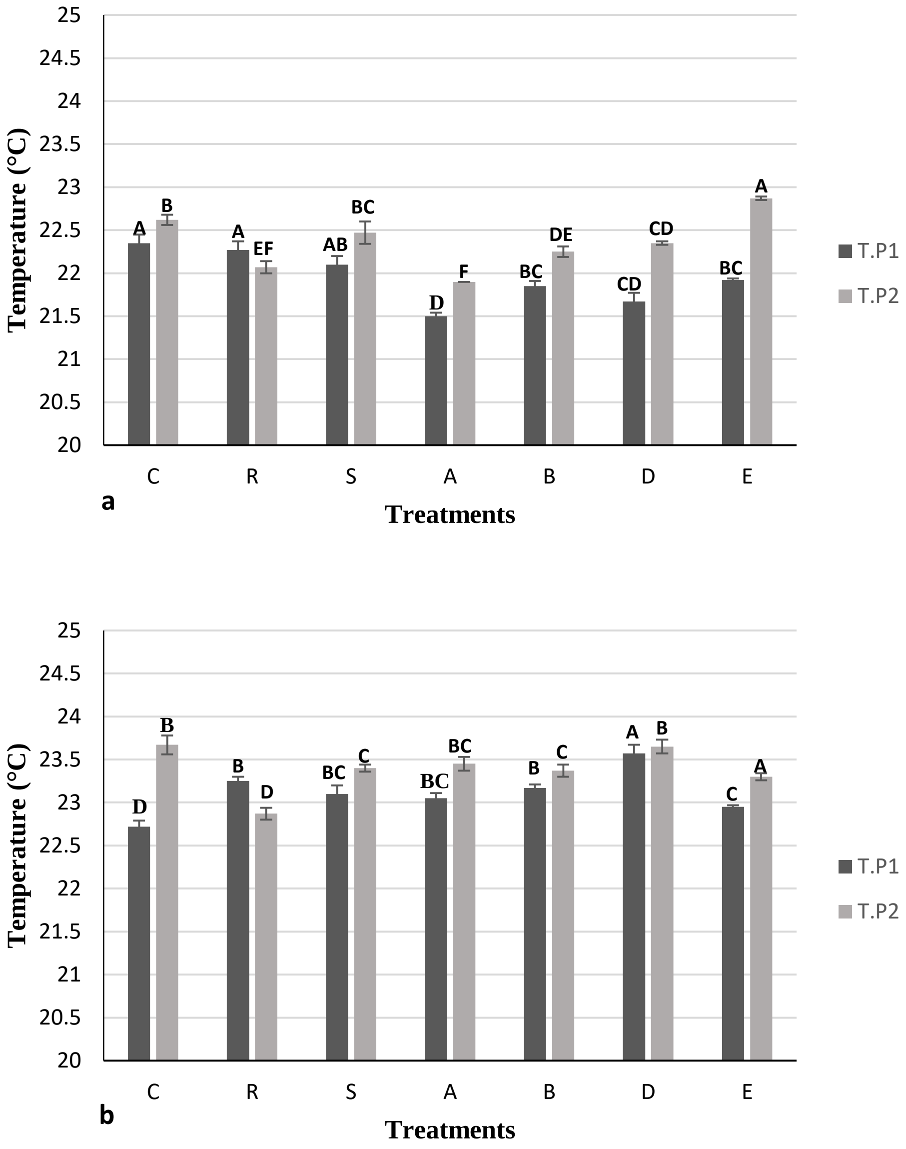


Figure 2.8. Effect of different treatments on canopy temperature (values are the mean from six biological replicates (mean ± SE (n=6), **a:** Canopy temperature under un-stressed condition; **b**: Canopy temperature under drought stressed condition. **Untreated drought stressed Control (C)**; *Rhizobium pisi* (R); salicylic acid (SA) (S); abscisic acid (ABA) (A); *Rhizobium pisi* along with salicylic acid (B); *Rhizobium pisi* with abscisic acid (D); *Rhizobium pisi* with both PGRs (SA and ABA) (E)

Uppercase alphabetic letters heading the bars exhibited significant differences within treatments, LSD significance difference test at $P \leq 0.05$). Time point 1= induction of 4 days of drought (TP1), Time point 2= induction of 8 days of drought stress (T.P$_2$).

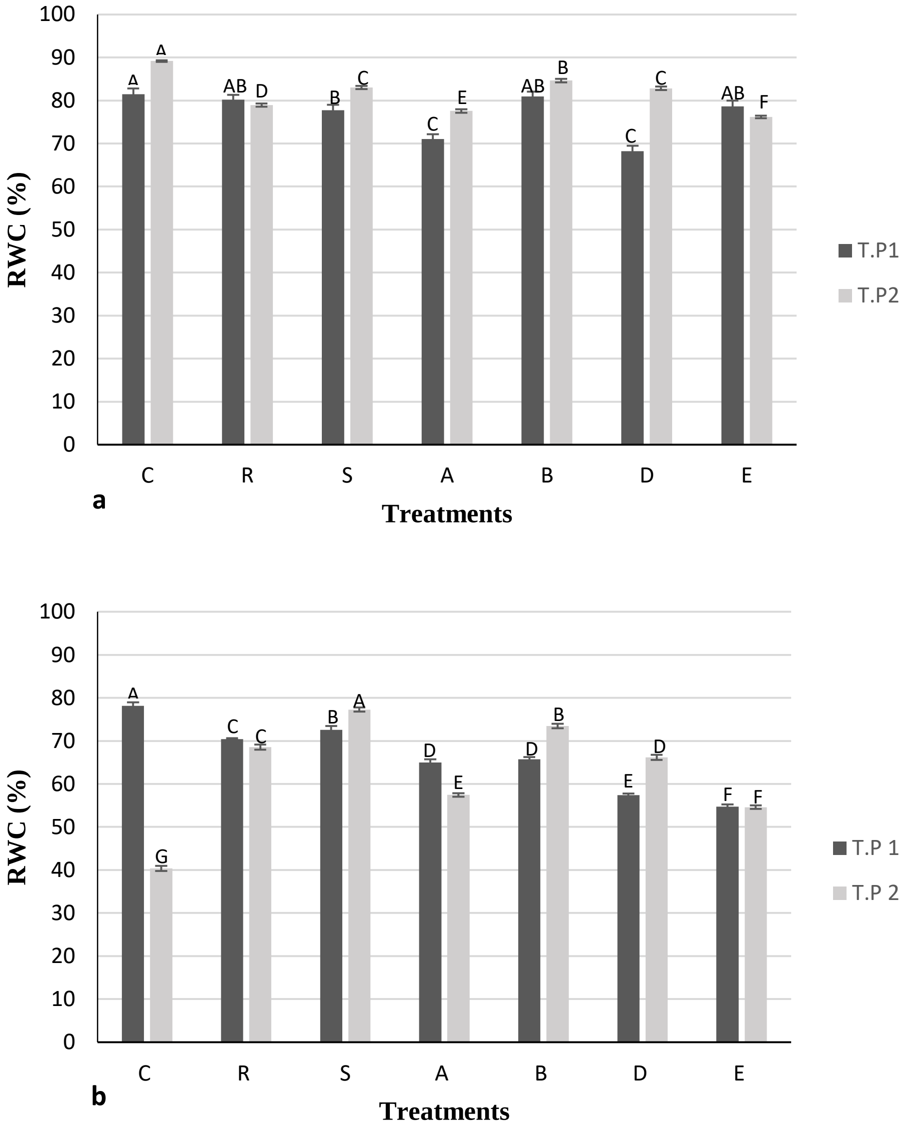


Figure 2.9. Effect of different treatments on relative water content (RWC) (values are the mean from six biological replicates (mean ± SE (n=6), **a:** Relative water content under un-stressed condition; **b**: Relative water content under drought stressed condition. **Untreated drought stressed Control (C)**; *Rhizobium pisi* (R); salicylic acid (SA) (S); abscisic acid (ABA) (A); *Rhizobium pisi* along with salicylic acid (B); *Rhizobium pisi* with abscisic acid (D); *Rhizobium pisi* with both PGRs (SA and ABA) (E)

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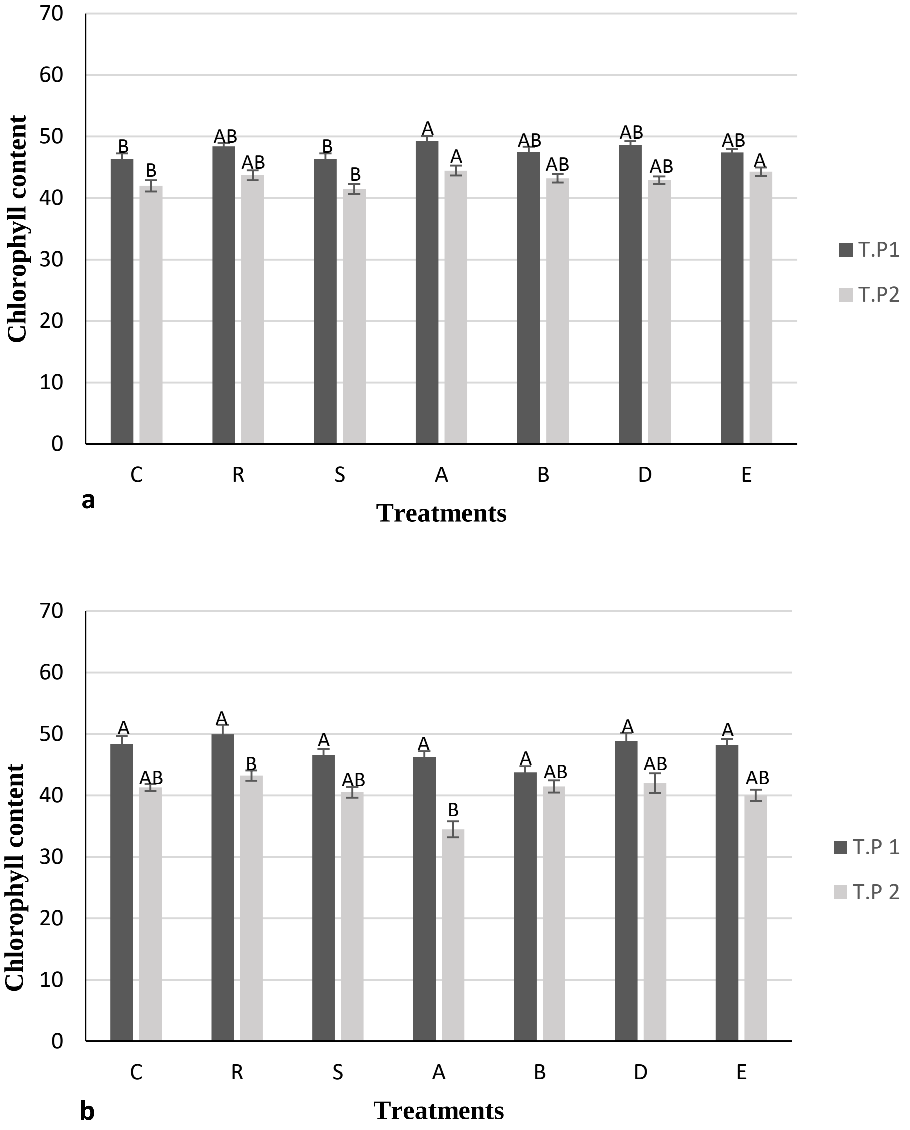

Figure 2.10. Effect of different treatments on chlorophyll content (values are the mean from six biological replicates (mean ± SE (n=6), **a:** Chlorophyll content under un-stressed condition; **b**: Chlorophyll content under drought stressed condition. **Untreated drought stressed Control (C)**; *Rhizobium pisi* (R); salicylic acid (SA) (S); abscisic acid (ABA) (A); *Rhizobium pisi* along with salicylic acid (B); *Rhizobium pisi* with abscisic acid (D); *Rhizobium pisi* with both PGRs (SA and ABA) (E)

Uppercase alphabetic letters heading the bars exhibited significant differences within treatments, LSD significance difference test at $P \leq 0.05$). Time point 1= induction of 4 days of drought (TP1), Time point 2= induction of 8 days of drought stress (T.P_2).

2.3.9. Chlorophyll fluorescence (PS II efficiency)

Under unstressed condition, no significant increase was recorded in treatments R, A and E over control at TP_1 (Figure 2.11). But, at TP_2 the treatments A, and B effectively increased Fv/Fm over C.

On induction of drought stress at TP_1 no significant effect of treatments was observed in the Fv/Fm over C but, treatments S, B, D and E showed significant increases in Fv/Fm over C. The maximum increase was due to treatment E.

2.3.11. Nutrient content of seedlings

Table 2 revealed that, under unstressed condition, the sodium (Na) content was increased due to *Rhizobium* inoculation and salicylic acid (SA) treatment. The maximum increase (605%) was due to SA treatment. ABA has no significant effect. The combined treatments of *Rhizobium* with SA or ABA or SA+ABA decreased the Na content as compared to control (C). Drought stress exhibited significant increase in the Na content. The maximum (1620%) increase was due to the combined treatment of the PGR (SA+ABA) with *Rhizobium*.

Under unstressed condition the **potassium** (K) content was observed higher in SA, ABA, *Rhizobium* + SA and *Rhizobium* + SA + ABA treatments. The maximum increase (205%) in the uptake of K was in SA over control. Drought stress demonstrated significant increase in the K uptake in all treatments. The maximum (184%) was in *Rhizobium* + SA + ABA treatment.

All the treatments demonstrated increase in Mg uptake. The maximum increase (24278%) was due to ABA treatment over control. The drought stress had significant increase in *Rhizobium* + SA (1406%) while the least increase in uptake (27%) was observed in *Rhizobium* inoculation over unstressed condition.

Calcium (Ca^{2+}) content was found maximum in all of *Rhizobium*, PGRs (ABA, SA) alone and combined treatments (B, D and E). The maximum increase (268%) was observed in treatment of *Rhizobium* inoculation with SA +ABA. Despite the fact, *Rhizobium* + SA treatment had no effect in the nutrient uptake. When compared with the drought, results divulged significant increase of 283% in the Ca^{2+} content uptake in ABA treatment but the decrease of 1% was noticed in the *Rhizobium* treatment.

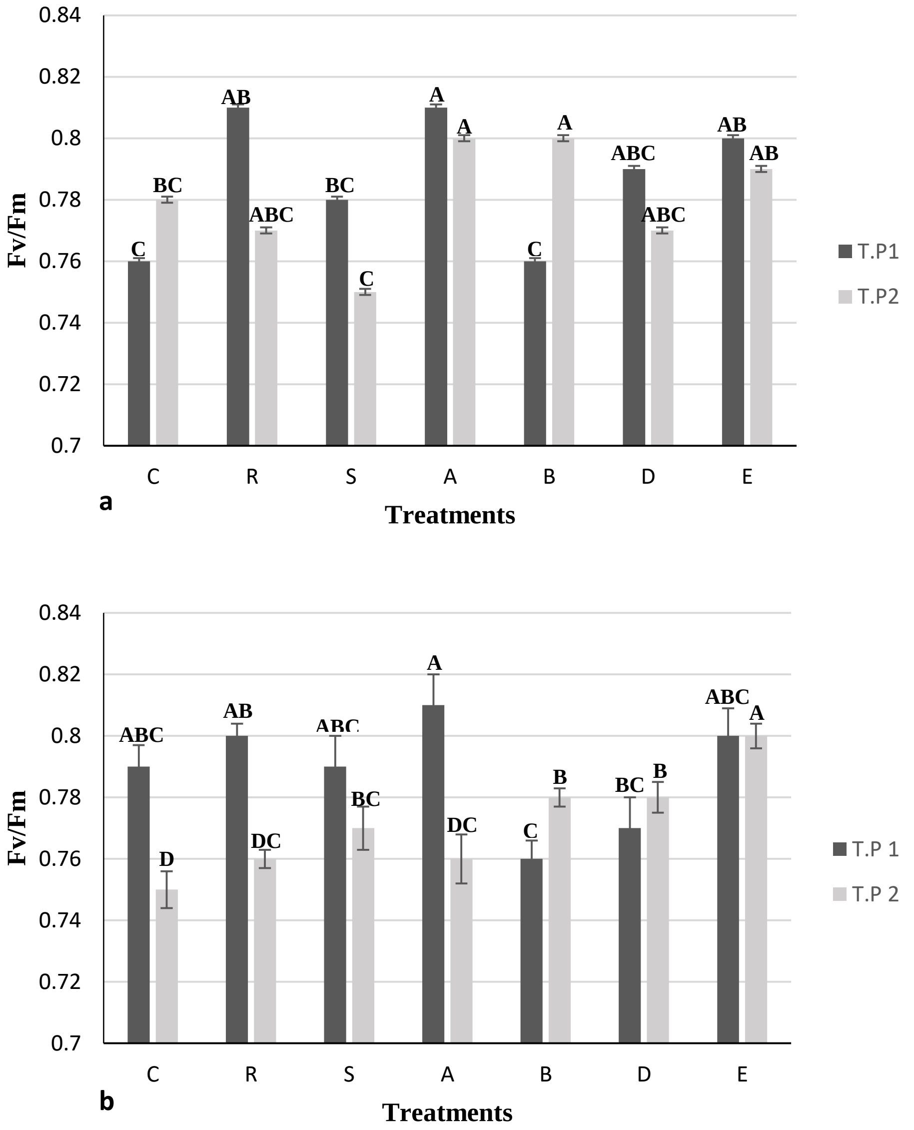

Figure 2.11. Effect of different treatments on photosynthetic efficiency (PSII) (values are the mean from six biological replicates (mean ± SE (n=6), **a:** Photosynthetic efficiency (PS II) under un-stressed condition; **b**: Photosynthetic efficiency (PS II) under drought stressed condition. **Untreated drought stressed Control (C)**; *Rhizobium pisi* (R); salicylic acid (SA) (S); abscisic acid (ABA) (A); *Rhizobium pisi* along with salicylic acid (B); *Rhizobium pisi* with abscisic acid (D); *Rhizobium pisi* with both PGRs (SA and ABA) (E)

Uppercase alphabetic letters heading the bars exhibited significant differences within treatments, LSD significance difference test at $P \leq 0.05$). Time point 1= induction of 4 days of drought (TP1), Time point 2= induction of 8 days of drought stress ($T.P_2$).

Iron (Fe) exhibited significant increase in uptake in SA alone treatment. Where, *Rhizobium* inoculation, *Rhizobium* + SA and ABA alone had least Fe uptake content.

Zinc (Zn) was increased under unstressed condition in SA (38%) and ABA (26%) treatments while the combined treatment *Rhizobium* + SA showed 3% increase in Zn content uptake over control. under unstressed condition Mn accumulation was increased by all the treatments SA > ABA > B > E > D > R over control. The maximum was (633%) with minimum uptake of (55%).

In comparison to unstressed condition, the drought stressed seedlings demonstrated a significant decrease of Zn content in all the treatments of *Rhizobium*, ABA and combine treatments; *Rhizobium* + ABA, and *Rhizobium* + SA + ABA. Though, exception to the SA and combine treatment of *Rhizobium* + SA where the content was significantly increase (84 and 33%) over unstressed condition. Drought induced increase in Mn accumulation in *Rhizobium*, ABA alone, *Rhizobium* + ABA and *Rhizobium* + SA + ABA treatment. Moreover, the minimum increase in uptake was recorded in combined *Rhizobium* + SA treatment that was 23% over the unstressed condition.

Table 2.2. Effects of macro and micro nutrients (mg/L) of seedlings under drought stress

		Macronutrients				Micronutrients		
		Na (mg/L)	**K (mg/L)**	**Mg (mg/L)**	**Ca (mg/L)**	**Fe (mg/L)**	**Zn (mg/L)**	**Mn (mg/L)**
Un-stressed	**C**	0.619 b ± 0.05	0.365 b ± 0.05	0.0014 d ± 0.000	18.34 e ± 0.58	0.226 b ± 0.056	0.069 b ± 0.000	0.009 c ± 0.000
	R	0.751 b ± 0.05	0.475 ab ± 0.05	0.0303 cd ± 0.006	22.46 c ± 0.62	0.205 b ± 0.054	0.035 c ± 0.004	0.014 c ± 0.003
	S	4.366 a ± 0.6	1.114 a ± 0.57	0.1475 bc ± 0.049	20.89 cd ± 0.56	0.469 a ± 0.051	0.095 a ± 0.020	0.066 a ± 0.004
	A	0.658 b ± 0.06	0.789 ab ± 0.05	0.3413 a ± 0.051	19.56 de ± 0.62	0.188 b ± 0.057	0.087 ab ± 0.005	0.044 b ± 0.003
	B	0.525 b ± 0.04	0.841 ab ± 0.05	0.1579 b ± 0.057	18.66 e ± 0.55	0.205 b ± 0.054	0.071 ab ± 0.005	0.04 b ± 0.004
	D	0.359 b ± 0.06	0.355 b ± 0.05	0.0479 bcd ± 0.029	24.83 b ± 0.08	0.243 b ± 0.026	0.067 b ± 0.004	0.021 c ± 0.004
	E	0.492 b ± 0.2	0.761 ab ± 0.05	0.0909 bcd ± 0.051	67.56 a ± 0.57	0.284 b ± 0.008	0.012 c ± 0.009	0.033 b ± 0.003
Drought stress	**C**	0.4204 c ± 0.05	0.3809 d ± 0.03	0.0215 d ± 0.00	14.493 e ± 0.37	0.209 c ± 0.02	0.033 b ± 0.002	0.013 b ± 0.003
	R	1.2023 b ± 0.12	0.6927 bc ± 0.05	0.0274 d ± 0.00	14.259 e ± 0.72	2.183 a ± 0.12	0.027 b ± 0.003	0.156 a ± 0.026
	S	0.6494 bc ± 0.05	0.7789 b ± 0.06	0.1195 c ± 0.05	15.419 de ± 0.63	0.245 c ± 0.05	0.061 b ± 0.005	0.025 b ± 0.002
	A	0.8914 bc ± 0.05	0.8195 b ± 0.05	0.2162 b ± 0.01	55.526 b ± 0.28	0.105 c ± 0.03	0.03 b ± 0.001	0.03 b ± 0.005
	B	0.5223 c ± 0.05	0.8095 b ± 0.06	0.324 a ± 0.02	16.615 d ± 0.31	0.073 c ± 0.01	0.044 b ± 0.002	0.016 b ± 0.002
	D	0.6623 bc ± 0.04	0.5608 c ± 0.06	0.0592 cd ± 0.00	19.524 c ± 0.27	0.332 c ± 0.05	0.032 b ± 0.001	0.044 b ± 0.004
	E	7.1531 a ± 0.55	1.0827 a ± 0.05	0.1016 c ± 0.00	66.552 a ± 0.60	1.465 b ± 0.31	0.231 a ± 0.028	0.03 b ± 0.001

Seedling nutrient content under stressed and unstressed condition. Effect of different treatments on seedling nutrient content (values are the mean from three biological replicates mean (n=3), Control (C); *Rhizobium pisi* (R); salicylic acid (SA) (S); abscisic acid (ABA) (A); *Rhizobium pisi* along with salicylic acid (B); *Rhizobium pisi* with abscisic acid (D); *Rhizobium pisi* with both PGRs (SA and ABA) (E). Lowercase alphabetic letters presented significant differences within treatments, LSD significance difference test at $P \leq 0.05$). The treatment means are with ±S.E of three replicates.

2.4. Discussion

The result revealed a distinct role of *Rhizobium* under drought stress which supercedes ABA in maintaining the water budget of the plant as evidenced by the RWC and fresh weight of the seedlings greater than the drought stressed treatment. Even under unstressed condition 15 days after sowing, the ABA treatment and *Rhizobium* inoculation maintained higher soil moisture content which demonstrates their ability in minimizing water loss in ABA treatment and hence the turgidly was better than the drought stress C (Yang et al., 2016; Staudinger et al., 2016; Ruggiero et al., 2017; Hussain et al., 2018). The maximum retention of soil moisture in ABA (A) treatment at TP1 may be attributed to the ABA enhanced WUE (water use efficiency) of the plant which reduces the rate of transpiration by closing the stomata (Saradadevi et al., 2017). Earlier studies validated the similar role of ABA (Aroca et al., 2006; Ngumbi and Kloepper, 2016) and *Rhizobium* (Figueiredo et al., 2008; Grover et al., 2011) on retention of soil moisture and water use efficiency. Noteworthy, the *Rhizobium* assistance to ABA at TP2 for improving RWC of leaves is demonstrated.

2.4.1. Fresh and dry weight and height of seedlings

Results demonstrated that *Rhizobium* is responsible for maintaining the turgidity of the plant in a much better way than ABA alone (Figure 1). On the imposition of drought stress ABA not only attenuated the inhibitory effect of drought stress but also significantly increased the fresh weight over the C at TP_2. ABA acts as an inhibitory hormone under unstressed condition, but induce tolerance to drought stress by minimizing water loss. The maximum increase in the fresh weight of seedlings under drought stress was due to *Rhizobium* inoculation; SA, when used in combination with *Rhizobium* further, augmented the fresh weight over the C under drought stress. *Rhizobium* with ABA (D) or *Rhizobium* with ABA and SA (E) showed significant decreases in fresh weight under drought stress at both time points. Fresh weight is associated with water and nutrient uptake. This suggests that R action was suppressed by the ABA and the SA was unable to alleviate this inhibition (Miura and Tada 2014).

Notably, ABA showed maximum inhibition in dry weight at both time points which may be attributed to ABA inhibition of cell division and cell differentiation. Previous studies revealed similar role of ABA (Aroca et al., 2006; Ngumbi and Kloepper, 2016) and *Rhizobium* (Figueiredo et al., 2008; Grover et al., 2011) on fresh biomass of seedlings which may be attributed to ABA-induced inhibition in the cell division and cell elongation (Melcher et al., 2010; Takatsuka and Umeda, 2014). Furthermore, the dry weight was significantly decreased in ABA treatments under stress even at TP_1 (Duan et al., 2007; Dhashnamurthi et al., 2013). The reduction in dry biomass demonstrates the growth inhibitory role of ABA. But under long term stress for 8d at TP2, ABA assisted the seedlings to withstand stress. The D and E treatments i.e. combined treatment of *Rhizobium* and *Rhizobium*, SA and ABA showed dry weight higher than ABA demonstrating the *Rhizobium* ability in the production of biomass, by augmenting cell division (Cohen et al., 2009).

The observed higher increase in the plant height in *Rhizobium* (R) or SA (S) treatment could be ascribed to *Rhizobium*-induced phytohormone production (Nagata and Suzuki, 2014; Fahad et al., 2015; Subramanium et al., 2015). ABA induced decrease in cell division may result in the observed reduction in plant height (Melcher et al., 2010; Ferguson and Mathesius, 2014).

2.4.2. Stomatal conductance and stomatal index

It was observed that water supply lead to considerably higher transpiration rate, stomatal conductance, and net-photosynthesis, (deSouza et al., 2005; Mafakheri et al., 2010). The ABA alone (A) and with *Rhizobium* (D) increased stomatal conductance at short term drought (TP_1). But, the value did not significantly differ at longer-term (TP_2) compared with *Rhizobium* treatment. The maintenance of higher RWC (%) of R treatment relative to ABA having similar stomatal index suggests the efficiency of treatment R at TP_2 for maintaining the water budget of plant under drought stress.

The studies evaluated canopy temperature emulation as a function of soil water status (Webber et al., 2015). The canopy temperature is a useful character utilized by breeders to choose lines tolerant to environmental stresses (Pino et al., 2010; Pinto and Reynolds, 2015). The canopy cooling appears to be associated with deeper roots in dry soils and substantial root biomass (Pino et al., 2010; Pinto and Reynolds, 2015). *Rhizobium* decreased the canopy temperature, possibly due to higher stomatal conductance and a hence higher rate of transpiration. The combination of ABA with R was unable to decrease the canopy temperature. This is evidenced by the observed decrease in RWC of the leaves of ABA treatment compared with S > R > B treatments under drought stress. Nevertheless, the combined treatments of R with ABA and SA or R with ABA have resulted in maximum Fv/Fm photosynthetic efficiency compared with other treatments.

2.4.3. Relative water content (RWC)

Leaf relative water content (RWC) is a significant criterion of water status in plants. It intends the equilibrium between water supply to the leaf tissue and transpiration rate (Lugojan and Ciulca, 2011). ABA treatment experiencing drought stress exhibited significantly higher RWC at TP_2. ABA has stomatal conductance much higher than the C facilitating the gaseous exchange. A significant increase (70%) in RWC was noticed in *Rhizobium pisi* treatment. Exogenous application of SA significantly improved the relative water content of the leaves under drought-stressed conditions (Hayat et al., 2010; Verma et al., 2017; Ahmad et al., 2017). The role of rhizobia is pronounced in maintaining water balance in leaves, nutrient balance and hormonal adjustment under drought stress (Naveed et al., 2015). The exogenous application of SA significantly increased the RWC under drought stress, hence maintained the turgidity of leaves (Shan and Wang, 2017; Sharma et al., 2018). Results depict that *Rhizobium* was more efficient in reducing the rate of transpiration as compared to ABA (Govindasamy et al., 2017; Fahad et al., 2017).

As the stomatal conductance at TP_2 under drought stress was reduced the dry weight of ABA treated plants were also reduced and the value was even lower than the C (Duan et al., 2007; Dhashnamurthi et al., 2013). Different strategies were adapted by *Rhizobium* which showed a significant increase in stomatal conductance over C at TP_2. However, it also showed higher RWC concomitant with the significant increase in fresh and dry weight at TP_2. Similar pattern of response was exhibited by SA.

2.4.4. Photosynthetic efficiency and chlorophyll content

The photosynthetic efficiency was significantly higher at TP_2 in treatments E > D > B > S demonstrating the synergistic role of *Rhizobium* with ABA and ABA and SA in augmenting photosynthetic efficiency under long term (TP2) drought stress.

2.4.5. Nutrient content

The availability of nutrients are primary factor for plant growth and productivity. Plants deficient in micronutrients may become prone to abiotic stresses and diseases (Ashraf et al., 2012). In the present study, R, SA, and ABA in various combinations were found more effective to reduce the Na accumulation under unstressed conditions while augmenting K accumulation. Drought stress though decreased the Na more than the K but the treatments with R, SA and ABA increased both Na + K. Notable increase was recorded in the combined treatment of R with ABA and SA. The R appears to assist both ABA and SA to augment Na + K accumulation (Grover et al., 2014; Sahin et al., 2015). K is a macronutrient intricate in the cellular turgor maintenance, movement of numerous enzymes (Locascio et al., 2019), regulation of opening and closing of stomata (Schroeder, 2003; Hurst et al., 2004) facilitates protein and starch synthesis and neutralizes organic and inorganic anions and macromolecules (Nieves-Cordones et al., 2016). Whereas, Na participates in the carbon cycle, chlorophyll synthesis and photosystem II activity (Quintero et al., 2011). R being more effective under drought stress to increase Na accumulation while SA was effective both under unstressed and drought stressed conditions to enhance K accumulation.

Calcium (Ca^{2+}) is the fifth most abundant element by mass in the earth's crust**.** Ca^{2+} is the secondary messenger (Bender and Snedden, 2013). Unstressed condition, Ca^{2+} accumulation was maximum in the combined treatment of *Rhizobium* + SA + ABA **(**Vivas et al., 2003; Han and Lee, 2005). Same was true under the drought stress the role of *Rhizobium* and ABA being more pronounced (Bano and Fatima, 2009; Mouradi et al., 2016). The increased uptake of Na, K, Mg, and Ca^{2+} were previously studied in tomato **(***Solanum lycopersicum* L.) and lettuce using PGPR under unstressed well-watered condition (Yang et al., 2009; Ullah et al., 2016). Drought significantly decreased plant growth and biomass (Rodriguez et al., 2004).

Magnesium (Mg) is a divalent cation (da Silva et al., 2011). The key function of Mg is a central atom of the chlorophyll molecule. Also involved in conversion and conservation of energy **(**Amtmann and Blatt 2009**)**, phosphorylation, de-phosphorylation, hydrolysis of a compound, structural stabilizer for nucleotide and protein synthesis **(**Merhaut, 2007). The drought increased the Mg accumulation over unstressed plants. ABA was again more effective both alone as well as under drought stress with *Rhizobium* to augment Mg accumulation over control. It was found that increased level of Ca^{2+} uptake by the foliar spray of ABA on banana plantlets, the drought stress induced remarkably increased concentration of Mg uptake in the plant leaf (Mahouachi, 2009).

Zinc (Zn) is the most deficient micronutrient in the soil (Imtiaz et al., 2010). It's application either as a soil amendment or foliar is recommended. The Zn, Mn, Ca^{2+}, and Na increased under well-watered normal seedlings due to *Rhizobium* and *Rhizobium* + SA + ABA treatment. However, drought stress reduces the nutrient content in all treatment with exemption to *Rhizobium* + SA + ABA treatment. Where the content was significantly high. The *Rhizobium* + SA + ABA treatment not only overcome the drought induced decrease in Zn, Mn and Fe but also enhanced the accumulation in leaves. Magnesium not only play an important role in plant development but also have a key function in human body. The biodegradable Mg and its alloys have acquired much consideration in regard to metabolic functions and especially in bone tissues (Chen et al., 2019). Magnesium has the maximum capacity of maintaining leaf nutrients under drought stress in chickpea as compared to pea, barely and oat (Neugschwandtner et al., 2015). Several studies on peanut, mung beans, chickpea and other legumes supported the evidence (O'Rourke et al., 2007; Imtiaz et al., 2010). SA was more effective for Zn and Mn accumulation whereas *Rhizobium* was more stimulatory for Fe accumulation.

Iron (Fe) is an pivotal micronutrient for several key processes; electron carrier, nitrogenase complex in legume plants (Rashid, 2005), haem synthesis, and nodule formation. The present study revealed SA and combined treatment of R with SA and ABA are more effective to enhance the uptake of Fe (Zhao et al., 2019; Movahhedi-Dehnavi et al., 2019; Barickman et al., 2019).

Manganese (Mn) aids in enzymatic activities. SA, ABA, PGPR and combined treatments of R with ABA and SA effectively enhanced Mn uptake under un-stressed and drought stress conditions as were discussed in earlier studies in sustainable agriculture (Khosdgoftarmanesh et al., 2007; Khosdgoftarmanesh et al., 2010). Conversely, R treatment significantly improves the accumulation under drought stress. The increased nutrient uptake for K, Mg, Ca^{2+}, and Mn^{2+} was observed in seedlings under drought conditions (Hu et al., 2008). The results demonstrated R alone or in combination with PGRs under drought stress conditions could attenuate the inhibition of plant growth.

Conclusion. It is inferred that *Rhizobium* inoculation may be more effective than that of ABA. The role of *Rhizobium* to mitigate drought stress supercedes that of SA and ABA but the combined treatment of *Rhizobium*, SA and R was found most efficient at TP_2 to ameliorate the inhibitory effects of drought stress on plant water status and photosynthetic efficiency. *Rhizobium* assisted ABA and SA in the induction of drought tolerance.

Chapter No. 3

Differential Expression analysis of Calmodulin (*CaM*) gene in Pea (*Pisum sativum* L.) seedlings under drought stress response

INTRODUCTION

3. Introduction to calmodulin (*CaM*) in plants

Plants are subjected to several environmental stresses which negatively affected the developmental processes by demarcating their genetic potential. To cope with such circumstances plants have gradually develop multiple processes to adapt to these environmental stresses (Zou et al., 2010; Wang et al., 2011). The calcium ion (Ca^{2+}) is considered as a well-established universal second messenger and is among one of the most primitive mechanisms that respond to abiotic and biotic stresses (Yang and Poovaiah, 2003; Hetherington and Brownlee, 2004; Ali et al., 2017). A number of extracellular stimuli elicit modifications in the cellular concentration of Ca^{2+} in plants (Reddy, 2001; Reddy et al., 2002a; Kudla et al., 2010). In higher plants, there are three major families of Ca^{2+} sensor and have been recognized that comprises; calmodulin (CaMs), CaM-like proteins (CMLs), Ca^{2+}-dependent protein kinases (CDPKs) and the Calcineurin B-like proteins (CBLs) (Luan et al., 2002; Reddy et al., 2002b; Bouche et al., 2005; Galon et al., 2010).

3.1. Structure of calmodulin (CaM)

The calmodulin (CaM) is a small acidic and extremely conserved protein in eukaryotes. It binds Ca^{2+} ions and acronym of calcium-modulated protein. It is contemplated as one of the best studied Ca^{2+} sensors (Popescu et al., 2007; Du et al., 2011; Reddy et al., 2011b). It binds to its target through the CaM binding domain (CBD). In the majority of the CBPs (CaM binding proteins) the prime protein sequence is not preserved (Defalco et al., 2010). The CaM and the prototype of CaM structure have 149 amino acids and having two globular domains in eukaryotes. Each domain comprised of two EF-hand motifs chain through a long flexible helix (Gifford et al., 2012; Villalobo et al., 2018). These proteins, in general, mechanisms one or more orthodox helix loop helix EF-hand motifs. The deciphering of stimulus-response coupled with a series of Ca^{2+} sensor proteins or calcium-binding proteins (CBPs) during the course of Ca^{2+} signalling (Ranty et al., 2006; Kudla et al., 2010). The calcium-binding proteins (CBP) have distinct structural composition, a large population and the diverse target proteins modulated through the Ca^{2+} elicitors reveal the intricacy of Ca^{2+} signalling that aid plants to acclimatize to the changing environ.

As, gradually many genomes have been sequences, it is explicit that CaM be associated with a small gene family. There are seven CaM genes encrypt for four highly conserved isoforms that is CaM1/4, CaM2/3/5, CaM6, and CaM7, reported in *Arabidopsis thaliana* (Landoni et al., 2010). These genes mainly varies in one to five amino acid residues. The expression of six CaM (SlCaM1, SlCaM2, SlCaM3, SlCaM4, SlCaM5 and SlCaM6) genes have reported in tomato (*Solanum lycopersicum*) (Peng et al., 2014).

3.1.1. Strategies to identify CaM proteins

The activity of CaM gives consideration to regulate the function of the target protein by relating to them, as they do not have any implicit catalytic activity (Hoeflich and Ikura, 2002). The CaMBD (CaM binding domain)/motif is not conserved in the target protein. But, the basic amphipathic α-helix generated by the target peptides holds hydrophobic residues on one side whereas basic residues on the other side. The hydrophobic section of the target peptide is generally held on to the hydrophobic pocket to anchor the target peptide. This in turn results in the interaction of basic section of the target peptide with the acidic groups of CaM protein. The exceptional adaptable trait of an extensive methionine residue and linker protein confers the conformational plasticity of CaM to adapt to various target peptides (Ikura and Ames, 2006). Despite that, the dectection of CaM target which is only based on the amino acid sequence of target proteins is unclear due to the disparity in the primary structure of CaMBDs (CaM binding domains). Several strategies has been proposed to identify the CaM proteins in plants such as yeast hybrid system (Perochon et al., 2010; Rodriguez-Caban et al., 2011), screening of cDNA libraries for CBPs (i.e., CaM binding proteins) in plants (Popescu et al., 2007; Ali et al., 2003), mRNA display approach (Shen et al., 2005; Lin et al., 2010).

3.1.2 CaM binding proteins in Pea (*Pisum sativum* L.)

Calcium (Ca^{2+}) is defined as a variable signalling molecule among all recognized molecules of its active member for cellular mechanisms. In plants, Ca^{2+} transduces signals by means of a neural network/system (Trewavas, 1999). It enables plants to respond to the abiotic and biotic stimulant (Knight and Knight, 2001). Various studies illustrated the inclusion of

Ca^{2+} fluxes in germination. The expression of PsCaM1 and the Ca^{2+} flux level in pea (*Pisum sativum* L.) seeds demonstrated a lower level of expression in dry seeds, cotyledons and embryonic axis. The PsCaM1 is thought to be comprise of three isoforms (PsCaM11, PsCaM12, and PsCaM13) that differ in single substitution of first helix. Furthermore, these isoforms are trimethylated (Duval et al., 2002). Out of these three isoforms; PsCaM11, and PsCaM12 are the major transcript that are linked with the quiescent and immature seeds in pea. Whereas, PsCaM13 nearly invisible till the later germination stage where the expression of PsCaM13 begin to upregulate. The expression of CaM/isoforms of CaM have commonly delineated with the specificity of tissues in *Vicia faba* (Ling and Assmann, 1992), *Zea mays* (Faichardet et al., 1996), *Triticum aestivum* (Yanget et al., 1998), radish (Negrini et al., 1995) and *Pisum sativum* (Allan and Trewaves, 1985). It is also hypothesised that the Ca^{2+} bind proteins not only work as sequester to stabilize the concentration of these proteins within the cell but also act as a signal transducer (Chin and Means, 2000). The distinctive trait of CaM within the single species of higher plants is the expression of multiple isoforms (Snedden and Fromm, 2001). Like, CaM is ciphered by 5 genes in Soybeans (Lee et al., 1995), 8 in *Solanum tuberosum* (Takezawa et al., 1995), and 9 genes in *Arabidopsis thaliana* (Zielinski, 2002). Initially, it was believed that the CaM isoform were inferred from cDNA that were deposited in the databases. However, these databases do not provide any clue of their actual expression.

3.1.3 Role of CaM in plant-microbe interactions

Calcium spiking occurred almost immediately after the perspicacity of symbionts by the host plants which is one of the initial cellular response of symbiosis. This particular process takes place in the perinuclear region of root hair and within the nucleus (Kosuta et al., 2008). Previous researches conferred a generalized symbiotic pathway, which comprised of 8 components containing; POLLUX/DMI1, SYMRK/DMI2, NENA, NUP 85, NUP133, CASTOR, CYCLOPS/IPD3, and CCaMK. All these components are essential for the development of arbuscular mycorrhizal symbiosis (AMS) and root nodulation symbiosis (RNS) (Oldroyd 2013; Singh and Parniske, 2012). The calcium-calmodulin kinase (CCaMK) carries a structural attribute facilitating it to link Ca^{2+}/CaM with ca^{2+}. It act as a decoder of the encoded signal of calcium (Levy et al., 2004; Mitra et al., 2004). So, its kinase activity is modulated through the both Ca^{2+} and Ca^{2+}/CaM (Poovaiah et al., 2013).

3.1.4 Function of CaM in plant development under abiotic stress

A number of techniques aimed at assimilating CML function, comprising genetic analysis, identification of downstream targets, protein biochemistry, and global expression profiling. These approaches are introducing to divulge the distinctive traits among the members of this wide protein family. The member of this family revealed their roles in plant development as well as in response to biotic and abiotic stress conditions (Bender and Snedden, 2013).

Various abiotic stress responses are arbitrated by Ca^{2+} signalling. As a result, the CML is not intricated in the allied signal transduction pathways. The modification in the expression level of several CMLs has been identified in the response to abiotic stress stimulants. The CML37 and CML38 promoter activities are receptive to various different treatments such as; drought, mechanical wounding, oxidative stress and salinity (Vanderbeld and Snedden, 2007). The CML multi-stress responsive gene2 (OsMSR2) originated from rice (*Oryza sativa*) is instigated by a number of abiotic stress stimulants. The overexpression of *OsMSR2* gene in *Arbidopsis* intensified the drought sensitivity to the exogenous application of ABA and tolerance to the salinity stress (Xu et al., 2011). The *OsMSR2* gene is an ortholog of *Arabidopsis thaliana* that includes; CML37, CML38, and CML39, conferring sustenance to the postulation that CMLs function in *Arabidopsis* respond under stress conditions. The transcript analysis, and promoter of CML24 unveiled an upregulation of expression levels in response to oxidative stress, exposure to ABA, as well as high/or low temperature (Delk et al., 2005). Comparably, the CML9 transcript levels altered in cold, dehydration, and NaCl responses. The salt responsive expression of CML9 gene is reliant on both; ABA signalling and synthesis (Magnan et al., 2008). It suggests that this CML takes part in the ABA dependent stress responsive pathway. Overall, the gene expression analysis suggested that various CMLs genes are stress responsive. Henceforth, these CMLs genes will be significant to ascertain transcription factor regulatory gene expression. Furthermore, they will enable to evaluate the degree of extent to which transcriptional activity linked to alters the CMLs protein level. Irrespective of this, the expression profiling has been verified as beneficial in conducting queries marked at cognizance the roles of stress induced CMLs.

Plant growth and survival are seriously intimidating due to the drought stress that is assessed as ubiquitous abiotic stress. The plant has the potential to adjust to drought conditions by enhancing their physiological process such as; retaining water within the cell, alleviating water uptake efficacy, and modulating water loss by means of transpiration (Yang et al., 2010a). Molecular studies conferred that numerous genes respond to the drought stresses in a spatial and temporal expression pattern (Yamaguchi-shinozaki and Shinozaki, 2006). Drought stress triggers signalling cascade implying TFs (i.e., AREBs and DREBs) and protein kinase phosphatases (such as; RPK1, SRK2C, CDPKs, and ABI1) which upregulates the production of molecules and chaperones. ABA is a phytohormone that acts a significant role in abiotic stresses particularly in drought and salinity. Drought stress stimulates the synthesis of ABA. Under drought, the level of ABA increased that lead to the closure of stomata in the guard cells and instigate expression of drought. A number of ABA responsive gene promoters comprise of cis-acting elements termed as ABRE (ABA-responsive elements) (Uno et al., 2000). The transcription of ABA entails more than two ABREs coupling elements in the promoter region for appropriate execution. Several Ca^{2+} regulated genes having such elements, signifying the fact that ABA regulates these particular responsive genes by changes in the cellular Ca^{2+} (Khan et al., 2015). In addition to this, different sites mediate ABA signalling for stress inducible genes (Shinozaki and Yamaguchi-Shinozaki, 2007). The drought responses can also be mediated through ABA-dependent pathways. These pathways are arbitrated through dehydration responsive element binding proteins (DREBs) (Agarwal et al., 2006; Seki et al., 2007).

Salicylic acid (SA) has usually recognized as a defense hormone. It regulates the plant defense system against the pathogen (Fu and Dong, 2013; Nimchuk et al., 2003). The SA activation pathway is under substantial regulation through Ca^{2+}/CaM-binding transcription factors (TFs) (Wang et al., 2009; Zhang et al., 2010; Wang et al., 2011). The TFs provides a location for the Ca^{2+} signal to establish the production of SA. The transcription of EDS1 and NDR1 which are considered as two critical genes are required for the stimulation of both C-terminal genes (i.e., CC-NB-LRR and TIR-NB-LRR) and R gene activated immunities. They are adversely controlled via. CaM regulated *AtSR1/CAMTA3* genes. These genes facilitates a solid control above the coalescence of SA that provides an operational method to evade mis-activation of PTI along with ETI, So SA is crucial for both (Nimchuk et al. 2003; Fu and Dong 2013). Moreover, Ca^{2+}/CaM may possibly be responsible for both positive and negative control through WRKY7, 11, 17, and 53 (Park et al., 2005; Journot-Catalino et al. 2006; Kim et al., 2006; Murray et al., 2007; Popescu et al., 2007). Though, their regulation and the direct downstream target genes activation by CaM persistently undetermined.

After the accrumulation of SA, the defense-related gene expression also appears to be regulated via. $Ca^{2+/}$CaM-mediated signalling. The interaction of CaM-binding to TGA3 develops with a target promoter (Szymanski et al., 1996). In addition, NPR1 interact with the TGA3, which is a critical transcription co-cofactor that intricate in the perception of SA and expression of a wide-ranging defense linked genes (Fu and Dong, 2013). They provide a potential alternative to modulate the productivity of defensive responses. Moreover, it might possibly inhibit the expression of the PR gene by the action of CBNAC transcription factor (Kim et al., 2012). It is quite entrancing to understand that the Ca^{2+}/CaM gene could employ a well-coordinated regulator even at the ultimate stage of defense reactions than the later stage of induced accumulation of SA (Poovaivah et al., 2013).

Aim of the study

The aim of the study was to investigate the expression level of PsCaM1 genes in pea by inducing different treatments of PGPR (*Rhizobium pisi*) and PGRs (ABA, and SA) under the influence of drought stress and to determine whether the gene could specifically expressed at the early vegetative phase.

3.2. Materials and Methods

The methods for growth conditions, sterilization of seeds, preparation of *Rhizobium* inocula subsequent to seed inoculation, seed inoculation with salicylic acid (SA) and abscisic acid (ABA), and the induction of drought stress was similar as described in chapter 2 (materials and methods section) at page 29.

3.2.1. RNA extraction

Unstressed and drought stressed plants for each time point were harvested in morning by 11:00 am. Seedlings leaf samples for each treatment were taken in replicates (approximately 6 biological replicates). Leaf sample of 0.5g (500mg) was harvested and immediately frozen in liquid N_2 for RNA extraction. RNA was extracted according to the AXYGEN manual (AXYGEN RNA extraction kit). The concentration of RNA was quantified via. NanoDrop-ND1000 spectrophotometer (Thermo Fisher Scientific Inc.) at a 1:10 (v/v) dilution.

3.2.2. cDNA synthesis for qRT-PCR

The cDNA libraries were prepared from the extracted purified RNA samples according to the manual using Thermo Scientific kit. One microgram of RNA was used for cDNA synthesis. PCR microtubes were sterilized and used. A total volume of reaction mixture for cDNA synthesis was of 20µL.The reaction mixture for cDNA synthesis comprised of mili-Q H_20 (11 µL), 1 µg RNA sample, Oligo (dT) primer (1µL), 5X reaction buffer (4µL), dNTP (10mM each) 2µL, Ribolock (1µL) and 1µL of RevertAid-Reverse Transcriptase polymerase (200U/µl) followed by quickly flicking and spinning the microtube. The reaction mixture was prepared on ice. Samples were first incubated at 65°C for 10 min and then were placed on ice for 2 min immediately. The later incubation was at 37°C for 60 min and 70°C for 15 min respectively. The synthesized cDNA samples were stored at -20° C.

3.2.3. qRT-PCR

The expression of PsCaM1 genes under drought stress was investigated using qPCR reaction which was done with 96 well plates (MicroAmp; Applied Biosystems) covered with optical adhesive covers (Applied Biosystems). The amplification of cDNA for qRT-PCR was performed via ABsolute^{TM} Blue QPCR low ROX-Mix (ThermoFisher Scientific) in an AriaMx

Real-Time qRT-PCR system (Agilent technologies, New South Wales, Australia) following the standard protocol. The reaction mix consisted of 4 µL (0.25 µM) each of primer (Eurofin, Thermo Scientific) and 1µL of diluted cDNA (30 ng), 5 µL of ABsolute Blue mix. The template for the Real-time PCR was 1:20 dilution of the cDNA synthesized and 1µl volume of the diluted template was used in 10 µl reaction volume. A control without the cDNA template was run as a negative control. Reaction conditions were as follows: one step at 95 °C for 10 min, and 40 cycles of 95 °C for 15s denaturation and 60 °C for 1 min annealing and extension. The data was analysed via. Agilent Aria-software. The expression of genes for each of the treatments i.e., PsAB13 for ABA (A) treatment, PsPR1for SA (S) treatment and PsNIN gene for *Rhizobium* (R) was normalize to the expression of housekeeping control gene actin (Table 3.1). Primers utilized for the expression analysis of PsCaM1 gene are listed below:

Table 3.1. List of genes along with the primers, primer sequences, target organism and expected size of the product.

Genes	**Gene name**	**Primer sequence (5'-3')**	**Target organism**	**Product size**
PsCaM1-F	CaM	AAGGACACCGACTCTGAGGA	*Pisum sativum*	753
PsCaM1-R		AGCAGCAGAGATGAATCCGT		
Actin-F	Actin	TCAGCACCTTCCAGCAGATG	*Pisum sativum*	
Actin-R		TCTGTGGACAATGGATGGGC		
PsDREB2A-F	DREB	GTTGTTCTTCGGTGGCAACA	*Pisum sativum*	200
PsDREB2A-R		AGGCTCATCCATTGGCTCTT		
PsABI3-F	ABI3	GGACTCCAAGAGGGTGATTTC	*Pisum sativum*	192
PsABI3-R		ATCCACCGCATCATTTCCAG		
PsPR1-F	PR1	GCTGCTGGTTATCAGTGTGG	*Pisum sativum*	189
PsPR1-R		TGGTTGAAGCTCAACGGAAC		
PsNin-F	Nin	AGAAGCCACGAGTATCCGC	*Pisum sativum*	159
PsNin-R		ATGATCGAGTTGTGGTCGGT		

3.3. Results

3.3.1 Relative Expression of PsCaM1 genes under drought stress

The expression analysis of PsCaM1 genes were analysed through drought *DREB* gene in *Pisum sativum* under different moisture regimes of 65% (for un-stressed control), 45% (drought stressed TP_1) and 40% (drought stressed TP_2) relative humidities using real-time qRT-PCR. The expression analysis of *CaM* gene was identified in *Pisum sativum* via specific PsCaM1 markers designed from *Pisum sativum* var. Alaska available sequence in NCBI. The imposition of drought were assessed after 4d (TP_1 45%) and 8d (TP_2 40%) respectively under low and high moisture content to instigate the expression of PsCaM1 at drought and un-stressed conditions. The amplification of housekeeping gene *Actin* was used as an internal control in qPCR reactions which had been consistently expressed in *Pisum sativum*.

Calmodulin genes (PsCaM1)

Figure 3.1 illustrated the relative expression of calmodulin gene in *Pisum sativum* under the influence of PGPR (*Rhizobium*) and PGRs (SA, ABA) alone and in combine treatments by inducing drought stress. It was discerned that *Rhizobium* (R) treatment exhibited a significant increase in the expression of PsCaM1 gene under drought stress in comparison to the un-stressed control condition. Under un-stressed condition, no significant increase was observed at both time points; TP_1 and TP_2 in the respective treatment. At long term drought stress 40% MC (TP_2), significant increase of ~1.5 folds in the relative expression was noticed. Whereas, at short term (TP_1) drought stress the increase in relative expression was of ~1 fold when compared with control treatment.

Similarly, the relative expression of PSCaM1 gene in treatment ABA (A) showed an increase of ~0.8 folds at TP_2 under un-stressed condition over control. However, no effect was recorded in the expression level at TP_1. Moreover, under drought stress condition a significant increase of ~1.5 and ~0.8 folds were observed at TP_1 and TP_2 respectively when compared with control.

It was also found that SA (S) treatment alone represented an enhanced level of expression of ~ 1 fold at TP_2 under un-stressed condition. While, a decrease in the expression level was examined at TP_1 (un-stressed condition ~0.1 folds), TP_1 (short term drought ~0.2 folds) and TP_2 (long term drought ~0.1 folds) over control.

Conversely to the sole treatments, the combine treatments of *Rhizobium* with ABA (D), *Rhizobium* with SA (B) and *Rhizobium* in combination with SA and ABA (E) showed a decrease (~0.2 folds) in the relative expression of gene at both time points; TP_1 and TP_2 under un-stressed and stressed condition in comparison to control. Though, ~0.8, ~0.5 and ~0.4 fold increase was observed in treatment B, D (short term drought TP1) and E (long term drought TP2) over control.

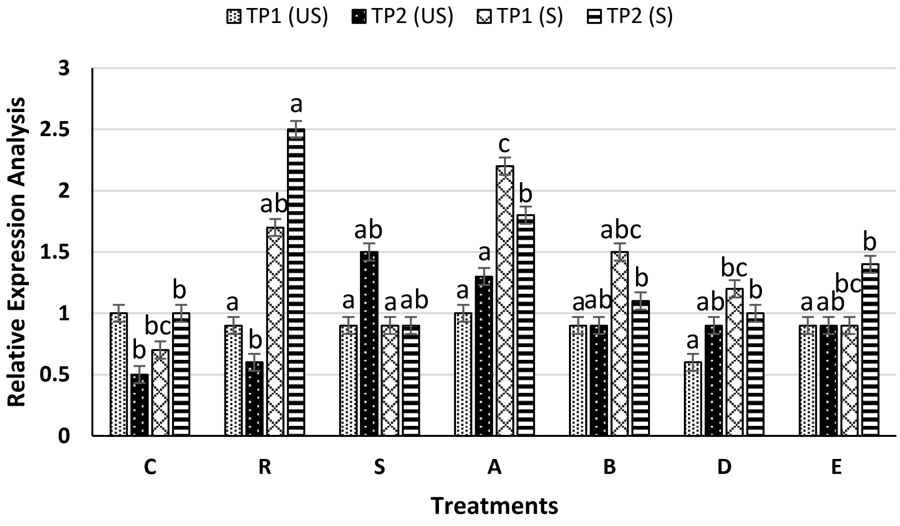

Figure 3.1. Relative expressions of Calmodulin (PsCaM1) gene of *Pisum sativum* under the effect of different treatments (values are the mean from six biological replicates (mean ± SE (n=6), Untreated drought stressed Control (C); *Rhizobium pisi* (R); salicylic acid (SA) (S); abscisic acid (ABA) (A); *Rhizobium pisi* along with salicylic acid (B); *Rhizobium pisi* with abscisic acid (D); *Rhizobium pisi* with both PGRs (SA and ABA) (E). LSD significance difference test at $P \leq 0.05$). Time point 1= induction of 4 days of drought (TP1), Time point 2= induction of 8 days of drought stress ($T.P_2$).

3.3.2 Expression of PsDREB2 genes

To specifically investigate the relative expression of Dehydration-responsive element binding protein 2 (DREB2) gene in *Pisum sativum* 28d old seedlings, specific primers for the respective gene were designed from NCBI. Results represented the highest expression level of PsDREB2 gene under the un-stressed and its expression was slightly drops down in treatments; B, E and S (60, 28, and 25% respectively) by higher/long term drought stress (Figure 3.2). Under the un-stressed condition, the expression pattern was downregulated in S, A, D and E treatments (25, 68, 75, and 81% respectively) over control at TP_1. While, *Rhizobium* treatment revealed a significant increase in the expression level of 37% and treatment B with 100% when compared with control. However, a significant decrease of 80% was observed in treatment B as compared to TP_1. All other treatments exhibited an enhanced expression pattern over control. The SA treatment under unstressed condition, slightly augmented the expression level at TP_2 but it was unable to sustain an increase in the expression under drought stress.

Furthermore, under stressed condition the expression level was equivalent to the expression pattern observed under un-stressed (well-watered seedlings) condition at TP_1. The significant least expression level was noticed in *Rhizobium* treatment which was 11% to that of control. On the other hand, the PsDREB2 expression level was evidently increased at long term drought stress (40% TP_2) than control. The intensification in the expression level was of 100% under all stress treatments, *Rhizobium* (R), ABA (A), and *Rhizobium* with ABA (D) (e.g., the approximation of 5fold increase was recorded at TP_2) with exception to SA (S) treatment which was increase about ~1.3 fold in comparison to control. Meanwhile, the expression pattern for the respective gene was significantly decrease under the drought stress condition in E and B treatments i.e., 25 and 60% respectively when compared with control.

The results demonstrated that PsDREB2 plays a pivotal role in long term drought (i.e., 40% moisture content), the expression level was ~6 folds higher in *Rhizobium*, ABA and D treatment than in control, well-watered plants and under drought stressed response of pea.

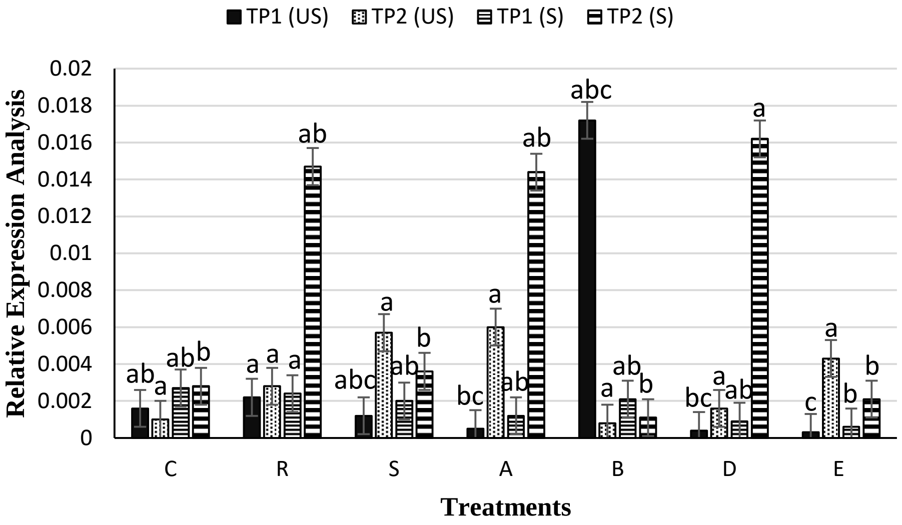

Figure 3.2. Relative expressions of Dehydration-responsive element binding protein 2 (PsDREB2) gene of *Pisum sativum* under the effect of different treatments (values are the mean from six biological replicates (mean ± SE (n=6), Untreated drought stressed Control (C); *Rhizobium pisi* (R); salicylic acid (SA) (S); abscisic acid (ABA) (A); *Rhizobium pisi* along with salicylic acid (B); *Rhizobium pisi* with abscisic acid (D); *Rhizobium pisi* with both PGRs (SA and ABA) (E). LSD significance difference test at $P \leq 0.05$). Time point 1= induction of 4 days of drought (TP1), Time point 2= induction of 8 days of drought stress (TP_2).

3.4. Discussion

Plant possess various types of calmodulin-related proteins that are unique from the other organisms. The notion of calmodulin related proteins and its isoforms are not clearly explicated so far (Reddy et al., 2011). The plant calmodulin related proteins family consists of members with varied numbers of projected EF-hand (ranges from three to six), and a few extend far off the standard length of calmodulin (Munir et al., 2016). The gene expression of Ca^{2+} sensors like the CaMs and CMLs is often instigated in response to a various abiotic stresses (Snedden and Fromm, 2001), as has been remarked for other Ca^{2+} sensors, such as CDPKs (Ludwig et al., 2004). Calcium (Ca^{2+}) has emanated as a noteworthy secondary messenger that controls the processes of hormonal and environmental indicators which are correlated with the abiotic and biotic stresses. In this study, the expression of gene encoding calmodulin (CaM) proteins in *Pisum sativum* were assessed under drought stress.

In the present study, the drought stress was induced on the different treatment of PGPR and PGRs in a way that seedlings were grown for 21 days. Later, drought stress was imposed by means of withholding the water supply. Pea seedlings were harvested for short term (TP_1 with 45% MC) and long term drought stress (TP_2 with 40% MC) at the same time of the day i.e., 11:00 AM. The reason to harvest all the samples at the same time of the day for two-time points was to avoid the potential impact of circadian clock. In accordance with the present experimental conditions, a number of researches were conducted to relate the changes that occurred in the gene expression during a period which reflects a close possible connection with the time frame. It was divulged from the studies that the time frame for the duration of stress have a visible effect on gene expression (Choi et al., 2004; Latini et al., 2007).

3.4.1 Expression of PsCaM1 in Pea seedlings

The purpose of the present work was to characterize the expression of PsCaM1 protein gene under drought stress as this Ca^{2+} sensor plays a fundamental role in the developmental and stress phenomenon possibly to be come across by plants. Most of the research work was done at PsCaM1 transcript levels which revealed that the expression of PsCaM1 is very minute in the dry seeds, amplified strikingly for the duration of early imbibition, in both axes and cotyledons (Holdsworth et al., 1999). To investigate whether PsCaM1 gene in pea perhaps implies in mediating the response to drought stress. So, the expression of the respective gene was evaluated after applying different treatments under drought stress by qRT-PCR. Results obtained from the qRT-PCR revealed that seeds inoculated with *Rhizobium pisi* have a noteworthy up-regulation in the relative expression of calmodulin (PsCaM1) gene at long term drought stress conditions (i.e., TP_2 with 40% moisture content (MC)). It was discerned that *Rhizobium* (R) treatment exhibited a significant increase of ~1.5 fold in the expression of PsCaM1 gene under drought stress in comparison to the un-stressed control well-watered condition. The up-regulation of a gene is possibly due to the PsCaM-mediated signaling activity which allows it to interact with the other transcription factor and modulate rehydration responsive genes (Abe et al., 2003; Yoo et al., 2005; Pandey et al., 2013). In the same way, it was found that ABA treatment up-regulated the relative expression pattern to ~0.8 and ~ 1.5 folds under short term (TP_1) and long term drought (TP_2) conditions singly over control and un-stressed control. Previous studies validated the present results by unfolding the potential role of ABA that is induced by abiotic stresses. Furthermore, it was stated that up-regulation of CaM gene in *Arabidopsis thaliana* (*AtCML9*) divulged a hypersensitive response to ABA in the course of germination, seedling growth phases and illustrated an alleviated tolerance to dehydration stresses (Yang and Poovaiah, 2002; Galon et al., 2010; Xu et al., 2011; Wang et al., 2015). Also, expression of various ABA-responsive gene were altered and transformed in rice to mitigate drought tolerance (Zeng et al., 2015).

The expression of CaM gene in SA (S) treatment was down-regulated in the stressed condition on both phases of drought (TP_1 and TP_2). While an enhanced expression level (~1 fold) was observed in un-stressed condition at TP_2 in comparison to control. The results of the study are not supported by the previous studies which ensued salicylic acid (SA) as an induced-defense compound and responsible for positive up-regulation of CaM (*AtCBP60g* CaM-binding TFs) gene in *Arabidopsis thaliana* in response to drought stress (Wang et al., 2009; Zhang et al., 2010; Wan et al., 2012). Despite the fact, recent researches reported that the down-regulation of CaM gene expression in SA is due to the failure of SA interaction with the CGCG box motif in the promoter region of EDS1 which suppresses the transcription factor and results in the negative regulation of SA-mediated defense plant immunity. Hence, SA has a negative influence on induced and basal biosynthesis of hormone regulated response in plants (Du et al., 2009; Ni et al., 2012; Qui et al., 2012).

The significant finding of the study was the upregulation of CaM gene in *Rhizobium* and ABA treatments under drought stress whereas down-regulation of expression in SA treatment alone. Whereas, the relative expression was positively up-regulated in B, D (TP_1), and treatment E (TP_2) under drought stress over control. The up-regulation of CaM pattern in the combined treatment is because of the synergistic effect of *Rhizobium* with plant growth regulators. It is determined from the present work that *Rhizobium pisi* has the potential to show variation in the PsCaM1 and PsDREB2 expression levels in *Pisum sativum* under drought stress. The PsCaM1 gene was induced in few treatments, while other treatments repressed in response to drought, signifying that members of the same gene families might execute different functions. Though, the expression level of PsCaM1 was slightly higher than the PsDREB2 gene. But, the entire data suggested the existence of fine regulation of the PsCaM11 gene in all treatments under long term drought stress and between the expression of the PsDREB2 gene and its downstream stress-response.

3.4.2. Expression of PsDREB2 gene in Pea seedlings

Plants have the capacity to respond to external stimuli to attain optimal plant growth and yield by discerning the external change and stabilizing the internal processes. Further, the expression of the right gene at the right time in the right tissue or cell is not the only cue to plant development and growth but as well as to the environmental responses (Reddy et al., 2011). Likewise, it was stipulated that signals sensed by cells are imparted through secondary messengers, for instance, Ca^{2+} ions, inositol polyphosphates, nitric oxide, cyclic nucleotide monophosphates, and other small molecules (Mazars et al., 2010).

The dehydration responsive element binding protein 2s (DREB2s) are the transcription factors. They act together with a cis-acting DRE (dehydration responsive element) sequence. They trigger the expression of downstream genes involve in heat shock, well-water stress reciprocation, and tolerance in *Arabidopsis thaliana* (Matsukura et al., 2009). The finding of DREB proteins and their role in abiotic stresses lead to the innovation of other homologue genes in other plants containing crops and legumes (Nayak et al., 2009). A comprehend information on the DREB1 class of transcription factor is accessible but the information on DREB2 class is confined. In the present study, the isolation of the DREB-related gene family named PsDREB2 was identified in pea (*P. sativum*). Although pea is the member of Viceae tribe and *M. truncula*ta be a member of Trifoliae tribe (Choi et al., 2004), the most apposite reported sequence of DREB2 in the model plant *Medicago trnculata* was used to design primer for the amplification of DREB2 gene in *P. sativum*.

The expression analysis of PsDREB2 results demonstrated that PsDREB2 plays a fundamental part in long term drought (i.e., 40% moisture content), the expression level was ~6 folds higher in Rhizobium, ABA and D treatment than in control, well-watered plants and under drought stressed response of pea. The present finding is supported by previous researches that at specific phase of dehydration/drought such as 40% MC, the expression level for PsDREB2 was 2 folds increased than in control, well-watered, un-stressed plants (Latini et al., 2007; Bieniawska et al., 2008; Saha and Vandemark, 2012). It was found that PGPRs have an impact on the expression of the stress responsive genes. As a result, they can regulate plant responses to stress conditions (Barnawal et al., 2017). However, the dehydration responsive element/C repeat (DRE/CT), work in conjunction with a chain of transcription factors recognized as *DREBs* proteins (DRE binding factor/C repeat binding factor; DREB/CBF). The DREBs protein induces a series of downstream dehydration responsive genes that enhance dehydration tolerance in plants (Yamaguchi-Shinozaki and Shinozaki, 2005; Jia et al., 2012; Gachomo et al., 2014).

Though the stress was applied at 21 days old pea seedlings and the period of drought was of 8 days. These conditions perhaps impeded the evaluation of expression patterns at this level to stress response. It was also observed that the expression of PsDREB2 slightly prompted when drought is applied, proposing that expression pattern changes with the drought stress periods (Liu et al., 2008). The expression of this specific gene reached a maximum of 40% MC. However, a reduced expression level was recorded at the early or short term drought stress which was at 45% MC. The down-regulation in the expression at early stages of the drought was evident in several studies, suggesting transcription factor might have a maximum role in roots at early stages that result in the reduction of expression (Agarwal et al., 2010; Lata and Prasad, 2011; Mizoi et al., 2012). ABA treatment induced a higher expression of the PsDREB2 gene. Previous studies exhibited that DREB2A is involved in the plant stress responses and a specific concentration of 100 µM ABA might induce the significant up-regulation of DREB2A gene expression (Jovanović et al., 2013). It seems like drought alone cannot lead to the upregulation of PsDREB2 in un-stress treatments until a specific drought period attained. In the meantime, the expression of PsDREB2 greatly enhanced in the treatment where seeds were inoculated with *Rhizobium*, primed with ABA, SA or in a combined effect.

Conclusion

The inoculation of *Rhizobium pisi* and priming of ABA and SA on the expression analysis of PsCaM11 under drought PsDREB2 had a positive upregulation of genes. The effect of Rhizobium was at par with ABA and showed an up-regulation of PsDREB2 under long term. The increased expression of the PsCaM11 gene in the treatments marks them as a potential candidate for stress tolerance to pea. However, the expression analysis of PsDREB2 exhibits that the gene is strikingly induced under long term drought. The combined treatments has further augmented the PsDREB2 gene. Thus, PsDREB2 could be a significant transcription factor that can be utilized for enhancing abiotic stress tolerance and for evaluating the disparity between genotypes.

Concluding Chapter

CONCLUDING CHAPTER

Plant growth promoting rhizobacteria (PGPR) are potentially active biological agents that enable plants to withstand in the extreme environment. PGPRs are considered to be effectual when applied in combination with other microorganisms or plant growth elicitors (PGRs). The results obtained during the present investigation divulged *Rhizobium pisi,* abscisic acid (ABA) and the combined treatment of *Rhizobium* with salicylic acid (SA) improved seedling biomass to mitigate drought-induced inhibition under the short period of drought.

Rhizobium pisi is the root nodulating bacteria and has a symbiotic association with the legumes. However, its association with *Pisum sativum* has not been investigated yet. Results from Pearson correlation revealed a strong correlation ($p < 0.05$) of the physiological as well as expression analysis of PsCaM1 and Ps DREB2 gene in pea seedlings induced by *Rhizobium pisi*. The best studied physiological parameters with enhanced growth were biomass and relative water content (RWC) for shorter and longer periods of drought. These results were further verified through biplots using the principle component analysis (PCA). The eigenvalues obtained from the varimax rotation loading factors (VFs) varies from ≥ 0.4-0.5 for a moderately strong correlation between the parameters.

Comparably to *Rhizobium,* ABA treatment has the potential role in tolerance against short term drought stress and was more pronounced. The treatment greatly improved the stomatal conductance (SC) by stomatal closure to reduce the water loss during drought and caused a significant reduction in the relative water content (RWC) and biomass of the seedlings. Pearson correlation delineated the strong correlation of the aforementioned physiological attributes under drought stress. The R measure strength and correlation for ABA treatment in Pearson correlation were + 0.92 which depicted a very strong positive correlation at the significance level of $p < 0.0001$. PCA analysis further validated the correlation results by having the eigenvalues of VFs 0.875 which is greater than the standard value for VFs (>0.75). However, the treatment is moderately correlated with the expression of PsCaM1 and PsDREB2 gene at short term and long term drought respectively.

The effect of combined treatments signified in *Rhizobium* + SA treatment where an ameliorated biomass, SC and RWC was higher but they were unable to sustain such effects in a longer run. However, the genes were upregulated at TP_2. Likewise, *Rhizobium* + ABA treatment exhibited the significant effects at par to *Rhizobium*. The expression of PsDREB2 augmented at long term drought (TP_2). From PCA analysis, under unstressed condition treatment D represented a high value of PsCaM1 value at TP_1. PC1 explains 29.06% of the total variance and PC2 25.50% explaining a lesser amount of variance, making it a reasonable summary measure. In addition to this, under stressed condition, principle component 1 (PC1) accounted for 39.7% of the variation, while PC2 contained 24.27% of the variation. Therefore, the plot of the PC scores (63.9%) of PC1 vs. PC2, making it a fairly good summary measure. Active observation "A" indicate a high value for PC2 and also identified a high value for SI TP_1 and CF TP_1. Strong association formed between "R" (FW TP_1 & TP_2). While "B" and "S" have low value with respect to PC2.

But, the remarkable finding of the study was the incredible enhancement of stomatal conductance (SC), chlorophyll fluorescence (CF) and stomatal index (SI) for the longer duration of drought. Furthermore, the expression of PsCaM1 and the stress responsive gene (PsDREB2) was upregulated greatly at TP_2. The correlation with the VFs was of > 0.789 and the R measures strength was 0.84 ($p < 0.0001$). In corroboration with the previous studies it was found that PGPR in combination with PGRs can formulate a consortium to maintain plant turgidity under drought stress (Seo and Park, 2010; Mittler and Blumwald, 2015; Suzuki et al., 2016; Tabassum et al., 2017; Khan et al., 2018).

Among the parameters, Pearson correlation results divulged an improved growth of seedlings, enhanced biomass production with significantly increased ($p < 0.05$) stomatal conductance under drought stress. The inoculation of seedlings with *Rhizobium* alleviated drought stress by exerting beneficial effects on plant growth and achieving nutrient availability and assimilation (Barnawal et al., 2019). The nutrient analysis depicted the augmented uptake of macro and micronutrients. Under drought stress, both Na and K were significantly increased in *Rhizobium*, *Rhizobium* + SA + ABA. The Mg content was alleviated in ABA, R + SA + ABA treatment and subsequently increased the Ca^{2+} content. Fe was significantly higher in *Rhizobium* and combined treatment R + SA + ABA. Similarly, Zn and Mn accumulation was also improved in *Rhizobium* treatment. The bacterial strain alone or in the consortium is responsible for the physiological, biochemical event in crop plants that led to ameliorate uptake of nutrients, and yield (Rêgo et al., 2014; Zhang et al., 2014; Jha and Subramanian, 2015). The stomatal conductance, stomatal index, RWC are positively correlated variables while chlorophyll content, fresh and dry biomass were somehow negatively correlated (Appendix 1).

Results from Pearson correlation for unstressed condition depicted a strong correlation between PsDREB2 to fresh weight (FW), relative water content (RWC) to plant height (PH), canopy temperature (CT) to PH, magnesium to stomatal conductance (SC) at TP_1, chlorophyll content (CC) TP_2 to CC at TP_1, CT to RWC at TP_1, PsCaM1 at TP_1 to stomatal index (SI) at TP_2, PH to FW, RWC to FW, calcium (Ca) to sodium (Na), zinc (Zn) to Na, manganese (Mn) to iron (Fe) and PsDREB2 to PsCaM1 at TP_2 (Table 4.1). However, a strong negative correlation was observed in; RWC to CF, SI to DW at TP_1, CC to FW, potassium (K) and Ca to FW, Ca to PH, Fe to SC, Zn to SC, RWC to CC and Na to RWC at TP_2 respectively. All the values were different from 0 with a significant level alpha ($\alpha = 0.05$) (Appendix 2).

Under stressed condition, Pearson correlation results delineated a strong correlation between; CC to DW, Ca to CF and SC, Fe to Na, Mn to K at TP_2, FW (TP_2) to FW (TP_1), DW (TP_2) to DW (TP_1), PH (TP_2) to PH (TP_1), SI (TP_2) to CF (TP_1), Ca (TP_2) to SI (TP_1), and PH (TP_1 and TP_2) to DW (TP_2) (Table 4.2). While, a negative correlation was recorded in CC to FW, SC to DW, PsDREB2 to SC at short term drought stress (TP_1), Mg to PH, Mg to CC, PsCaM1 to CC, Zn to Ca at long term drought stress (TP_2), SC (TP_1) to DW (TP_2), RWC (TP_1) to CF (TP_2), PsDREB2 (TP_1) to SC (TP_2), SC (TP_2) to DW (TP_1) and Zn (TP_2) to SI (TP_1) respectively (Appendix 3). The results from PCA analysis along with Pearson correlation revealed that *Rhizobium* at par to SA and ABA treatments have positive impact on the seedling at short term drought stress but with the combined treatments of *Rhizobium* + SA and *Rhizobium* + SA + ABA had significantly higher effects on growth under long term drought.

Rhizobium showed maximum upregulation of the PsCaM1 gene under stress. Though, ABA is more effective than *Rhizobium* at TP_1 but unable to sustain the upregulation of gene at long term stress. The upregulation of the PsDREB2 gene was maximum in *Rhizobium* + ABA followed by *Rhizobium* and ABA treatment alone under long term drought stress. Under the unstressed condition, SA slightly augmented PsDREB2 gene expression at TP2 but SA was unable to withstand the expression level of the respective gene under drought stress at any of the time points.

It is concluded from the data that *Rhizobium* alone or in association with SA used to mitigate drought induced inhibition on plant growth, biomass chlorophyll content, stomatal conductance, RWC, and expression level of PsCaM1. At short term drought stress, the individual treatments of *Rhizobium* and SA exhibited better growth in relation to stress responsive gene effects on pea seedlings. While at long term drought stress, *Rhizobium* assisted SA and ABA mitigate drought induced adverse effects. It is inferred that combined treatments of *Rhizobium* + SA + ABA serve to mitigate drought stress in pea (*Pisum sativum* L.) in an effective manner that the sole treatments for a longer period of stress.

Future prospects

- The study needs to be extended to have an insight into the synergistic role of AB, SA also polyamines and a range of plant growth promoting rhizobacteria (PGPR) in consortium with *Rhizobium* to elucidate the role under drought and other stresses.
- The mechanism of action for PGPR with other PGR e.g. polyamine need to be studied in detail for the sustainable crop production under drought stress.

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Appendices

APPENDIX 1

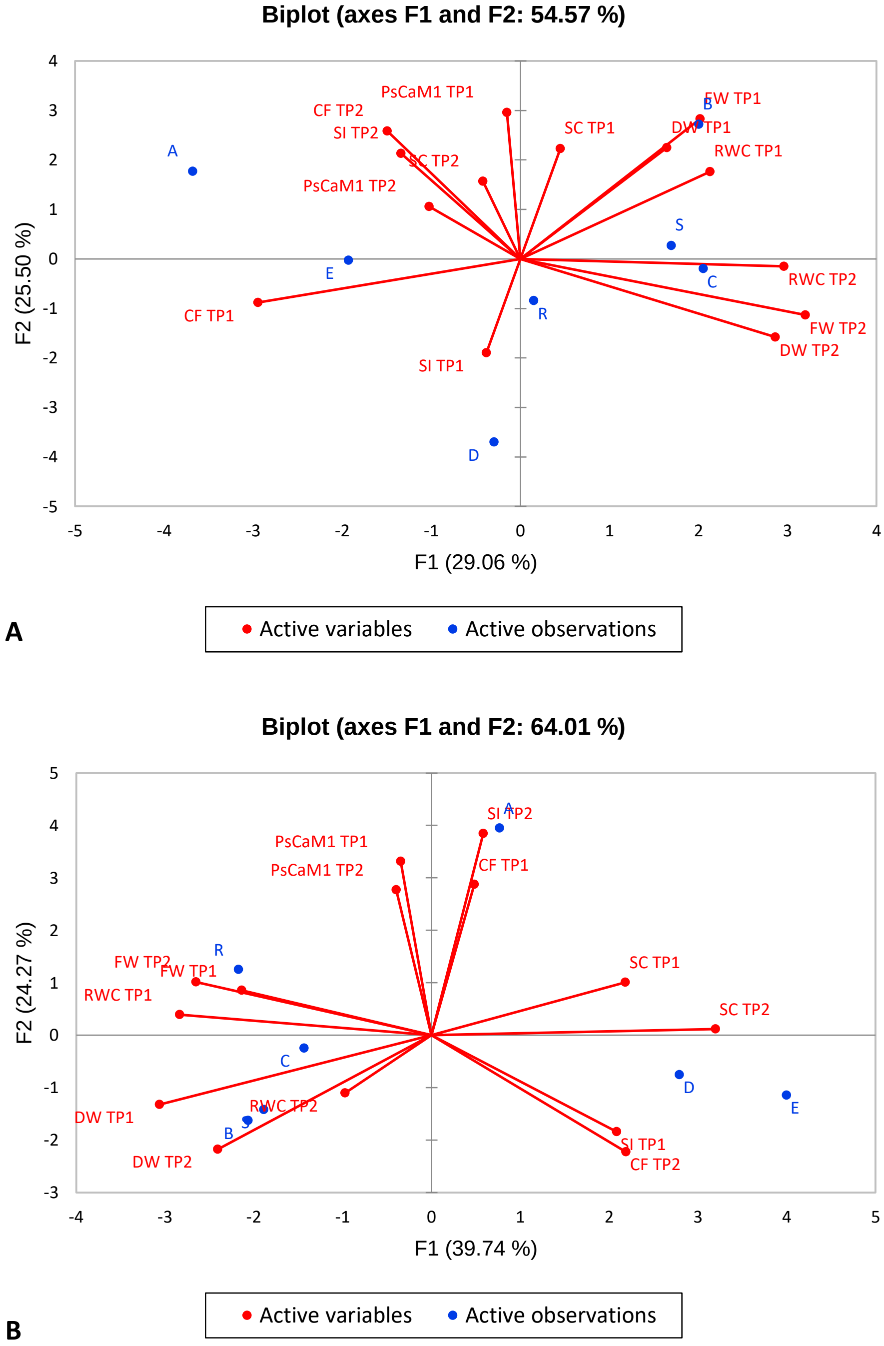
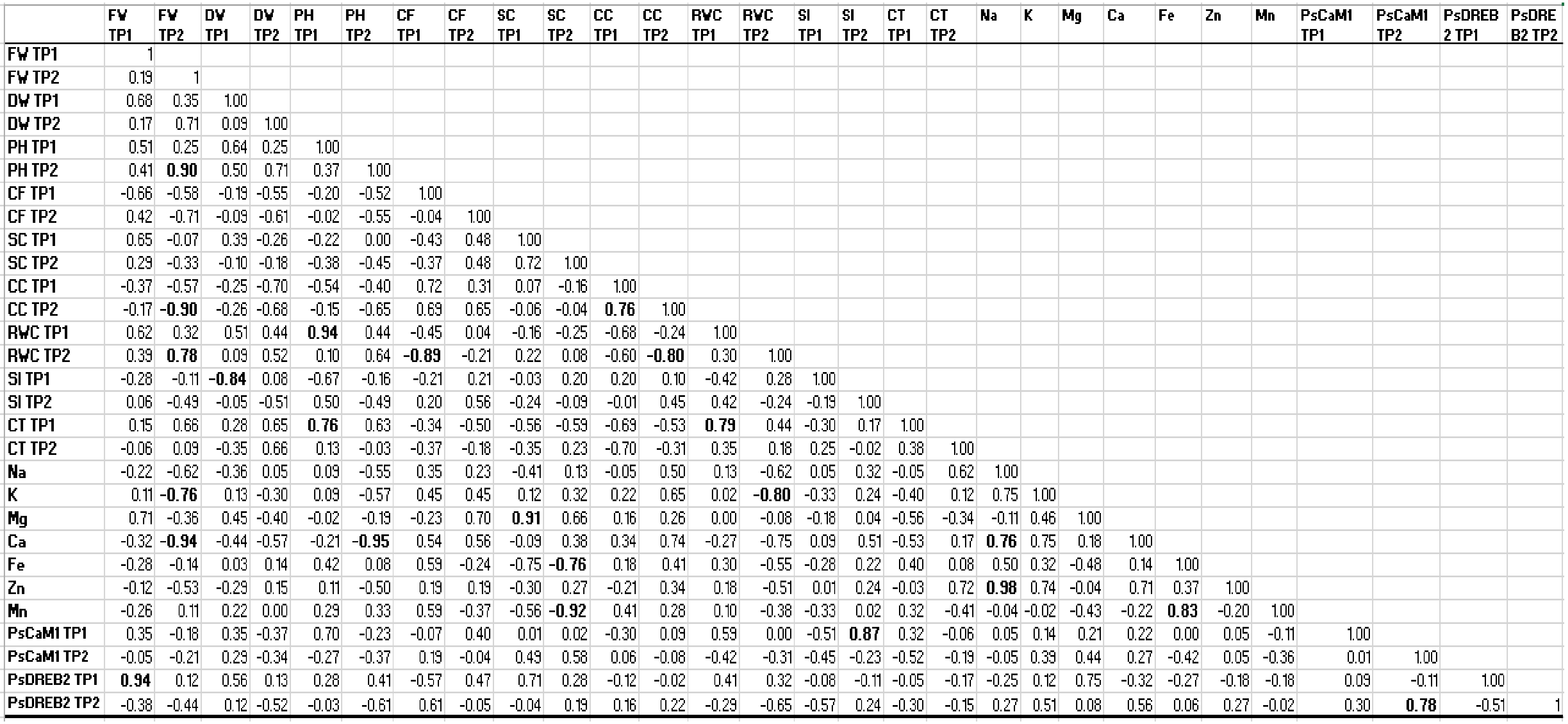


Figure 4.1. Pearson correlation between fresh weight (FW), dry weight (DW), stomatal conductance (SC), stomatal index (SI), chlorophyll fluorescence (CF) and PsCaM1 gene expression determined by principal component analysis (PCA). **(A):** The biplot among axes, F1 and F2, 54.57% for unstressed; **(B):** the biplot axes F1 and F2 was 64.01% for stressed variations respectively. The positively correlated variables are in the same quadrates. Active variables are denoted with red lines whereas active observation are the treatments and explicated with blue dots.

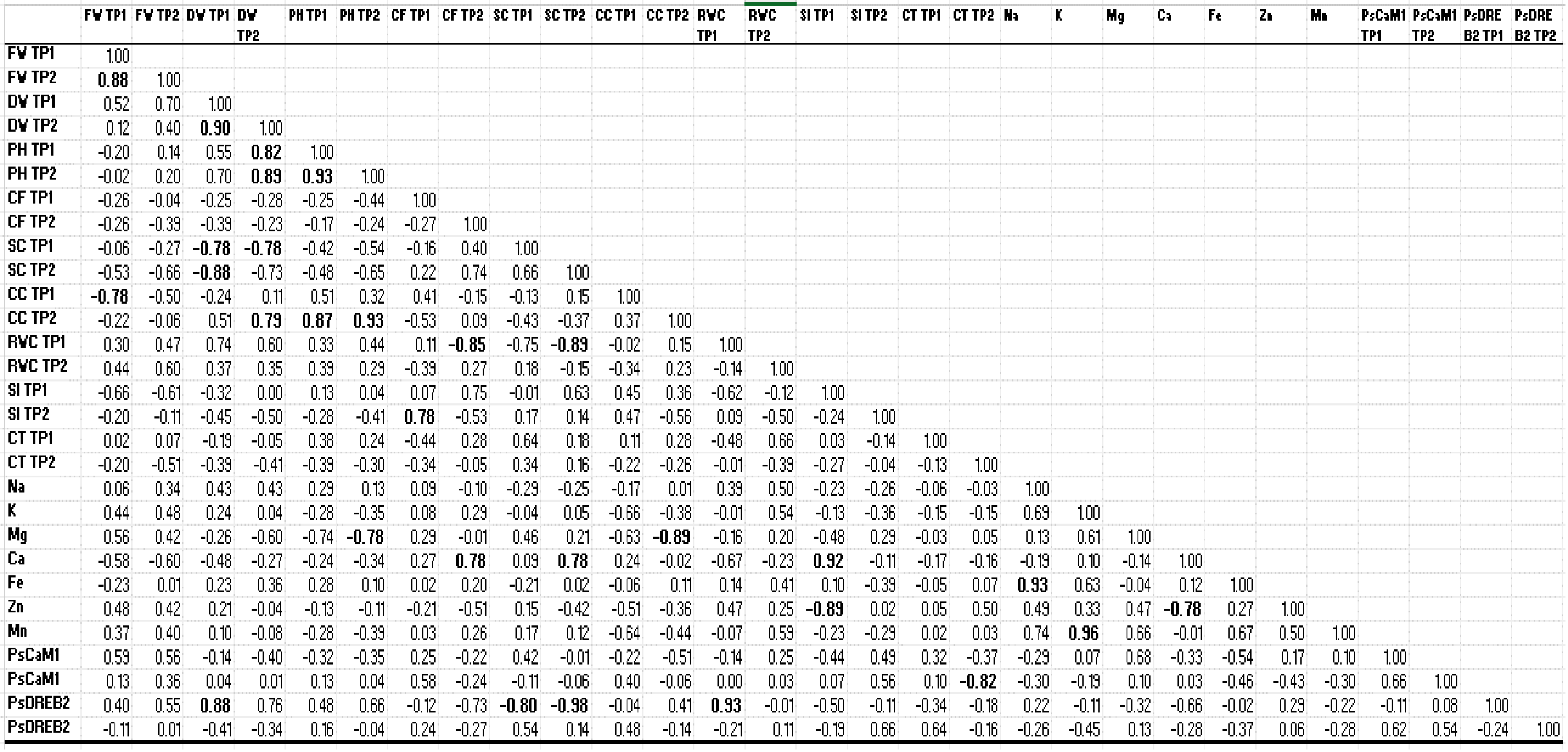
Table 1. Pearson correlation matrix for physiological, nutrients and molecular attributes under unstressed condition.



	FW TP1	FW TP2	DW TP1	DW TP2	PH TP1	PH TP2	CF TP1	CF TP2	SC TP1	SC TP2	CC TP1	CC TP2	RWC TP1	RWC TP2	SI TP1	SI TP2	CT TP1	CT TP2	Na	K	Mg	Ca	Fe	Zn	Mn	PsCaM1 TP1	PsCaM1 TP2	PsDREB2 TP1	PsDREB2 TP2
FW TP1	1																												
FW TP2	0.19	1																											
DW TP1	0.68	0.35	1.00																										
DW TP2	0.17	0.71	0.09	1.00																									
PH TP1	0.51	0.25	0.64	0.25	1.00																								
PH TP2	0.41	**0.90**	0.50	0.71	0.37	1.00																							
CF TP1	-0.66	-0.58	-0.19	-0.55	-0.20	-0.52	1.00																						
CF TP2	0.42	-0.71	-0.09	-0.61	-0.02	-0.55	-0.04	1.00																					
SC TP1	0.65	-0.07	0.39	-0.26	-0.22	0.00	-0.43	0.48	1.00																				
SC TP2	0.29	-0.33	-0.10	-0.18	-0.38	-0.45	-0.37	0.48	0.72	1.00																			
CC TP1	-0.37	-0.57	-0.25	-0.70	-0.54	-0.40	0.72	0.31	0.07	-0.16	1.00																		
CC TP2	-0.17	**-0.90**	-0.26	-0.68	-0.15	-0.65	0.69	0.65	-0.06	-0.04	**0.76**	1.00																	
RWC TP1	0.62	0.32	0.51	0.44	**0.94**	0.44	-0.45	0.04	-0.16	-0.25	-0.68	-0.24	1.00																
RWC TP2	0.39	**0.78**	0.09	0.52	0.10	0.64	**-0.89**	-0.21	0.22	0.08	-0.60	**-0.80**	0.30	1.00															
SI TP1	-0.28	-0.11	**-0.84**	0.08	-0.67	-0.16	-0.21	0.21	-0.03	0.20	0.20	0.10	-0.42	0.28	1.00														
SI TP2	0.06	-0.49	-0.05	-0.51	0.50	-0.49	0.20	0.56	-0.24	-0.09	-0.01	0.45	0.42	-0.24	-0.19	1.00													
CT TP1	0.15	0.66	0.28	0.65	**0.76**	0.63	-0.34	-0.50	-0.56	-0.59	-0.69	-0.53	**0.79**	0.44	-0.30	0.17	1.00												
CT TP2	-0.06	0.09	-0.35	0.66	0.13	-0.03	-0.37	-0.18	-0.35	0.23	-0.70	-0.31	0.35	0.18	0.25	-0.02	0.38	1.00											
Na	-0.22	-0.62	-0.36	0.05	0.09	-0.55	0.35	0.23	-0.41	0.13	-0.05	0.50	0.13	-0.62	0.05	0.32	-0.05	0.62	1.00										
K	0.11	**-0.76**	0.13	-0.30	0.09	-0.57	0.45	0.45	0.12	0.32	0.22	0.65	0.02	**-0.80**	-0.33	0.24	-0.40	0.12	0.75	1.00									
Mg	0.71	-0.36	0.45	-0.40	-0.02	-0.19	-0.23	0.70	**0.91**	0.66	0.16	0.26	0.00	-0.08	-0.18	0.04	-0.56	-0.34	-0.11	0.46	1.00								
Ca	-0.32	**-0.94**	-0.44	-0.57	-0.21	**-0.95**	0.54	0.56	-0.09	0.38	0.34	0.74	-0.27	-0.75	0.09	0.51	-0.53	0.17	**0.76**	0.75	0.18	1.00							
Fe	-0.28	-0.14	0.03	0.14	0.42	0.08	0.59	-0.24	-0.75	**-0.76**	0.18	0.41	0.30	-0.55	-0.28	0.22	0.40	0.08	0.50	0.32	-0.48	0.14	1.00						
Zn	-0.12	-0.53	-0.29	0.15	0.11	-0.50	0.19	0.19	-0.30	0.27	-0.21	0.34	0.18	-0.51	0.01	0.24	-0.03	0.72	**0.98**	0.74	-0.04	0.71	0.37	1.00					
Mn	-0.26	0.11	0.22	0.00	0.29	0.33	0.59	-0.37	-0.56	**-0.92**	0.41	0.28	0.10	-0.38	-0.33	0.02	0.32	-0.41	-0.04	-0.02	-0.43	-0.22	**0.83**	-0.20	1.00				
PsCaM1 TP1	0.35	-0.18	0.35	-0.37	0.70	-0.23	-0.07	0.40	0.01	0.02	-0.30	0.09	0.59	0.00	-0.51	**0.87**	0.32	-0.06	0.05	0.14	0.21	0.22	0.00	0.05	-0.11	1.00			
PsCaM1 TP2	-0.05	-0.21	0.29	-0.34	-0.27	-0.37	0.19	-0.04	0.49	0.58	0.06	-0.08	-0.42	-0.31	-0.45	-0.23	-0.52	-0.19	-0.05	0.39	0.44	0.27	-0.42	0.05	-0.36	0.01	1.00		
PsDREB2 TP1	**0.94**	0.12	0.56	0.13	0.28	0.41	-0.57	0.47	0.71	0.28	-0.12	-0.02	0.41	0.32	-0.08	-0.11	-0.05	-0.17	-0.25	0.12	0.75	-0.32	-0.27	-0.18	-0.18	0.09	-0.11	1.00	
PsDREB2 TP2	-0.38	-0.44	0.12	-0.52	-0.03	-0.61	0.61	-0.05	-0.04	0.19	0.16	0.22	-0.29	-0.65	-0.57	0.24	-0.30	-0.15	0.27	0.51	0.08	0.56	0.06	0.27	-0.02	0.30	**0.78**	-0.51	1

The bold values are different from the 0 value and are significant at α = 0.05. Fresh weight (FW), dry weight (DW), plant Height (PH), chlorophyll fluorescence (CF), stomatal conductance (SC), chlorophyll content (CC), relative water content (RWC), stomatal index (SI), canopy temperature (CT), *Pisum sativum* calcium-modulating gene (PsCaM1), *Pisum sativum* dehydration-responsive element binding gene (PsDREB2) with time point 1 (TP_1) and time point 2 (TP_2).

Table 2. Pearson correlation matrix for physiological, nutrients and molecular attributes under stressed condition.

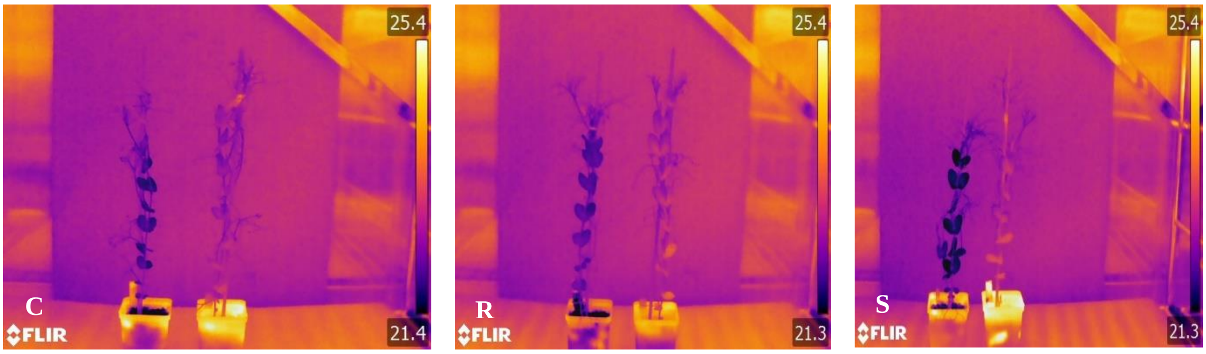


	FW TP1	FW TP2	DW TP1	DW TP2	PH TP1	PH TP2	CF TP1	CF TP2	SC TP1	SC TP2	CC TP1	CC TP2	RWC TP1	RWC TP2	SI TP1	SI TP2	CT TP1	CT TP2	Na	K	Mg	Ca	Fe	Zn	Mn	PsCaM1 TP1	PsCaM1 TP2	PsDRE B2 TP1	PsDRE B2 TP2
FW TP1	1.00																												
FW TP2	**0.88**	1.00																											
DW TP1	0.52	0.70	1.00																										
DW TP2	0.12	0.40	**0.90**	1.00																									
PH TP1	-0.20	0.14	0.55	**0.82**	1.00																								
PH TP2	-0.02	0.20	0.70	**0.89**	**0.93**	1.00																							
CF TP1	-0.26	-0.04	-0.25	-0.28	-0.25	-0.44	1.00																						
CF TP2	-0.26	-0.39	-0.39	-0.23	-0.17	-0.24	-0.27	1.00																					
SC TP1	-0.06	-0.27	**-0.78**	**-0.78**	-0.42	-0.54	-0.16	0.40	1.00																				
SC TP2	-0.53	-0.66	**-0.88**	-0.73	-0.48	-0.65	0.22	0.74	0.66	1.00																			
CC TP1	**-0.78**	-0.50	-0.24	0.11	0.51	0.32	0.41	-0.15	-0.13	0.15	1.00																		
CC TP2	-0.22	-0.06	0.51	**0.79**	**0.87**	**0.93**	-0.53	0.09	-0.43	-0.37	0.37	1.00																	
RWC TP1	0.30	0.47	0.74	0.60	0.33	0.44	0.11	**-0.85**	-0.75	**-0.89**	-0.02	0.15	1.00																
RWC TP2	0.44	0.60	0.37	0.35	0.39	0.29	-0.39	0.27	0.18	-0.15	-0.34	0.23	-0.14	1.00															
SI TP1	-0.66	-0.61	-0.32	0.00	0.13	0.04	0.07	0.75	-0.01	0.63	0.45	0.36	-0.62	-0.12	1.00														
SI TP2	-0.20	-0.11	-0.45	-0.50	-0.28	-0.41	**0.78**	-0.53	0.17	0.14	0.47	-0.56	0.09	-0.50	-0.24	1.00													
CT TP1	0.02	0.07	-0.19	-0.05	0.38	0.24	-0.44	0.28	0.64	0.18	0.11	0.28	-0.48	0.66	0.03	-0.14	1.00												
CT TP2	-0.20	-0.51	-0.39	-0.41	-0.39	-0.30	-0.34	-0.05	0.34	0.16	-0.22	-0.26	-0.01	-0.39	-0.27	-0.04	-0.13	1.00											
Na	0.06	0.34	0.43	0.43	0.29	0.13	0.09	-0.10	-0.29	-0.25	-0.17	0.01	0.39	0.50	-0.23	-0.26	-0.06	-0.03	1.00										
K	0.44	0.48	0.24	0.04	-0.28	-0.35	0.08	0.29	-0.04	0.05	-0.66	-0.38	-0.01	0.54	-0.13	-0.36	-0.15	-0.15	0.69	1.00									
Mg	0.56	0.42	-0.26	-0.60	-0.74	**-0.78**	0.29	-0.01	0.46	0.21	-0.63	**-0.89**	-0.16	0.20	-0.48	0.29	-0.03	0.05	0.13	0.61	1.00								
Ca	-0.58	-0.60	-0.48	-0.27	-0.24	-0.34	0.27	**0.78**	0.09	**0.78**	0.24	-0.02	-0.67	-0.23	**0.92**	-0.11	-0.17	-0.16	-0.19	0.10	-0.14	1.00							
Fe	-0.23	0.01	0.23	0.36	0.28	0.10	0.02	0.20	-0.21	0.02	-0.06	0.11	0.14	0.41	0.10	-0.39	-0.05	0.07	**0.93**	0.63	-0.04	0.12	1.00						
Zn	0.48	0.42	0.21	-0.04	-0.13	-0.11	-0.21	-0.51	0.15	-0.42	-0.51	-0.36	0.47	0.25	**-0.89**	0.02	0.05	0.50	0.49	0.33	0.47	**-0.78**	0.27	1.00					
Mn	0.37	0.40	0.10	-0.08	-0.28	-0.39	0.03	0.26	0.17	0.12	-0.64	-0.44	-0.07	0.59	-0.23	-0.29	0.02	0.03	0.74	**0.96**	0.66	-0.01	0.67	0.50	1.00				
PsCaM1	0.59	0.56	-0.14	-0.40	-0.32	-0.35	0.25	-0.22	0.42	-0.01	-0.22	-0.51	-0.14	0.25	-0.44	0.49	0.32	-0.37	-0.29	0.07	0.68	-0.33	-0.54	0.17	0.10	1.00			
PsCaM1	0.13	0.36	0.04	0.01	0.13	0.04	0.58	-0.24	-0.11	-0.06	0.40	-0.06	0.00	0.03	0.07	0.56	0.10	**-0.82**	-0.30	-0.19	0.10	0.03	-0.46	-0.43	-0.30	0.66	1.00		
PsDREB2	0.40	0.55	**0.88**	0.76	0.48	0.66	-0.12	-0.73	**-0.80**	**-0.98**	-0.04	0.41	**0.93**	-0.01	-0.50	-0.11	-0.34	-0.18	0.22	-0.11	-0.32	-0.66	-0.02	0.29	-0.22	-0.11	0.08	1.00	
PsDREB2	-0.11	0.01	-0.41	-0.34	0.16	-0.04	0.24	-0.27	0.54	0.14	0.48	-0.14	-0.21	0.11	-0.19	0.66	0.64	-0.16	-0.26	-0.45	0.13	-0.28	-0.37	0.06	-0.28	0.62	0.54	-0.24	1.00

The bold values are different from the 0 value and are significant at α = 0.05. Fresh weight (FW), dry weight (DW), plant Height (PH), chlorophyll fluorescence (CF), stomatal conductance (SC), chlorophyll content (CC), relative water content (RWC), stomatal index (SI), canopy temperature (CT), *Pisum sativum* calcium-modulating gene (PsCaM1), *Pisum sativum* dehydration-responsive element binding gene (PsDREB2). Whereas time point 1 is TP_1 and time point 2 as TP_2.

Annex 2. Growth phases from germination to vegetative stage in walk-in-chamber with control conditions

Annex 3. Plants before harvesting



Annex 4. Assessment of canopy temperature for all treatments under unstressed and stressed condition. Where; C: control; R: rhizobia; S: salicylic acid (SA)

List of Publications

Author List of Publication

List of Ph.D. Research

1. Tasmia Bashir, Shumaila Naz, Asghari Bano. 2020. **Plant Growth Promoting Rhizobacteria with Plant Growth Regulators Attenuate the effect of Drought Stress**. Pakistan Journal of Botany. 52(3): 783-792**.**

List of Other Publications

1. Muhammad Asad Ali, Khushi Muhammad, Aftab Ahmad Anjum, Mansur-ud-Din Ahmad, Masood Rabbani, Muhammad Zubair Shabbir, Arfan Ahmad, Muhammad Nawaz, Muhammad Tasleem Ghori, Javed Muhammad, Haroon Rashid Chaudhry, Tariq Jamie, Muhammad Haisem, **Tasmia Awan**, Rais Ahmad, Bhushan M Jayarao. 2017. Association of soil chemistry and other factors with spatially distributed Burkholderia mallei DNA in Punjab province, Pakistan. IEEE. DOI: 10.1109/IBCAST.2017.7868058.

2. Barkat Ali, Younas Sohail, **Tasmia Bashir** and Abdul Samad Mumtaz. 2016. Biogeography of Rust Fungi and their Hosts in Pakistan. Science International (Lahore). 28(5): 4777-478.

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PLANT GROWTH PROMOTING RHIZOBACTERIA IN COMBINATION WITH PLANT GROWTH REGULATORS ATTENUATE THE EFFECT OF DROUGHT STRESS

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Abstract

The present study evaluates the effects of plant growth hormones (PGR), salicylic acid (SA), abscisic acid (ABA) and plant growth promoting rhizobacteria (PGPRs) *Rhizobium pisi* (DSM 30132 strain) applied alone and in combination, on pea (*Pisum sativum* L.) cv. Florida plants under well-watered and drought stressed conditions. Prior to sowing seeds were soaked for 5h in broth culture (10^8 cfu/ml) of *Rhizobium pisi* and SA /ABA. Seeds were soaked for 6h in distilled water, ABA, SA solutions. Plants were subjected to drought stress on 21 days old seedlings by withholding the supply of water at two different time points; for 4d (TP_1) and for 8d (TP_2). Rhizosphere soil of abscisic acid treated plants exhibited higher retention of soil moisture at TP_1. Abscisic acid decreased the fresh and dry weight of plants under unstressed condition but increased the fresh weight as well as relative water content under drought stress. The response of *Rhizobium* and SA were at par. *Rhizobium* and SA ameliorated the adverse effects of drought stress more effectively than ABA. The *Rhizobium* inoculation reduced the stomatal conductance under unstressed condition but significantly increased stomatal conductance under drought stress at TP_2. SA alone and in combination with *Rhizobium* stimulated the stomatal conductance under unstressed condition. Under drought stress, at TP_1 all the treatments alone and in combination increased the relative water content (RWC) significantly over drought stressed plants. The FV/FM ratio was increased in SA treatment or in combination with SA, *Rhizobium* and ABA.

It is inferred from the data that *Rhizobium* alone or in association with SA may be used to mitigate drought induced inhibition on plant growth and biomass. At TP_1 the individual treatments of *Rhizobium*, ABA and SA exhibited better growth effect on pea plants. At TP_2, *Rhizobium* assisted SA and ABA to mitigate drought induced adverse effects over control. The combined application of PGPR and PGRs can be substantiated more effectively on crop plants under drought stressed condition. Furthermore, integrating these approaches in the cropping system can contribute to maintaining soil fertility status, with better economic returns for future use.

Key words: PGPRs, Salicylic acid, Abscisic acid, Abiotic stress, Pea

Introduction

Pea (*Pisum sativum* L.), a cool season food legume is a versatile crop cultivated worldwide (Mendler-Drienyovszki & Dobra´nszki, 2011; Nisar *et al*., 2008). The water requirements of pea is relatively high during growing season; the critical stages are the initial germination and the flowering. During the pod-filling phase the sensitivity of peas to drought stress is much less (Harrison, 2018). The drought stress induced during flowering stage reduces the number of pods per plant resulting in significant reduction in yield (Harrison, 2018).

Crop yield can be retained to a specific level by utilization of specific plant growth-promoting rhizobacteria (PGPR) that interact with crops (Glick 2012; Sandhya *et al*., 2010; Araus *et al*., 2008), in the manifestation of suboptimal environments including; drought and high salinity (Glick, 2014). Recent studies revealed various nods of convergence between stress responsive hormonal and ROS mechanisms that lead to biotic and abiotic stresses (Sewelam *et al*., 2016; Glombitza *et al*., 2004). Plant growth regulators (PGRs) ABA has a fundamental importance under drought stress and increases 55 fold of the original. ABA interacts with SA signalling pathways in an intricate manner. The use of PGPR has been demonstrated as a solution for the sustainability of agro-ecosystem under stresses. These strains are responsible for alleviating the plant growth from biotic/abiotic stress responses.

Globally, the preceding climate changes are expected to have a considerable repercussion on precipitation, intensifying the drought stress. There is a dire need to improve drought tolerance in crops in order to enhance their growth and yield using a number of PGPRs and PGRs (Khan *et al*., 2019). Previous studies demonstrated the favourable effects of PGPRs and PGRs on wheat and maize crops alleviated drought stress (Khan *et al*., 2018; Mega *et al*., 2019; Kumar *et al*., 2019). However, literature is scanty on pea plants. The present study was aimed to assess the role of PGPR (*Rhizobium pisi*) and PGRs (SA and ABA) on the growth of pea under drought stress.

Materials and Methods

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