Epigenetic and targeted metabolic changes in microbial biostimulant-treated maize plants under drought stress conditions

by

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DEDICATION

In memory of my father and to my mother, thank you for your endless love, sacrifices, prayers, support and advices, I hope that this achievement will complete the dream that you had for me for all those years when you chose to give me the best education you could.

To God, my Almighty creator who has been my source of inspiration and knowledge, from whom I drew my strength from throughout this journey.

Table of Contents

List of figures

List of tables

Preface

The content of this dissertation has been presented (or partially presented) in local and international seminars and conferences, and is currently under preparation for publication as review and research articles in peer-reviewed international scientific journals.

Local and International Seminar and Conferences

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Publications

Tugizimana F, Nephali L, Lephatsi M, Chele K, Steenkamp P, Buthelezi N, Opperman H, Huyser J. 2020. Decoding the metabolic landscape of maize responses to experimentally controlled drought stress: a greenhouse case study. *Environmental Metabolomics: Approaches, Challenges and Future Perspectives*. Eds. Beale D and Jones O. Elsevier (In production). A book chapter

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Summary

Drought stress is one of the major limiting factors in agriculture globally, hampering crop yields in approximately 70% of arable farmlands. In this regard, microbial-biostimulants, such as plant growth-promoting bacteria (PGPR)-based formulations, have been proven to provide sustainable and economically favorable solutions that could introduce novel approaches to improve agricultural practices and crop productivity under adverse environmental conditions. However, to devise these novel biostimulants-based agricultural strategies, there is a necessity to firstly understand the physiology and biochemistry governing the interactions between biostimulants and plants. Herein, targeted metabolomics, epigenetics and gene expression analyses were employed to elucidate molecular mechanisms governing plant growth-promotion, stress priming and enhanced drought stress responses induced by a microbial-based biostimulant formulation (a consortium of five *Bacillus* sp. strains) in maize (*Zea mays*) plants. The findings evidenced that this microbial-based biostimulant promotes growth of maize plants and renders the latter proactively adapted to drought conditions *via* multi-layered molecular mechanisms involving alterations in both primary and secondary metabolism, a reprogramming of DNA methylome and differential stress-related gene expression profiles. This fundamental (and actionable) knowledge generated from this work contributes to ongoing scientific efforts to decode modes of action of biostimulants (at both cellular and molecular levels), and provides a necessary step in the biostimulant industry for novel formulations and agricultural strategies for sustainable food production.

Chapter 1

General Introduction

Index

1.1 Problem statement

As sessile organisms, plants are constantly exposed to potentially stressful conditions that are often unfavourable for growth and development, and therefore have evolutionarily developed mechanisms that enable them to respond and adapt (Pandey *et al.*, 2016). These adverse environmental factors pose a growing threat to food security, affecting agronomically important crop plants (Koyro *et al.*, 2012; Zhu, 2016). Furthermore, the global population is estimated to reach 9.8 billion by 2050. Subsequently, to provide adequate food supply and meet the projected demands from the globally rising population, agricultural production must significantly increase (Jorge & António, 2018). However, achieving this imperative, *i.e.* increasing agricultural productivity, is currently an immense challenge due to decreased arable land and increasing adverse environmental conditions due to both biotic and abiotic stresses. The latter, such as drought, salinity and extreme temperatures, reduce the majority of crop yields by over 50% and are being intensified due to climate change (Araus *et al.*, 2002; Singhal *et al.*, 2016).

Although improved adaptation to these abiotic stresses has long been a pursuit of crop plant breeders, this is understandably difficult to achieve since abiotic stress resistance is a multigenic and quantitative trait controlled by multi-layered cellular and molecular events; considering also the increasing drought severity and expansion (Wani *et al.*, 2013; Onaga & Wydra, 2016; Zivcak *et al.*, 2016). Hence, innovative and efficient strategies to improve crop quality and tolerance against abiotic stresses are imperatively required. Biostimulants, such as plant growth-promoting rhizobacteria (PGPR)-based formulations, represent potentials to provide sustainable and economically favourable solutions that could introduce novel approaches to improve agricultural practices and crop productivity (Singh, 2013; Kumari *et al.*, 2019; Aamir *et al.*, 2020). Microbial biostimulants, particularly PGPR-based formulations and the focus of this work, have been successfully used to improve agricultural productivity in a sustainable manner (Yakhin *et al.*, 2017; Fleming *et al.*, 2019). However, to effectively establish and devise novel biostimulant-based agricultural strategies, there is a necessity to firstly understand the physiology and biochemistry governing the interactions between biostimulants and plants at both cellular and molecular levels. This knowledge gap – decoding molecular and physiological mechanisms underlying biostimulant action – is one of the main bottlenecks that hamper the biostimulant field and industries from implementing and

maximising the value of (traditional and novel) such formulations in agronomic practices (Yakhin *et al.*, 2017; Rouphael & Colla, 2020). Hence, exploring both the chemical and gene space of maize plants, the current work (reported in this dissertation) is part of ongoing scientific efforts to elucidate molecular mechanisms activated/stimulated by biostimulants (in crop plants) towards growth promotion and enhanced adaptability to abiotic stress condition.

1.2 Background

Plant beneficial microorganisms, such as PGPR have been shown to promote plant growth through various direct and indirect mechanisms such as enhanced nutrient acquisition (nitrogen, phosphorus and other essential minerals), modulation of plant hormones, antibiotic and lytic enzymes production, induced systemic resistance and the production of antimicrobial metabolites such as volatile organic compounds, siderophores and lipopeptides, just to mention a few (Ahemad & Kibret, 2014; Kundan *et al.*, 2015). Furthermore, PGPR induce protective mechanisms in plants including increased antioxidant metabolism, osmotic adjustment, ethylene inhibition, indole-3-acetic acid (IAA) production and abscisic acid (ABA) synthesis, which augment the plant's immune system and defences to confer protection against abiotic stresses (Ngumbi & Kloepper, 2016; Kumar *et al.*, 2019; Goswami & Deka, 2020).

One of the PGPR-induced protective phenomena is the preconditioning of plant defences and resistance against (a)biotic stresses, known as priming (Bruce *et al.*, 2007). The latter is a physiological state in which defence responses are activated faster and more efficiently (Beckers & Conrath, 2007). Priming mechanisms are described at different levels: modification of key regulatory transcript or protein levels, accumulation of intermediate compounds in the cells, metabolic reprogramming (metabolic memory), and (epi)genetic mechanisms resulting in epigenetic memory (Schwachtje *et al.*, 2019). Some of these epigenetic remodelling outputs involved in the priming phenomenon include DNA methylation and histone modification, which are long lasting mechanisms (Tugizimana *et al.*, 2018). On the other hand, at the transcript and protein level, priming events involve reprogramming of transcripts, activation and regulation of the expression of defence-related genes, and modulation of enzyme activities (Hilker *et al.*, 2016). These priming-related

reconfigurations (at cellular level) of both gene- and chemical space define long-term stress memory, and can lead to enhanced transgenerational tolerance against abiotic stresses. This transgenerational (epigenetic) stress memory is mechanistically characterised by histones and DNA modifications or chromatin remodelling without any alterations of the nucleotide sequence (Madlung & Comai, 2004; Tricker, 2015).

Generally, plants have evolved a plethora of epigenetic mechanisms that contribute to normal developmental growth in plants. Furthermore, emerging studies are revealing the role of the plant epigenome involvement in regulating plant defence responses to abiotic stress, thereby influencing and redefining plant survival, adaptation and evolution of plant resistance mechanisms (Boyko & Kovalchuk, 2008; Mirouze & Paszkowski, 2011; Eichten *et al.*, 2014; Yaish, 2017). However, such studies and knowledge-base are still the tip of an iceberg, and the ramifications and roles of epigenetic alterations in abiotic stress responses remain enigmatic. For instance, the elucidation of the molecular framework underlying the epigenetic memory in abiotic stress conditions is still elusive; the effects of biostimulants on the plant epigenome (under normal and/or stress conditions) and subsequent physiological reprogramming are still unknown. Furthermore, the holistic and mechanistic understanding of the spatial and temporal synchronisation of chromatin remodelling, DNA methylation and other epigenetic modifications, and the downstream phenotypical plasticity, in both primed and naïve plant abiotic stress responses is still missing (Sudan *et al.*, 2018; Rehman & Tanti, 2020; Chang *et al.*, 2020). Thus, unravelling mechanistic details governing the events pertaining to the epigenetic regulation of biostimulant-treated plants, under normal and abiotic stress conditions, is an imperative step towards generating actionable fundamental knowledge to complete the molecular puzzle defining the biostimulant-plant interactions.

As echoed in subsequent chapters of this dissertation, epigenetic alterations are also regulated and translated at the metabolome level. The latter is highly dynamic and the final readout of genotype × environment × phenotype relationship of an organism (Fiehn *et al.*, 2000; Fiehn, 2001, 2002). Measuring and assessing plant metabolite profiles and dynamics, under specific physiological conditions, thus theoretically encrypt the multi-layered chemical and biochemical processes involved in the plant metabolism (Král'ová *et al.*, 2012; Obata & Fernie, 2012; Piasecka *et al.*, 2019). Metabolomics – the qualitative and quantitative analysis of all measurable metabolites in a biological system – has thus positioned itself as a central

pillar in systems biology to interrogate cellular and organismal metabolism at a global level (Fernie, 2003; Kopka *et al.*, 2004). Furthermore, owing to the inherent sensitivity of the metabolome to genetic and environmental perturbations, alterations in a system's metabolism can be assessed and thereby provide mechanistic insights (Bino *et al.*, 2004). Thus, generated metabolic profiles and fluxes reflect the integrated output of the molecular machinery and biochemistry of a biological system under consideration (Tugizimana *et al.*, 2013; 2018; 2020). The application of plant metabolomics approaches in exploring the molecular and biochemical mechanisms underlying defence responses to fluctuating environmental conditions has increased over the past decade (Sarabia *et al.*, 2018; Razzaq *et al.*, 2019; Castro-Moretti *et al.*, 2020). These studies provide a promising basis for expanding and refining our understanding of the plant's plasticity to reconfigure both primary and secondary metabolism as well as the degree by which plants tolerate abiotic stresses.

Metabolomics has, therefore, undoubtedly become a powerful research tool to study the biochemical mechanisms underlying plant growth and development in the context of plant metabolic responses to abiotic stresses (Jorge *et al.*, 2016; Jorge & António, 2018). Furthermore, metabolomics offers unique opportunities to decode mechanistic frameworks that define modes of action of microbial biostimulants towards plant growth promotion and enhancement of defences against abiotic stresses (Tugizimana *et al.*, 2018; Adeniji *et al.*, 2020; Kellogg & Kang, 2020). As mentioned above, one of the phenomena *via* which biostimulants can improve plant protective machinery against environmental stresses is priming. The latter is a promising alternative approach due to the long-term and broad-spectrum resistance it provides against abiotic stress, providing an effective mechanism for crop protection under abiotic stresses (Martinez-Medina *et al.*, 2016). Accordingly, a comprehensive and mechanistic understanding (at molecular and cellular levels) of the beneficial effects of the biostimulants involved in priming (*e.g.* PGPR-based biostimulants) could pave ways to design novel strategies that will aid plants in adverse environmental conditions, thus contributing to sustainable food security. The current work therefore intends to profile both metabolic changes and global DNA methylation (elucidate epigenetic traits), underlying PGPR–maize interactions and the subsequent potentiation against drought stress. The results from this study will contribute in ongoing efforts to unravel the molecular and cellular events that explain the positive effects of biostimulant-plant interactions, pointing to

specific epigenetic changes and metabolic traits, and subsequently defining potential PGPR-induced 'memory markers' of enhanced responses to abiotic stresses.

1.3 Aim and objectives

The **main aim** of this work was to study PGPR-induced changes in the epigenome and metabolome of maize plants to elucidate the PGPR-induced biochemical events involved in plant growth promotion and priming against drought stress. Thus, the research question of this study, which can be classically described as a **hypothesis**, reads that "PGPR-based biostimulants can enhance plant growth as well as induce drought stress tolerance through differential changes in both metabolic and epigenetic profiles of maize plants". To achieve the aim of this work in examining the postulated research question, the main actionable **objectives** included (**1**) conducting a literature review on plant abiotic stress responses, and the potentiation of these responses through microbial-biostimulants treatment, (**2**) applying a targeted metabolomics approach to elucidate PGPR-induced metabolic landscape that define plant growth promotion, priming and drought stress tolerance mechanisms in maize plants, and (**3**) profiling differential epigenetic modifications (DNA methylation) and gene expression analysis, under PGPR and drought stress treatments in maize.

1.4 Outline of the Dissertation

This dissertation is subdivided into five self-standing units, thematically-linked chapters, inclusive of this general introduction (**Chapter 1**), with **Chapters 3** and **4** presented as complete studies, each comprising of a literature review, methodology description, discussion of the results, and conclusion. **Chapter 2** provides an overview of the existing literature on abiotic stress responses from signal perception to the activation of defence responses. It further assesses the current knowledge and gaps regarding the use of microbial-based biostimulants in enhancing plant growth, development and stress tolerance. Furthermore, **Chapter 2** highlights epigenetics and metabolomics as comprehensive tools to investigate the underlying biochemical mechanisms in biostimulant-plant interactions. To investigate the differential metabolic changes induced by PGPR in the primary and secondary metabolism of maize plants, a targeted liquid chromatography-mass spectrometry metabolomics study was conducted and reported in **Chapter 3**. PGPR-induced metabolic reprogramming, pointing to

enhanced plant growth, priming and drought stress tolerance in maize plants under mild and severe drought conditions, was investigated. Additionally, morphophysiological modifications induced by PGPR-based biostimulant and drought treatment were assessed. **Chapter 4** reports on global DNA methylation profiles in PGPR-treated maize plants, under well-watered, mild and severe drought stress conditions. An ELISA-based technique was employed to determine the relative global DNA methylation levels. The differences in the latter, among different treatment groups, were analysed and described. Furthermore, to complement the generated global methylation profiles, the expression of selected defence-related genes was assessed. **Chapter 5** is a general conclusion that integrates the findings of this work, highlighting key fundamental findings, that are actionably relevant for the biostimulant industry, and pointing out the potential use of PGPR-based biostimulants for sustainable agricultural practices.

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Chapter 2

Plant Responses to Abiotic Stresses, Microbial Biostimulants and Plant Priming: Metabolomics and Epigenetic Perspectives

Index

Chapter 2

Plant Responses to Abiotic Stresses, Microbial Biostimulants and Plant Priming: Metabolomics and Epigenetic Perspectives

Summary

Abiotic stresses are prime factors limiting plant growth and crop productivity. Considering the dual pressures of a booming population and detrimental abiotic stress effects on agricultural productivity, it is vital to decode the biological processes that regulate plant growth, development and productivity. In response to abiotic stresses, plants mount comprehensive stress-specific responses which mediate signal transduction cascades, transcription of relevant responsive genes and the accumulation of numerous different stress-specific transcripts and metabolites, as well as coordinated stress-specific biochemical and physiological readjustments. These natural mechanisms employed by plants are however not always sufficient to ensure plant survival under abiotic stress conditions. Biostimulants such as plant growth promoting rhizobacteria (PGPR) are emerging as plant priming agents and novel strategies in improving crop quality, yield and resilience against adverse environmental conditions. Additionally, biostimulants are therefore progressively being integrated into production systems, with the goal of modifying underlying plant physiological and biochemical processes in order to enhance stress tolerance and productivity. To successfully formulate these microbial-based biostimulants and design efficient application programs, the understanding of molecular and physiological mechanisms that govern biostimulant-plant interactions under environmental perturbations is imperatively required. Systems biology is a comprehensive analysis that uses different 'omics' approaches providing insights of the complex regulatory molecular networks in biological systems. The integration of different omics approaches (epigenetics and metabolomics) can unravel insights on the complex network of plant-PGPR interactions allowing for identification of molecular targets responsible for stress tolerance. This review chapter highlights the current knowledge on plant defence responses to abiotic stresses, from perception to the activation of cellular and molecular events. The chapter further reviews current knowledge on the application of microbial biostimulants and the use of epigenetics and metabolomics approaches to elucidate mechanisms of action of microbial biostimulants.

Keywords: Abiotic stress · Biostimulants · DNA methylation · Histone modifications · Epigenetics · Metabolomics · Priming · Memory

2.1 Introduction

As sessile organisms, plants are constantly exposed to adverse environmental perturbations, such as abiotic stresses. Anthropogenic contributions and increasing climate change continuously exacerbate the detrimental effects of these stresses on crop productivity, thereby posing a threat to global food security. Evolutionarily, plants have developed a multi-layered, complex and highly regulated immune system that involves sensing various danger signals and integration of this information to produce appropriate responses to diverse challenges, ensuring growth and development (Espinas *et al.*, 2016). These response mechanisms that are induced by stress exposure result in gene expression reprogramming and phenotypic modifications which, in turn, give rise to acquired memorisation, that can either be transient or long lasting (He & Li, 2018). Since plants are repeatedly exposed to different adverse environmental conditions, it is advantageous for plants to be able to remember past stress occurrences for adaptation and defence. In the last decade, progress has been made in elucidating and describing stress memory mechanisms in plants. One of these systems is known as defence priming, which sensitises and prepares the plant for future (a)biotic stress conditions (Pastor *et al.*, 2014).

Priming or pre-conditioning (of plant defences and adaptive mechanisms) as stress memory is a state in which plants are rendered more resistant to subsequent stresses, displaying faster and more efficient defence responses (Conrath et al., 2006; Beckers & Conrath, 2007; Conrath, 2011). Multiple examples of stress memory in response to stimuli such as drought, salinity and cold in higher plants have been shown across several species and discussed in great details (Herman & Sultan, 2011; Walter *et al.*, 2011). In this regard, numerous molecular mechanisms underpinning plant memory have been elucidated to date. One mechanism thereof is sustained alterations in levels of key signalling transcription factors, enzymes and/or proteins, which provides an insight of how the plant metabolism is altered and maintained by exposure to various stresses (Conrath *et al.*, 2006; Santos *et al.*, 2011; Pecinka & Mittelsten Scheid, 2012; Kinoshita & Seki, 2014; Vriet *et al.*, 2015). Another probable avenue could be chromatin alterations, such as DNA methylation, histone tail modifications or paused RNA polymerase II, which play an additional role in the coordinated changes in the patterns of gene expression that underpin memory responses (Chinnusamy *et al.*, 2008; Mirouze & Paszkowski, 2011; Eichten *et al.*, 2014; Avramova, 2015).

Consequently, the underlying mechanisms of these phenomena are the subject of much research. Hitherto, the potential impact of DNA methylation induced by priming as a stress memory has been reported in numerous studies (e.g., Lämke & Bäurle, 2017; He & Li, 2018), however, open questions still remain around the specificity of epigenetic marks and their stability throughout mitosis resulting in stress memory maintenance. Moreover, the exact mechanisms linking DNA modifications to transcriptional responses under abiotic stress are still enigmatic (Asensi-Fabado *et al.*, 2017). During stress encounters, metabolic perturbations are induced as part of the defence phenomenology, and some of these metabolic responses may persist following recovery, where the plant physiology returns to equilibrium (Hemme *et al.*, 2014; Pagter *et al.*, 2017). This change in metabolite levels such as the accumulation of signalling compounds has been shown to play a major role in stress memory under abiotic stress (Bruce et al., 2007). For example, it has been reported that the accumulation of abscisic acid (ABA) is involved in short term drought stress memory (Ding *et al.*, 2012). Alterations in the epigenome and metabolism remodel molecular circuits and networks that define stress memory mechanisms in plants. DNA methylation and metabolic reprogramming resultant from biostimulant treatment may therefore provide predictive understanding of priming mechanisms under drought stress.

Biostimulants are substances which, when applied in low concentrations not only mitigate stress, but also promote plant growth (du Jardin, 2015). Although the physiological effects of biostimulants have been documented (Conrath, 2011), a comprehensive understanding of modes of action of biostimulants at the cellular and molecular levels is still required to better value and develop formulations that are effective and science-based credible. Such in-depth molecular studies will aid improvement of the efficacy of biostimulants and will help optimise their applications in agriculture.

Metabolomics is a comprehensive ‘omics’ approach for the qualitative and quantitative analysis of metabolites in a biological system under certain physiological conditions (Tugizimana *et al.*, 2013). Being a final recipient and translation of biological information, changes in the environment resulting in changes in gene expression and protein expression, are directly reflected in the metabolome, thus making it more complex when compared to other ‘-omes’ (Nalbantoglu, 2019). Moreover, the metabolome being a convolution of all upstream biological information (from genome to proteomic level) and closest to the

phenotype, metabolomics –the interrogation of the metabolome of a biological system– provides thus a holistic signature of the physiological state of a biological system as well as knowledge on its biochemical processes (Fiehn *et al.*, 2000; Fiehn, 2001, 2002).

In the post-genomic era, comprehensive analysis using different systematic 'omics' approaches (systems biology) has provided insights of the complex regulatory molecular networks associated with stress adaptation and tolerance in plants. Molecular components (transcripts, proteins and metabolites) of an organism are placed into functional networks or models which describe the dynamic activities of the organism under different environmental conditions. For example, transcriptomics, proteomics and metabolomics which analyse RNAs and their expression, protein modifications and metabolites, respectively, have offered an unprecedented understanding of plant regulatory networks (Cramer *et al.*, 2011). Consequently, integration of the different 'omics' approaches can unravel insights on the complex network of plant-microbial interactions allowing for robust identification of molecular targets responsible for stress tolerance. Genetic modifications have been widely used for enhanced stress tolerance, however, the exploration of epigenetic modifications and metabolomic changes for potential crop improvement is still at an early stage. Thus, this **Chapter** provides an overview of the literature context of the work presented in this dissertation – to address some of these knowledge gaps.

2.2 Stress perception, signalling and plant responses

To counteract the adverse effect of environmental perturbations, plants have evolved comprehensive defence mechanisms that help them tolerate abiotic stresses by means of physical adaptation as well as integrated molecular and cellular responses. The initial and crucial step in abiotic stress defence mechanisms is the perception of the stress signals and their transduction in order to activate the relevant adaptive molecular responses to ensure survival.

2.2.1 Abiotic stress perception and downstream signalling

Perception of the stress signals is performed by receptors/sensors such as histidine kinases (HKs) and receptor-like kinases (RLKs) (Shiu & Bleecker, 2001; Xu *et al.*, 2008; Nongpiur

et al., 2012). Numerous receptors containing leucine-rich repeats (LRR), associated with abiotic stress have been identified in plants. These receptors are classified as either receptor-like kinases or proteins (RLKs or RLPs). For the perception of abiotic stress, there is growing evidence that suggests RLKs as main regulators of environmental stress regulation. For example, receptor-like protein kinase1 (RPK1), proline-rich-extensin-like rlk4 (PERK4), guard cell hydrogen peroxide-resistant1 (GHR1) and calcium/calmodium-regulated cysteine-rich rlk (CRK36) are involved in sensing drought and cold stress, and have been reported to regulate water stress signalling in *Arabidopsis* (Osakabe *et al.*, 2005, 2010, 2013; Yang *et al.*, 2010; Hua *et al.*, 2012). Furthermore, a variety of RLKs regulate a wide range of processes including root and shoot development, symbiosis and cellular differentiation (De Smet *et al.*, 2009). Following stress perception by the receptor proteins present on cell surfaces, the signal is transduced into different downstream signalling networks – a phenomenon known as signal transduction. A generic signal transduction pathway is initiated by perception, followed by the generation of secondary messengers resulting in the activation of a phosphorylation cascade that targets proteins involved in the regulation of stress defence genes. Early response signals have been unfolded and include cytosolic calcium (Ca^{2+}) elevation (Knight & Knight, 2001; Steinhorst & Kudla, 2013), reactive oxygen species (ROS) (Boscaiu *et al.*, 2008; Suzuki *et al.*, 2012; Choudhury *et al.*, 2013), and mitogen-activated protein kinase (MAPK) cascade activation (**Figure 2.1**).

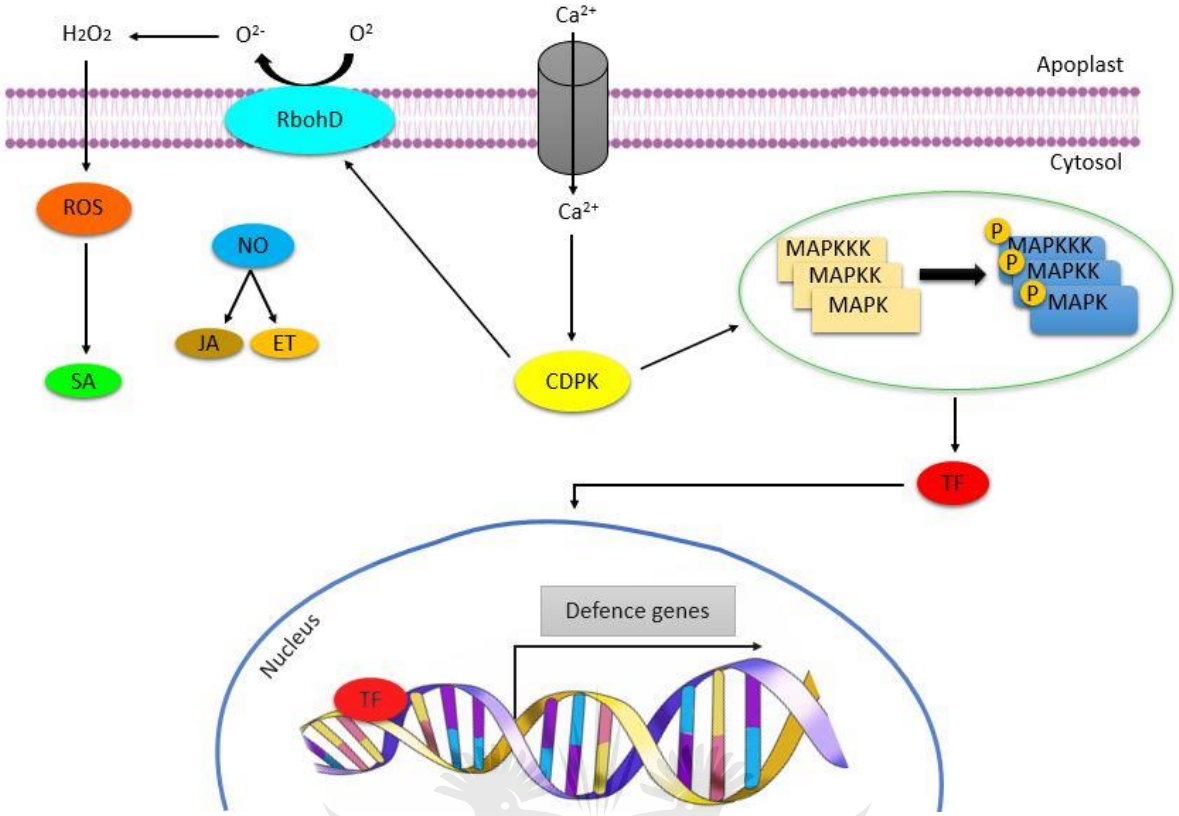

Figure 2.1: **Overview of defence signalling in plants under abiotic stress**. Upon perception of stress, several secondary messengers such as ROS and Ca^{2+} and NO are activated, which then induce different kinases such as CDPKs and MAPKs, resulting in the activation of transcription factors, enzymes and proteins which, in turn, activate the transcription of defence-related genes. Phytohormones, SA, JA, and ET, are also induced and contribute to plant immunity. **Abbreviations**: ROS, reactive oxygen species; Ca^{2+}, calcium; NO, nitric oxide; CDPK, calcium dependent protein kinases; MAPK, mitogen-activated protein kinase; SA, salicylic acid; JA, jasmonic acid; ET, ethylene (generated by the author of this dissertation).

2.2.1.1 Calcium signalling in response to abiotic stress

Ca^{2+} signalling plays a vital role in the specificity of the plants' cellular responses towards stress (Sanders *et al.*, 2002; Dodd *et al.*, 2010), and each stimulus perception is followed by a rapid increase in intracellular content of the said ions. Under normal physiological conditions, resting cytosolic Ca^{2+} ($[Ca^{2+}]$cyt) is maintained at nanomolar concentration levels *via* active transportation into the calcium stores or outside the cells into the apoplast, resulting in Ca^{2+} gradients of numerous magnitudes (Steinhorst & Kudla, 2014). Changes in the cytosolic Ca^{2+} influx and efflux patterns are evoked upon various stimuli perception as a response mechanism, and this is controlled through different channels and pumps. These $[Ca^{2+}]$cyt fluctuations by stimuli can occur in a repetitive manner in which the frequency and the amplitude of the signal are dependent on the type of the stimulus, thus making this a

signature (Dodd *et al.*, 2010). Each calcium signature encodes information that is specific to a stimulus through the type of tissue, subcellular location, size and the frequency (Miwa *et al.*, 2006; McAinsh & Pittman, 2009) and, therefore, defines the type of defence response. For Ca^{2+} signals to be decoded, calcium signal sensors that can sense any variations in levels and relay the information depicted within the signatures to activate relevant signalling cascades, are mandatory. Numerous studies have identified and characterised the prominent calcium sensor proteins defined by the calmodulin (CaM) and calmodulin-like protein (CMLs) family, calcineurin-B like proteins (CBLS), the calcium and calmodulin-dependent protein kinase (CCaMK) and calcium-dependent protein kinase (CDPK) family, which are known to occur in numerous gene families forming complex signalling networks in plants (Harper *et al.*, 2004; Ranty *et al.*, 2006; Yang *et al.*, 2010; Hashimoto & Kudla, 2011; Perochon *et al.*, 2011). These proteins are very diverse since they exhibit numerous affinities for Ca^{2+} ions. The binding of calcium to these sensors induces a conformational change that activates downstream targets, thereby contributing to an additional layer of specificity and resulting in transduction of the initial stimuli perception into specific biological responses (Hashimoto & Kudla, 2011).

2.2.1.2 Reactive oxygen species signalling in response to abiotic stress

ROS production is an early response mechanism directed towards abiotic stress and serves as an important secondary messenger in plants. These molecules defined as atmospheric oxygen intermediates which have a high biological significance in plants, include hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), singlet oxygen ($^1O_2$) and superoxide anion (O_2^-) formed through definite pathways. Under normal plant growth conditions, ROS are present in moderate levels through the action of antioxidants and enzymes that keep the levels in balance, thereby rendering these molecules as excellent signalling transducers (Mittler *et al.*, 2011). However, abiotic stresses induce excessive ROS production known as the oxidative burst, which acts as a signal in adjusting the plant cellular machinery in the activation of defence responses under abiotic stress (KrishnaMurthy & Rathinasabapathi, 2013). Although the production of ROS differs between different cellular compartments, the generated ROS signal is still considered a signature.

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The generation thereof is common in all stress encounters in plants and a combination of different stresses is likely to result in different ROS levels, therefore different sensors can be utilised to decode these signatures and create a signal that is specific to each stress (Choudhury *et al.*, 2013, 2017). NADPH oxidases (NOXs) catalyses the formation of superoxides and in plants are known as respiratory burst oxidase homologs (RBOHs) (Sagi & Fluhr, 2001; Sumimoto, 2008; Marino *et al.*, 2012). Phosphorylation of receptor kinases under stress encounters results in elevated Ca^{2+} which, in turn, activates the respiratory burst oxidase homolog D (RbohD). Consequently, this induces excess production of ROS that causes depolarisation of plant cells walls (Jeworutzki *et al.*, 2010). ROS spread through the entire plant in what is known as a 'ROS wave', and concomitantly triggering cell-to-cell communication that results in systemic signal(s) activation (Miller *et al.*, 2009; Mittler *et al.*, 2011). Calcium and ROS enhance the induction of the each other during stress encounters in a phenomenon known as a mutual interplay, which results in the fine tuning of signalling (Görlach *et al.*, 2015). For example, during salt stress, the superoxide produced activates calcium channels, which activate the vacuolar calcium channel TWO PORE CHANNEL1 (TPC1). TPC1 then transports the Ca^{2+} from the vacuole and induces the activation of RBOH protein D. This feedback loop is responsible for the propagation of the ROS and Ca^{2+} waves resulting in an efficient acclimation response (Evans *et al.*, 2016).

2.2.1.3 MAPKs pathway activation in response to abiotic stress

In addition to rapid systemic signalling induced by Ca^{2+} and ROS secondary messengers, kinase cascades of the MAPKs similarly play a crucial role in plant signalling of several environmental cues. The MAPK cascade is a result of a series of phosphorylation events that activate relevant genes in response to different stresses. In this system, the induced stress signals are transported from the receptors to specific effectors, thereby resulting in the regulation of relevant genes, different cellular activities and proteins involved in development and adaptation processes (Ligterink & Hirt, 2001; Galletti *et al.*, 2011; Sinha *et al.*, 2011; Moustafa *et al.*, 2014; Yi *et al.*, 2015). Signal transduction by MAPK cascades encompasses three types of kinases namely mitogen-activated protein kinase (MAPK), mitogen-activated protein kinase kinase (MAPKK) and mitogen-activated protein kinase kinase kinase (MAPKKK). Firstly, the MAPKKK, located downstream of specific receptors, is activated in response to extracellular stimuli which, in turn, activates a downstream MAPKK *via*

phosphorylation of its two serine or threonine residues located in the activation loop. Subsequently, MAPKK behaves as a specific kinase and phosphorylates a MAPK on its tyrosine or threonine residues located in the activation loop (Pitzschke *et al.*, 2009; Moustafa *et al.*, 2014; Jagodzik *et al.*, 2018). The latter eventually leads to the activation of various effector proteins located in the nucleus or cytoplasm as well as additional protein kinases, enzymes or transcription factors involved in plant stress-driven signalling pathways (**Figure 2.1**). Furthermore, MAPK cascades activated upon abiotic stresses such as drought, cold and salt show cross-talks with ROS, abscisic acid (ABA) and ethylene (ET) signalling (Menges *et al.*, 2008; An *et al.*, 2010; Chang *et al.*, 2012).

2.2.1.4 Phytohormone production in response to abiotic stress

During stress exposure, plants can amplify the initial stress signals, depending on the type of stress encountered and they do this by making of use of phytohormones. These phytohormone-driven signalling can either trigger new signalling events that are similar to those of the initial signal, or they can initiate an entirely different signalling event with different components (Huang *et al.*, 2012). The most-reported plant defence response phytohormone against abiotic stresses is ABA. Phytohormone accumulation has been linked to early plant stress signalling events such as rapid ROS production, showing the importance of the early and conventional plant responses in phytohormonal regulation that is dependent on the nature of the stress.

For instance, the accumulation of ABA under water deficit and high salinity is dependent on ROS production *via* the NADPH oxidase (Kwak *et al.*, 2003). This ABA-induced ROS accumulation can enter guard cells and activate Ca^{2+} channels which results in an increase in cytosolic Ca^{2+} and thus induce stomatal closure (Pel *et al.*, 2000). ABA is first sensed by the cells *via* the ABA receptors RCAR/PYR1/PYL (Regulatory Components of ABA-receptor/pyrabactin resistant protein/PYR-like proteins) (Ma *et al.*, 2009), resulting in the activation of open stomata 1 (OST1). OST1 is a member of the SNF1-related protein kinase 2 (SnRK2) family that mediates ABA-induced stomatal closing and the regulation of ROS production through the phosphorylation of the NADPH oxidase (Sirichandra *et al.*, 2009). MAPK cascades have also been implicated in ABA-mediated stress responses that are either upstream or downstream of ROS production (Xing *et al.*, 2008).

2.2.2 Abiotic stress responses: cellular and molecular events

Plant defence responses aiding in adaptation to abiotic stresses are coordinated and fine-tuned by changes in growth and development as well as cellular and molecular mechanisms. Significant progress has been made in elucidating these defence response mechanisms to environmental perturbations, which generally comprise alterations in the plant transcriptome, proteome and metabolome (Bokhari *et al.*, 2007; Fernandes *et al.*, 2008; Li *et al.*, 2008; Shulaev *et al.*, 2008). Following signal perception and transduction, adaptive responses are activated and result in the expression of stress-related genes regulated by TFs at the transcriptional level. A single TF can regulate the expression of numerous genes through the specific binding thereof to the *cis*- and *trans*-element in the promoters of target genes and this type of transcriptional regulation is termed regulon (Nakashima *et al.*, 2009).

Numerous regulons that are activated in response to abiotic stress have been identified in plants, and are components of ABA, a principal phytohormone involved in the regulation of abiotic stress in plants by regulating an intricate gene regulatory system that permits plants to tolerate environmental perturbations (Cutler *et al.*, 2010; see **section 2.2.1**). Myeloblastosis oncogene (MYB)/myelocytomatosis oncogene (MYC) and the ABA-responsive element binding protein/ABA-binding factor (AREB/ABF) regulons function in ABA-dependent gene activation pathways (Saibo *et al.*, 2009), whereas dehydration-responsive element binding protein 1 (DREB1)/C-repeat binding factor (CBF), DREB2, NAC (CUC, NAM, and ATAF) and the zinc-finger homeodomain (HD) regulons function in ABA-independent gene expression (Nakashima *et al.*, 2009) (**Figure 2.2**). The different TFs involved in stress tolerance normally function independently of each other, however, it has been shown that the ABA-dependent and ABA-independent pathways converge at several points representing transcriptional repressors and enhancers which may interact directly or indirectly with the DREB and AREB, and hence initiate synergistic interactions between cold, drought and salinity stress (Baena-González & Sheen, 2008; Huang *et al.*, 2012; Kimotho *et al.*, 2019).

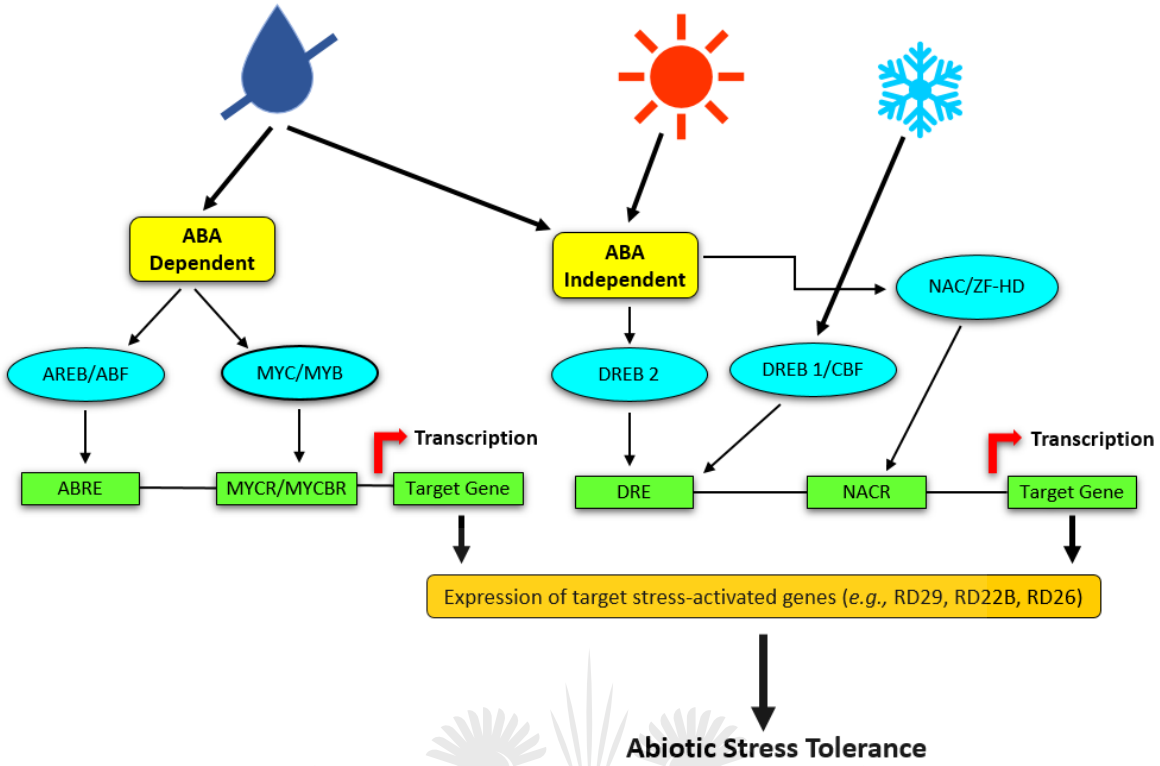

Figure 2.2: Transcriptional regulatory networks of abiotic stress signals. Signal transduction pathways in drought, heat and cold-stress responses are either ABA-dependent or ABA independent. In the ABA-dependent pathway, ABRE functions as a main ABA-responsive element. MYB2 and MYC2 function in ABA-inducible gene expression of the RD22 gene. MYC2 also functions in JA-inducible gene expression. The RD26 NAC transcription factor is involved in ABA and JA-responsive gene expression in stress responses. DRE is mainly involved in the regulation of genes not only by drought and salt but also by cold stress. DREB1/CBFs are involved in cold-responsive gene expression. DREB2s are important transcription factors in dehydration and high salinity stress-responsive gene expression. Another ABA-independent pathway is controlled by drought and salt, but not by cold. **Abbreviations**: MYB, myeloblastosis oncogene; MYC, myelocytomatosis oncogene; AREB, ABA-responsive element binding protein; ABF, ABA-binding factor; DREB, Dehydration-responsive element binding protein; CBF, C-repeat binding factor; ZF-HD, zinc-finger homeodomain (generated by the author of this dissertation).

Abiotic stress-inducible genes that are regulated by the different regulons include late embryogenesis abundant (LEA) class genes (RD29B, RAB18), cell cycle regulator genes (ICK1) and PP2Cs (ABI1 and ABI2). RD22 and RD26 have received special attention as potential targets for improvement of abiotic stress tolerance (Arbona *et al.*, 2017). These stress-regulated genes together with their products have important roles in abiotic stress responses and tolerance. As previously mentioned in the above paragraphs, much progress has been made in the understanding of signal transduction, transcriptional regulation and gene expression in plant responses to different abiotic stresses (Hu *et al.*, 2008; Yu *et al.*, 2016; Jin *et al.*, 2017). In transgenic *Arabidopsis thaliana* for example, the overexpression *Glycine soja* NAC TF, designated as *GsNAC019*, induced alkaline stress tolerance at both the

seedling and mature stages, even though the transgenic plants had reduced sensitivity to ABA (Cao *et al.*, 2017). In addition to all the plant defence responses induced by abiotic stress previously mentioned, plants can be primed for more rapid and stronger defence responses towards stress.

2.3 Microbial biostimulants and enhancement of plant responses to abiotic stresses

Plant growth and development regulation, together with the alleviation of detrimental effects of abiotic stresses, are crucial factors that determine the productivity of cultivated plants. Abiotic stresses are well known to negatively affect plant growth and development, and are responsible for crop losses globally. Despite the huge progress on plant abiotic stress research over the past decades that have paved the way to advance our knowledge on molecular and cellular mechanisms underlying plant tolerance to adverse conditions, much is yet to be understood. Furthermore, biostimulants are increasingly being integrated into production systems, with the goal of modifying underlying plant physiological and biochemical processes in order to enhance stress tolerance and productivity. By definition, biostimulants are diverse substances or microorganisms that stimulate the plant's natural processes to enhance nutrient uptake, nutrient efficiency, stress tolerance and/or crop quality when applied to plants, irrespective of their nutrient content (du Jardin, 2015). Additionally, biostimulants foster plant growth and development throughout the plant's life cycle from seed germination until maturity, improve the plant's metabolism, improve stress tolerance, facilitate nutrient assimilation, translocation and utilisation, enhance soil physiochemical properties and drive the development of complementary soil microorganisms (Calvo *et al.*, 2014).

Plant biostimulants are available in a wide range of formulations with varying constituents, but are generally categorised into four major groups based on their source and content. These groups include amino acid-containing products (AACP), hormone-containing products (HCP), humic substances (HS) and plant growth-containing microorganisms (du Jardin, 2015). Additionally, numerous categories of biostimulants have been extensively studied such as protein hydrolysates (Colla *et al.*, 2015), seaweed extracts (SWE) (Battacharyya *et al.*, 2015), silicon (Savvas & Ntatsi, 2015), humic and fulvic acids (Canellas *et al.*, 2015), arbuscular mycorrhizal fungi (Rouphael *et al.*, 2015) and plant growth-promoting rhizobacteria (PGPR) (Ruzzi & Aroca, 2015). In this regard, the potential effects of some

these of biostimulants in ameliorating abiotic stress in various plants have been extensively reviewed (**Table 2.1**).

Table 2.1: Summary of different biostimulants and their abiotic stress-alleviating effect in plants.

Biostimulant	Crop	Stress Tolerance	Reference
Azospirillum brasilense	*Triticum aestivum*	Drought tolerance	(Pereyra *et al.*, 2012)
Azotobacter chrococcum	*Zea mays*	Salt tolerance	(Rojas-Tapias *et al.*, 2012)
Azotobacter chrococcum	*Triticum aestivum*	Temperature tolerance	(Egamberdiyeva & Höflich, 2003)
Azospirillum lipoferum	*Triticum aestivum*	Salt tolerance	(Bacilio *et al.*, 2004)
Ascophyllum nodosum	*Kappaphycus alvarezii*	Cold tolerance	(Loureiro *et al.*, 2014)
Ascophyllum nodosum	*Camellia sinensis*	Drought tolerance	(Spann & Little, 2011)
Burkholderia phytofirmans	*Vitis vinifera*	Cold tolerance	(Fernandez *et al.*, 2012)
Flavobacterium glaciei	*Solanum lycopersicum*	Cold tolerance	(Subramanian *et al.*, 2016)
Pantoea dispersa	*Triticum aestivum*	Cold tolerance	(Selvakumar *et al.*, 2008)
Fulvic and humic acids	*Festuca arundinacea*	Drought tolerance	(Zhang & Schmidt, 2000)
Fulvic and humic acids	*Agrostis palustris*	Drought tolerance	(Zhang & Ervin, 2004)
Humic acid and phosporous	*Capsicum annuum*	Salt tolerance	(Çimrin *et al.*, 2010)
Humic acids	*Oryza sativa*	Oxidative and drought stress	(García *et al.*, 2012)
Humic acids	*Phaseolus vulgaris*	Salt tolerance	(Aydin, 2012)
Protein hydrolysates	*Zea mays*	Salt tolerance	(Ertani *et al.*, 2013)
Protein hydrolysates	*Lactuca sativa*	Salt tolerance, cold tolerance	(Botta, 2013; Lucini *et al.*, 2015)
Seaweed extracts	*Arabidopsis thaliana*	Cold tolerance	(Rayirath *et al.*, 2009)
Seaweed extracts	*Agrostis stolonifera*	Heat tolerance	(Zhang & Ervin, 2008)
Seaweed extracts	*Spinach oleracea*	Drought tolerance	(Xu & Leskovar, 2015)
Seaweed extracts	*Zea mays*	Cold tolerance	(Bradáčová *et al.*, 2016)

Several possible key mechanisms of action induced by biostimulants in relation to abiotic stress alleviation have been elucidated and include ROS scavenging, membrane stability, osmoprotection, stomatal regulation, ion homeostasis, nutrient availability and metal chelation (Van Oosten *et al.*, 2017), however, the explicit underlying modes of action responsible for these effects remain largely unknown (Yakhin *et al.*, 2017; Carolina Feitosa de Vasconcelos & Helena Garófalo Chaves, 2019).

2.3.1 PGPR-based biostimulants and defence priming against abiotic stresses

PGPR are increasingly being used as biostimulant formulations, showing potentials for improving plant health, development and sustainable increased yield. These soil bacteria that inhabit the rhizosphere interact symbiotically with the plant host to enhance plant growth. This chemical communication is translated into physiological benefits through various mechanisms. A detailed account of the complexity of the rhizosphere, its densely and diverse population, and molecular signalling web (Pineda *et al.*, 2010, 2013; Pieterse *et al.*, 2014; Venturi & Keel, 2016) is beyond the scope of this study. Although the rhizosphere chemistry remains largely unknown, and the establishment of plant-rhizomicrobiome mutualistic interactions is still poorly characterised, emerging studies have reported that various PGPR species enhance improvement in agronomic yields through direct or indirect mechanisms (Kevin, 2003; Niu *et al.*, 2018; Barnawal *et al.*, 2019), which include nitrogen fixation, production of growth-stimulating phytohormones and solubilisation of mineral phosphates (Singh, 2013). Similarly according to Barnawal *et al.* (2019) and other recent studies (**Table 2.2**), the improvement in agronomic yields by PGPR (or PGPR-based biostimulants) is due to the production of growth-stimulating phytohormones (indole-3-acetic acid (IAA), zeatin, abscisic acid (ABA), ethylene (ET) and gibberellic acid (GA)), secondary metabolites (siderophores, lipopeptides and N-acyl homoserine lactone) and volatile organic compounds (hydrogen cyanide, acetoin and 2,3 butanediol). Most recently, it has been demonstrated that *Azospirillum brasilense* Sp245 lipopolysaccharides (LPS) stimulates growth (fresh weight and root length) in *Arabidopsis thaliana* (Méndez-Gómez *et al.*, 2020) and this further suggest that PGPR-derived MAMPs also play a role in plant growth stimulation.

Table 2.2: Plant growth-promoting mechanisms induced by rhizobacteria

Strain	Mechanism	Plant	References
***Enterobacter aerogenes* (LJL-5), *Pseudomonas aeruginosa* (LJL-13)**	1-aminocyclopropane-1-carboxylic acid (ACC) deaminase	Alfalfa	(Liu *et al.*, 2019b)
***Burkholderia* sp. MTCC 12259**	IAA, ACC deaminase	Rice	(Sarkar *et al.*, 2018)
***Bacillus aryabhattai* MCC3374**	ACC, IAA, N_2 fixation, siderophore	Rice	(Ghosh *et al.*, 2018)
***Streptomyces* sp. VITMS22**	IAA	Mustard	(Kizhakedathil & Devi C, 2018)
***Azotobacter chroococcum*CAZ3**	IAA, siderophores, ammonia, and ACC deaminase	Maize	(Rizvi & Khan, 2018)
***Enterobacter* sp.**	ACC deaminase, IAA, siderophore, N_2 fixation	Rice	(Mitra *et al.*, 2018)
***E. aerogenes* MCC 3092**	IAA production, ACC deaminase, nitrogen fixation, and P solubilisation	Rice	(Pramanik *et al.*, 2018)
Bacillus safensis	IAA, ACC deaminase	Wheat	(Chakraborty *et al.*, 2018)
***Enterobacter cloacae* HSNJ4**	IAA	*Brassica napus* L. (rapeseed)	(Li *et al.*, 2017)
***Acinetobacter* strain RSC7**	IAA	*Vigna radiate* (mung bean)	(Patel *et al.*, 2017)
***Enterobacter ludwigii* PS1**	Auxin, siderophore, Hydrogen cyanide	Sea buckthorn	(Dolkar *et al.*, 2018)

Application of PGPR to induce abiotic stress tolerance in plants is extensively explored as an attractive strategy to regulate plant stress (Dimkpa *et al.*, 2009; Kasim *et al.*, 2013; Ben Rejeb *et al.*, 2014), and several mechanisms through which these organisms induce stress tolerance have been deciphered (**Figure 2.3**). In addition to the plant growth mechanisms mentioned above, PGPR can also induce abiotic stress tolerance in plants by modifying phytohormonal activity, maintaining iron homeostasis and osmotic balance (Vandana *et al.*, 2020).

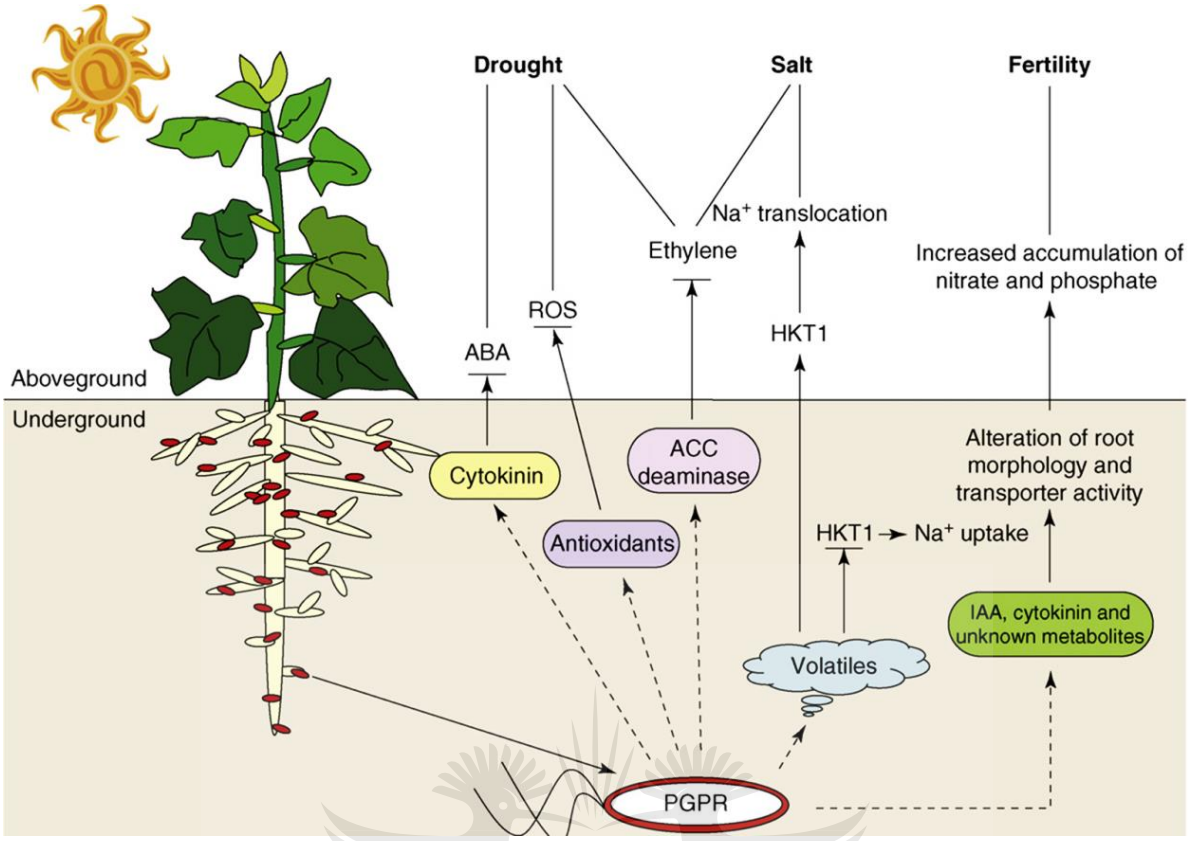

Figure 2.3: **Overview of induced systemic tolerance elicited by PGPR against drought, salinity and fertility stresses.** Broken arrows indicate compounds secreted by PGPR under stress including cytokinin, antioxidants, ACC deaminase and volatiles. Cytokinins and antioxidants result in ABA accumulation and ROS degradation, respectively. ACC deaminase degrades ACC and inhibits ethylene production. The volatiles emitted by PGPR downregulate HKT1 expression in roots but upregulation in shoot tissue, resulting in the recirculation of Na^+ in the whole plant under high salt conditions. **Abbreviations**: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylate; HKT1, high-affinity K^+ transporter 1; IAA, indole acetic acid; IST, induced systemic tolerance; PGPR, plant growth-promoting rhizobacteria; ROS, reactive oxygen species (adapted from Yang *et al.*, 2009).

Under abiotic stresses PGPR employ different mechanisms to help plants survive under such environment and these include: (i) production of ACC deaminase which lowers ethylene levels in plants (Glick, 2014; Gamalero & Glick, 2015); (ii) osmolytes secretion (proline, choline and trehalose) which acts as osmoprotectants; (iii) bacterial volatile secretion to induce stress tolerance (i.e 2R,3R-butanediol induces stomatal closure); (iv) secretes phytohormones (IAA, gibberelins and cytokinins) which stimulates lateral roots and root hairs formation, thus, increase water and nutrient uptake; (v) changes root cell membrane elasticity and improve membrane stability; (vi) exopolysaccharide secretion, which improve permeability by increasing soil aggression and maintaining high water potential around plant roots. Besides these know mechanisms, PGPR can trigger physiological events/processes in the interacting plant that lead to stress tolerance/priming (Mhlongo et al., 2018).

2.3.2 PGPR-induced priming as strategy towards enhanced abiotic stress tolerance

PGPR-plant interactions lead to enhanced resistance against abiotic stresses *via* PGPR-induced preconditioning of the plant immunity and is known as priming. In this state, the plant responds more rapidly and/or robustly following exposure to stress, thereby resulting in better stress tolerance when compared to non-primed plants (**Figure 2.4**) (Conrath *et al.*, 2002; Bruce *et al.*, 2007; Beckers & Conrath, 2007). This condition of preparedness achieved termed the 'primed state' has been linked to efficient activation of the defence responses which result in enhanced stress resistance. Priming can be induced by different factors including infection by pathogens, colonisation of roots by beneficial microbes, synthetic or natural chemicals, alteration of the primary metabolism and perception of volatile compounds (Conrath *et al.*, 2006). Even though priming mechanisms are not fully understood yet, numerous hypotheses have been proposed and include the accumulation of inactive proteins involved in signal amplification such as MAPKs (Beckers *et al.*, 2009), activation of transcription factors that enhance transcription of defence-related genes following stress perception (Conrath *et al.*, 2006) and epigenetic changes involving DNA modifications, histone modifications or chromatin alterations (Madlung & Comai, 2004). Plant priming has been considered as a promising strategy for the control of stress because it enhances defence responses without affecting the overall fitness of a plant, and the resultant stress resistance or tolerance cannot be overcome by microbes, subsequently providing long-term resistance (Alagna *et al.*, 2020).

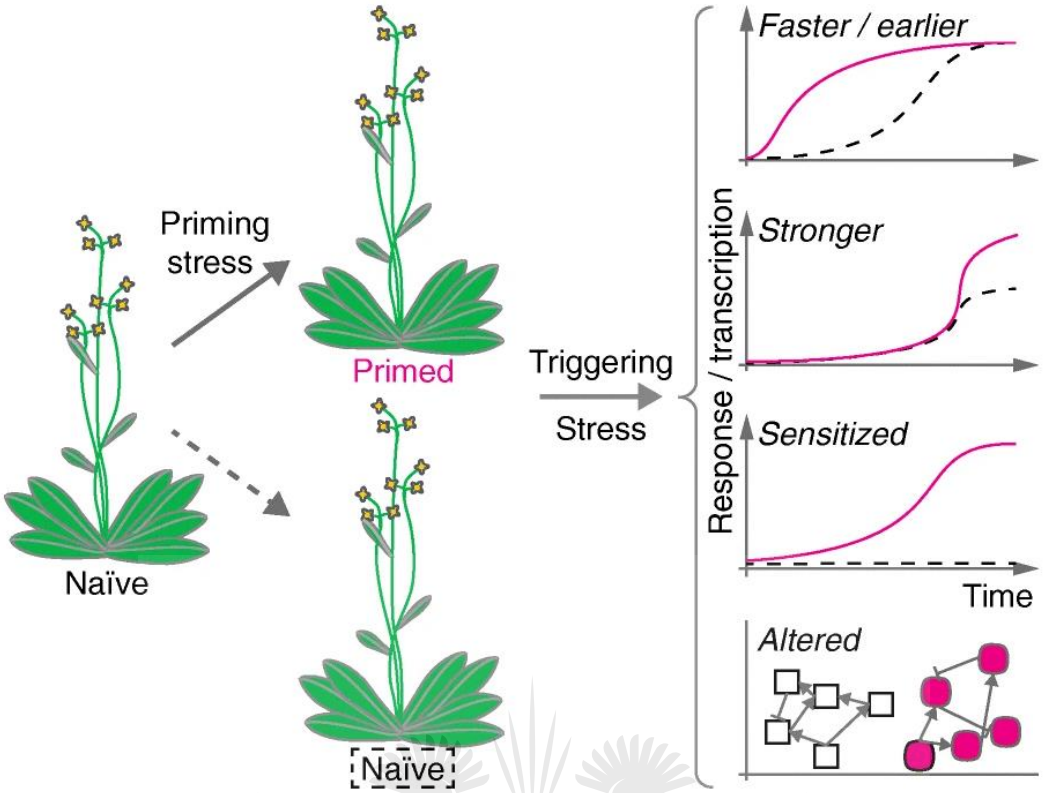

Figure 2.4: **Priming modifies responses upon stress encounter**. A naïve plant may be primed by either exposure to stress or other priming factors such as microbes. Response patterns differ in primed and naïve plants; the primed plant may respond to inducing stress more rapidly or more robustly than a naïve plant. It may also be sensitised so that the response is triggered at a lower fitness cost. The primed plant may further modify its response mechanisms to regulate a network of genes that is different from that found in a naïve plant (adapted from Lämke & Bäurle, 2017).

The rhizosphere chemistry remains largely unknown, and the establishment of plant-rhizomicrobiome mutualistic relations is still poorly characterised, however, emerging studies have reported that various PGPR species can pre-condition the plants for augmented defence responses against abiotic stresses (Smékalová *et al.*, 2014; García-Cristobal *et al.*, 2015; Abd El-Daim *et al.*, 2019; Brahim *et al.*, 2019; Zubair *et al.*, 2019). The molecular mechanisms underlying the rhizobacteria-related defence priming show that this induced state suggests a reprogramming in the cellular metabolism and regulatory machinery of the plants. Current insights propose that, preceding environmental perturbations, primed plants re-programme supporting metabolic pathways by altering the biosynthesis of different compounds such as sugars, amino acids and organic acids (Gamir *et al.*, 2014; Pastor *et al.*, 2014). The knowledge of biochemical and molecular mechanisms in defence priming is, however, still largely unknown, and a detailed mechanistic description of the various layers driving the priming events is still limited (Tugizimana *et al.*, 2019a). Nevertheless, despite these

limitations, this potentiation of the immune system and stress adaptability is unquestionably a fundamental means that plants have evolved as an adaptive strategy: by memorising past stress encounters in order to amplify defensive capacity upon subsequent stresses. As such, defence priming represents a promising and complementary alternative strategy that can provide new opportunities for plant protection against abiotic stress.

Priming mechanisms are described at different levels, and they can either be long- or short-lived. DNA methylation and histone modification observed at the epigenetic level are long-lasting mechanisms (Tugizimana *et al.*, 2018), and modulation of enzyme activities and changes in the abundance of transcripts observed at the transcript or protein level are short-lived mechanisms (Hilker *et al.*, 2016). A comprehensive and mechanistic understanding (at molecular and cellular levels) of the beneficial effects of the biostimulants (*e.g.* PGPR-based biostimulants) would pave ways to design novel strategies that will aid plants in adverse environmental conditions, thereby contributing to sustainable food security. Thus, the current study intends to investigate both epigenetic and metabolic changes related to the effects of biostimulant applications (*e.g.* PGPR-based formulations) in maize under drought stress.

2.4 Plant epigenetic mechanisms and their role in abiotic stress responses

Epigenetics refers to heritable changes in gene expression without alterations in the underlying DNA sequence and a growing number of studies postulate such regulations to be part of the underlying mechanisms of priming effects (Bruce *et al.*, 2007; Jaskiewicz *et al.*, 2011; Conrath *et al.*, 2015). This epigenetic remodelling includes histone modification as well as small RNAs and DNA methylation events which participate in the regulation of stress-responsive genes at both the transcriptional and post-transcriptional levels by altering the chromatin status of the genes. Moreover, epigenetic modifications play crucial roles in the formation of stress memory, which may be inherited by the progeny resulting in enhanced stress tolerance. Some of these modifications persist longer and are considered as transgenerational 'memory marks', whereas others are short-lived, dynamic modifications which are quickly removed again – *i.e.* chromatin marks (Pecinka & Mittelsten Scheid, 2012; Gaydos *et al.*, 2014; Hilker *et al.*, 2016; Kishimoto *et al.*, 2017; Schwachtje *et al.*, 2019). Although the molecular workings that define the transgenerational primed state are still largely unknown, epigenetic modifications and imprints are key components of this cellular

and molecular phenomenology of stress memory for storing and retrieving stress-related information (Lämke & Bäurle, 2017). As such, epigenetic transgenerational memory in plants is defined as a memory mark that extends from a generation under stress exposure to the first generation not exposed to the same stress (Lämke & Bäurle, 2017) (**Figure 2.5**).

Pecinka & Mittelsten Scheid (2012) cogently argued that the evidence of transgenerational epigenetic inheritance resultant from abiotic stress requires long-lasting changes of two generations or more that significantly influenced the plant's stress responses and adaptation. Conversely, Yaish (2017) suggested that there is evidence for long-lasting epigenetically-induced changes in stressed plants, however, it is difficult to spot. Consequently, for the past few years, several studies have reported on how plants acquire new traits induced by stress cues due to changes in epigenetic marks observed in the transposable elements (TEs) (Boyko *et al.*, 2010), promoters (Bilichak *et al.*, 2012) and gene coding regions (Jiang *et al.*, 2014).

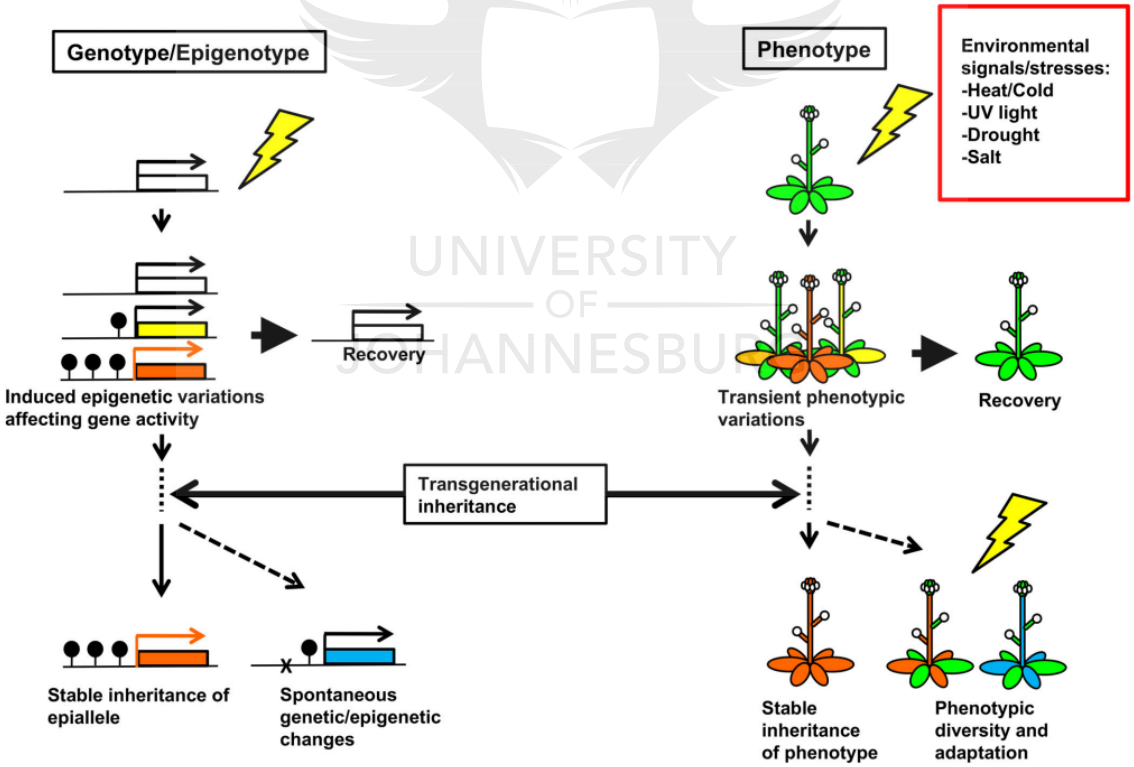

Figure 2.5: **A model of transgenerational epigenetic memory**. Perception of environmental stress by plants may induce epigenetic modifications in the plant genome, while a loss of existing modifications can also occur. These epigenetic variations can be transient and revert to the initial epigenetic state. In some cases, induced epigenetic variations may be transmitted transgenerationally and may become adaptive if the offspring experiences environmental signals similar to the previous generation (adapted from Miryeganeh & Saze, 2020).

It has been proposed that transgenerational inheritance of epigenetic marks and stress tolerance form part of the adaptive process in plants (Kinoshita & Seki, 2014) through the transfer of epigenetic modifications from the parental genome to the progeny without any reprogramming occurring in the gametes and embryos. The degree of this reprogramming is, however, still blurred, thus leaving a question of how much of the stress-induced epigenetic marks induced by priming are transferred to the progeny. Key common molecular features underlying epigenetic modifications associated with priming in abiotic stresses have been reported in numerous studies and include transcriptional memory, histone methylation and DNA hypo- and/or hypermethylation (Ding *et al.*, 2012; Singh *et al.*, 2014; Feng *et al.*, 2016; Wibowo *et al.*, 2018) (**Table 2.3**). Zheng *et al.* (2017) reported the improvement of drought adaptability in rice plants due to multi-generational drought exposure. They identified drought induced epimutations, which could maintain altered DNA methylation levels in the subsequent generations. Analysis of the drought-associated genes revealed that the DNA methylation level of the genes was modified by the multigenerational drought stress. These results therefore suggest that epigenetic mechanisms play imperative roles in plant's adaptations to environmental stresses. Consequently, the heritable epigenetic variations having morphological, physiological and ecological consequences can be considered important resources in plant improvement which may help improving adaptation and tolerance in crop plants for the adverse environments.

Table 2.3: Epigenetic mechanisms induced in different crop species under abiotic stress.

Crop	Abiotic stress	Epigenetic mechanism	References
Arabidopsis thaliana	Salt and drought stress	Histone acetylation	(Zheng *et al.*, 2016)
Arabidopsis thaliana	High salinity stress	Histone acetylation	(Sako *et al.*, 2016)
Arabidopsis thaliana	Cold stress	Hypermethylation	(Kenchanmane Raju *et al.*, 2018)
Arabidopsis thaliana	Salinity	Hypomethylation	(Arıkan *et al.*, 2018)
Hordeum vulgare	Terminal drought stress	Hypermethylation	(Surdonja *et al.*, 2017)
Beta vulgaris	Salt stress	Histone acetylation	(Yolcu *et al.*, 2016)
Hydrilla verticillata	Metal (copper) stress	Hypermethylation	(Shi *et al.*, 2017)
Zea mays	Heat	Histone acetylation	(Wang *et al.*, 2015)
Zea mays	Cold	Hypomethylation	(Hu *et al.*, 2012)
Zea mays	Cold	Histone acetylation	(Hu *et al.*, 2011)
Zea mays	Cold	DNA demethylation	(Steward *et al.*, 2002)
Populas	Drought stress	Hypermethylation	(Liang *et al.*, 2014)
Oryza sativa	Salt stress	Demethylation,	(Zhu *et al.*, 2015)
Vicia faba	Drought stress	Demethylation	(Abid *et al.*, 2017)
Triticum aestivum	Salt stress	Hypermethylation	(Kumar *et al.*, 2017)

2.4.1 DNA methylation and plant responses to abiotic stresses

DNA methylation is an epigenetic mark which involves the transfer of a methyl group from S-adenosyl methionine (SAM) to the fifth carbon of the pyrimidine ring of cytosine nucleotide without altering the underlying DNA sequence (Robertson, 2005). The modification machinery involves a highly regulated series of enzymatic reactions and complex molecular rearrangement events. DNA methylation is generally catalysed by a variety of enzymes termed DNA methylases (MTases), comprising two main groups: (i) *de novo* MTases, which catalyse the transfer of a methyl group to unmethylated cytosines, and include methyltransferase 1 (MET1), chromomethylase 3 (CMT3) and domains rearranged methyltransferase (DRM); and (ii) maintenance MTases, which maintain methylation that has already been established (Zhu, 2008). This epigenetic mechanism is involved in various biological processes such as development, stress adaptation and genome stability and evolution. Methylation of cytosine bases can be symmetric CG and CHG, and asymmetric CHH (where H = A, C or T) (Law & Jacobsen, 2011; Matzke & Mosher, 2014; Wang *et al.*, 2014).

Symmetrical methylation involves the recruitment of a MET to hemi-methylated daughter strands following DNA replication. Conversely, asymmetric methylation is determined *de novo* after every replication cycle and does not possess any inheritance mechanisms (Lämke & Bäurle, 2017). In plants, *de novo* methylation is catalysed by DRM2, and maintained by three different pathways: CG methylation by MET1, CHG methylation by CMT3, a plant specific DNA methyltransferase, and asymmetric CHH methylation through persistent *de novo* methylation by DRM2 (Chan *et al.*, 2005). Studies have revealed that in plants, methylation occurs predominantly on the CG, then CHG and CHH context, respectively (Cokus *et al.*, 2008). In addition, DNA methylation in plants is usually restricted to CGs located within the gene body while TE sequences tend to be methylated at most of their CG, CHG, and CHH sites (Gehring & Henikoff, 2008). The highly abundant methylation on repetitive DNA sequences suggests that one of the main functions of this epigenetic mechanism is to suppress the activity of transposons.

TEs make up a considerable proportion of the plant's genome, therefore the regulation thereof is essential because they are potentially highly mutagenic and their accumulation

limits survival (Saze *et al.*, 2012). Moreover, DNA methylation is also observed in gene coding regions in plants and often assembled in regulatory regions of genes such as promoters where it plays a vital role in regulating the gene expression. Numerous studies have reported that methylation in the promoter region causes transcriptional silencing, suggesting that changes in methylation could lead to novel transcriptional regulation of the associated genes (Dowen *et al.*, 2012; Du *et al.*, 2015). As such, changes in DNA methylation contribute tremendously to the plant's capability to conquer and respond to adverse conditions (Boyko & Kovalchuk, 2008). Under stress conditions, changes in hypermethylation of DNA (an increase in epigenetic DNA methylation) or hypomethylation of DNA (a decrease in methylation of DNA) (Chinnusamy & Zhu, 2009) result in gene expression alterations (activation/suppression) and are indicative of stress defence mechanisms, however, this is dependent on the type of stress response induced and varies between species. Given the changes orchestrated by DNA methylation, which result in gene silencing/activation, plants possess enzymes that counteract the activity of MTases to remove the methylation – a process known as DNA demethylation. DNA demethylases demethylate TEs or transcription start sites for gene expression regulation. These enzymes include demeter (DME), repressor of silencing 1 (ROS1) and demeter-like (DML) proteins (DML2 and DML3), which initiate demethylation *via* a base excision repair (BER) mechanism (Ikeda & Kinoshita, 2009; Zhu, 2009; Wu & Zhang, 2010).

Several examples have indicated that DNA methylation can either increase or decrease in response to stress and it appears that demethylation, which leads to gene expression activation, is a prompt response common in plants. In maize seedlings under cold stress exposure, hypomethylation was observed in root tissue following the expression of *ZmMI1* and after seven days of cold exposure (recovery), the cold-induced decrease in methylation levels was not restored to basal levels (Steward *et al.*, 2002). Additionally, in *Populus trichocarpa,* an increase in methylation was observed under drought stress, with 10.04% compared to the watered plants with a 7.75% increase (Liang *et al.*, 2014). Herein, it has been reported that there is a correlation between stress-induced gene expression and methylation levels under drought stress, with an up-regulation of 7329 genes together with hypermethylation as well as 10 322 down-regulated genes with hypomethylation shown (Liang *et al.*, 2014). Methylation status/level differs between species and genotypes under different stress factors. For example, an increase in DNA methylation levels was reported in

different rice genotypes of 20% in Nagina-22 and 37% in IR-64-DYT1.1 under drought stress (Kumar & Singh, 2016). The accumulated knowledge on DNA methylation over the years has been a big accomplishment in plant biology and to understand this phenomenon better, the question to ask is whether adaptation and stress memory are determined by particular site-specific methylation or methylated regions, and on how these regions are regulated by defence signalling.

Furthermore, emerging studies have evidently pointed to DNA methylation as one of the key components of stress priming and memory (Crisp *et al.*, 2016; Wibowo *et al.*, 2016, 2018; Lämke & Bäurle, 2017; He & Li, 2018). Recently, Kuźnicki *et al.* (2019) provided evidence on how DNA methylation alterations impact on the regulation of stress-responsive gene expression for intergenerational resistance to *Phytophthora infestans* induced by β-aminobutyric acid (BABA)-primed potato. Plants that were treated with BABA rapidly experienced DNA hypermethylation. This *de novo* induced DNA methylation correlated with the up-regulation of CMT3, DRM2, and ROS1 genes in potato. BABA transiently activated DNA hypermethylation in the promoter region of the *R3a* resistance gene triggering its downregulation in the absence of the stress. However, in the successive stages of priming, this DNA hypermethylation state changed into demethylation with the active involvement of potato DNA glycosylases. Interestingly, the methylation variations were transmitted to the next generation in the form of intergenerational stress memory and the progeny of the primed potato showed a higher transcription of *R3a* that associated with an augmented intergenerational resistance when compared to the inoculated progeny of unprimed plants. This epigenetic plasticity is postulated to mediate stress memory, although the open questions remain on the exact mechanisms of maintained memory.

2.4.2 Histone modifications, small RNAs and abiotic stress responses

In addition to alterations of DNA molecules through methylation or other processes (**section 2.4.1**), chemical modification of histones is another main component of the epigenome. Histones are essential proteins involved in packing and ordering of the DNA molecule into a fundamental structural unit of chromatin called a nucleosome. The latter consists of ~ 147 bp of DNA wrapped around a histone octamer that contains two copies each of H2A, H2B, H3 and H4, and accessibility of genomic DNA is regulated at this core particle (Luger *et al.*, 1997). The N-terminal regions (or tails) of the histones protrude from the larger structure and

are prone to various reversible, chemical post-transcriptional modifications (PTMs) that affect chromatin structure and function (Eichten *et al.*, 2014). Histone PTMs involved in enhancing/repressing gene expression include acetylation, phosphorylation, ubiquitination, biotinylation, de-acetylation, sumoylation, carbonylation and glycosylation catalysed by various enzymes. These histone modifications thus alter gene accessibility for transcriptional machinery (Tariq & Paszkowski, 2004; Kouzarides, 2007; Liu *et al.*, 2010; Lauria & Rossi, 2011; Berr *et al.*, 2012).

In the context of plant-environment interactions, studies have revealed that histone modifications that are closely related to plant responses to abiotic stress exposure are histone acetylation, de-acetylation, methylation and demethylation, which are catalysed by histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs) and histone demethylases (HDMs), respectively (Zhang, 2008; Luo *et al.*, 2017). The trimethylation of H3K4 is reported to be associated with transcription activation whereas dimethylation of H3K9 and H3K27 represses gene transcription (Zhang *et al.*, 2007). For instance, in *Arabidopsis thaliana*, a reference plant generally used for epigenetic studies in plant responses to abiotic stresses, under prolonged cold stress, an increase in H3K9 and H3K27 dimethylation and a decrease in H3K4 trimethylation was observed (Amasino & Sung, 2004). Furthermore, histone acetylation takes place at the flowering locus C (FLC), a flowering repressor. These histone modifications result in stable repression of FLC that permits flowering in winter-annuals types of *A. thaliana* that exhibit flowering delays in the first season (Amasino & Sung, 2004). Sokol *et al.* (2007), using western blotting to study the response of Arabidopsis T87 and tobacco BY-2 cell lines nucleosomes, demonstrated that phosphorylation and phosphoacetylation of histone H3 Ser-10, as well as acetylation of histone H4 lys14, were increased under cold stress and high salinity conditions.

Similarly to DNA methylation (**section 2.4.1**), histone modifications as probable marks of priming and stress memory have been reported in several studies (Jaskiewicz *et al.*, 2011; Sani *et al.*, 2013; Liu *et al.*, 2014; Feng *et al.*, 2016; Lämke *et al.*, 2016). A recent study by Liu *et al.* (2019) reported that extreme heat stressed *Arabidopsis* plants showed accelerated flowering which was also observed in the unstressed offspring, however, the mechanism remains unknown (Migicovsky *et al.*, 2014). They further showed that the heat-induced heat shock transcription factor (HSFA2) activated the H3K27me3 demethylase relative of early

flowering 6 (REF6) which de-repressed HSFA2. The REF6 and HSFA2 form a loop that activates transgenerational degradation by the suppressor of gene silencing 3 (SGS3)-interacting protein 1 (SGIP1) which, in turn, leads to the biosynthesis of trans-acting siRNA (tasiRNA) inhibition. The REF6-HSFA2 loop induces early flowering but decreases immunity. This feedback loop is a form of long-term epigenetic memory of heat that is maintained by the transgenerational 'ON' state of HSFA2. In addition to histone modifications, the stalling of RNA polymerase II was suggested as a drought stress-induced memory mark in *A. thaliana* (Ding *et al.*, 2012) and could provide a chromatin content that is active, and prepares genes involved in development and stimuli responses for appropriate expression (Wu & Snyder, 2008).

Target gene repressions by small non-coding RNAs have also been found to be engaged during plant abiotic stress and transcriptional gene silencing (TGS) by 24 nucleotides heterochromatic small interfering RNAs (hc-siRNAs), *via* RNA directed DNA methylation (RdDM), which is reported as an epigenetic mechanism of gene regulation in plants (Martínez de Alba *et al.*, 2013; Matzke & Mosher, 2014). RdDM in plants is exclusive to small RNA-mediated chromatin modifications because it depends on a particular transcriptional machinery that is fixated around two plant-specific RNA polymerase II (Pol II)- related enzymes called Pol IV and Pol V (Haag & Pikaard, 2011). In brief, the canonical view of RdDM involves the following steps: (i) transcripts from Pol IV are first copied into long double-stranded RNAs (dsRNAs), (ii) processed by dicer-like 3 (DCL3) into siRNAs and transported to the cytoplasm, (iii) following loading of one strand of these siRNAs onto Argonaute (AGO4), they are re-imported to the nucleus, where the siRNA guides the targeting of transcripts from Pol V by sequence complementarity and (iv) ultimately, this targeting recruits DNA methyltransferase activity to mediate *de novo* methylation of cytosines in all classes of sequence contexts (Matzke & Mosher, 2014). Numerous examples of environmentally responsive siRNAs as key mechanisms for priming and stress have been reported (Boyko *et al.*, 2010; Luna *et al.*, 2012; Rasmann *et al.*, 2012), however, the link and correlation between epigenetic modifications and acclimation is still an ongoing challenge.

In addition to epigenetic modifications that result from the complex multi-layered defence responses induced by abiotic stress (**section 2.4**), primary and secondary metabolites are also altered as a form of metabolomic adaptation. As such, the crosstalk between epigenetics and

metabolism are fundamental aspects of cellular adaptation to abiotic stress. The epigenome is dynamically regulated by the metabolome and alterations arising from abiotic stress cues may therefore co-ordinately drive aberrant gene expression which, in turn, contributes to adaptation and stress memory (epigenetic and metabolomic memory).

2.5 Metabolomics and the elucidation of plant responses to abiotic stresses

Various cellular and biochemical changes induced by abiotic stress are defined at the plant metabolism level. In a simplified description, under abiotic stresses, the plant metabolism is perturbed due to factors such as inhibition of enzymes or increased demand for compounds required for normal growth and development. Consequently, the plant's metabolic network must continually readjust under these conditions to maintain the normal metabolomic homeostasis and allow for the production of defence-related compounds that aid in stress tolerance. Accordingly, the current implementation of metabolomic approaches provides a thorough analysis of vital components of the plant's defence responses to abiotic stress. Here, metabolomics is a powerful tool that provides an overview of how an organism's metabolic network is regulated in response to stimuli. Metabolomics is a rapidly expanding omics science that has been widely applied in different fields. This multidisciplinary 'omics' science is defined as the comprehensive, qualitative and quantitative analysis of all the small molecules, termed metabolites, in a biological system (Fiehn, 2001; Dettmer *et al.*, 2007).

The metabolome can be described as a pool of low-molecular-weight metabolites usually less than 1500 Da, together with their precursors and intermediates of the corresponding biosynthetic pathways. These metabolites are considered final products of cellular regulatory processes (gene expression and protein activity), linking different biological information levels (genome to metabolome). Consequently, metabolite profiles potentially indicate the functional state of an organism and provide a holistic mark of the physiological state of the system under consideration (Fernie, 2003; Kopka et al., 2004; Bino et al., 2004; Tugizimana et al., 2013) (**Figure 2.6**).

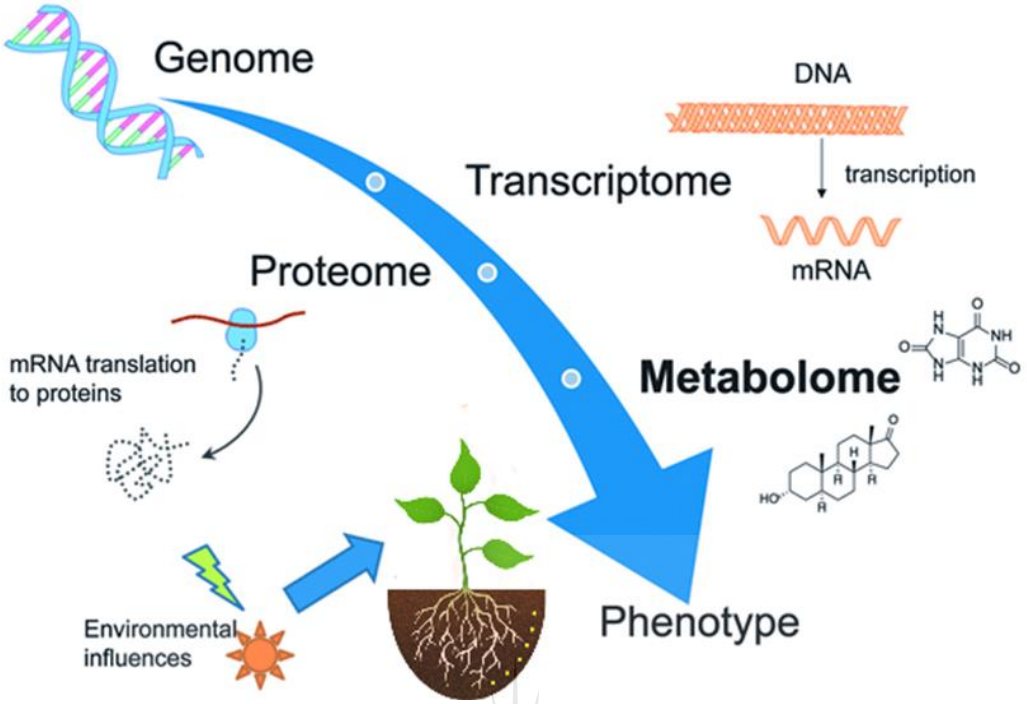

Figure 2.6: Overview of the omics cascades illustrating the flow and link from the genome to the metabolome. The metabolome is the downstream output representing the physiological state of the phenotype resulting from direct flow of biological information. The downstream output represented by the metabolome not only reflects the integration of the genome, transcriptome and proteome output, but also the input from environmental influences such as abiotic stress (modified from Steuer *et al.*, 2019).

Additionally, these low-weight molecules form an indispensable part of the plant metabolism, influencing all biological processes such as plant defence or tolerance to abiotic stresses (Obata & Fernie, 2012). As a high-throughput technology, metabolomics has been extensively used for various studies ranging from drug discovery, enzyme discovery, nutrigenomics, microbial biotechnology, toxicology, to crop and stress tolerance improvement in plants (Gomez-Casati et al., 2013).

2.5.1 Metabolomics as an investigatory tool in abiotic stress responses and defence priming

Metabolomics is a multidisciplinary 'omics' science that has proven indispensable in interrogating cellular biochemistry and metabolism, and has established itself as a powerful research tool to address biological questions related to plant-environment interactions. Metabolite profiling of plants growing under abiotic stress conditions has provided crucial information about changes at biochemical and molecular levels underlying plant growth and

adaptation. It is worth mentioning that metabolomic changes that have been reported in plants subjected to stress conditions are dependent on different causes; thus, these metabolic alterations have different significance and are expected to differently correlate with stress tolerance. Metabolomic reprogramming due to adverse environmental conditions involves complex and highly regulated molecular events some of which include (1) the stability and catalytic activity of enzymes involved in the biosynthesis/degradation of particular metabolites, (2) the adjustment of metabolite concentrations to re-establish homeostasis and normal metabolic fluxes and (3) the accumulation of compounds involved in mediating stress tolerance mechanisms (Genga *et al.*, 2011).

Under stress conditions, the total number, concentration, and types of metabolites are significantly altered. The alteration in gene expression is directly reflected in the metabolite profiles of plants. Acquiring knowledge about these differential metabolite profiles (which play a vital role in the growth, development, survival of the plant), and their modulation upon the onset of various abiotic stresses is highly fundamental. Reported metabolite changes have opened up the scope for the identification of viable metabolic markers which are important for abiotic stress tolerance of plants (Obata & Fernie, 2012; Kumar *et al.*, 2016; Freund & Hegeman, 2017; Parida *et al.*, 2018). Numerous studies have reported on the use the of metabolomics to study metabolite fluctuations in plants under stressful conditions (Urano *et al.*, 2009; Zhou *et al.*, 2011; Bowne *et al.*, 2012; Srivastava *et al.*, 2013; Figueroa-Pérez *et al.*, 2014; Zhao *et al.*, 2014; Shen *et al.*, 2016). Thus, metabolomics has become an indispensable tool in comprehending molecular mechanisms underlying abiotic stress responses.

For example, upon exposure to abiotic stresses such as temperature, salinity and drought, plants accumulate a wide of compatible osmolytes which primarily function to maintain turgor. The accumulated solutes which vary among species include sugars (glucose, sucrose and fructose), polyols, betaines and amino acids such as proline (Chen & Murata, 2002; Shulaev *et al.*, 2008). Some of these compounds are known to play roles as osmoprotectants, low molecular weight chaperones, photosystem II complex stabilisers and ROS scavengers (Szabados & Savouré, 2010). Additionally, some metabolites may act as chelating agents (sequestering toxic metals and ions), energy sources and signalling molecules under abiotic stress (Alcázar *et al.*, 2010; Szabados & Savouré, 2010). Metabolite fluctuations in response to individual abiotic stresses such as drought, salinity or heat have been widely studied, and

comprehensive reviews on this topic can be found in the literature cited here in (Obata & Fernie, 2012; Jorge *et al.*, 2016; Jorge & António, 2018).

The accumulation of various metabolites is one of the key mechanisms that plants use to cope with abiotic stresses; however, these natural mechanisms are not always adequate to ensure plant survival in all abiotic stress conditions; hence, the exploration of plant priming for more rapid and robust defence responses. As previously mentioned, priming mechanisms are described at different levels ranging from the epigenome to the metabolome. However, the metabolome as a key mediator of priming is largely unexplored. Additionally, deciphering of the priming event which leads to intense and faster defence responses is far from being fully fathomed at biochemical and molecular levels, consequently, dissecting metabolomic changes induced by priming may provide insight on some of the key underlying priming mechanisms.

Evidence on plant metabolic changes that occur as a result of priming have been reported and include the reprogramming of the primary metabolism and differential biosynthesis of secondary metabolites (Conrath *et al.*, 2015; Mhlongo *et al.*, 2016; Tenenboim & Brotman, 2016; Schwachtje *et al.*, 2018; Tugizimana *et al.*, 2019b), which are stored in a form of 'metabolic memory' or 'metabolic imprint', resulting in rapid and robust defence responses upon subsequent challenges (Mauch-Mani *et al.*, 2017; Schwachtje *et al.*, 2019). For example, the accumulation of sugars, amino acids and hormones or their conjugates as key metabolic events during the priming phase has been reported to render the plant in a state of alertness upon subsequent environmental challenges (Gamir *et al.*, 2014; Balmer *et al.*, 2015). Additionally, a study by Pastor *et al.* (2014) reported on metabolic changes that take place during the priming phase. Following chemical priming by BABA to determine if priming pre-conditions the plant for attack by activating relevant metabolic pathways, *A. thaliana*'s primary metabolism was found to be boosted through alterations of the Tricarboxylic acid (TCA) cycle intermediates, namely citrate, fumarate, malate and oxoglutarate. Furthermore, the amplification of the phenylpropanoid biosynthesis and the octadecanoic pathway was also observed.

The metabolic reprogramming of the primary and secondary metabolism indicates that multiple metabolic pathways are involved in the priming phenomenon. The

interconnectedness of these metabolic pathways has been reported to have feedback loops that allow for rapid activation of cellular defences to potential adverse conditions such as abiotic stress (Tugizimana *et al.*, 2018). Despite the exponentially increasing efforts to elucidate these metabolic changes, gaps still exist in understanding the comprehensive molecular and biochemical mechanisms involved in the priming phenomenon due to its complexity. Regardless of these limitations, defence priming is unquestionably one of the key adaptive mechanisms plants employ under constantly fluctuating environmental conditions. Two approaches exist for investigating the metabolome: untargeted - and targeted analysis, with the typical workflow comprising of three main experimental steps namely sample preparation, data acquisition and data mining (Trygg *et al.*, 2007; Verpoorte *et al.*, 2008; Kim & Verpoorte, 2010; Tugizimana *et al.*, 2013). An untargeted analysis is used for discovery-driven studies such as the depiction of metabolomic changes induced by specific treatments, disease or genetic changes (Patti *et al.*, 2012), simultaneously measuring as many metabolites as possible in the system in an unbiased manner. By contrast, a targeted analysis aims at identifying and quantifying metabolites in selected biochemical pathways, or specific classes of compounds in a hypothesis-driven approach (Dudley *et al.*, 2010), and is the approach that will be employed to elucidate plant defence responses and priming mechanisms under mild and severe drought stress conditions (**Chapter 3**).

The plant kingdom is reported to contain a diverse array of between 200,000 and 1,000,000 metabolites which vary per their classes, chemical structures and polarities (Saito & Matsuda, 2010; Obata & Fernie, 2012). Owing to this wide chemical diversity, as well as the wide concentration range, there is no single analytical platform currently for the comprehensive examination of the entire metabolome *in toto* (Bino *et al.*, 2004). Consequently, a combination of different analytical platforms is often employed in plant metabolomics to detect and characterise these diverse compounds as holistically as possible. Current plant metabolomics approaches are reliant on either mass spectrometry (MS) or nuclear magnetic resonance (NMR) based approaches. Several comprehensive protocols on these approaches have been published (Lisec *et al.*, 2006; De Vos *et al.*, 2007; Tohge & Fernie, 2010), along with several excellent reviews (Fiehn *et al.*, 2000, 2015; Cajka & Fiehn, 2014; Lu *et al.*, 2017; Rodrigues *et al.*, 2019). Additionally, MS systems are often coupled to chromatographic platforms such as gas chromatography – mass spectrometry (GC-MS) and liquid chromatography – mass spectrometry (LC-MS); and such analytical systems have

become popular in metabolomics studies, providing more sensitive detection of metabolites and wide coverage of the metabolome under consideration (Tugizimana *et al.*, 2013).

2.6 Conclusion

The present climate change is depleting natural resources and exerting negative impacts on crop production, thus threatening food security. Modern agriculture is shifting toward improved crop yield and quality by exploiting the natural priming phenomenon in plants. In this context, the exploitation of biostimulants to enhance abiotic stress tolerance has increasingly become a strategy worth pursuing among the scientific community. Biostimulants could pose as feasible alternatives in enhancing plant growth and increased stress tolerance. Numerous reports have elucidated the potential effects of biostimulant applications using various microorganisms such as PGPR; however, their comprehensive modes of action and underlying molecular mechanisms in relation to their priming effects and growth promotion are still enigmatic. This **Chapter** reviews and discusses the growing literature and the current knowledge on plant defence responses to abiotic stresses and the integration of biostimulants in agricultural production systems, pointing out some knowledge gaps, some of which are addressed by the work reported in this dissertation. The increasing use of biostimulants has demonstrated the positive effects of these formulations which include the modification of plant physiological and biochemical processes to enhance stress tolerance and productivity. This literature review **Chapter** further briefly highlights the application of metabolomics and epigenetic approaches in studying plant defence responses to abiotic stresses and defence priming. Thus, the metabolomics and epigenetics work presented in this dissertation (**Chapters 3** and **4**) contributes towards the on-going efforts of identifying the key underlying biochemical and molecular mechanisms induced by biostimulants in crop plants under abiotic stress conditions, such as drought stress, for improved growth and enhanced drought tolerance.

2.7 List of References

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Chapter 3

Altered Metabolic Profiles Associated with Microbial Biostimulant-Mediated Growth Enhancement and Drought Stress Tolerance in Maize: A Targeted Metabolomics Study

Index

Chapter 3

Altered Metabolic Profiles Associated with Microbial Biostimulant-Mediated Growth Enhancement and Drought Stress Tolerance in Maize: A Targeted Metabolomics Study

Summary

Microbial-based biostimulants are emerging as effective strategies to improve agriculture productivity; however, the modes of action of such formulations are still largely unknown. Thus, the present work aimed at elucidating metabolic changes in maize (*Zea mays*) leaves conferring growth promotion and drought stress tolerance induced by a microbial-based biostimulant (a consortium of plant growth-promoting rhizobacteria, PGPR) treatment. Measurement of the selected physiological stress markers representing the antioxidant system machinery together with relative shoot, root and total dry biomass of the various treatments revealed a significant increase in enzymatic regulators of oxidative stress and biomass in PGPR-treated plants compared to naïve plants. Also, a liquid chromatography-mass spectrometry-based targeted metabolomics approach revealed differential quantitative profiles in amino acid, phytohormone, flavonoids and phenolic acid levels in maize plants treated with PGPR under well-watered, mild, and severe drought stress conditions. These metabolic alterations point to the ability of this microbial-based biostimulant to promote plant growth and defence preconditioning. These measured metabolic changes, which gravitate towards growth promotion and enhanced drought stress responses, are translated in key biochemical and physiological events that include: (1) growth promotion of roots, leaves and shoots; (2) enhanced photosynthetic capacity; (3) energy production through amino acid recycling; (4) nutrient and water uptake; (5) ethylene inhibition through 1-aminocyclopropane-1-carboxylic acid degradation; (6) production of osmolytes; (7) protein biosynthesis; and (8) augmented antioxidant capacity. The findings of this study therefore unravelled key molecular mechanisms underlying the biostimulant-induced drought tolerance in maize plants, providing insights into biostimulant-based priming and growth promotion.

Keywords: Biostimulants, drought stress, metabolomics, MRM, PGPR, *Zea mays*

3.1 Introduction

As echoed in **Chapter 2**, drought remains one of the most severe environmental stresses that reduce crop productivity, hindering agricultural productivity globally. In the past years, drought stress has diminished yields of important cereals by over 10% (Lesk *et al.*, 2016) and it is still the main limiting factor of food production in numerous countries (Lau & Lennon, 2012). Drought negatively affects several crop plants such as maize (*Zea mays* L.), a primary food crop in South Africa, and an economically important cereal crop worldwide (Aslam *et al.*, 2015). Generally, plants have evolved multi-layered defence or resistance mechanisms for survival in the ever-changing environment (**Chapter 2**). Plant adaptation or tolerance against drought stress conditions can be described into three strategies: escape, avoidance and tolerance; and each of these strategies may evolve either as inherent responses that occur independently of drought or can evolve as heritable plastic responses that are dependent on one or more environmental cues (Kooyers, 2015). Drought escape occurs when plants complete their life cycle before drought conditions become severe by efficiently using up the water stored in the soil for growth and development. Conversely, drought avoidance occurs when plants minimise water loss and/or maximise water uptake from the roots by reducing the transpiration rate, increasing root growth, or limiting vegetative growth to circumvent dehydration during drought. Lastly, drought-tolerant plants can endure water deficit by lowering osmotic potential inside the cells and maintaining cell turgor through the accumulation of ions and compatible solutes (Farooq *et al.*, 2009, 2012; Sanders & Arndt, 2012).

Water deficit negatively impacts plant growth and development by inducing an array of changes at molecular and cellular levels, translated into alterations in plant physiology and morphology (Osakabe *et al.*, 2014). These changes include stomatal closure, leaf senescence, decreased net photosynthesis and a decline in photosynthetic pigments, impaired nutrient metabolism and carbohydrate metabolism, reduced nutrient uptake, translocation and respiration and ultimately reduced yield production (Lawlor & Cornic, 2002; Chaves *et al.*, 2003; Farooq *et al.*, 2009; Agustí *et al.*, 2012) (**Figure 3.1**). The extent of these deleterious effects of drought stress on plant growth and development is highly dependent on the intensity and duration of the stress. At the molecular level, drought stress induces the accumulation of abscisic acid (ABA) which, in turn, induces drought-responsive genes *via*

different ABA pathways (Shinozaki & Yamaguchi-Shinozaki, 2007). To overcome these deleterious effects of drought stress on plants, several strategies have been employed. For the past decade, researchers relied on molecular tools and conventional breeding programs for genetics improvement to enhance environmental stress tolerance of different plant genotypes (Cattivelli *et al.*, 2008); however, these techniques have limitations such as cost, and the controversy over genetically modified crops. Additionally, the use of fertilisers, fungicides and pesticides for environmental stress management pose negative effects on both the environment and the consumers. Consequently, alternative approaches that are environmental-friendly are considerably imperative at present time.

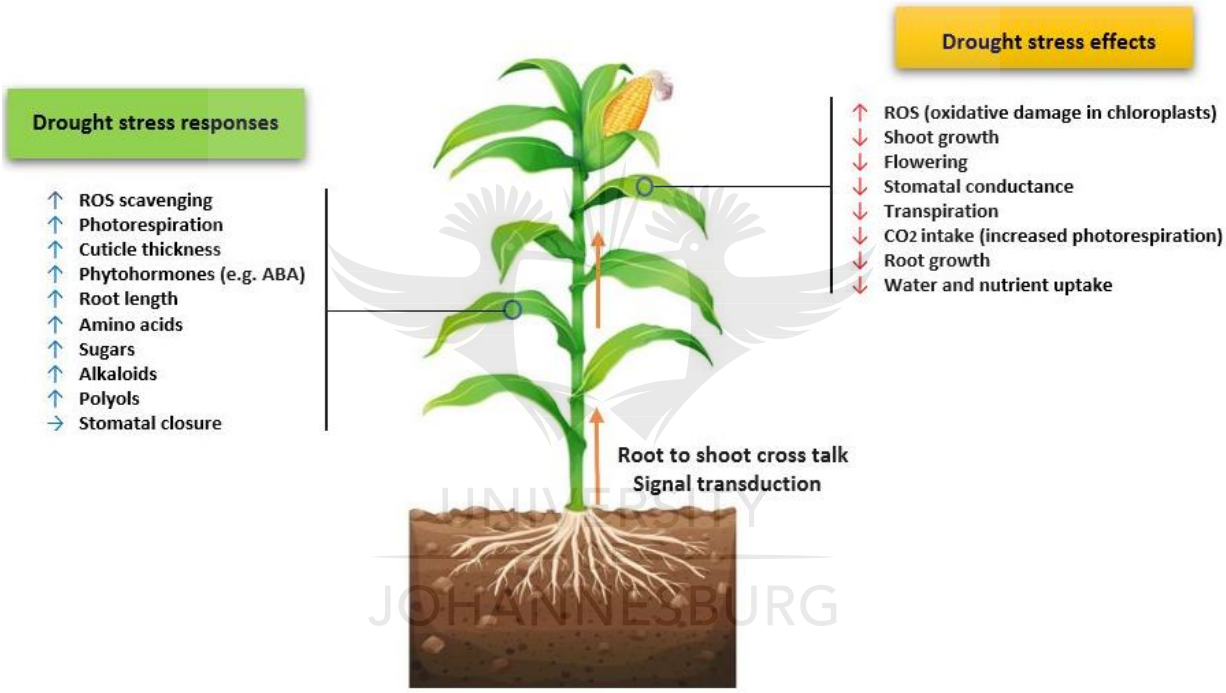

Figure 3.1: **A schematic model showing some of the drought stress effects and plant responses**. Drought stress affects multiple plant growth processes and to counteract these effects, plants display a wide range of defence mechanisms which mediate plant growth and development (Generated by the author of this dissertation).

As mentioned in **Chapter 2** (**section 2.3.1**), recently, attention has been drawn to the use of beneficial microbes that mediate drought tolerance; and the application of plant growth-promoting rhizobacteria (PGPR) to induce abiotic stress tolerance is being extensively studied as an attractive approach to control plant stress (Conrath *et al.*, 2006; Kasim *et al.*, 2013). The ability of microbes to mitigate drought stress was first reported by Timmusk & Wagner (1999) in which *Arabidopsis thaliana* inoculated with *Paenibacillus polymyxa* PGPR under

drought stress were found to be tolerant to subsequent drought stress challenges. Following this, various groups have reported on how PGPR can alleviate drought stress and induce stress tolerance (Arvin *et al.*, 2012; Lim & Kim, 2013; Yogendra *et al.*, 2015; Barnawal *et al.*, 2017; Rubin *et al.*, 2017; Mutumba *et al.*, 2018; Jochum *et al.*, 2019; Khan *et al.*, 2019b; Lin *et al.*, 2020). PGPR are diverse in their chemical and physical mechanisms of promoting plant growth and several mechanisms have been proposed for PGPR-induced drought stress tolerance in various plants. In general, these mechanisms include: (1) production of phytohormones such as abscisic acid (ABA) and indole-3-acetic acid (IAA); (2), 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase to reduce ethylene synthesis; (3) accumulation of osmolytes; (4) exopolysaccharide production and (5) alteration of root morphology. Despite all these mechanisms that have been proposed, a comprehensive and mechanistic understanding of PGPR (or microbial-based biostimulant)–induced mechanisms (at molecular and cellular levels) in enhancing stress tolerance is still not fully understood. As previously mentioned in **Chapter 2** (**section 2.5**), metabolomics, a multidisciplinary omics science, can provide actionable insights defining biochemical mechanisms underlying plant defence responses to abiotic stresses.

Metabolomics has emerged as an indispensable tool that aims to quantify a set of metabolites in an organism at any developmental stage, in each cell type, tissue, or organ (Fiehn, 2001), thus providing the signature of the phenotype. This multidisciplinary omics science has been widely applied in various fields. In plant sciences, one of the domains in which metabolomics has been utilised is to investigate plant responses to environmental stress conditions (Bundy *et al.*, 2009), decoding reconfigurations in both plant primary and secondary metabolism that underlie adaptive mechanisms against abiotic stresses (**Figure 3.2**). Plant primary metabolites are involved in all basic physiological processes that govern growth and development in plants, and they encompass components of processes such as photosynthesis, glycolysis, and the citric acid cycle and associated pathways. These metabolites include amino acids, tricarboxylic acids, sugars, and phytohormones. Conversely, secondary metabolites are synthesised by the plant through precursors derived from the plant primary metabolism (Thirumurugan *et al.*, 2018); and these secondary metabolites are generally involved in plant responses to environmental factors. Profiling of the plant metabolome under stress conditions proposed that pools of different metabolites such as sugars, amino acids, polyols and organic acids play an essential role in drought tolerance (Farooq *et al.*, 2012). For example,

metabolites such as amino acids (proline and glutamine), amines (polyamines and glycine betaine, GB), and γ-amino-N-butyric acid (GABA) can confer osmoprotection under drought stress conditions.

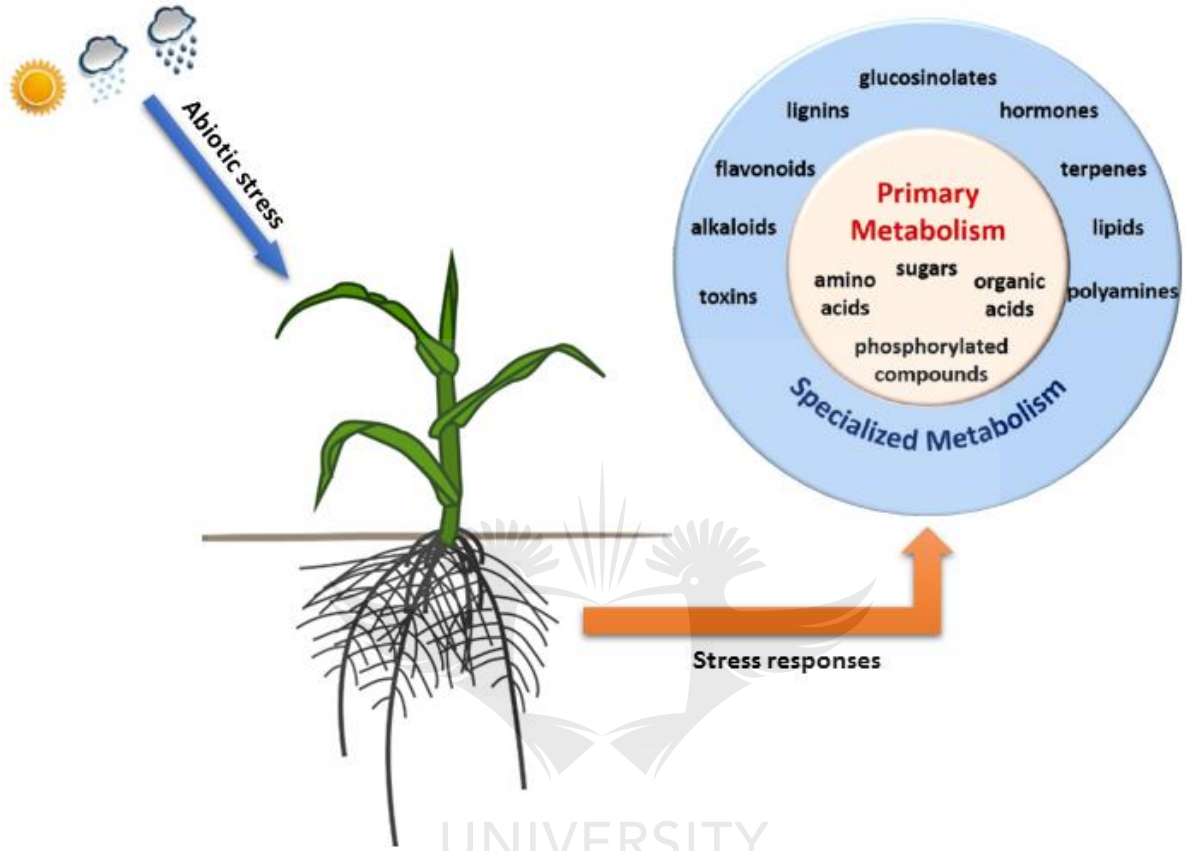

Figure 3.2: **Schematic representation of changes in plant metabolism in response to abiotic stresses**. The primary and secondary metabolism of the plant is reprogrammed upon stress encounter, resulting in stress tolerance and sustained growth. This remodelling of the plant metabolism under stressful conditions spans a wide spectrum of metabolite classes including amino acids, sugars, organic acids, phytohormones, polyamines and flavonoids (modified from Moretti *et al*., 2020).

Targeted metabolomic approaches allow for the analysis of certain groups of metabolites either cognate to a specific metabolic pathway or a class of compounds in a biological sample, providing absolute quantification (Lu *et al.*, 2008; Wei *et al.*, 2010; Begou *et al.*, 2017). Multiple reaction monitoring (MRM) is the most common mode of tandem mass spectrometry employed for targeted metabolomics analysis in which numerous different precursor-product ion pairs are monitored in a single analysis using a triple quadrupole (QqQ)-equipped MS instrument. In this mode, the metabolite precursor ion with a given *m/z* is selected in the first quadrupole (Q1). The precursor ion is then fragmented in the second quadrupole (q2, *i.e.* collision cell) through a process known as collision-activated dissociation

(CAD) or collision-induced dissociation (CID). Lastly, fragmented ions are accelerated into the third quadrupole (Q3), where the product ion with a specified *m/z* is selected and introduced to the detector (Roberts *et al.*, 2012; Courant *et al.*, 2014) (**Figure 3.3**).

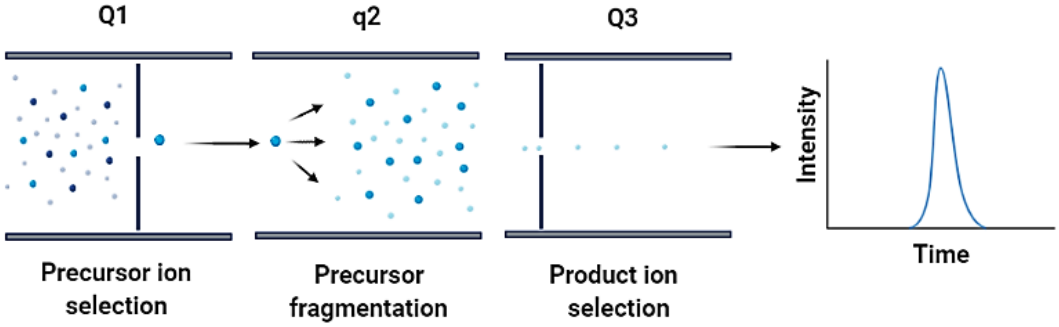

Figure 3.3: **Overview of multiple reaction monitoring (MRM)**. MRM analyses specific precursors producing specific products by holding the precursor and product static at the given *m/z* in the two mass analysers, Q1 and Q3 respectively. Precursor ion fragmentation is induced *via* collision-activated dissociation (CAD) or collision-activated dissociation (CID) in q2 (collision cell), resulting in the product ion formation (Generated by the author of this dissertation).

When coupled with separation techniques such as liquid chromatography (LC), or gas chromatography (GC), MRM-based analyses provide high sensitivity, selectivity, reproducibility, absolute quantification and cover a broad dynamic range of metabolites providing optimal analysis of complex biological samples (Morin *et al.*, 2013). Thus, using liquid chromatography coupled to triple quadrupole mass spectrometry (LC-QqQ-MS)-based approach, the current study aims to investigate quantitative changes in selected metabolite classes – amino acids, phytohormones and phenolics – associated with the application of a microbial-based biostimulant on maize plants under well-watered, mild- and severe drought stress conditions. Such quantitative interrogation of selected metabolites can reveal key molecular events and alterations in plant metabolism, induced by the application of a biostimulant, that point to growth promotion and enhancement of drought resistance in maize. Thus, the study (reported in this **Chapter 3**) contributes towards the generation of a fundamental knowledgebase describing the molecular mechanisms underlying the biostimulant effects on plants. Such insights are necessary for advancement of the biostimulant industry and global food security at large.

3.2 Materials and methods

3.2.1 Chemicals

All the chemicals utilised for sample analyses were of analytical grade, highest purity and were obtained from different international providers. Methanol and acetonitrile were LC-MS grade from Romil (SPS, Cambridge, UK). Leucine enkephalin and formic acid from Sigma Aldrich (Munich, Germany). Water was purified using a Milli-Q Gradient A10 system (Siemens, Fahrenburg, Germany).

3.2.2 Plant Materials, Growth conditions and Treatments

Maize (*Zea mays* L.) plants, PAN 3Q-240, were cultivated in 15 L pots (8 seeds per pot), each filled with slightly acidic (pH 5.2) sandy soil (17 kg). The pots were placed in a completely randomised design (CRD) order in a greenhouse at Omnia facilities in Sasolburg, Free-State, South Africa. An experimental study design was developed in which all different conditions (control and treated), were described as treatment (T) (**Table 3.1**). The control groups were represented by T2 (well-watered without PGPR), T5 (mild drought without PGPR) and T6 (severed drought without PGPR); and the treated groups were represented by T1 (well-watered with PGPR), T3 (mild drought with PGPR), T4 (severe drought with PGPR). However, for the rest of the chapter, they will be referred to as control (C), mild drought without PGPR (MD), severe drought without PGPR (SD), well-watered with PGPR (PGPR), mild drought with PGPR (MD-PGPR) and severe drought with PGPR (SD-PGPR) respectively (**Table 3.1**).

Table 3.1: Description of treatment conditions used to study the effect of PGPR-based biostimulant application on mild and severe drought stressed plants.

Treatment	PGPR rate of 2 billion cfu per ml (L ha^{-1})	Treatment description
T1	2	Well-watered with biostimulant (**PGPR**)
T2	0	Well-watered without biostimulant (Control; **C**)
T3	2	Mild drought with biostimulant (**MD-PGPR**)
T4	2	Severe drought with biostimulant (**SD-PGPR**)
T5	0	Mild drought without biostimulant (**MD**)
T6	0	Severe drought without biostimulant (**SD**)

A PGPR-based biostimulant formulation (Omnia Group Ltd, Bryanston, South Africa) containing five *Bacillus* strains was used in this study. The formulation comprised of one strain of *Bacillus amyloliquefaciens*, two strains of *Brevibacillus laterosporus* and two strains of *Bacillus licheniformis* (Omnia Group Ltd, South Africa); however, the comprehensive description of how the formulation was prepared cannot be disclosed in this dissertation due to the Omnia trademark and commercialisation purposes. The PGPR-based biostimulant treatment diluted 100 times to 8 mL per pot was evenly applied at a rate of 2 L per Hectare at planting stage (**Figure 3.4**) using a micropipette in the furrow with the seed. Following the emergence, the 8 seedlings cultivated per pot were thinned to five plants per pot, by selecting uniform and healthy plants.

Each pot was considered as a biological replicate and contained five plants at the harvesting time. Four biological replicates (i.e. four pots) per treatment were harvested at each time point (**section 3.2.3**). All pots were irrigated to 90% plant available water (PAW) to allow for good germination. Drought stress was imposed at the 2-leaf stage (2 weeks after emergence, WAE) by a withholding water method where the water level was allowed to drop to 50% PAW then maintained at that level for the mild drought stress group, and dropped to 20% PAW for the severe drought conditions. The well-watered plants were maintained at the 90% PAW throughout the study (**Figure 3.4**). Greenhouse conditions that were measured daily include temperature (midday, 28 ± 3 °C and night 12 ±2 °C), relative humidity (45 ± 8%) and (midday) light intensity (738 ± 41 µmole $m^{-2}s^{-1}$).

3.2.3 Plant material harvesting

Leaf tissue harvesting for all treatments and biological replicates (**Table 3.1**; **subsection 3.2.2**) was performed at two different time points; four and six weeks after emergence (WAE) and following mild and severe drought application referred to as (4 WAE and 6 WAE respectively) (**Figure 3.4**). The plant leaves were cut off and rapidly immersed in liquid nitrogen to quench any enzymatic reactions that can result in metabolite fluctuations caused by the handling and wounding of the plant during harvesting. This was then followed by the storage of the leave material at −20 °C prior to morphophysiological analysis and metabolite extraction.

Figure 3.4: **Cultivation and different growth stages of maize plants.** PGPR treatment was applied at planting stage, and mild drought and severe drought stress initiated at 2 weeks after emergence. Harvesting was done at two time points, 4 and 6 weeks after emergence (Generated by the author of this dissertation).

3.2.4 Morphophysiological analysis

3.2.4.1 H_2O_2 content

The H_2O_2 content was assayed according Brennan and Frenkel (1977). One hundred mg of chilled leaf tissue was macerated in 4 mL cold acetone and the homogenate was filtered through a Whatman No. 1 filter paper. Two mL of this filtrate were treated with 1 mL of titanium reagent (20% titanium tetrachloride in concentrated HCl, 32% v/v) and 1 mL of concentrated ammonia solution to precipitate the titanium-hydroperoxide complex. After centrifugation (at 5000 × g for 30 min) the precipitate was dissolved in 2 N H_2SO_4 and the absorbance was obtained at 415 nm. The H_2O_2 content was calculated from a standard curve prepared in a similar way and expressed as μmol.g^{-1} fresh mass (fm).

3.2.4.2 Total ascorbate content

The ascorbate (AsA) content was assayed according to the method described by Hodges *et al.*, (1996). To determine the total ascorbate content, 200 µL of the supernatant (from homogenization of 5 g of fresh weight leaf tissue and centrifuged) was added to 500 µL of a 150 mM K_2PO_4 buffer solution (pH 7.0) containing 5 mM EDTA and 100 µL of 10 mM di-thiothreitol (DIT) to reduce DHA to AsA. The reaction was allowed to continue for 15min, after which 100 µL of a 0.5% N-ethylmaleimide solution was added to the reaction mixture at 25 °C to quench the excess DIT. The solution was colored by adding 400 µL of a 44% o-phosphoric acid solution, 400 µL of a 10% trichloroacetic acid (TCA) solution, 200 µL of 30 $g.L^{-1}$ $FeCl_3$ solution and 400 µL of a,a'-dipyridyl in 70% (v/v) ethanol solution. The solution was kept at 40 °C for 60 min after which the absorbance at 525 nm was measured spectrophotometrically. The concentration was estimated by using a standard curve.

3.2.4.3 Malenoaldehyde content

Malenoaldehyde was measured spectrophotometrically using the thiobarbituric (TBA) method according to Dhindsa *et al.* (1981). A volume of 2 ml of the extract was added to a solution containing 1 mL of a 20% trichloroaceticacid (TCA) and 0.5% thiobarbituric (TBA). The mixture was heated in a water bath at 95 °C for 30 min. The solution was allowed to cool to room temperature and centrifuged at 14,000 rpm for 10 min. The absorbance was read at 532 nm and the non-specific absorbance at 600 nm was subtracted from the measured absorbance value. The MDA content was calculated by using an extinction coefficient of 155 mM^{-1} cm^{-1}.

3.2.4.4 Extraction of antioxidant enzymes

Frozen (−80 °C) leaf tissue (0.5 g) was homogenized in 1.5 mL of a 50 mM potassium phosphate buffer (PBS, pH 7.8) containing 1 mM EDTA, 1 mM di-thiotreitol (DIT) and 2% (w/v) polyvinylpyrrolidone (PVP) using a chilled mortar and pestle kept on ice. The homogenate was centrifuged at 15,000 ×g at 4 °C for 30 min. The clear supernatant was used for superoxide dismutase enzyme assays. For measuring ascorbate peroxidase activity, the tissue was separately ground in 50 mM PBS (pH 7.8) supplemented with 2 mM ascorbate, 1 mM EDTA, 1 mM DIT and 2% (w/v) PVP. All assays were done at 25 °C.

3.2.4.5 Enzyme analysis

3.2.4.5.1 Ascorbate peroxidase (APX) activity

Ascorbate peroxidase (APX) (EC 1.11.1.11) was assayed according to Nakano and Asada (1981). This was done by taking 3 mL of a reaction mixture (described above) containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2 and 0.1 mL enzyme extract and following the hydrogen peroxide-dependent oxidation of ascorbate by measuring the decrease in the absorbance at 290 nm (E = 2.8 mM^{-1} cm^{-1}). Ascorbate peroxidase activity was expressed as µmol ascorbate oxidized.$min^{-1}mg^{-1}$ protein.

3.2.4.5.2 Superoxide peroxidase (SOD) activity

Superoxide dismutase (EC 1.15.1.1) activity was assayed using the kit (A001-1) provided by Elabscience, Total superoxide dismutase (T-SOD) activity assay kit, WST-1 method, which is based on the method described by Beyer and Friedovich, (1987). One unit of SOD activity was defined as the amount of enzyme required for 1 mg tissue proteins in 1 ml of a reaction mixture to raise SOD inhibition rates to 50% at 550 nm, expressed as µg.mg^{-1} protein.

3.2.4.5.3 Catalase (CAT) activity

Catalase (EC 1.11.1.6) activity was assayed using an assay kit provided by Elabscience, CAT-activity kit. Catalase activity was estimated as the amount of enzyme that decomposes 1 µmol H_2O_2 at 405 nm sec^{-1} in 1 mg fresh tissue proteins, expressed as µg.mg^{-1} protein. During stress, a plant will show an increase in the non-enzyme compounds such as well as the activity of detoxification enzymes. If the stress is not too severe the natural antioxidant systems will be able to keep the oxidant species to a level where the damage to cellular structures is not severe to an extent that leads to large scale cell death. The severity of cellular damage can be followed by analysing the breakdown products of lipid membrane components. A popular marker that is used is malenoaldehyde (MDA) concentration. An increase in malenoaldehyde concentration is an indication of increase lipid peroxidation, which is indicative of increase membrane lipid damage (Yanling *et al*., 2015).

3.2.5 Extraction of metabolites

Extraction of metabolites was initiated by adding liquid nitrogen to the frozen leave tissues and grinding them into a fine powder using a pestle and mortar. To avoid any chance of sample crossover, the pestle and mortar were cleaned (washed using dH_2O and rinsed with 80% aqueous methanol) between samples. Following this, two grams (2 g) of the powder was weighed in a sterile Falcon tube and 20 mL of 80% cold methanol was added in a 1:10 m/v ratio. The mixture was then homogenised for 2 min using an Ultra-Turrax homogeniser and sonicated for 30 s using a probe sonicator (Bandelin Sonopuls, Germany) set at 55% power. The homogenizer and the probe were cleaned with 80% aqueous methanol between samples to avoid sample crossover. The resulting homogenates were centrifuged at 5100 rpm for 20 min at 4 °C. The supernatants were placed in 50 mL round-bottom flasks, evaporated to 1 mL at 55 °C using a Büchi Rotavapor R-200, and dried to completeness with a speed vacuum concentrator (Eppendorf, Merck, South Africa) set at 45 °C. Resuspension of the extracts was done using 500 µL LC-MS grade methanol : Milli-Q water (1:1, v/v), followed by filtration through 0.22 µm nylon filter into pre-labelled HPLC glass vials fitted with 500 µL inserts (Shimadzu, South Africa). The filtered samples were then stored at 4 °C pending LC-ESI-QqQ-MS analysis.

3.2.5.1 Preparation of standards and multiple reaction monitoring (MRM) method development

Amino acid -, phytohormone -, flavonoid - and phenolic acid standards used in this study were of ≥98% purity, obtained from Merck (Germany), Sigma (United States of America) and BDH (England) manufacturers. Thirty-eight metabolites including internal standard (D-flourophenylalanine) were selected and quantified, and these include amino acids, osmolytes, phytohormones, flavonoids and phenolics (**Tables S3.1**). The amino acids, phytohormones and osmolytes, flavonoids and phenolics working solutions were over the concentration ranges of 25–1000 µg/L, 43.7-8.7×10^{-5} nM, 10–1000 µg/L and 7.78-250 nM respectively. The working solutions were all prepared in 50% aqueous methanol (Romil, Cambridge, UK) and stored at 4 °C. The analysis was performed using a triple quadrupole mass spectrometry (LCMS-8050 (Shimadzu, Kyoto, Japan)), equipped with an electrospray ionisation (ESI) source and ultra-fast liquid chromatography (UFLC) as a front-end. The MRM-MS method

was used for absolute quantification of the targeted metabolite classes. MRM-MS conditions (**Table S3.1**) were developed and optimisation was done by direct infusion into the ionisation source (ESI); and the MRM optimisation method tool (an integral component of LabSolutions LCMS software, Shimadzu Corporation) was used for collision energy (CE) optimisation for all the transitions, by collecting product ion scan data and finding the optimum CE for each transition.

3.2.5.2 LC-ESI-QqQ-MS metabolite profiling by ultra-fast liquid chromatography

The prepared samples and standards as described in **sections 3.2.3** and **3.2.4** were analysed on the ultra-fast liquid chromatography (UFLC) system, equipped with a Shim-pack GIST C18 column (2 µm; 100 × 2.1 mm l.D) (Shimadzu, Kyoto, Japan), thermostatted at 40 °C. Chromatographic separation was achieved using a gradient elution system consisting of eluent A (MilliQ water with 0.1% formic acid) and eluent B (methanol with 0.1% formic acid) (Romil Chemistry, UK) at a constant flow rate of 0.2 mL min^{-1}. Each metabolite class (amino acids, phytohormones, flavonoids and phenolics) had a specific elution gradient (**Table 3.2**).

Table 3.2: Stepwise gradient elution profile for amino acids, phytohormones, flavonoids and phenolic acids with MilliQ water and 0.1% formic acid (eluent A), and methanol with 0.1% formic acid (eluent B).

Amino acids		
Time (min)	**Mobile phase A (%)**	**Mobile phase B (%)**
1	98	2
2	95	5
2	90	10
2	50	50
1	98	2
Phytohormones		
3	98	2
3	90	10
24	80	20
8	95	5
2	98	2
Flavonoids		
2	70	30
10	70	30

18	95	5
1	98	2
Phenolic acids		
3	95	5
3	80	20
8	70.2	20.8
3	50	50
7	70	30
5	95	5

The total chromatographic run time was 10, 40, 31 and 30 min; and injection volume 3, 1, 2, and 3 µL for amino acids, phytohormones, flavonoids and phenolic acids, respectively. The MRM-MS detection parameters developed and optimised as described in **section 3.2.4** (**Table S3.1**) were then applied, and the MS conditions were as follows: nitrogen gas was used as a drying gas and a nebulising gas at flow rates of 10 L min^{-1} and 3 L min^{-1} respectively. The heating gas flow was set at 10 L min^{-1}, interface temperature at 300 °C, interface voltage at 4 kV, DL temperature at 250 °C, and heat block temperature at 400 °C.

3.2.5.3 Data Analysis: Processing, pre-treatment and chemometric analysis

LabSolution Quant Browser™ (Shimadzu, Kyoto, Japan) was used to process the LC-MRM-MS data acquired, from which the calibration curves were constructed to obtain the concentrations of the unknown samples expressed in ppb (for amino acids and phenolics) and nM (for hormones and flavonoids), which were then converted to ng/g to create a concentration data matrix. MetaboAnalyst 4.0 (Chong *et al.*, 2018), a comprehensive web-based tool, was used for processing, analysing, visualising and interpreting the data. Prior to data analysis, MetaboAnalyst performs a data integrity check by assessing the data labels (class and concentration values), pair specifications, and detecting the presence of missing values or features using its integral algorithms. The tool has a default method of replacing missing values using small numbers (one-fifth of the minimum positive values of their corresponding variables in the data) which assumes that the missing values are a result of low signal intensity metabolites that are below the detection limit; however, no values were replaced in this study. Following missing values replacement is the data filtering option which aims to identify and remove low-quality data points that have an improbable contribution to the modelling of the data, thus improving performance and reducing the false

discovery rate (FDR) for downstream statistical analysis (Hackstadt & Hess, 2009; Bourgon *et al.*, 2010). Data filtering is highly recommended for large datasets (untargeted analysis) due to the large amount of noise present in the data (Xia *et al.*, 2012a) and often omitted for quantitative datasets, therefore it was not performed for this particular dataset.

Subsequent to the data integrity check, data normalisation, a data pre-treatment method was applied. The selected pre-treatment methods which were deemed appropriate for metabolite concentration adjustment in this study were transformation and Pareto scaling with no row-wise normalisation. Data analysis was performed using chemometric analysis employed by MetaboAnalyst 4.0 collection of statistical and machine learning algorithms that are highly robust for multidimensional data analysis.

Initially, unsupervised multivariate statistical methods such as principal component analysis (PCA) was performed to explore the structure of the data (trends, groupings), allowing the identification of any similarities or differences between and within the samples. Secondly, supervised multivariate methods including partial least squares – discriminant analysis (PLS-DA) and orthogonal partial least squares – discriminant analysis (OPLS-DA) were then performed, driven by the knowledge obtained from the unsupervised analysis. From these, important features (metabolites) that discriminate the compared groups (*e.g.* control *vs.* treated) were selected using variable importance in projection (VIP) scores and loadings S-plots respectively. Since PLS-DA and OPLS-DA are prone to data over-fitting, the computed chemometric models were validated using a 10-fold cross-validation (CV) method and a permutation test, where only statistically significant models were used in data mining. The former was applied as a tuning procedure during computation and expressed with Q^2 metrics as measured performance (predictive ability of the model) and the latter as an estimation of the distribution of the performance measure between the data (*X*) and the newly permuted class labels (*Y*). Lastly, for quantitative analysis and biological interpretation, hierarchical cluster analysis (HCA) was performed and (Pearson's correlation distance measure) visualised using heatmaps, boxplots, bar graphs, metabolite-metabolite correlation analysis (MMCA) and pathway analysis using MetaboAnalyst 4.0. Additionally, radar plots constructed using Microsoft excel were used to show the calculated logarithmic fold changes from the absolute quantification of each metabolite in different targeted classes, where the control group in each plot is set at zero.

3.3 Results and discussion

As highlighted in **Chapter 2** and **section 3.1** of this **Chapter**, adaptation or resistance to abiotic stresses like drought is an important acquirement of agriculturally relevant crops like maize. Hence, development of strategies, such as the exploration and application of microbial-based biostimulants, to enhance plant resistance or tolerance to drought stress is imperatively essential. However, for the realisation of sustainable, efficient and innovative biostimulant formulations, decoding molecular choreography and cellular events that define the complex and multi-layered mechanisms of PGPR-based biostimulants, for growth promotion and defence priming, is a necessary step. Hence, the work presented in this **Chapter 3** is a scientific effort to contribute to understanding microbial biostimulant-induced physiological and metabolic reprogramming that gravitates towards plant growth promotion and enhancement of drought tolerance in maize plants. Thus, this section which articulates the findings of this metabolomics study (**Chapter 3**) is subdivided into three main subsections: **(i)** morphophysiological changes regarding PGRP treatment under well-watered and drought stress conditions, (**ii**) a quantitative metabolome map correlated to growth promotion and priming effects of PGPR-based biostimulant, and (**iii**) a post-challenge metabolic landscape of maize treated with PGPR under mild and severe drought stress conditions.

3.3.1 Morphophysiological changes in naïve and PGPR-treated maize plants under well-watered and drought stress conditions

Drought-induced changes in the plant metabolism are highly complex, coordinated and translated at various cellular and organismal levels. Some of the drought-induced physiological changes are related to oxidative stress. The latter is mostly due to decreasing stomatal conductivity that confines CO_2 influx into the leaves. This leads to the formation of ROS, the excess of which has deleterious effects on plant cells *via* oxidation of cellular components such as proteins, DNA and lipids (Mittler, 2002). The physiological responses to this drought-induced harmful ROS comprise an array of antioxidant machinery (**Table 3.3**) involving non-enzymatic elements (*e.g.* AsA, MDA) and an enzymatic component (*e.g.* CAT, SOD and APX).

Table 3.3: Selected physiological stress markers of the plant cellular milieu. Different letters indicate statistically significant differences between treatments; $p \leq 0.05$.

Level / Concentration	Without PGPR			With PGPR		
	Control	MD	SD	Control	MD	SD
Non-enzymatic stress markers (µmol. g FW^{-1})						
Leaf H_2O_2	61.7^{a}	91.2^{a}	137.9^{b}	56.5^{a}	79.7^{a}	101.3^{ab}
Leaf malondialdehyde (MDA)	25.3^{a}	34.6^{a}	63.9^{b}	23.7^{a}	28.9^{a}	42.1^{c}
Leaf ascorbate (AsA)	227.5^{a}	329.3^{b}	598.1^{c}	241.1^{a}	409.7^{d}	634.8^{c}
Enzyme activities (µmol. $min^{-1}.mg^{-1}$ protein)						
Superoxide dismutase (SOD)	54.7^{a}	67.9^{b}	89.8^{c}	61.2^{ab}	91.9^{c}	118.6^{d}
Ascorbate peroxidase (APX)	40.4^{a}	46.7^{b}	59.8^{c}	43.6^{ab}	49.8^{b}	72.7^{d}
Catalase (CAT)	4.1^{a}	7.6^{b}	11.5^{c}	4.4^{a}	8.2^{b}	15.4^{d}

From the results, it is clear that the maize plants employed both non-enzymatic and enzymatic strategies to combat the osmotic (drought) stress conditions. The severe drought stress treatment had a much more significant impact on the stress biomarkers, which is to be expected. Apart from the leaf H_2O_2 and MDA concentration in the moderate drought stress treatment, all of the other biomarkers increased significantly ($p \leq 0.05$) relative to the control (unstressed) treatment. This points to the fact that the plants were still managing the moderate stress, with no significant damage to cellular structures. Under the severe stress treatment, the MDA concentration increased 2.5-fold (**Table 3.3**), which indicates significant damage to membrane structures.

PGPR have been identified and studied extensively during the last few decades, and recent studies suggest that these organisms can help plants to cope with drought stress (Yang *et al.*, 2016). The physiological parameters analysed during this current study revealed that one mechanism by which this is achieved is the influence of PGPR on the antioxidant capacity of plants. As mentioned earlier, plants employ enzymatic and non-enzymatic strategies to help fight oxidative stress. Various studies including the studies by Khan *et al.*, (2019b) and Yang *et al.*, (2016), have shown that PGPR have a positive effect on the antioxidant capacity of various plant species. During this study, we found that the PGPR consortium used had a positive influence on both the enzymatic and non-enzymatic strategy of *Zea mays* plants

during drought stress conditions. This was apparent under moderate and severe drought stress conditions (**Table 3.3**).

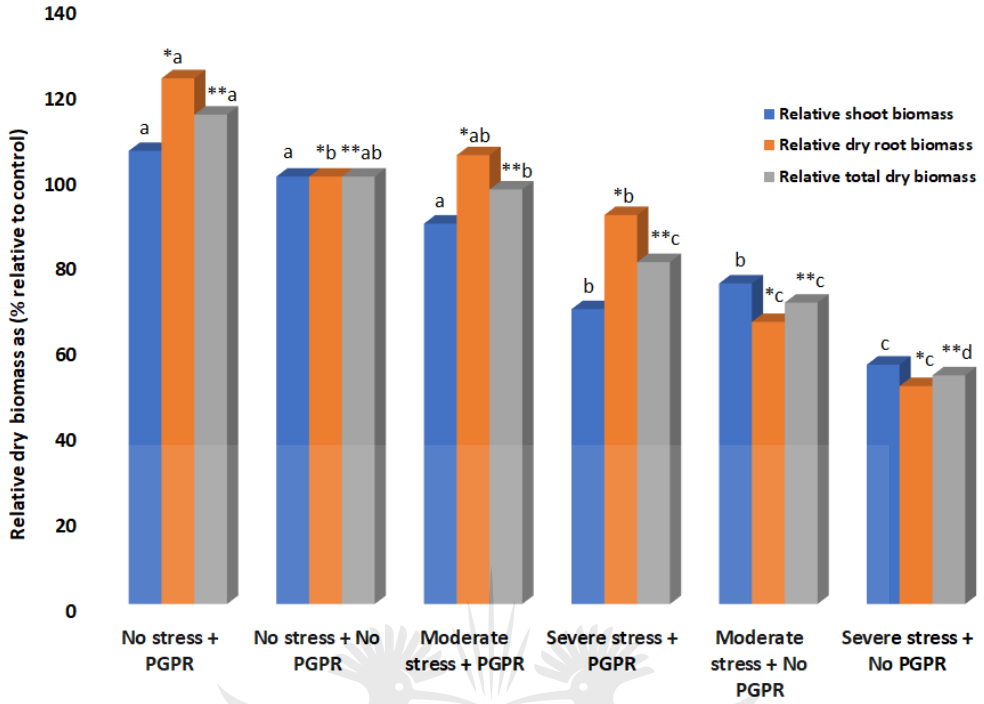

Figure 3.5: **Morphological changes**: Relative shoot, root and total dry biomass of the various treatments. Treatments containing the same letters are not significantly different from one another ($p \leq 0.05$); LSD dry shoot biomass = 24.1, *LSD dry root biomass = 20.8, **LSD total dry biomass = 16.7.

When comparing PGPR-treated plants with non-treated plant in terms of the popular stress marker, leaf H_2O_2, the PGPR consortium did not have a statistically significant ($p \leq 0.05$) effect on the leaf H_2O_2 concentration for moderately drought stress maize plants, but plants subjected to severe drought stress showed a significant ($p \leq 0.05$) reduction (27%) in leaf H_2O_2 concentration (**Table 3.3**). The decrease in H_2O_2 concentration also led to a significant ($p \leq 0.05$) decrease (35%) in MDA concentration for the severe drought stressed plants, suggesting that significantly less damage to membranes, lipids and proteins were encountered in PGPR drought stressed plants. The enzymatic detoxification processes in the plants also showed marked improvement when the plants were treated with the PGPR consortium. The PGPR caused significant ($p \leq 0.05$) increases in the SOD activity of 35% and 32% over the non-PGPR treated moderately and severely stressed plants, respectively (**Table 3.3**). The APX and CAT activity was also significantly ($p \leq 0.05$) increased in the PGPR-treated severely stressed plants, with respective increases of 21% and 34% over non-PGPR-treated severely stressed plants (**Table 3.3**).

The increase in cellular detoxification led to significantly more biomass accumulation in the PGPR-treated plants (**Figure 3.5**). This is to be expected due to the decrease in damage to membranes, lipids, proteins *etc*. According to the results, the PGPR-treated moderately stressed plants showed a 19%, 59% and 38% increase in the shoot, roots and total dry biomass, respectively. The severely stressed plants also showed a 23%, 78% and 49% increase in the shoot, roots and total dry biomass, respectively (**Figure 3.5**). All of these increases were significant at $p \leq 0.05$. These morphophysiological changes phenotypically demonstrate the effects of PGPR in enhancing drought-related defence responses resulting in enhanced growth promotion and stress tolerance. At the molecular level, these phenotypic reflections of PGPR effects are defined by PGPR-induced reprogramming of the primary and secondary metabolism in maize plants under naïve (well-watered) and drought stress conditions.

3.3.2 PGPR-induced reprogramming in primary and secondary metabolism for growth promotion and defence sensitisation

To understand and further explain these phenotypically observed PGPR-induced effects related to growth promotion and enhancement of maize responses to drought stress conditions (**section 3.3.1**), the maize metabolism was interrogated. The targeted approach applied (**subsection 3.2.5**) offered opportunities to quantitatively examine a selected chemical space of primary and secondary metabolism, defined by amino acids, phytohormones, flavonoids and phenolic acids (**Figure 3.6**).

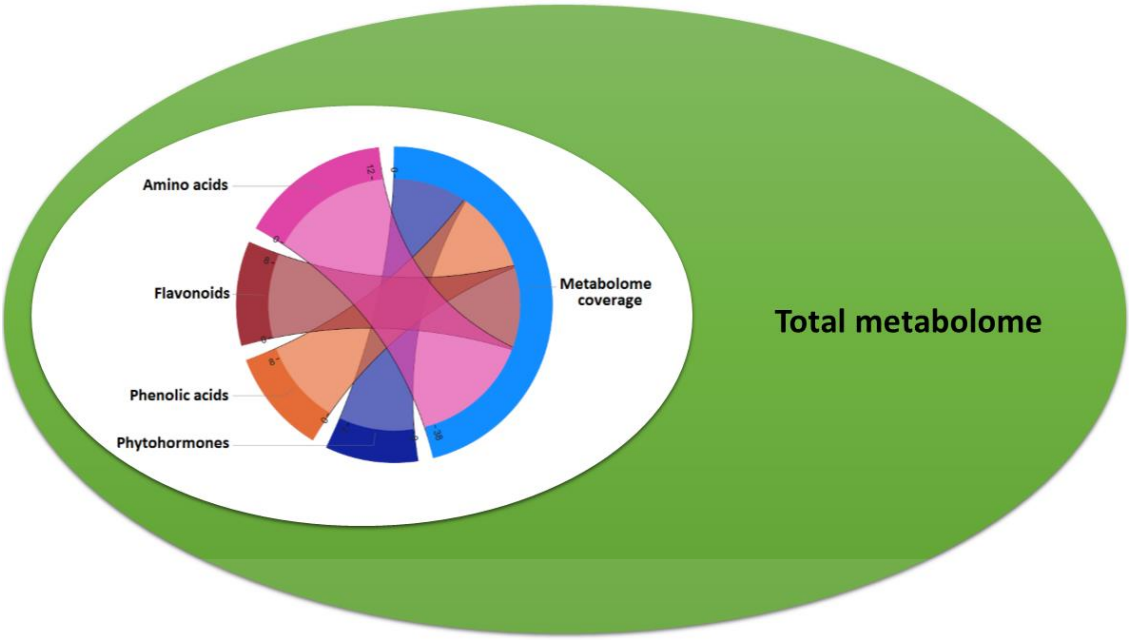

Figure 3.6. Metabolome coverage: A Chord diagram showing metabolites classes targeted in this study. The diagram displays infographically the fraction of the metabolome (metabolite classes extracted from the different treatments) covered in this study, situating the studied metabolome in the 'undefined dimension' of the maize's chemical space.

Previous studies (Touraine *et al.*, 2013; Saia *et al.*, 2015; Ahanger *et al.*, 2018; Khan *et al.*, 2019a; Tugizimana *et al.*, 2019; Mhlongo *et al.*, 2020) utilising untargeted metabolomics approaches have demonstrated/revealed that one of the key characteristics of plant growth promotion and priming induced by microbial biostimulants is a reconfiguration of plant metabolism that spans a wide range of (bio)chemistries entailing amino acids, phytohormones and secondary metabolites. Thus, to further zoom-in into the reported hypothetical metabolomic reprogramming in response to PGPR application leading to enhanced plant growth and priming, a targeted metabolomics approach was employed in the current study, covering a fraction of the maize metabolome (**Figure 3.6**), with diverse biological functionalities. Targeted metabolomics provide a more quantitative evaluation of the pre-defined groups as a confirmation and elaboration for the novel findings obtained from untargeted analysis (Alonso *et al.*, 2015).

3.3.2.1 PGPR-induced alterations in amino acids and phytohormones levels – growth promotion and priming

Chemometrics models, PCA and PLS-DA, by summarising the variation in multivariate space into a smaller number of latent components, revealed distinct treatment-related sample groupings (**Figures 3.7** and **S3.1**) and allowed the description of differential quantitative metabolic changes in naïve (T2) and PGPR-treated (T1) plants. These models revealed that PGPR induced differential metabolomic changes in non-stressed plants as infographically depicted in a distinct separation in the two groups (**Figure 3.7A**). As previously mentioned (**section 3.2)**, targeted analysis was performed at two different time points (4 and 6 WAE). It is worth noting that PLS-DA modelling revealed a clear distinction of the two time points separated in the first component and the naïve and PGPR-treated groups (T1) were clearly separated in the second component. The use of OLPS-DA loading S-plots and variable importance in projection (VIP) plots (**Figure 3.7B**) further allowed the extraction of statistically significant variables driving the differentiation between naïve plants and PGPR-treated plants.

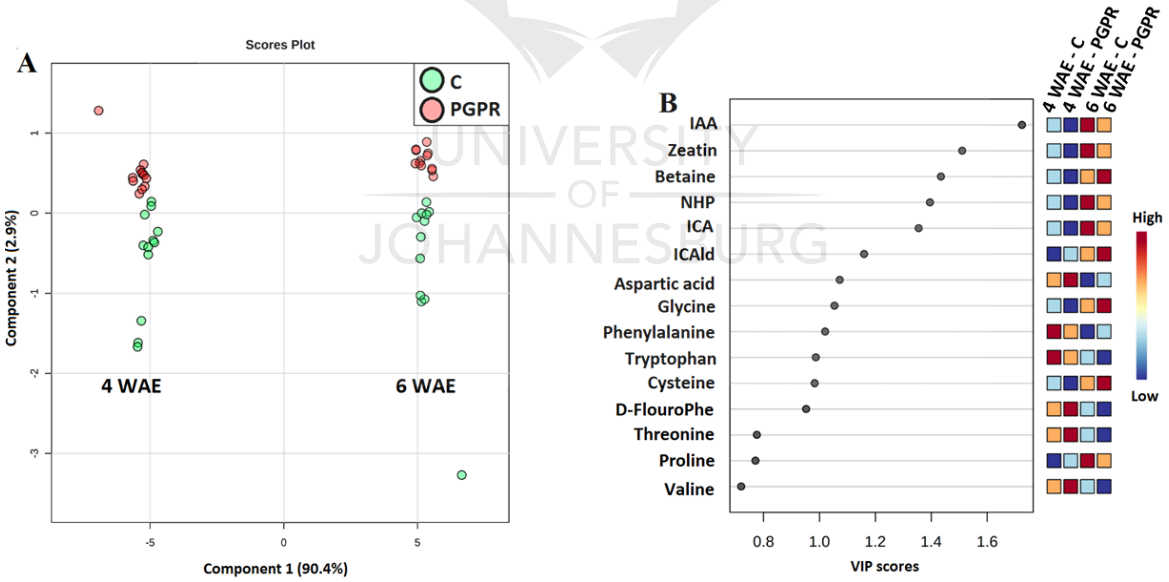

Figure 3.7. Models describing the comparative quantification analysis of amino acid and phytohormones. (**A**) Partial least squares-discriminant analysis (PLS-DA) score plot. (**B**) Variable importance projection plot showing the discriminant metabolite (VIP > 1) responsible for the class separation. **Abbreviations**: C, control; PGPR, refers to PGPR-treated plants; WAE, weeks after emergence; IAA, Indole-3-acetic acid; NHP, N-Hydroxyethylpthalimide; ICA, Indole-3-carboxylic acid; and ICAld, Indole-3-carboxyaldehyde.

Overall differential changes of amino acid, phytohormone and betaine (an osmolyte) profiles induced by PGPR in non-stressed plants are represented by radar plots (**Figure 3.8**) at 4 and 6 WAE. These infographics depict the logarithmic fold changes of the measured metabolites, reflecting quantitative changes associated with the PGPR treatment in maize plants, pointing to PGPR-induced reprogramming of primary metabolism. Hierarchical clustering heatmap analysis allowed for the visualisation of these quantitative changes of both phytohormones and betaine (**Figure 3.9A**) and amino acids (**Figure 3.9B**) between the two comparisons (C and PGPR).

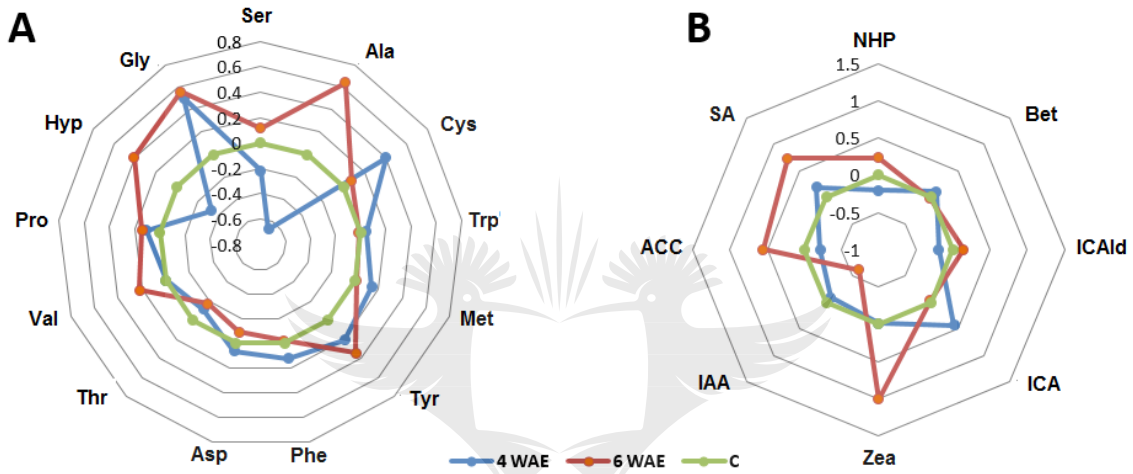

Figure 3.8: **Radar plots showing quantitative ratio changes of amino acids, phytohormones and osmolyte profiles under well-watered conditions with and without PGRP treatment**. Plots show the calculated logarithmic fold changes from the quantification of each metabolite. **(A)** Amino acid and **(B)** phytohormone and Betaine profile from well-watered conditions (T2) and well-watered conditions with PGPR (T1) showing metabolite changes at 4 WAE and 6 WAE. **Abbreviations**: Ser, Serine; Gly, Glycine; Hyp, Hydroxyproline; Pro, Proline; Val, Valine; Thr, Threonine; Asp, Aspartic acid; Phe, Phenylalanine; Tyr, Tyrosine; Met, methionine; Trp; Tryptophan; Cys, Cysteine; Ala, Alanine; NHP, N-Hydroxyethylpthalimide; SA, Salicylic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; IAA, Indole-3-acetic acid; Zea, Zeatin; ICA, Indole-3-carboxylic acid; ICAld, Indole-3-carboxyaldehyde; Bet, Betaine.

Quantitative measurement of selected phytohormones showed indole-3-carboxylic acid (ICA), salicylic acid (SA), indole-3-carboxaldehyde (ICAld), 1-aminocyclopropane-1-carboxylic acid (ACC), N-hydroxyphthalimide (NHP), indole-3-carboxylic acid (ICA) and zeatin (Zea) to be signatory makers of PGPR treatment in maize plants (**Figure 3.7B** and **Figure S3.2**). PGPR treatment revealed an accumulation of phytohormones; SA, and ICA at 4 WAE and Zea, ACC and SA at 6 WAE (**Figure 3.9A**). Primary metabolites such as carbohydrates, amino acids, fatty acids, phytohormones and organic acids are considered as imperative for plants in their growth and development including biological processes such as

respiration and photosynthesis. Phytohormones are signalling molecules produced in minuscule quantities that also regulate every aspect of plant growth and development, as well as adaptation under constantly changing environments (Srivastava, 2002; Peleg & Blumwald, 2011). Phytohormone biosynthesis has been reported as one of the direct mechanisms employed by PGPR for enhanced plant growth promotion and which correlates with the findings of this study (**Figure 3.9A**) in which an increase in SA, ICA and Zea is observed when compared to naïve plants. SA is a signalling molecule involved in plant defence mechanisms by regulating physiological and biochemical functions; and several studies have suggested SA as a growth regulator. An increased accumulation of SA induced by PGPR treatment (**Figure 3.9A**) is associated with enhanced plant growth due to SA's ability to modulate specific plant responses such as seed germination, vegetative growth and respiration. Additionally, SA enhances photosynthetic rate, increasing plant energy production (Mateo *et al.*, 2006; Janda *et al.*, 2012, 2014) which is utilised by biological processes governing plant growth and development. This increase in SA induced by PGPR results in enhanced plant growth and energy production, which serves as a priming mechanism for future stress encounters in which the plant development has been fully established to withstand any environmental cues. Several PGPR strains have been reported to have the ability to induce increase in the SA levels (Bakker *et al.*, 2014), and this phenomenon has been linked to rhizomicrobe-induced plant priming (Mhlongo *et al.*, 2018) in tomato plants.

Zea is one of the most common forms of naturally occurring cytokinins in plants. Cytokinins are essential plant hormones that do not only regulate plant immunity but also influence various traits of plant growth and development (Kieber & Schaller, 2014). Cytokinins stimulate cell division, leaf shoot and growth, differentiation and growth of axillary buds. Biogenesis of chloroplasts and the rate of photosynthesis are highly dependent on cytokinins. The inhibition of leaf senescence which is associated with chlorophyll breakdown, photosynthetic machinery disintegration and oxidative damage is modulated by cytokinins (Hönig *et al.*, 2018). In roots, cytokinins control root morphology and uptake and translocation of nutrients from the soil. The metabolism and transport of amino acids, carbohydrates and macromolecules such as nitrogen, iron, phosphorus and sulphur which are vital for plant growth (Argueso *et al.*, 2009). Additionally, mediation of ROS production

through the enhancement of the antioxidant system has been reported in cytokinins (Synková *et al.*, 2006; Aremu *et al.*, 2014; Brizzolari *et al.*, 2016).

The cytokinin content and activities of several enzymes have been elucidated with the aim of determining cytokinin effect on the plant antioxidant system. Synková *et al.* (2006), reported an increase in the activity of SOD, ascorbate peroxidase (APOD) which was correlated to the increased Zea content. As highlighted in **section 3.3.1**, these antioxidative enzymes scavenge H_2O_2 resulting in the detoxification of any active ROS species. PGPR treatment induced the accumulation of antioxidative enzymes (**section 3.3.1**) and therefore the observed accumulation of Zea functionally correlates to the measured differential levels of these enzymes, as Zea can act as an enhancer of the antioxidative enzymes. Thus, it can be postulated that the inhibition of ROS by PGPR application protects cell membranes and photosynthetic machinery from oxidative damage, resulting in enhanced plant growth. Additionally, the established antioxidant system may serve as a priming mechanism in PGPR-treated plants through the maintenance of the redox state upon abiotic stress encounters in which ROS production is accelerated. Several reports have elucidated the capacity of PGPR to synthesise cytokinins (Timmusk & Wagner, 1999; Sandhya *et al.*, 2010; Asari *et al.*, 2017) correlated to enhanced plant growth and development. The mode of action induced by PGPR-based biostimulant for enhanced plant growth pointing to the 'primed state' through the accumulation of Zea therefore involves shoot and leave initiation and growth, increase in the rate of photosynthesis, enhanced nutrient uptake and antioxidant system establishment.

PGPR-induced accumulation of ICA (**Figure 3.9A**) can be linked to the catabolism of indole-3-acetic acid (IAA) into ICA, which has been recognised as a priming secondary metabolite (Gamir *et al.*, 2012). Auxins, primarily IAA, are endogenous plant hormones known for their regulatory role in plant growth and development such as root growth promotion. Previous studies have demonstrated how plants have evolved a complex system that regulates IAA levels, including its synthesis from the tryptophan-dependent pathway (Mashiguchi *et al.*, 2011; Stepanova *et al.*, 2011). IAA can be catabolised *via* two processes: the decarboxylative and non-decarboxylative pathways. Catabolism of IAA in plants is usually performed through the non-decarboxylative pathway with major degradation products being indole-3-carboxylic acid (ICA), indole-3-aldehyde (IAld), 2-oxindole-3-acetic acid (oxIAA), and indole-3-

carbinol (I3C) (Ljung *et al*., 2002). Low concentrations of IAA generally stimulate root growth, and therefore the PGRP-induced catabolism of IAA into ICA and other intermediates is a plant growth promotion mechanism employed by this PGPR-based biostimulant: the application of this biostimulant leads to catabolism of IAA into ICA, resulting in increased root growth. This increased root growth ultimately promotes nutrient uptake from the soil which are essential for normal plant development. Additionally, the stimulation of root growth may pose as a priming mechanism resulting in the enhanced uptake of nutrients and water under limiting environmental stress conditions (Sanchez-Calderon *et al*., 2013). The accumulation of ICA complements the observed decrease in ICAld because previous studies have reported that ICAld can be oxidised to ICA (Böttcher *et al*., 2014; Müller *et al*., 2019).

Furthermore, PGPR treatment resulted in a downregulation of NHP, ICAld and ACC at 4 WAE (**Figure 3.9A**). In this regard, 1-aminocyclopropane-1-carboxylic acid (ACC), is an immediate precursor of ethylene, involved in the regulation of plant homeostasis, development (Yoon & Kieber, 2013; Vanderstraeten & van Der Straeten, 2017) and defence responses (Tsang *et al*., 2011). ACC is degraded by ACC deaminase into nitrogen and α-ketobutyrate (can be converted to succinyl-CoA, a TCA cycle intermediate required for energy production). The measured decreased level of ACC in PGPR-treated plants can be postulated to be linked to its degradation, providing a nitrogen source and energy for plant growth and development. Previous studies have reported the ability of certain PGPR to produce ACC deaminase enzyme which degrades ACC, resulting in shoot and root growth promotion which enhanced plant growth and development by reducing ethylene present in plants (Zahir *et al*., 2011; Gamalero & Glick, 2015; Singh *et al*., 2015). Ethylene is a plant hormone that accumulates in plants under a variety of abiotic stresses (Cheng *et al*., 2007; Indiragandhi *et al*., 2008; Pourbabaee *et al*., 2016) inhibiting plant growth through increased abscission and premature plant senescence. The production of ACC deaminase by PGPR to inhibit ethylene production can therefore prime plants under environmental stress by modulating its negative effects on plant growth, leading to enhanced organ development and stress tolerance.

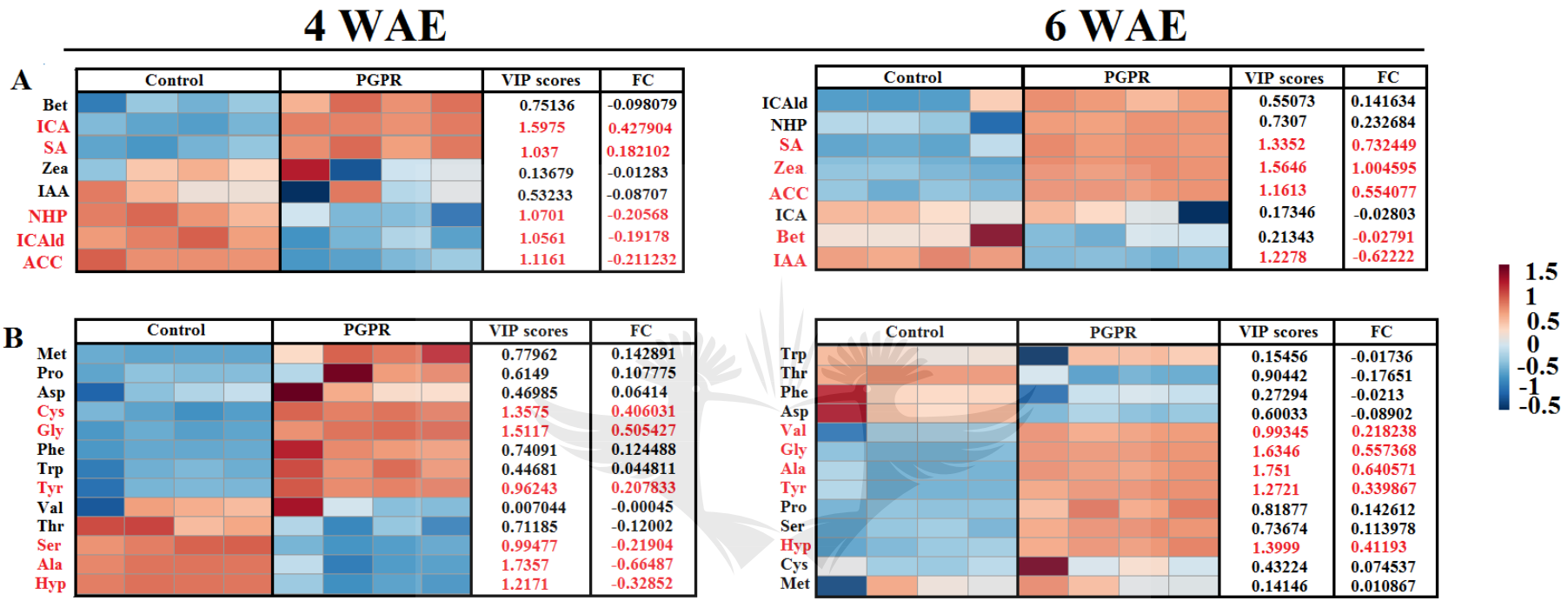

Figure 3.9: **Quantitative analysis of amino acids and phytohormone abundances in naïve plants (Control, T2) and PGPR-treated plants (T1)**. Hierarchical clustering heatmap displayed with VIP scores and logarithmic fold changes of (**A**) phytohormones and (**B**) amino acids at 4 and 6 WAE. Discriminant metabolites obtained from both OPLS-DA modelling and VIP scores plots are highlighted in red.

Quantitative analysis of the selected amino acids revealed an accumulation of cysteine (Cys), tyrosine (Tyr), glycine (Gly) and serine (Ser) at 4 WAE and Ser, alanine (Ala), Cys, Tyr, valine (Val), hydroxyproline (Hyp) and Gly at 6 WAE to be signatory makers of PGPR treatment in maize (**Figure 3.9B**). Amino acids are the basic building blocks of proteins and they fulfil multiple functions in the plants including the stimulation of plant growth. Following degradation, amino acids' carbon skeletons can serve as key metabolic intermediates. The skeletons are generally converted into precursors or intermediates of the tricarboxylic acid (TCA cycle) (Hildebrandt *et al.*, 2015) - a central metabolic hub required for ATP production. Amino acids therefore contribute to the mitochondrial metabolism and ATP production. The energy produced from the TCA cycle is utilised for biological processes that aid in plant growth and development such as fatty acids biosynthesis and protein synthesis (Cleveland & Morris, 2015). The carbon skeleton of all amino acids can be funnelled into only seven TCA intermediates namely: acetyl CoA, pyruvate, acetoacetyl CoA, α-ketoglutarate, succinyl CoA, fumarate, and oxaloacetate (**Figure 3.10**), driving the production of energy. PGPR-induced accumulation of Tyr, Cys, Gly at 4 WAE and Ser, Ala, Cys, Tyr, Val, Hyp and Gly at 6 WAE (**Figure 3.9B**) is therefore suggested to be associated with the increased flux of their catabolism into TCA intermediates for ATP production, thereby enhancing the fuelling of a wide-range of energy-demanding biochemical processes involved in plant growth and development such as gene expression, mobility and metabolism. Cellular ATP has been reported to regulate numerous processes involved in abiotic stress responses such as ROS regulation under abiotic stress (Sun *et al.*, 2012). The increased ATP production induced by PGPR treatment through the amino acid accumulation can be postulated to be a form of pre-conditioning for subsequent environmental stress encounter by powering plant defence responses aiding survival and tolerance. Interestingly, the number of amino acids accumulated due to PGPR treatment differed between the two time points. At 6 WAE, all the signatory amino acids (Ser, Ala, Cys, Tyr, Val, Hyp and Gly) were increased, whereas under 4 WAE (Tyr, Cys and Gly), Ser and Ala were decreased. This therefore points to the dynamism in cellular metabolism and suggests that energy production induced by PGPR treatment is enhanced as the plant develops due to increased energy demand for normal plant growth and development processes.

PGPR treatment induced the accumulation of Tyr, common to both time points in non-stressed plants (**Figure 3.9B**). Tyr is an aromatic amino acid (AAA) involved in the synthesis

of proteins (Parthasarathy *et al.*, 2018), which can be catabolised into fumarate and serve as a precursor of secondary metabolite such as auxins, phytoalexins, alkaloids and flavonoids (Facchini *et al.*, 2004; Hudson & Prabhu, 2010). Increased levels of Tyr is due to its degradation into precursors of the phenylpropanoid pathway, where it is a direct precursor of coumarate (MacDonald & D'Cunha, 2007). Tyr can also serve as a precursor for the biosynthesis of tocopherols such as vitamin E through its transamination into homogentisate from which vitamin E is synthesised from (DellaPenna & Pogson, 2006; Mène-Saffrané & DellaPenna, 2010; DellaPenna & Mène-Saffrané, 2011). Vitamin E can modulate ROS production, and therefore its accumulation may be beneficial to plant growth and survival by mitigating oxidative stress resulting from environmental cues.

PGPR-induced increase in the level of phenylalanine (Phe), an additional AAA, was also observed (**Figure 3.9B**). Phe can be catabolised into phenylpropanoid pathway precursors. The phenylpropanoid pathway plays a significant role in plant development and it confers resistance to environmental stresses such as ROS scavenging. Considering its functional role and involvement in plant defence, the PGPR-induced activation of this pathway could be a form of pre-conditioning, preparing the plant for stress conditions through enhanced defence responses. Additionally, Garcia-Seco *et al.*, (2015) and Mandal, (2019) have reported the upregulation of the phenylpropanoid pathway leading to the biosynthesis of flavonoids as part of an induced systemic response (ISR) induced by PGPR. The activation of ISR by PGPR is crucial for enhanced tolerance against abiotic stress conditions.

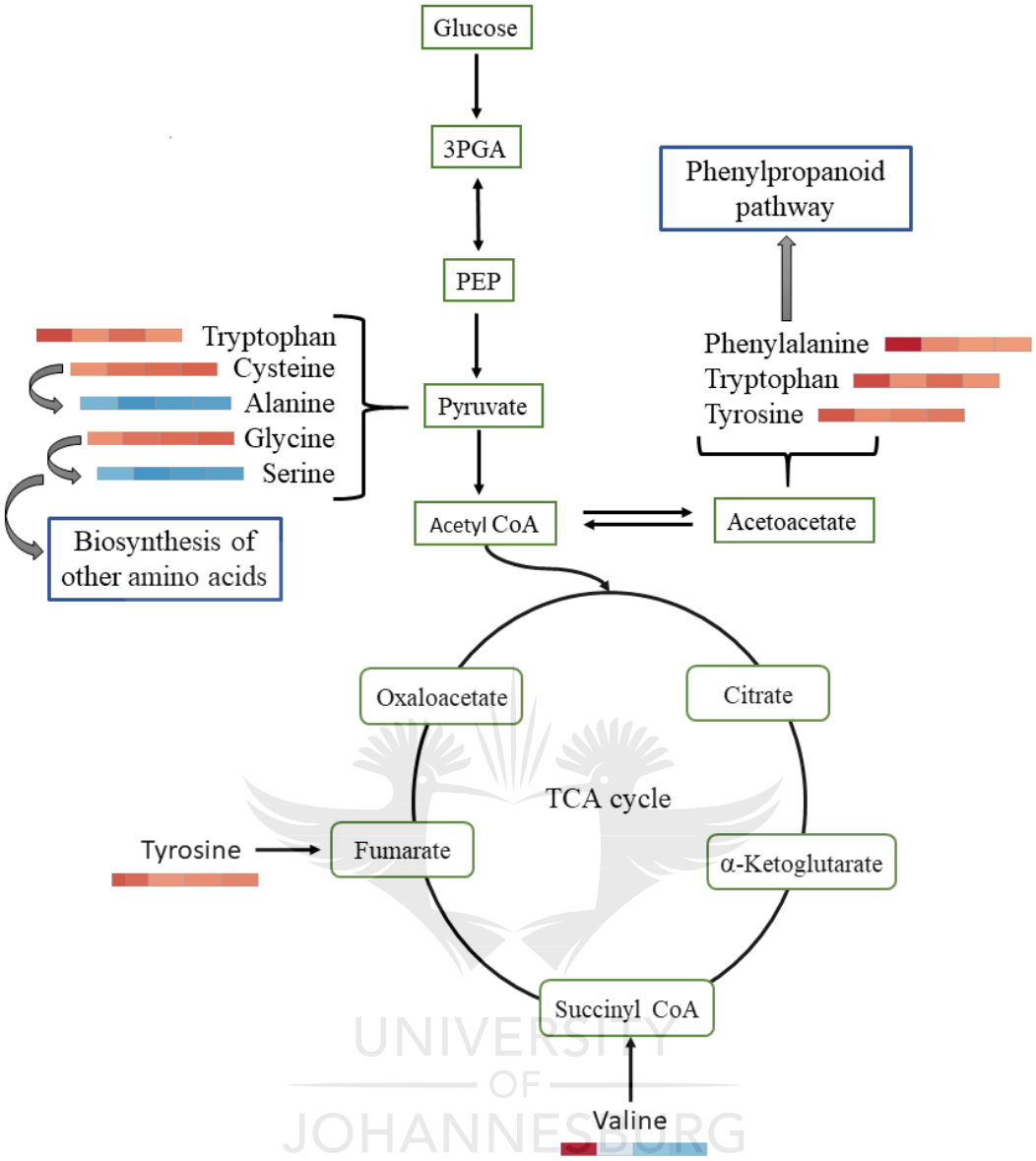

Figure 3.10. **Catabolism of the amino acid carbon skeletons into TCA intermediates**. The carbon skeleton of the signatory amino acids can be fed directly into the TCA cycle by being degraded to acetyl CoA, pyruvate, acetoacetyl CoA, succinyl CoA, and fumarate for energy production. The heatmaps depict the quantitative levels of the amino acids, pointing to enhanced energy production through increased metabolic flux of amino acid degradation. **Abbreviations**: TCA, tricarboxylic acid cycle; 3PGA, 3-phosphoglyceric acid; PEP, phosphoenolpyruvate.

To further describe the interrelationships between the different measured metabolites, based on their quantitative profiles, a correlation analysis was employed, pointing to the biochemical modifications and framework underlying the PGPR-induced growth promotion and defence priming. Metabolite-metabolite correlation analysis (MMCA) was performed in which the magnitude and direction of the correlation between the metabolites was computed, revealing underlying biologically functional interconnectivities of measured metabolites, an

atlas of cellular dynamisms in response to experimental conditions (Hu *et al*, 2014). A positive correlation indicates an increase/decrease in the metabolite levels, and a negative correlation indicates an inversely proportional relationship between the metabolites, where if one metabolite increases, the other decreases (Rosato *et al.*, 2018). Furthermore, the exploitation of the metabolite ratios represented by each box on the correlation-heatmaps can indicate the direction of the quantitative metabolic profiles in a biochemical pathway.

Ala and Ser both have a negative correlation with Gly, Cys, Val, and Phe, and Zea, Bet, SA, IAA, Gly, Cys, Trp and Phe respectively (**Figure 3.11**), indicating that these metabolites can be degraded into their respective carbon skeletons to drive the metabolic flux towards the synthesis of other amino acids (Hildebrandt *et al.*, 2015; Hildebrandt, 2018). Additionally, the general level of correlation was relatively low between the amino acids and phytohormones; however, there were a number of clusters that indicated highly positive or negative correlations among the metabolites. The clusters that were most prominent, showing a positive correlation were amino acids. Strong correlation among amino acids reflects their mutual constrained relationship (Du *et al.*, 2003). The observed positive correlation between different amino acids under PGPR treatment in well-watered conditions therefore suggests that different amino acids have mutual functions and feed-forward interactions involved in plant growth promotion and defense preconditioning.

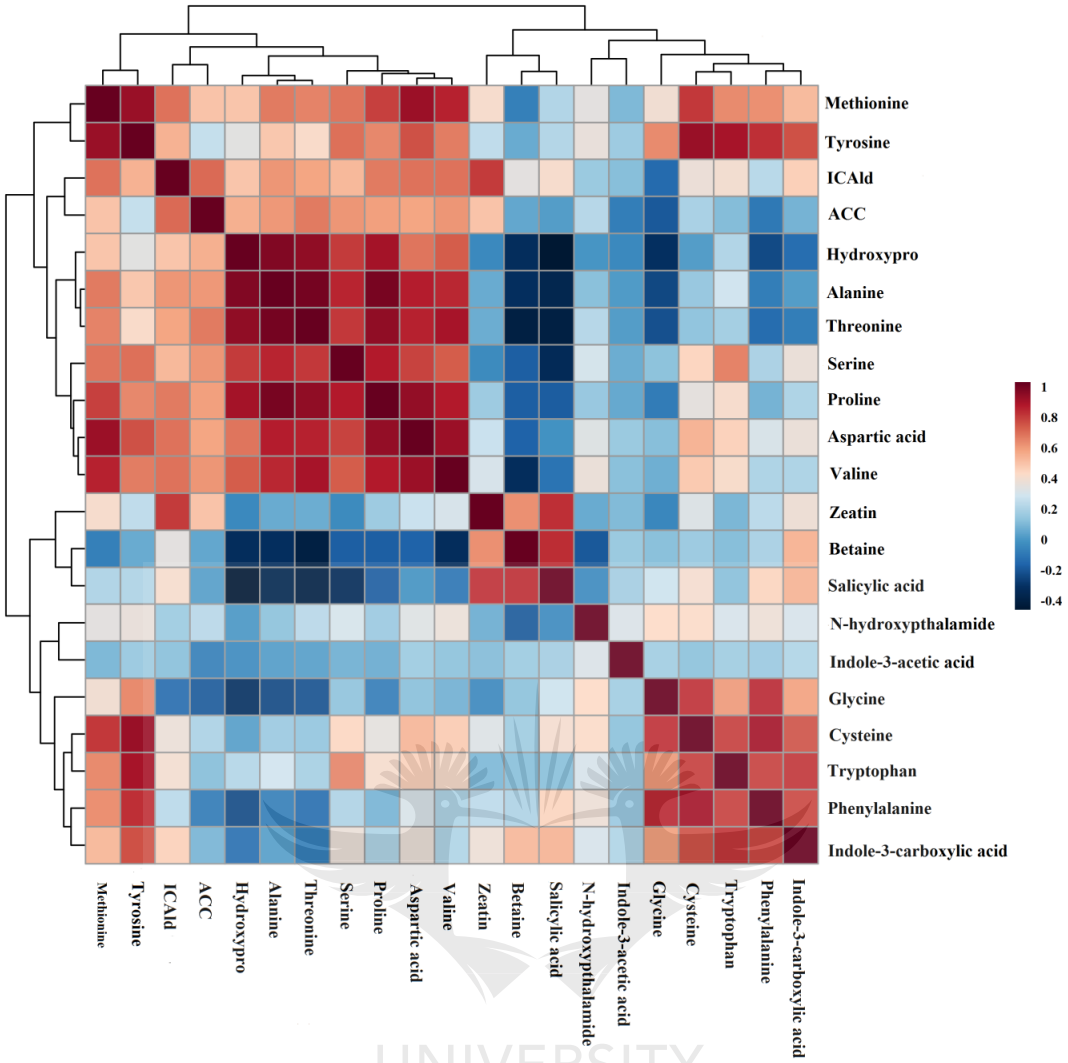

Figure 3.11: **Metabolite-metabolite correlation analysis heatmap**. Correlation analysis heatmap based on Pearson's *r* distance measure where red represents positive correlation and blue negative correlation between naïve- and PGPR-treated maize plants, both under well-watered conditions. **Abbreviations**: ACC, 1-aminocyclopropane-1-carboxylic acid.

Furthermore, to situate the observed metabolic reprogramming induced by PGRP treatment, in a metabolome view context, a pathway analysis was carried out using the metabolic pathway analysis (MetPA) – an integral module of MetaboAnalyst 4.0 (Chong *et al.*, 2019). Pathway enrichment analysis refers to the quantitative enrichment analysis directly using the metabolite concentration values, as compared to compound lists used by over-representation analysis. As a result, it is highly sensitive and has the potential to identify subtle but consistent changes amongst different metabolites under study that are involved in the same biological pathway. Metabolites are mapped and visualised to known and most relevant biochemical pathways (Xia *et al.*, 2012b, 2015; Alonso *et al.*, 2015; Chong *et al.*, 2018). The selected amino acids and phytohormones were uploaded into MetPA for the overview

representation and pathway topological analysis based on a hypergeometric test algorithm and relative-betweenness centrality respectively. MetPA revealed ten significant metabolomic pathways with impact score > 1.0, out of a total of 30 pathways that were uniquely altered by PGRP treatment. The significant pathways included Trp metabolism, Gly, Ser and The metabolism, Phe metabolisms, isoquinoline alkaloid metabolism, Cys and Met metabolism and Tyr metabolism (**Table 3.4** and **Figure 3.12**), with Trp and Gly, Ser and Thr metabolism pathways showing the highest hits.

Table 3.4: Significant metabolic pathways involved in response to PGRP treatment in non-stressed *Zea mays*.

No	Pathway	Total	Expected	Hits	Impact
1	Tryptophan metabolism	23	0.32787	2	0.5862
2	Glycine, serine and threonine metabolism	33	0.47042	6	0.5134
3	Phenylalanine metabolism	12	0.17106	1	0.4230
4	Isoquinoline alkaloid biosynthesis	6	0.085531	1	0.4117
5	Cysteine and methionine metabolism	46	0.65574	5	0.2012
6	Tyrosine metabolism	18	0.25659	1	0.1675
7	Arginine and proline metabolism	28	0.39914	2	0.1601
8	Alanine, aspartate and glutamate metabolism	22	0.31361	2	0.1259
9	Glyoxylate and dicarboxylate metabolism	29	0.4134	2	0.1194
10	Aminoacyl-tRNA biosynthesis	46	0.65574	12	0.1111

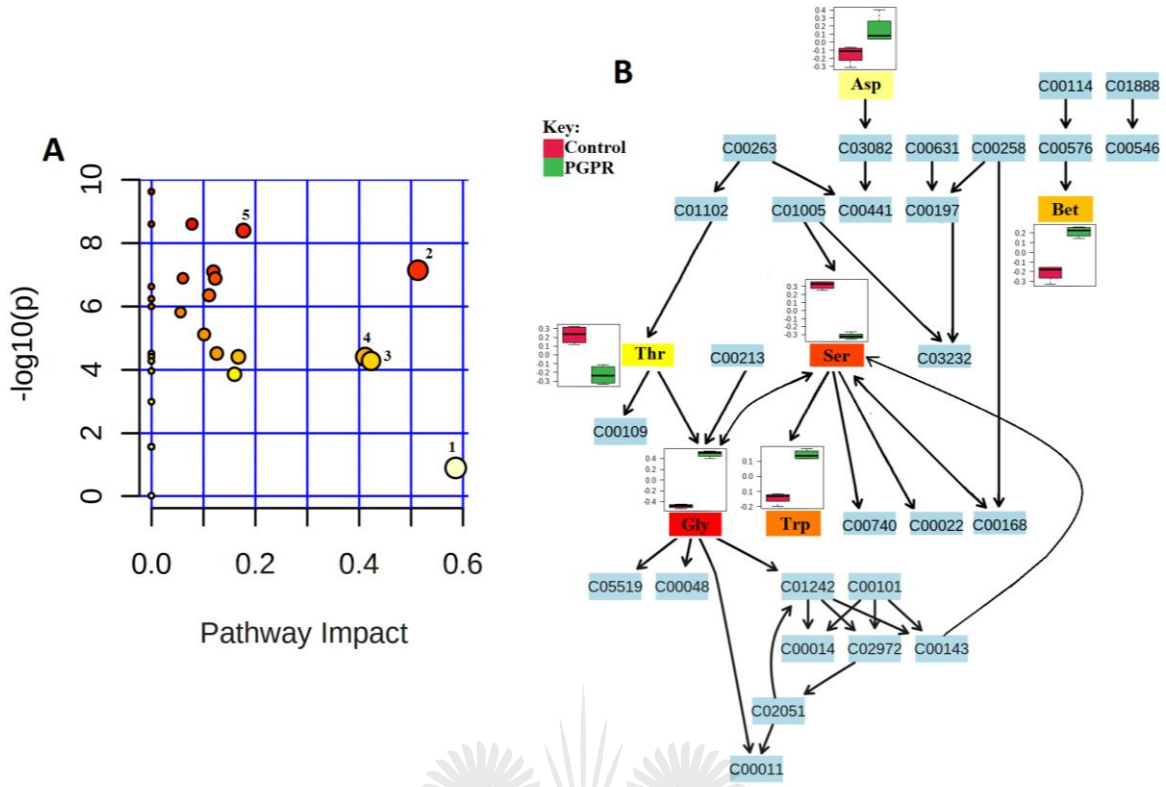

Figure 3.12. **Summary of pathway analysis with MetPA**. Representation of MetPA-computed metabolic pathways and their significance/pathway impact under control (T2) and PGPR treatment (T1). (**A**) The graph, "metabolome view" contains all the matched pathways (the metabolome) arranged by *p*-values (pathway enrichment analysis) on the *y*-axis, and the pathway impact values (pathway topology analysis) on the *x*-axis, with the numbers corresponding to the mapped pathways listed in **Table 3.4**. The node colour is based on the *p*-value and the node radius is defined by the pathway impact values. The latter is the cumulative percentage from the matched metabolite nodes. (**B**) Topology map of glycine, serine and threonine metabolism displaying altered amino acids in response to PGPR treatment

As highlighted above, amino acids serve as precursors for secondary metabolites, and Phe/Trp are precursors of the phenylpropanoid pathway which play a crucial role in the interconnection between the primary and secondary metabolism in plants, regulating plant growth and development. Phe metabolism is of paramount importance for plants under drought stress. Phe is used as a building block for proteins, and also serves as a precursor for numerous plant compounds that are crucial for plant growth, development and abiotic stress tolerance (Tohge *et al.*, 2013; Pascual *et al.*, 2016). The Gly, Ser, and Thr pathway which was also significantly altered plays a key role in the synthesis of amino acids including Lys, Thr, Met, and isoleucine (Ile) (Hildebrandt *et al.*, 2015) under stress conditions. Consistent with this finding, Ser and Gly were the most significantly affected metabolites in the topology map (**Figure 3.12B**). A recent study by Khan *et al.* (2019b) reported on different metabolic pathways induced by PGPR under drought stress. The authors revealed Trp

metabolism, Phe metabolism and Gly, Ser and Thr metabolism as one of the significantly altered pathways which are involved in conferring drought stress tolerance in chickpea (*Cicer arietinum* L.).

3.3.2.2 PGPR-induced changes in secondary metabolism – growth promotion and priming

Chemometric models, PCA (**Figure S3.3**) and PLS-DA (**Figure 3.13**), revealed distinct treatment-related sample groupings and allowed the description of differential quantitative metabolic changes on flavonoids and phenolic acids in naïve (T2) and PGPR-treated (T1) plants. These models revealed that PGPR induced differential metabolomic changes in non-stressed plants through a distinct separation in the two groups (**Figure 3.13A**). The computed PLS-DA models revealed a clear separation of the groups; two time points separated in the first component, and the naïve and PGPR-treated groups separated in the second component. The use of OLPS-DA loading S-plots and variable importance in projection (VIP) plots (**Figure 3.13B**) further allowed the extraction of statistically significant variables driving the differentiation between naïve plants and PGPR-treated plants.

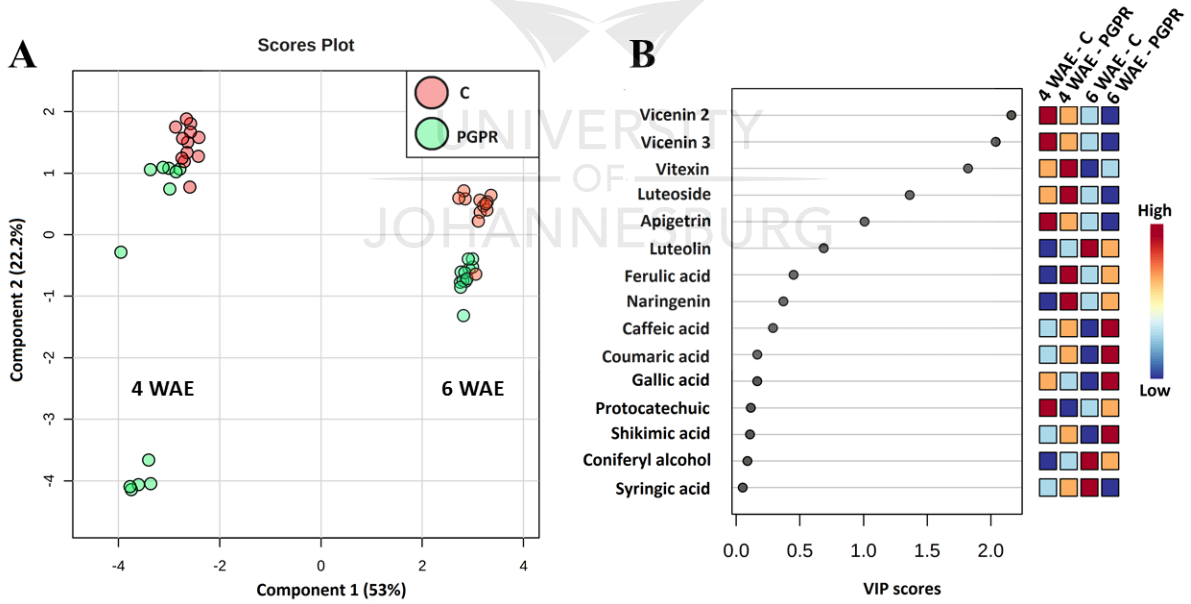

Figure 3.13. Chemometric models of comparative quantification analysis of flavonoids and phenolic acids. (**A**) Partial least squares-discriminant analysis (PLS-DA) score plot. (**B**) Variable importance projection plot showing the discriminant metabolite (VIP > 1) responsible for the class separation. **Abbreviations**: C, control; PGPR refers to the biostimulant used; WAE, weeks after emergence.

Overall quantitative changes (logarithmic fold changes) in flavonoids and phenolic acid profiles induced by PGPR treatment are represented by radar plots at 4 and 6 WAE (**Figure 3.14**). The observed changes indicate that PGPR treatment influences the plant's secondary metabolism to mediate plant growth and development. PGPR treatment revealed an accumulation and downregulation of the selected flavonoids and phenolic acids (**Figure 3.14**) for time points 4 and 6 WAE.

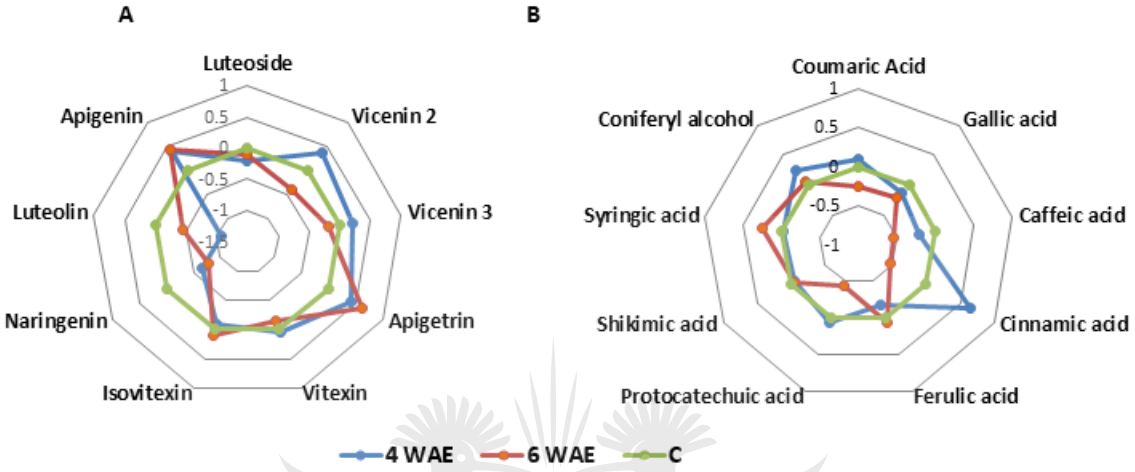

Figure 3.14: **Radar plots showing quantitative ratio changes of flavonoid and phenolic acid profiles under well-watered conditions with (T1) and without PGRP treatment (T2)**. Plots show the calculated logarithmic fold changes from the quantification of each metabolite. (**A**) Flavonoid and (**B**) phenolic acid profiles from well-watered conditions with PGPR showing metabolite changes at 4 WAE and 6 WAE.

Hierarchical clustering heatmap analysis allowed for the visualisation of these quantitative changes of both flavonoids (**Figure 3.15A**) and phenolic acids (**Figure 3.15B**) between the two treatments (C and PGPR, T2 vs. T1). Quantitative measurement of selected flavonoids revealed apigenin, vicenin-2 (apigenin-6,8-di-C-β-D-glucoside), vicenin-3 (apigenin-6-C-β-D-glucoside-8-C-β-D-xyloside), apigetrin (apigenin-7-O-β-D-glucoside), luteoside, naringenin, and luteolin to be signatory markers of PGPR treatment (**Figure 3.15**; **Figure S3.4**). As previously mentioned in **section 3.1**, secondary metabolites are various chemical compounds produced by plants through metabolic pathways and functionally linked to the primary metabolism. These secondary metabolites are widely distributed in plants and are usually classified based on their biosynthetic pathways and three major families are generally considered: alkaloids, terpenes/steroids and phenolics (Bourgaud *et al.*, 2001). Phenylpropanoid compounds are a class of secondary metabolites that are synthesised from primary metabolites – Phe/Tyr, through a series of enzyme-catalysed reactions. They have

been suggested to play various roles in plant development, including regulation of cell physiology and signalling. Phenylpropanoids can be divided into five groups depending on their chemical structures and this includes flavonoids, phenolic acids, coumarins, and monolignols (Liu *et al.*, 2015).

Flavonoids are polyphenol containing compounds that are ubiquitously present in plants, and their roles in plant growth and development have been widely discussed. They are found in numerous modified forms, synthesised through hydroxylation, methylation, acylation and glycosylation enzyme catalysed reactions (Lepiniec *et al.*, 2006; Saito *et al.*, 2013), among which glycosylated flavonoids are by far the most common natural compounds which may occur as C-glycosyl flavanones (Rauter *et al.*, 2007). Flavonoids have been reported to have diverse functions that have evolved in plants to regulate growth and development. These functions include control of respiration and photosynthesis (Cushnie & Lamb, 2005), antioxidant and chelating capacity (Agati *et al.*, 2012; Stolarzewicz *et al.*, 2013), drivers of symbiosis between plants and rhizobacteria (Weston & Mathesius, 2013), regulators of auxin transport and catabolism. The accumulation of apigenin, apigetrin and vicenin induced by PGPR (**Figure 3.15**) enhances photosynthesis and respiration capacity providing energy for plant growth and maintenance. The produced energy indicates PGPR-induced priming mechanisms in which the increased energy pool can be utilised under environmental stress encounters to ensure sustained plant growth and development. As highlighted (**Chapter 2, section 2.2.2**), ROS are present in moderate levels under normal plant growth conditions, however, stress induces excess production of ROS, resulting in deleterious effects such as cell death. Accumulation of flavonoids can inhibit the generation of ROS, by scavenging ROS and hindering the production of ROS producing enzymes, thus maintaining a redox state inside the cells. This indicates a priming mechanism induced by PGRP, to pre-condition the plants' antioxidant system resulting in a stronger defence system following stress cues. Martinez *et al.* (2016), reported a decrease in oxidative damage due to the accumulation of flavonoids in tomato plants and this, correlates to the findings of the current study.

PGPR treatment induced a decrease in the levels of naringenin when compared to the control group (**Figures 3.15** and **3.16**). Naringenin is a general precursor for the synthesis of isoflavones, flavones and flavonols. Flavonols are synthesised from naringenin through the action of flavone synthase I (FSI) or flavone synthase II (FNSII) (Martens & Mithöfer, 2005).

The expression profile of *FNSII* gene is investigated in **Chapter 4** (**section 4.3.3**). The down-regulation effect induced by PGPR on naringenin may possibly be due to its degradation/conversion into flavones apigenin and its glycosides (vicenin-2, vicenin-3, and apigetrin) (**Figure 3.15A**). Several bacteria have been reported to degrade flavonoids for various functions such as utilizing them as carbon or detoxification sources. Pillai & Swarup (2002) reported the ability of *Pseudomonas putida* (PGPR) to catabolise naringenin and quercetin. It is therefore logical to postulate that the consortium of PGPR strains (*Bacillus* spp.) used in this study promotes plant growth by inducing the degradation of naringenin into flavones which have been identified as the most crucial class of flavonoids that participate in plant defence mechanisms (Pollastri & Tattini, 2011). Naringenin has also been reported to suppress plant growth in several species such as *Spinacia oleracea*, *Lactuca sativa* and *Glycine max* L. (Wei *et al*., 2004; Bido *et al*., 2010), through its inhibitory effect on 4-coumarate: CoA ligase, a key enzyme in the phenylpropanoid pathway involved in the synthesis of phenolics and other products such as lignin. The observed decrease in naringenin levels induced by PGPR (**Figures 3.15A** and **3.16**) may therefore suggest the inhibition of naringenin accumulation associated with plant growth suppression. A decrease in naringenin may result in low concentrations with no inhibitory effect on4-coumarate: CoA ligase, and promotion of secondary metabolites biosynthesis through the phenylpropanoid pathway.

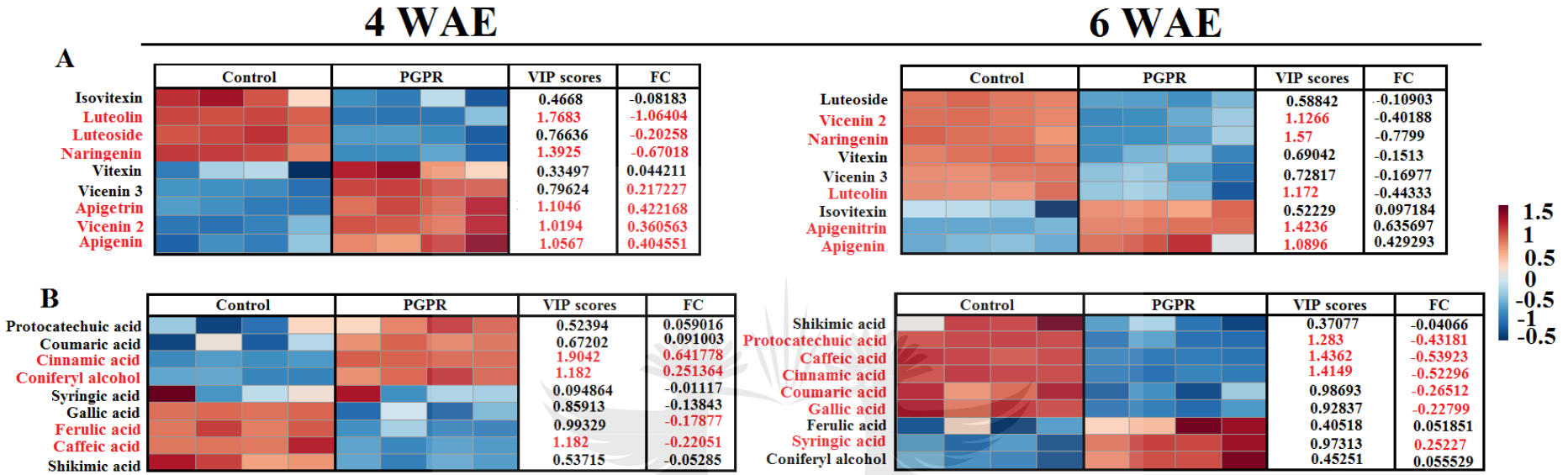

Figure 3.15. **Quantitative analysis of flavonoids and phenolic acids abundances in naïve plants (Control, T2) and PGPR-treated (T1) plants**. Hierarchical clustering heatmap displayed with VIP scores and logarithmic fold changes of (**A**) Flavonoids and (**B**) Phenolic acids at 4 and 6 WAE. Discriminant metabolites obtained from both OPLS-DA modelling and VIP scores plots are highlighted in red.

PGPR treatment led to an accumulation of signatory phenolic acids: coumaric acid, cinnamic acid, protocatechuic acid, coniferyl alcohol at 4 WAE; syringic acid, ferulic acid and coniferyl alcohol at 6 WAE (**Figure 3.15B**). Phenolic acids are extensively spread-out through the plant kingdom and form a diverse group of plant metabolites including hydroxybenzoic and hydroxycinnamic acids (Mandal *et al.*, 2010). The accumulation of phenolic acids induced by PGPR has been widely reported (Singh *et al.*, 2002, 2003, 2012; Ahemad & Kibret, 2014). These secondary metabolites mediate plant growth and reproduction by influencing physiological processes related to growth and development including cell division, seed germination and synthesis of photosynthetic pigments (Tanase *et al.*, 2019). The degradation products of phenolic acids contribute to nitrogen mineralisation and formation of humus. The latter thus actively provides the plant with essential nutrients required for growth and development (Halvorson *et al.*, 2009; Kefeli *et al.*, 2012; Min *et al.*, 2015). Phenolic acids also chelate metals and improve soil porosity, enhancing absorption sites and increasing the mobility and availability of elements, such as potassium, calcium, magnesium, copper, zinc, manganese and iron for plants (Seneviratne & Jayasinghearachchi, 2003).

The observed accumulation of the various phenolic acids induced by PGPR treatment (**Figure 3.15B** and **3.16**) indicates a PGPR-induced enhancement of plant growth and development through the increased flux of organic and inorganic nutrient uptake by the plant. The increased nutrient availability ensures plant survival under adverse environmental conditions, which indicates a possible priming mechanism induced by PGPR treatment. Phenolic acids are produced in moderate levels under optimal conditions in plants and in excess under suboptimal conditions in response to environmental perturbations (Kefeli *et al.*, 2012; Cheynier *et al.*, 2013), and numerous studies have reported on the antioxidant activity of these compounds (reducing agents, quenchers of singlet oxygen formation and free radical scavengers) related to plant adaptation under abiotic stress (Michalak, 2006; Ghasemzadeh & Ghasemzadeh, 2011; Lee *et al.*, 2013; Król *et al.*, 2014). The accumulation of phenolic acids resultant from PGPR treatment can serve as a priming mechanism through increased antioxidant capacity, resulting in the mitigation of oxidative stress under stressful conditions.

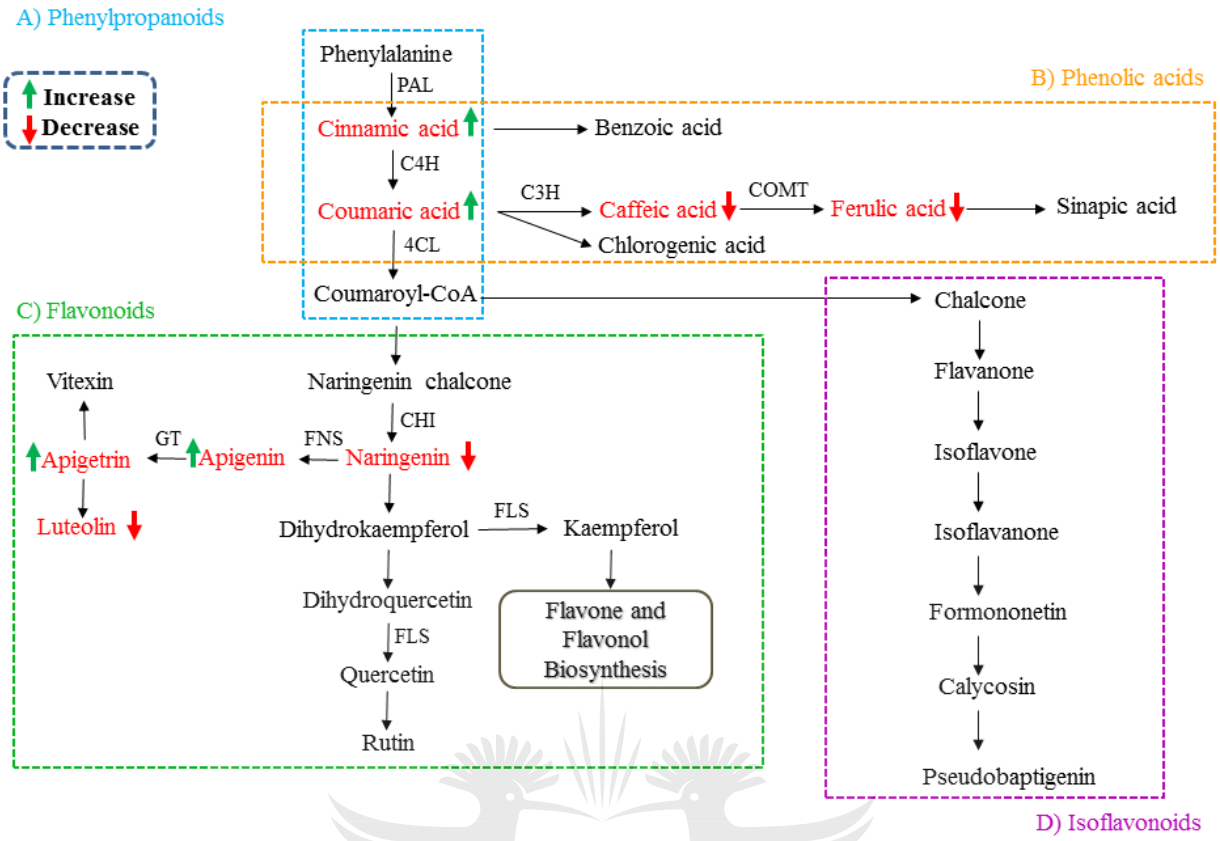

Figure 3.16. **General phenylpropanoid and related metabolomic pathways**. The simplified scheme comprises of the phenylpropanoid pathway branching into phenolic acids, flavonoids and isoflavonoids biosynthesis. The pathways show the measured quantitative changes of phenolic acids and flavonoids induced by PGPR treatment in well-watered plants and the inter-connection of the metabolites in different pathways. **Abbreviations**: CHI, chalcone isomerase; FLS, flavonol synthase; PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate CoA ligase; C3H, coumarate 3-hydroxylase COMT, Caffeic acid/5-OH ferulic acid O-methyltransferase; GT, glycosyltransferase.

Cinnamic acid was the most abundant when compared to the rest of the upregulated phenolic acids (**Figure S3.5**). As previously mentioned, phenolic acids together with flavonoids are synthesised from primary metabolites - Phe/Tyr, through a series of enzyme-catalysed reactions *via* the phenylpropanoid pathway. Cinnamic acid formed from the deamination of phenylalanine through the action of phenylalanine ammonia lyase (PAL) is a pivotal branch of primary and secondary metabolism, representing a crucial regulatory step in the formation of numerous phenolic acids (Mandal *et al.*, 2010; Fraser & Chapple, 2011; Yin *et al.*, 2012). It is converted into phenolic acids including coumaric acid and ferulic acid. The increased accumulation of cinnamic acid induced by PGPR is therefore to drive the flux for the biosynthesis of additional phenolic acids required for sustained plant growth and development (**Figure 3.16**). A decrease in caffeic acid, ferulic acid, coumaric acid, gallic

acid, and syringic acid and protocatechuic acid was observed due to PGPR treatment. This downregulation can also drive the accumulation of their immediate precursors such as cinnamic acid and ferulic acid, in order to sustain homeostasis (**Figure 3.16**). Moreover, the accumulation of some phenolic acids is phytotoxic. Phenolic acids including gallic acid have been reported to have a phytotoxic effect by inhibiting either seed germination or root growth in various plant species (Li *et al.*, 2010; Goleniowski *et al.*, 2013) and the maintenance of this phenolic acids at moderate levels will enhance root growth. This growth mechanism can therefore pre-condition the plant by facilitating increased nutrient uptake under adverse environmental conditions.

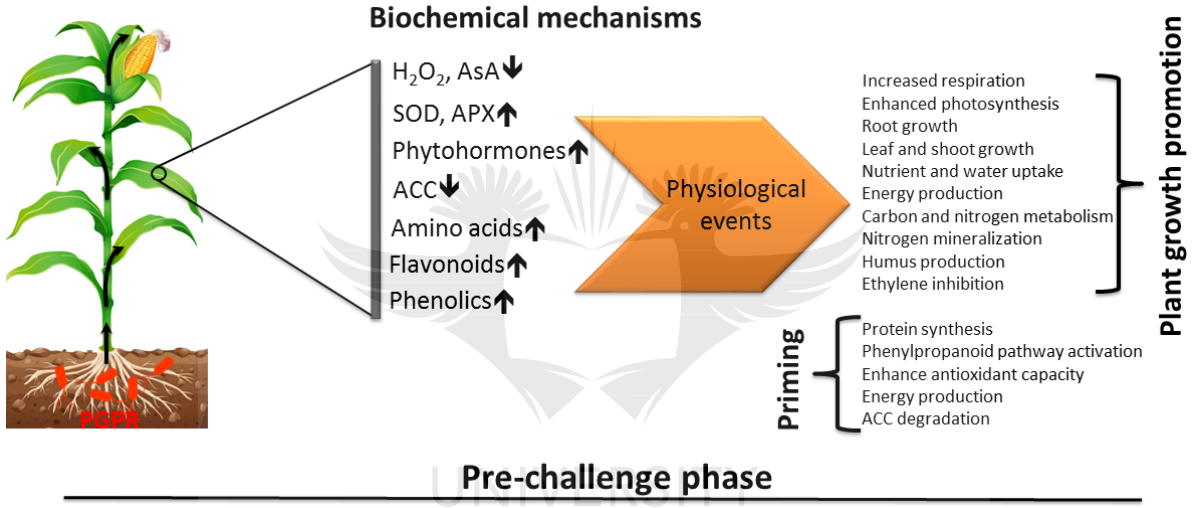

Figure 3.17. **Proposed schematic diagram representing key mechanisms induced by microbial biostimulants in plant growth promotion and priming based on the finding reported above**. In the pre-challenge phase (well-watered conditions), PGPR treatment induced biochemical mechanisms which are associated to plant growth promotion and defence priming, such as reprogramming of plant morphophysiology and changes in levels of amino acids, flavonoids, phytohormones and phenolic acids. **Abbreviations**: H_2O_2, Hydrogen peroxide; MDA, Malenoaldehyde; AsA, Ascorbate; SOD, superoxide dismutase (SOD), ascorbate peroxidase (APX); catalase, (CAT); and ACC; 1-aminocyclopropane-1-carboxylic acid.

Thus, the decoded differential (quantitative) metabolic profiles, described in a metabolism view and cellular language, reveal key molecular and physiological processes (in maize plants) activated by the microbial-based biostimulant for growth promotion and preconditioning of defences (**Figure 3.17**). The model emerging from these results suggests that the microbial-based biostimulant promotes the development of maize plants *via* altering levels of amino acids, phytohormones and phenolics. These quantitative metabolic changes span a wide range of metabolic pathways (in both primary and secondary metabolism),

activating and positively rerouting physiological events towards growth and healthily (organismal) development. These physiological events include increased respiration, enhancement of photosynthesis machinery, favouring nutrient and water uptake (**Figure 3.17**). Furthermore, the elucidated metabolic landscape points to biostimulant-induced preconditioning of maize defences against drought stress conditions. In addition to physiological events directly related to plant growth and development, the microbial-based biostimulant activated biochemical and physiological events of a 'stress-preparedness' state (**Figure 3.17**): increased pool of amino acids, activation of a stress-related hormonal network, a pre-conditioned antioxidant capacity and a regulation of protein synthesis machinery.

3.3.3 Metabolomic changes induced by PGPR treatment under mild and severe drought stress conditions and their impact on drought tolerance

Chapter 3 (**section 3.3.2**) of this dissertation decoded some of the key metabolic changes characterising the pre-challenge (non-stressed) phase induced by PGPR treatment in the primary and secondary metabolism. This subsection therefore extensively focuses on the quantitative changes of amino acids, hormones, flavonoids and phenolic acids induced by PGPR-based biostimulant under mild and severe drought stress conditions (T3 and T4 *vs.* T5 and T6). To explicitly evaluate the effects of PGPR treatment on drought stressed plants, naïve plants (C), mild drought (MD) and severe drought (SD) stressed plants (**Table 3.1**; **subsection 3.2.2**) were initially investigated, followed by PGPR-treated drought stressed plants (MD/SD-PGPR). PCA (**Figures S3.6** and **S3.7**) and PLS-DA (**Figure 3.18**) modelling, by summarising the variation in multivariate space into a reduced number of latent components, revealed distinct treatment-related sample groupings, allowing for the description of differential quantitative metabolic changes in naïve plants, drought stressed plants and PGPR-treated stressed plants.

These models revealed that drought stress and PGPR induced differential metabolomic changes in maize plants, as infographically depicted by distinct treatment-related sample groupings in scores space (**Figures 3.18A** and **B**). Interestingly, for amino acids and phytohormones analysis (**Figure 3.18A**), there is a clear discrimination between the naïve group and all the treatments (the drought stressed and PGPR-treated drought stressed groups). This therefore implies that the metabolism of naïve plants and primed plants is readjusted due to mild and severe drought stress treatment. Conversely, PLS modelling of the flavonoids and

phenolic acids only showed a clear discrimination of the control group from the other treatments which were all grouped into one (**Figure 3.18B**). To identify key signatory metabolites driving the separation the different classes under study, OLPS-DA loading S-plots (**Figures S3.8 – S3.11**) and variable importance in projection (VIP) plots were employed for amino acids and phytohormones (**Figure 3.18C**), and flavonoids and phenolic acids (**Figure 3.18D**).

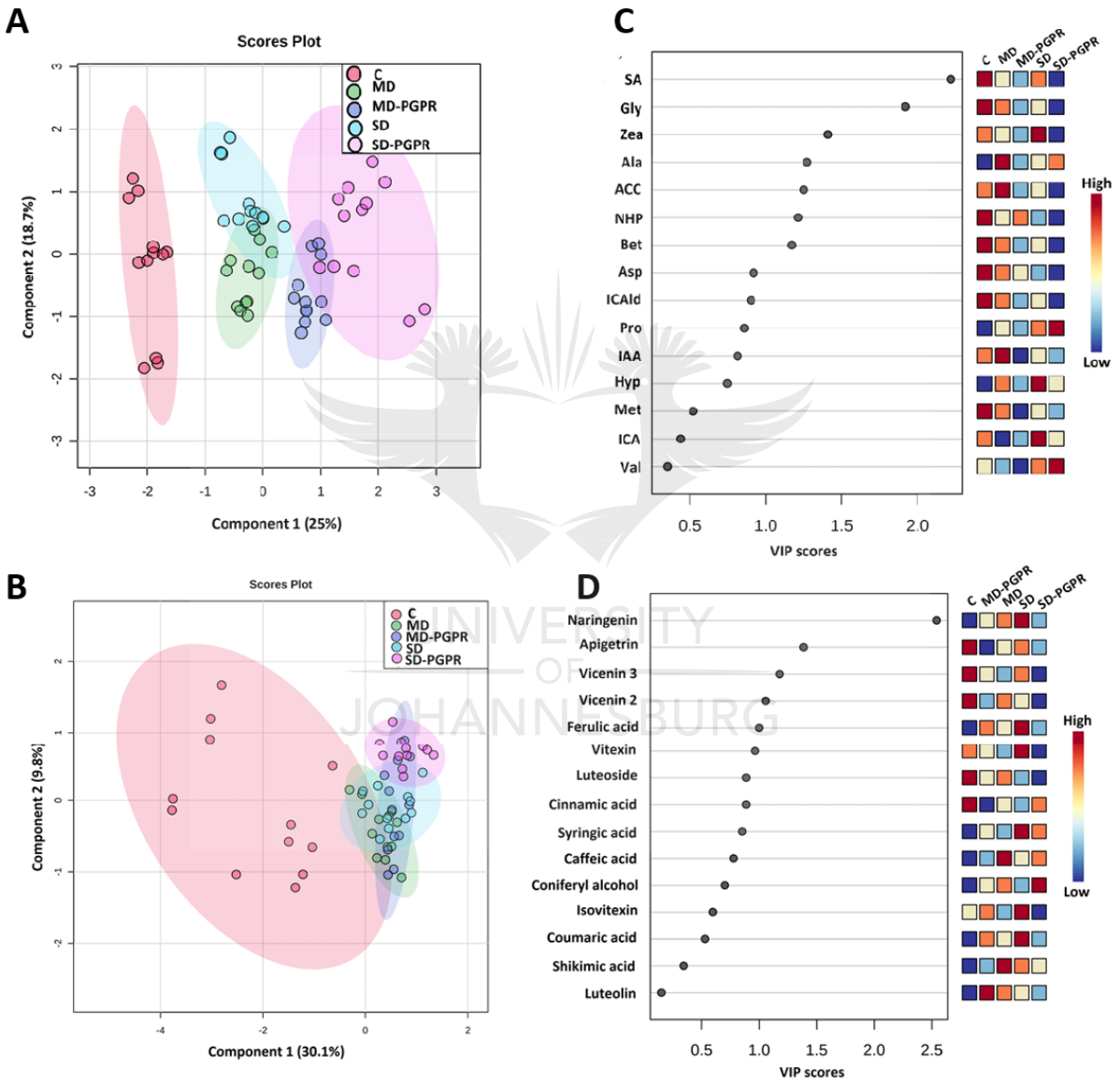

Figure 3.18. **Chemometric models of comparative quantification analysis of the primary and secondary metabolites**. Partial least squares-discriminant analysis (PLS-DA) scores plot (**A**) Amino acids and phytohormones, (**B**) Flavonoids and phenolic acids, and variable importance in projection plot showing the discriminant metabolite (VIP scores > 1) responsible for the class separation (**C**) Amino acids and phytohormones, (**D**) Flavonoids and phenolic acids. **Abbreviations**: C, control; PGPR, plant growth-promoting rhizobacteria; MD, mild drought; SD, severe drought.

Overall differential changes of amino acids, phytohormones, flavonoids and phenolic acids profiles induced by mild and severe drought stress conditions in naïve plants are represented by radar plots (**Figure 3.19**) at 4 WAE. The evaluation of the metabolic changes indicates that drought stress influences both the primary and secondary metabolism by differentially altering quantitative changes in the different metabolite classes. Hierarchical clustering heatmap analysis allowed for the visualisation of these quantitative changes for amino acids and phytohormones in both naïve- and PGPR-primed maize plants under mild- and severe drought stress conditions (**Figure 3.20**).

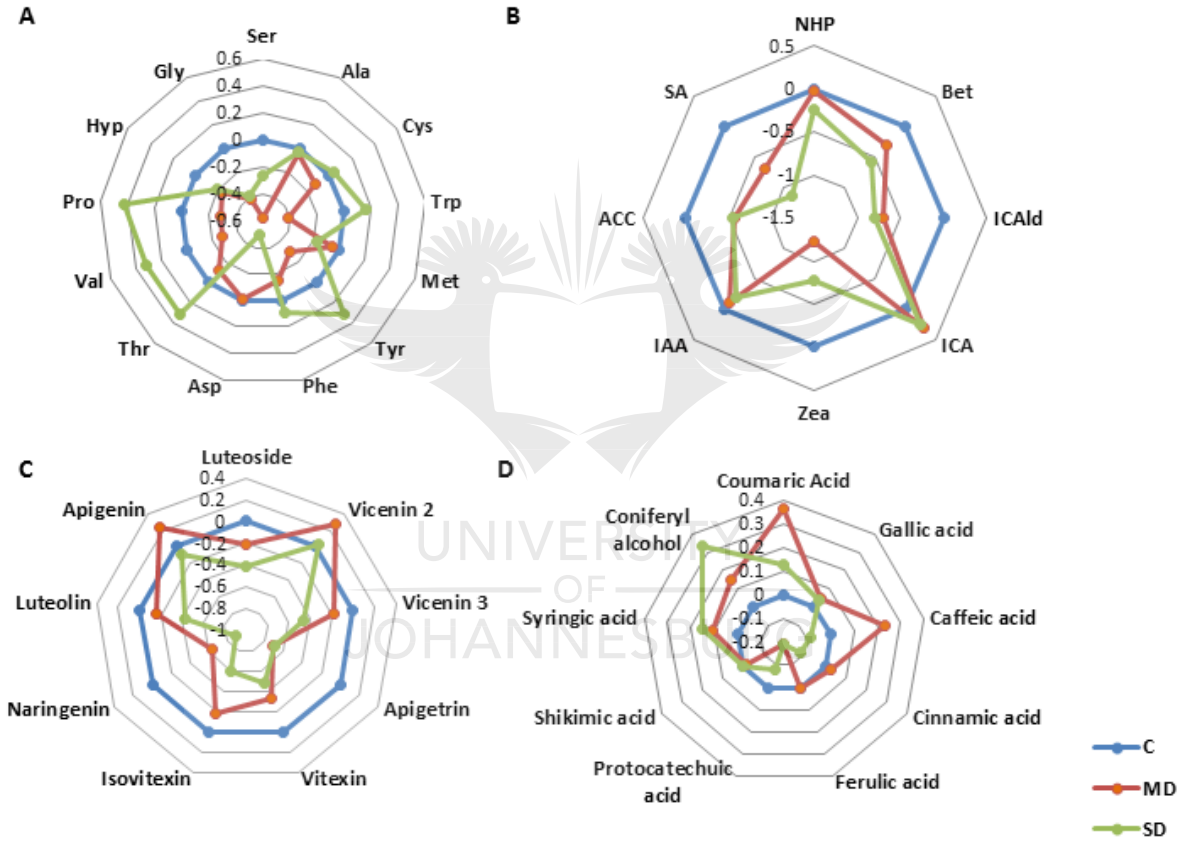

Figure 3.19: **Radar plots showing quantitative ratio changes of metabolites under well-watered (T2) conditions with and drought treatment (T3, T4).** Plots show the calculated logarithmic fold changes from the quantification of each metabolite (**A)** Amino acids **(B)** Hormones **(C)** Flavonoids and **(D)** Phenolic acids showing metabolite changes at 4 WAE.

Signatory amino acids that showed decreased levels under MD conditions included Ser, Ala, Cys, Gly, and HyP; and for SD conditions included Ser, Met, Asp, Gly, and Hyp (**Figure 3.20**). The decrease in levels of Ser, Gly, and Hyp evident in both MD and SD may suggest a common drought stress response mechanism induced in plants to combat mild or severe

drought stress. Under drought stress, these amino acids can be catabolised into TCA intermediates for increased energy production required for sustained plant growth as highlighted in **section 3.3.2**. The downregulation of Gly is coupled to increased energy production due to low Gly concentrations' inhibitory effect on photorespiration, an energy wasteful process that inhibits photosynthesis in plants (Eisenhut *et al.*, 2007; Kang *et al.*, 2018). Ala and Cys unique to MD which were also downregulated can also serve as TCA cycle intermediates for increased energy production. Met downregulation under severe drought conditions has been reported as a drought induced defence mechanism in plants (Larrainzar *et al.*, 2014), and this correlates with the findings of the present study. Not only is Met catabolised into acetyl-CoA – a TCA cycle precursor, but it can also be adenosylated into S-adenosyl methionine (SAM), a common precursor for ethylene signalling and polyamine biosynthesis (Pandey *et al.*, 2000; Amir, 2010).

The production of polyamines upregulates the biosynthesis of osmolytes such as soluble sugars under severe drought conditions. In plants, the synthesis of osmolytes serves as an effective mechanism for water deficit. Osmolytes are frequently used by cells to accommodate osmotic pressure within the plant cells by acting as osmoprotectants which directly stabilise protein and membrane structures under dehydration conditions (Iqbal, 2018). It is worth noting that the decrease in the concentrations of the overall amino acids differed in MD and SD conditions. Under MD, more amino acids were downregulated when compared to SD conditions (**Figure 3.20**). This finding implies that under MD conditions, the plant can downregulate several amino acids for the induction of numerous proteins, whereas under SD conditions, protein biosynthesis is limited due to the severity of the drought stress. A recent proteomics study by Iqbal (2018) reported on protein accumulation under mild, moderate and severe drought stress conditions in which differential increase in proteins involved in carbohydrate energy metabolism, protein homeostasis, transcription, cell structure, cell membrane transport, signal transduction, stress and defence responses was observed. Since drought stress often gives rise to protein denaturation, plants therefore need to balance between synthesis and degradation of proteins to maintain normal cellular metabolic activities required for plant growth and defence responses. (Reinbothe *et al.*, 2010; Hildebrandt *et al.*, 2015).

In contrast to the normal defence mechanisms employed by plants under mild and severe drought stress discussed above, PGPR-primed plants induced an accumulation of numerous

signatory amino acids (Gly, Cys, Ser, Thr, Val, HyP, and Pro) under mild drought stress conditions and (Gly, Cys, and Met) under severe drought stress conditions (**Figure 3.20**). This suggests that the application of microbial-based biostimulants activates cellular and molecular defence mechanisms under drought stress which involve dynamic changes in amino acid levels. Numerous studies have reported the increase of the amino acid pool as one of the PGPR-based mechanisms in enhancing drought tolerance (Vardharajula *et al.*, 2011; Ahemad & Kibret, 2014b; Khan *et al.*, 2019b). As previously mentioned, these amino acids can be catabolised into TCA intermediates for energy production, and therefore the increase in their pool may point to this need of energy production. The latter can then be used for sustained plant growth under unfavourable drought stress conditions. PGPR treatment revealed the accumulation of Pro, HyP, and Tyr (also observed in MD with no PGPR) under mild drought stressed plants. The accumulation of osmolytes is a general mechanism plants deploy to cope with water deficit, and is documented as a PGPR-based priming mechanism for enhanced growth under drought in several plants (Paul *et al.*, 2008; Sandhya *et al.*, 2010, 2015; Ansary *et al.*, 2012; Shintu & Jayaram, 2015). Pro accumulation under drought stress is a key adaptive plant response mechanism under drought stress (Szabados & Savouré, 2010; Fàbregas & Fernie, 2019). This amino acid is an osmolyte that regulates water potential in plant cells, enabling plants to sustain normal growth under drought stress conditions. Moreover, it can act as a signalling molecule that modulates mitochondrial functions, influence cell proliferation and trigger the expression of genes crucial for plant recovery following stress encounters (Szabados & Savouré, 2010; Meena *et al.*, 2019). Additionally, Pro can act as a free radical scavenger, cell redox balancer, source of carbon, nitrogen and energy, stabiliser for cellular structures and membranes, and an activator of detoxification pathways (Kishor *et al.*, 2005; Trovato *et al.*, 2008; Hayat *et al.*, 2012).

Interestingly, additional osmolytes Hyp and Bet accumulation increased in PGPR-treated plants under both mild and severe drought stress conditions. Bet enhances drought stress by maintaining membrane integrity, cell osmolarity, and protecting the photosynthesis systems. Additionally, due to its zwitterion state, it can interact with protein complexes and membranes, protecting them from ROS (Gupta *et al.*, 2014). The Ser content, which also increased under PGPR treatment in drought stressed plants (**Figure 3.20**), is involved in the biosynthesis of glycine betaine – an osmoprotectant. Ser can be catabolised into choline through the decarboxylation reactions to produce choline which then undergoes oxidation

reactions to form glycine betaine (Sakamoto & Murata, 2001, 2002), which has been reported to be one of the most efficient osmoprotectants (Kurepin *et al.*, 2015). The quantitative increase in Ser content in PGPR-treated stressed plants can be therefore be channelled towards the biosynthesis of glycine betaine conferring drought stress tolerance. For example, Gou *et al.* (2015) reported an increase in betaine under drought stress in maize plants, induced by a PGPR strain *Pseudomonas fluorescens*, and an improvement of water retention and plant growth under drought stress conditions was observed. Additionally, Gagné-Bourque *et al.* (2016) reported an accumulation of serine in *B. subtilis* B26 treated plants under drought stress.

In contrast to the accumulation of the amino acids highlighted above, PGPR treatment also induced a decrease in quantitative levels of signatory metabolites Tyr, Trp, Phe, Val and Thr. As deciphered in **subsection 3.3.2.1**, AAAs Tyr, Trp, and Phe are central molecules in plant metabolism (**Figure 3.20**). Aside from their function as protein building blocks, these amino acids serve as precursors for a variety of plant hormones (**Figure 3.20**), as well as for a very wide range of aromatic secondary metabolites with multiple biological functions. The degradation of these amino acids initiates the phenylpropanoid pathway which produces numerous plant defence-related compounds such as flavonoids, lignin, vitamin E and suberin. PGPR treatment therefore drives the degradation of these amino acids to the biosynthesis of phenylpropanoids, resulting in increased production of defence-related compounds to enhance drought stress tolerance The AAAs levels were higher in PGPR-primed plants when compared to PGPR-primed stressed plants (**subsection 3.3.2.1**). Thus, this indicates that during the PGPR-priming phase AAAs synthesis is preconditioned to provide a pool of precursors for the synthesis of defence related metabolites under drought stress.

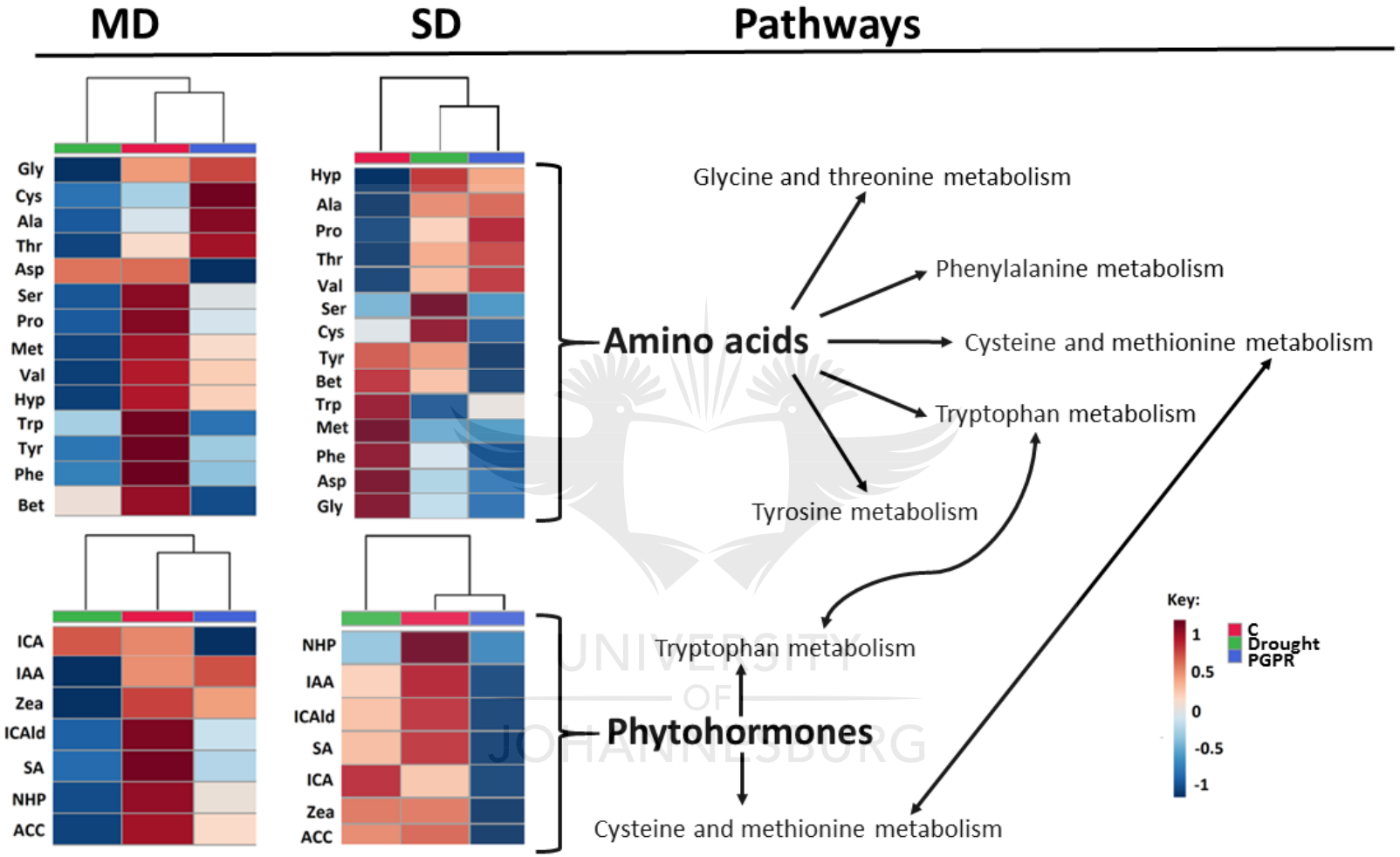

Figure 3.20. Quantitative analysis of amino acids and phytohormones. Heatmap hierarchical cluster analysis displaying metabolite abundances under control (T2), mild /severe drought stress (T5, T6) and mild /severe drought stress conditions with PGRP treatment (T3, T4), together with differentially altered metabolic pathways**. Abbreviations**: Mild drought, MD; Severe drought, SD; and Control, C.

The chemometric modelling (**Figure 3.18A**) revealed that the signatory metabolites related to drought stress conditions are NHP, Bet, ICAld, Zea, ACC and SA (**Figure 3.20**), where mild and severe drought stress conditions led to a decrease in levels of these phytohormones. Plant development is regulated and coordinated through the action of several hormones which may act either close to or distant from their sites of synthesis to mediate responses to environmental stimuli (Davies, 2010). Plants can modify their biochemistry and physiology in rapid responses to fluctuations in their environment through the action of phytohormones, a critical requirement for their survival. Environmental perturbations therefore elicit changes to the production, distribution or signal transduction of growth hormones as well as stress hormones, which may promote specific protective mechanisms. The downregulation of phytohormones under drought stress may therefore suggest a form of stress tolerance for enhanced survival through the regulation of hormonal homeostasis directed towards normal conditions (naïve plants with no stress). As previously mentioned **(section 3.3.2**), ICAld can be catabolised into ICA and IAA, supported by the observed accumulation of ICA associated with enhanced stress tolerance. This was also phenotypically observed where the biomass (root and shoot) of PGPR-primed stressed plant was found to be higher than that of naïve stressed plants or that of PGPR-primed plants compared to naïve plants (**section 3.3.1; Figure 3.5**). Conversely, PGRP treatment induced ICAld accumulation under drought stress which may suggest increased flux in IAA biosynthesis through indole-3-acetaldehyde dehydrogenase action. Drought stress generally stimulates ACC (an ethylene precursor), which increases ethylene synthesis in plants (Wang *et al.*, 2003). Apart from ethylene being a positive regulator of many physiological responses in plants, it is also associated with reduced plant growth and senescence.

The downregulation of ACC under drought may suggest a stress response mechanism by the plant, to inhibit the production of ethylene. Additionally, under mild and severe drought stress conditions with PGPR treatment, ACC levels were upregulated (**Figure 3.20**). PGPR have been reported to synthesise ACC deaminase enzymes that degrade ACC and lower ethylene levels by converting ACC into nitrogen and α-ketobutyrate (Gamalero & Glick, 2015; Olanrewaju *et al.*, 2017; Nascimento *et al.*, 2018). However, the high ACC content observed in PGPR-primed stressed plant, suggest that PGPR-priming preconditions ACCs pathway for enhance the synthesis of ethylene to initiated plant defence response/stress tolerance. Recent studies have shown that ACC also act as a signalling molecule that

regulates plant growth and development independent of ethylene. For example, (Xu *et al.*, 2008; Tsang *et al.*, 2011) reported ACC as part of the plant signalling mechanisms where root cell elongation was observed following ACC application. Thus, the high levels of ACC may indicate a PGPR induced drought tolerance mechanism in primed plants which enhances root growth under drought stress.

NHP quantitative levels were decreased in naïve plants under both mild and drought stress conditions (**Figure 3.20**). N-substituted phthalimides have been reported to mimic multiple gibberellins (GAs) effects on growth and development (Li *et al.*, 2015; Jiang *et al.*, 2017). The GA signalling pathway has been shown to be a major target for stress responses and the central role of GA in response to abiotic stress has become increasingly evident. For example, Achard *et al.* (2006) and Colebrook *et al.* (2014) showed that GAs negatively/inversely affect drought stress responses in plants. Here, the authors demonstrated that GA over accumulation leads to water sensitivity in wild type plants, whereas, GA-deficient mutants showed an increased water deficit tolerance. Additionally, Plaza-Wüthrich *et al.* (2016) has reported on how GA deficiency confers drought tolerance in staple crops tef (*Eragrostis tef* Zucc.) and finger millet (*Eleusine coracana* Gaertn). The drought stress-induced decrease in the levels of NHP may therefore be an adaptive mechanism employed by plants under drought stress for survival.

Furthermore, PGPR treatment induced an increase in the levels of Zea, SA, ICAld and NHP as in maize plants under mild and severe drought stress conditions (**Figure 3.19**). Zeatin stimulates cell division, root hair proliferation, controls differentiation of root meristem, and regulates water balance and growth under drought stress. PGPR treatment induced the accumulation of Zea under both mild and severe drought stress conditions, suggesting its role in drought stress tolerance through enhanced root development, osmotic adjustment and growth. The increased accumulation of cytokinins by different PGRP strains under drought stress has been reported in previous studies (Timmusk & Wagner, 1999; Pospíšilová *et al.*, 2005; Liu *et al.*, 2013). SA on the other hand is a phytohormone that has diverse roles in plant development, such as mitigating oxidative stress under drought stress. It also influences stomatal closure through SA-mediated ROS accumulation and activated mitogen-activated protein kinases (MAPKs) thereby initiating the expression of key enzymes and drought stress responses. A recent study by Jochum *et al.* (2019) reported elevated levels of SA and

increased root growth in wheat and maize plants inoculated with *Bacillus* spp. and *Enterobacter* spp. under drought stress. Interestingly, under both mild and severe drought conditions, PGPR treatment induced the accumulation of all phytohormones except ICA and NHP for mild stress and severe drought stress treatment, respectively. The association of PGPR with profound changes in the plant's hormone homeostasis as well as growth and development related to abiotic stress tolerance has been well established. These changes encompass, but are not limited to enhanced growth, root and shoot architecture modifications, and synthesis of secondary metabolites (Spaepen *et al.*, 2014; Verbon & Liberman, 2016). The accumulation of the phytohormones reported in this study therefore reveals one of the key drought tolerance mechanisms through which PGPR acts to mediate drought stress.

The metabolic reprogramming in naïve and primed (PGPR-treated) plants under mild and severe drought stress also involved differential changes in the levels of flavonoids and phenolic acids (**Figure 3.21**). Under mild drought stress, an accumulation of signatory metabolites namely flavonoids (vicenin, apigenin) (**Figure 3.21**) and phenolic acids (coniferyl alcohol, syringic acid, cinnamic acid, caffeic acid and coumaric acid) (**Figure 3.21**) was observed. In response to the constantly fluctuating environmental conditions such as drought, plants have evolved the capacity to synthesise various phenolic acids (Caldwell *et al.*, 2003) and flavonoids. Under stress conditions, the phenylpropanoid and flavonoid metabolic pathways are altered resulting in the increased accumulation of different flavonoids and phenolic acids. Drought stress disturbs the balance between ROS generation and scavenging, and thus accelerates ROS propagation which damages vital macromolecules such as nucleic acids and proteins, ultimately leading to cell death. ROS induce protein damage by oxidising amino acid residues resulting in irreversible carbonylation in side chains (Kristensen *et al.*, 2004). Additionally, ROS limit CO_2 fixation in chloroplasts and react with chlorophyll during photosynthesis to form the chlorophyll triplet state which can rapidly generate singlet oxygen ($^1O_2$), thus causing damage to photosynthetic complexes (Asada, 2006; Buchert & Forreiter, 2010).

As previously mentioned in **subsection 3.3.2.2**, the accumulation of flavonoids and phenolic acids has been linked to strong radical scavenging activity. These antioxidants therefore contribute to the mitigation of oxidative stress induced by drought stress. This defence mechanism employed by plants under mild and severe drought stress conditions is indicative

of drought stress tolerance through antioxidant production. Numerous studies in different plant species have reported phenolic acids as indicators of drought tolerance through increased antioxidant capacity (Caldwell *et al.*, 2003; Weidner *et al.*, 2009; Robert *et al.*, 2010; Gharibi *et al.*, 2016; Varela *et al.*, 2016; Sarker & Oba, 2018; Laxa *et al.*, 2019). Contrary to the increased accumulation of flavonoids and phenolics under mild and severe drought stress conditions, most of the flavonoids (apigetrin, vitexin, naringenin, vicenin, isovitexin, and luteolin) (**Figure 3.21**) and phenolic acids (protocatechuic acid, ferulic acid, cinnamic acid and caffeic acid) (**Figure 3.21**) were downregulated under severe drought stress conditions in non-primed plants. Since plants depend on the antioxidant system (flavonoids and phenolic acids) to counteract the effect of ROS induced by drought stress, the downregulation of these compounds indicates that the plants' natural defence system may be overwhelmed, resulting in plants succumbing to death due to severe drought as observed in **section 3.3.1** (**Figure 3.5**), where non-primed stressed plants had lower antioxidant markers and root and shoot biomass when compared to the PGPR-primed stressed plants.

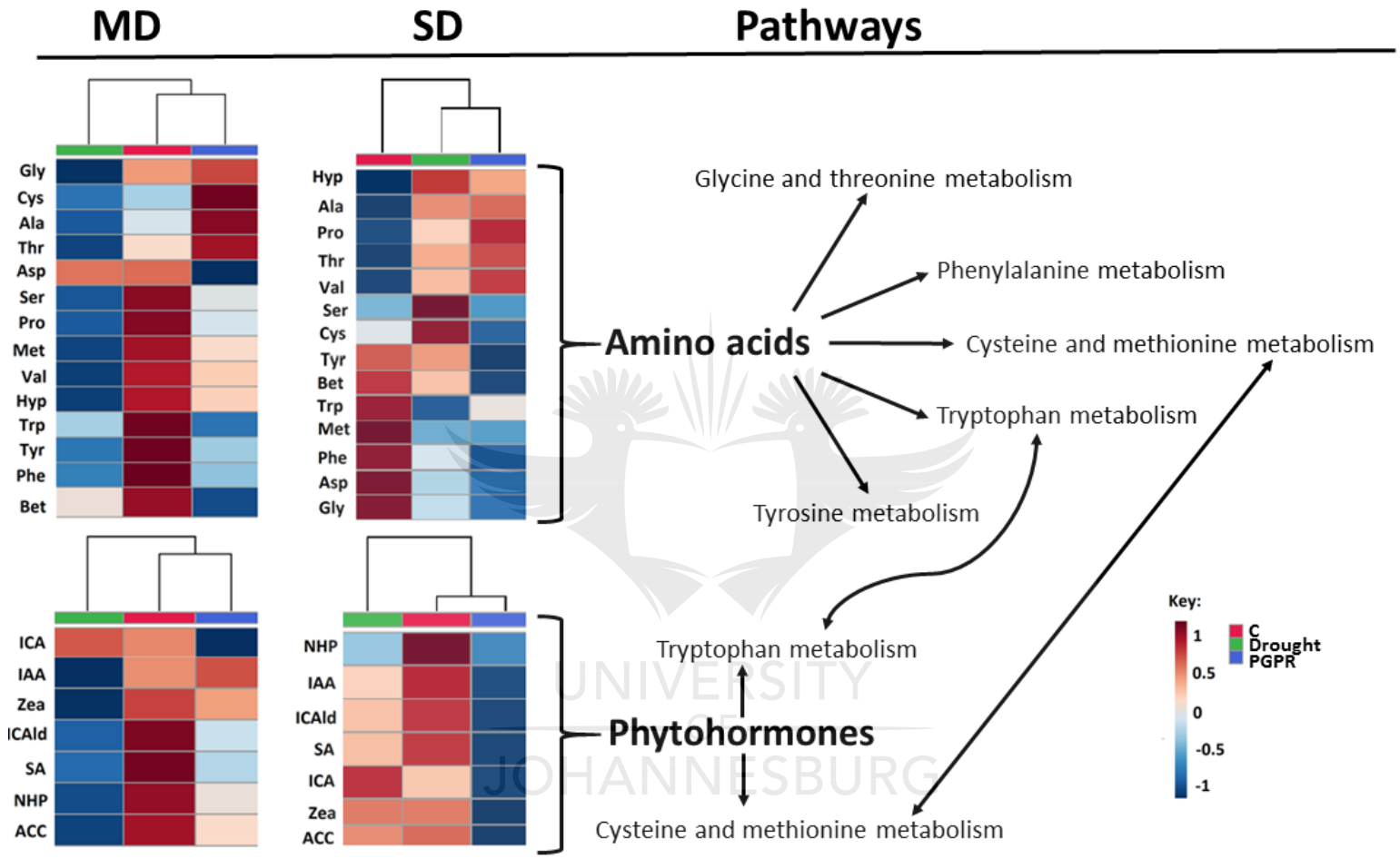

Figure 3.21. **Quantitative analysis of flavonoids and phenolic acids**. Heatmap hierarchical cluster analysis displaying metabolite abundances under control, mild /severe drought stress and mild / severe drought stress conditions with PGRP treatment (T5/T6 *vs*. T3/T4), together with differentially altered metabolic pathways. **Abbreviations**: Mild drought, MD; severe drought, SD; and control, C.

It is worth noting that the downregulation of apigetrin and naringenin was the highest when compared to the other flavonoids. Since naringenin is a general precursor for the synthesis of flavonoids, it can be degraded to drive the synthesis of other flavonoids involved in stress responses (Petrussa *et al.*, 2013). Moreover, coumaric acid and caffeic acid had the highest accumulation rate when compared with other phenolics and this is due to these molecules serving as upstream precursors for the rest of the phenolic acids (**Figure 3.21**). Metabolite-metabolite correlation analysis was employed (**Figure 3.22**) to unravel the inter-relationship of these metabolites using the magnitude and direction of the correlation. Naringenin and apigetrin both displayed a strong negative correlation between numerous phenolic acids and flavonoids including gallic acid, coumaric acid, shikimic acid, coniferyl alcohol, protocatechuic acid, cinnamic acid syringic acid and coniferyl alcohol. Conversely, coumaric acid showed a negative correlation between syringic acid, apigenin, vicenin, isovitexin and naringenin (**Figure 3.22**).

As previously mentioned in **section 3.3.2**, a negative correlation is indicative of an inversely proportional relationship between metabolites where an increase in another metabolite may result in the decrease of the other. The decrease in quantitative levels of naringenin, apigetrin, coumaric acid and caffeic acid induced by mild and severe drought stress suggest that these metabolites serves as precursors, for the biosynthesis of other phenolic acids and flavonoids with increased antioxidant capacity for drought stress tolerance. The overall metabolite-metabolite correlation analysis (**Figure 3.22**) revealed a negative correlation between flavonoids and phenolic acids as indicated by the blue clustering patterns. The weak correlation could be associated to the independent functions of these metabolite classes under drought stress or the biosynthesis of flavonoids from upstream phenolic acids, resulting in the rise of flavonoids concentrations causing a decrease in the concentrations of phenolic acids or vice versa.

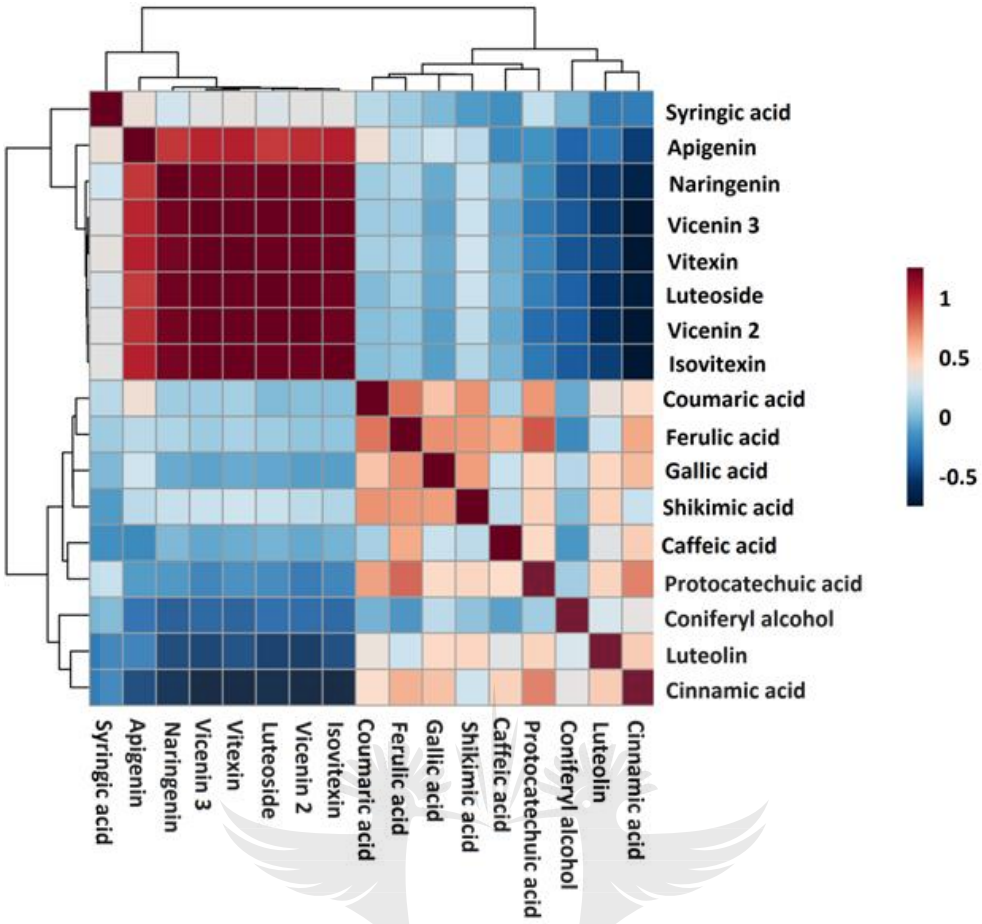

Figure 3.22. **Metabolite-metabolite correlation analysis heatmap**. Correlation analysis heatmap based on Pearson's *r* distance measure where red represents positive correlation and blue negative correlation between drought stressed without PGPR plants (T5/T6) and drought stressed plants with PGPR (T3/T4) under both mild and severe drougth conditions.

PGPR-primed maize plants, on the other hand, had different profiles of these secondary metabolites, pointing to enhanced drought stress tolerance. The primed plants showed an accumulation of naringenin and ferulic acid, and a decrease of isovitexin, luteolin, luteoside, coniferyl alcohol, cinnamic acid, caffeic acid and coumaric acid under mild drought stress (**Figure 3.21**). As mentioned above, naringenin upregulation is suggested to be due to the metabolite being a primary intermediate in flavonoid synthesis in which it can be degraded into other flavonoid classes resulting in enhanced suppression of ROS production and drought stress. A recent study by Yildiztugay *et al.* (2020) reported naringenin as an alleviator of short term osmotic stress by controlling the toxicity of hydrogen peroxide in the chloroplast. Similarly, ferulic acid is an upstream intermediate in the biosynthesis of plant secondary metabolites. This suggests enhanced drought stress tolerance induced by PGPR under drought conditions. Accumulation of numerous flavonoids and phenolic acids indicates

a more enhanced antioxidant capacity resulting in drought stress tolerance. The accumulation of these defence related metabolites can be linked to the morphological changes observed in **Figure 3.2**, where PGPR-primed plants exposed to mild/severe stress were found to have higher relative biomasses (shoot, root and dry) when compared to naïve plants. Moreover, the antioxidant markers were found to be higher in PGPR-primed stressed plants when compared to naïve stressed (**Table 3.3**), indicating that PGPR-primed plants have an augmented antioxidant system to negate the effects of excess ROS production induced by drought stress. During plant priming, the underlining molecular mechanisms leading to defence related metabolite accumulation are pre-conditioned. Thus, the high accumulation of phenolic acids (Singh *et al*., 2002, 2003, 2012; Ahemad & Kibret, 2014a) and flavonoids (Alexander *et al*., 2019; Asghari *et al*., 2020; Mirzaei *et al*., 2020) in PGPR-primed plants under severe drought stress is mechanism in which primed plant used to withstand drought. Phenolic acids and flavonoids are known to act as antioxidants that suppress ROS by scavenging free radicals, inhibition of pro-oxidant enzymes, potent metal chelators of metals involved in ROS production and recycling of other antioxidants in the plant.

To further understand the functional impact of PGPR treatment under drought stress and situate this differential changes in a metabolome view, MetPA was employed. The quantitative metabolomic profiles of amino acids, phytohormones, flavonoids and phenolic acids for treatments SD, MD, SD-PGPR and MD-PGPR were analysed using the following parameters: (1) enrichment analysis using the global test method, and (2) centrality measurement using relative betweenness. The most significantly altered pathways with an impact score greater than 0.1 related to PGPR treatment under drought stress include Phe metabolism, Trp metabolism, Tyr metabolism, Gly, Ser and Thr metabolism, and Cys and Met metabolism, with Trp and Cys and Met metabolism common to both amino acids and phytohormones quantitative levels (**Figure 3.20**). In contrast, flavonoid biosynthesis, flavone and flavonol biosynthesis, Tyr metabolism, Trp metabolism, phenylalanine metabolism and phenylpropanoid biosynthesis pathways were significantly altered due to PGPR treatment under drought stress (**Figure 3.21**). Additionally, Khan *et al.* (2019b,a) similarly reported Phe metabolism as one of the key pathways altered in response to the application of PGPR and plant growth regulators (PGRs) in chickpea under drought stress. As underlined in **subsection 3.3.2.1**, Phe and Trp are upstream precursors for various secondary metabolites such as phenylpropanoids, which play essential roles in plant growth and defence against

drought stress. Additionally, the phenylpropanoid pathway which was significantly altered due to PGPR treatment can, in turn, induce flavonoid biosynthesis (**Figure 3.21**), thereby enhancing the drought stress tolerance through over accumulation of antioxidants shown *via* increased quantitative levels of flavonoids in this study. Trp metabolism has also been reported as one of the most enriched pathways in the tolerant genotype of maize kernels (Wang *et al.*, 2019) and spring wheat (Michaletti *et al.*, 2018) than sensitive genotype under drought stress, indicating that this pathway is essential in heightened drought adaptation and plant development.

In summary, PGPR induced differential morphophysiological and metabolic changes, enhanced physiological events that govern drought stress tolerance in the post-challenge phase (**Figure 3.23**). PGPR treatment in mild and severe drought stress conditions mediated key mechanisms involved in drought stress tolerance as shown in **Figure 3.23**. These key mechanisms include increased levels of amino acids, flavonoids, hormones, phenolic acids and antioxidant markers. The observed changes when situated in the maize metabolome spanned key impacted pathways including Phe metabolism, Gly metabolism, and flavonoid biosynthesis associated with enhanced drought stress tolerance.

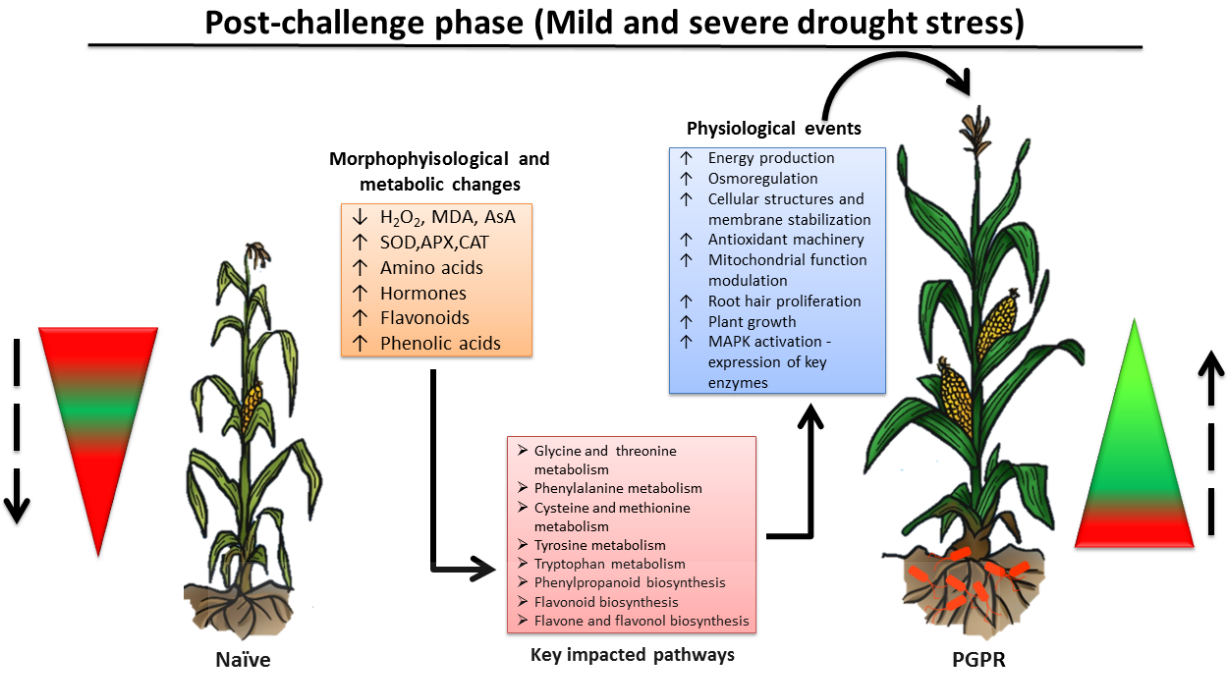

Figure 3.23. Schematic diagram of a model representing key mechanisms induced by microbial biostimulants in enhancing drought stress tolerance. PGPR treatment induced morphophysiological and metabolic changes which are associated to enhanced drought stress tolerance, such as accumulation of antioxidant markers, reprogramming of amino acids, flavonoids, phytohormones and phenolic acids metabolic profiles. These changes induced by PGPR point to physiological events that enhance drought stress tolerance whereas naïve plants display low drought stress responsive mechanisms and thus cannot tolerate drought stress. **Abbreviations**: H_2O_2, Hydrogen peroxide; MDA, Malenoaldehyde; AsA, Ascorbate; SOD, superoxide dismutase; APX, ascorbate peroxidase; and CAT, catalase.

PGPR-mediated drought stress tolerance mechanisms elucidated herein include enhanced (i) energy production facilitated by amino acid degradation into TCA intermediates, (ii) osmoregulation, (iii) cellular and membrane stabilization, (iv) antioxidant machinery, (v) root hair proliferation and (vi) activation of MAPK cascades pointing to enhanced expression of drought stress responsive genes. Naïve plants however do not efficiently exhibit the drought stress tolerance mechanisms mediated by PGPR and therefore cannot negate the deleterious effects of drought stress and succumb to the stress.

3.4 Conclusion

Plant-microbe interactions are complex, dynamic and very crucial, particularly in terms of crop agriculture, and it is of specific interest in plant metabolomics for abiotic stress control. Numerous studies have stressed the amplitude of the importance of microbial biostimulants, predominantly PGPR-based formulations, in plant-microbe interactions under various environmental conditions. PGPR have also been shown to improve overall plant growth and development and stress tolerance mechanisms under abiotic stress conditions. As illustrated in the introduction, this study focused on elucidating PGPR-based biostimulant-induced morphological and metabolic differentiations in maize plants, indicatory of probable mechanisms by which the microbial-based biostimulant stimulates plant growth and enhances drought stress tolerance through priming. In terms of morphological results, under PGPR treatment, non-enzymatic markers were decreased whereas enzymatic stress markers were elevated. This upregulation of antioxidant enzymes revealed one key mechanism through which PGPR alleviates oxidative stress in both unstressed and drought stressed plants. Additionally, PGPR induced increase in relative shoot, root and dry biomass could be attributed to PGPR enhancing the plants overall element and nutrient uptake. Targeted metabolomics approach employed in this study revealed metabolic reconfigurations of the primary and secondary metabolism induced by PGPR under well-watered, mild and severe drought stress conditions. These differential changes in amino acids, phytohormones, flavonoids and phenolic acids observed in this study elicited by PGPR revealed the probable mechanisms employed by PGPR in promoting plant growth and priming mediation resulting in enhanced drought stress tolerance (a model summarized in **Figures 3.17** and **3.23**).

These mechanisms include increased energy production (amino acid channelling into TCA intermediates), increased root, leaf and shoot growth, enhanced photosynthesis, nutrient and water uptake, ACC degradation, increased osmolyte production, increased protein biosynthesis, increased secondary metabolite biosynthesis and oxidative stress mitigation. The observed differential metabolic profiles were mapped onto various metabolic pathways linked to the priming phenomenon and plant growth promotion. Results drawn from this study therefore suggest PGPR as a potential priming agent, enhancing plant growth and development as well as mild and severe drought stress tolerance. The work presented herein is therefore a contribution to the ongoing efforts of elucidating the biochemical and molecular

complex mechanisms underlying plant-PGPR interactions in enhancing drought stress tolerance through the exploitation of targeted metabolomics analysis. Metabolomics therefore offers opportunity to decode the molecular mechanisms that explain the modes of actions of biostimulants.

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3.7 Supplementary Materials

The supplementary figures and tables are provided as additional information to support the main results reported here in **Chapter 3**. All the experimental raw data, figures and tables that are not included in this dissertation are available in the Department of Biochemistry at the University of Johannesburg; and accessible with permission from Dr. Fidele Tugizimana

Table S3.1: Development and optimisation of MRM parameters by direct infusion and optimisation of the collision energy (CE) for each compound transition using the MRM optimisation method tool.

Compound name	Rt (min)	Ion mode	*m/z*	Transition	CE (eV)	Quadrupole 1 (V)	Quadrupole 3 (V)	Dwell time (ms)
Amino acids								
Proline	1.50	$[M+H]^+$	116.20	116.20>70.15 116.20>43.10	-18.0 -28.0	-14.0 -13.0	-11.0 -15.0	17.0 17.0
Cysteine	1.31	$[M+H]^+$	241.20	241.20>151.90	-14.0	-12.0	-15.0	37.0
Serine	1.34	$[M+H]^+$	106.20	106.20>59.95 106.20>88.10	-13.0 -13.0	-12.0 -12.0	-10.0 -18.0	17.0 17.0
Alanine	1.40	$[M+H]^+$	90.20	90.20>44.05 90.20>44.90	-13.0 -30.0	-10.0 -18.0	-15.0 -16.0	17.0 17.0
Threonine	1.40	$[M+H]^+$	120.20	120.20>56.05 120.20>74.10	-16.0 -12.0	-13.0 -13.0	-20.0 -28.0	17.0 17.0
Aspartic acid	1.41	$[M+H]^+$	134.05	134.05>74.10	-15.0	-10.0	-13.0	37.0
Valine	1.73	$[M+H]^+$	118.20	118.20>72.10 118.20> 55.05	-12.0 -23.0	-14.0 -14.0	-12.0 -23.0	17.0 17.0
Methionine	2.14	$[M+H]^+$	150.20	150.20>60.90 150.20>56.10	-17.0 -24.0	-10.0 -11.0	-20.0 -24.0	17.0 17.0

Tyrosine	3.29	$[M+H]^+$	182.00	182.0>136.10	-14.0	-13.0	-24.0	37.0
Phenylalanine	5.93	$[M+H]^+$	166.00	166.00>120.10	-14.0	-12.0	-21.0	131.0
Tryptophan	6.77	$[M+H]^+$	205.20	205.20>188.05 205.20>146.10	-11.0 -17.0	-14.0 -14.0	-19.0 -14.0	64.0 64.0
Glycine	1.40	$[M+H]^+$	76.10	76.10>76.10	-13.0			100
Betaine* (Osmolyte)	1.55	$[M+H]^+$	118.05	118.05	-25.0	-	-	100
Hormones								
N-hydorxyethylpthalimide*	30.08	$[M+H]^+$	192.15	192.15>192.15	-10.0	-	-	100
Indole-3-carboxyaldehyde*	16.60	$[M+H]^+$	146.05	146.05	-25.0	-	-	100
Indole-3-carboxylic acid*	22.00	$[M+H]^+$	161.95	161.95>161.95	-15.0	-	-	100
Indole-3-acetic acid (IAA)	26.81	$[M+H]^+$	176.10	176.10>130.10 176.10>77.20 176.10>103.10	-15.0 -43.0 -30.0	-20.0 -12.0 -12.0	-20.0 -20.0 -22.0	65.6 65.6 65.6
Zeatin	12.98	$[M+H]^+$	220.15	220.15>202.05 220.15>136.00 220.15>119.00	-19.0 -24.0 -34.0	-10.0 -11.0 -10.0	-19.0 -24.0 -11.0	100.0 100.0 100.0
Salicylic acid (SA)	22.60	$[M-H]^-$	137.00	137.00>92.95 137.00>65.00 137.00>75.05	15.0 28.0 32.0	20.0 14.0 14.0	20.0 10.0 27.0	65.6 65.6 65.6
1-Amino-cyclopropane carboxylic acid (ACC)	1.58	$[M+H]^+$	101.60	101.60>56.20 101.60>28.15 101.60>30.20	-14.0 -23.0 -37.0	-18.0 -18.0 -18.0	-21.0 -10.0 -30.0	65.6 65.6 65.6
Flavonoids								
Luteoside	9.98	$[M+H]^+$	449.0	449.0>287.15 449.0>417.15 449.0>153.10	-21.0 -9.0 -54.0	-11.0 -13.0 -11.0	-19.0 -21.0 -29.0	100.0 100.0 100.0
Vicenin 2	4.36	$[M+H]^+$	595.0	595.0>324.90 595.0>475.25 595.0>379.20	-35.0 -17.0 -30.0	-22.0 -20.0 -22.0	-22.0 -16.0 -27.0	100.0 100.0 100.0

Vicenin 3	5.99	$[M-H]^-$	563.0	563.0>353.0 563.0>383.05 563.0>473.20	30.0 34.0 29.0	28.0. 28.0 20.0	17.0 26.0 14.0	100.0 100.0 100.0
D-fluorophenylalanine (internal standard)	1.50	$[M+H]^+$	184.0	184.0>138.15 184.0>91.15 184.0>118.15	-14.0 -30.0 -22.0	-12.0 -10.0 -11.0	-26.0 -17.0 -11.0	100.0 100.0 100.0
Apigetrin	15.04	$[M-H]^-$	431.0	431.0>268.10 431.0>269.15 431.0>210.90	35.0 25.0 51.0	20.0 11.0 15.0	29.0 29.0 20.0	100.0 100.0 100.0
Isovitexin	6.21	$[M-H]^-$	431.0	431.0>311.15 431.0>341.00 431.0>283.10	22.0 22.0 36.0	20.0 20.0 15.0	21.0 16.0 29.0	100.0 100.0 100.0
Vitexin	6.22	$[M-H]^-$	431.0	431.0>311.15 431.0>283.05 431.0>341.20	21.0 33.0 20.0	20.0 15.0 20.0	21.0 19.0 24.0	100.0 100.0 100.0
Naringenin	7.19	$[M-H]^-$	271.0	271.0>151.05 271.0>119.10 271.0>106.95	16.0 26.0 24.0	13.0 13.0 13.0	26.0 21.0 19.0	100.0 100.0 100.0
Luteolin	16.80	$[M-H]^-$	285.0	285.0>132.95 285.0>151.15 285.0>175.15	34.0 26.0 24.0	13.0 10.0 10.0	23.0 29.0 19.0	100.0 100.0 100.0
Apigenin	18.37	$[M-H]^-$	269.0	269.0>117.05 269.0>88.95 269.0>151.10	34.0 20.0 23.0	18.0 28.0 13.0	19.0 15.0 26.0	100.0 100.0 100.0
Phenolics								
Coumaric acid	10.24	$[M-H]^-$	163.0	163.0>119.10 163.0>93.05 163.0>117.15	17.0 17.0 18.0	16.0 30.0 29.0	22.0 16.0 20.0	100.0 100.0 100.0
Gallic acid	1.92	$[M-H]^-$	169.20	169.20>125.15 169.20>79.15 169.20>80.95	20.0 18.0 15.0	11.0 20.0 12.0	12.0 15.0 22.0	100.0 100.0 100.0
Caffeic acid	6.75	$[M-H]^-$	179.05	179.05>135.10 179.05>134.15	18.0 18.0	11.0 23.0	24.0 23.0	100.0 100.0

				179.05>79.0	20.0	19.0	12.0	100.0
Cinnamic acid	19.33	$[M+H]^+$	149.10	149.10>77.05 149.10>42.90 149.10>92.85	-12.0 -17.0 -11.0	-27.0 -13.0 -16.0	-28.0 -15.0 -17.0	100.0 100.0 100.0
Ferulic acid	15.69	$[M-H]^-$	193.20	193.20>134.10 193.20>178.30 193.20>149.10	13.0 17.0 13.0	15.0 17.0 10.0	29.0 21.0 15.0	100.0 100.0 100.0
Protocatechuic acid	1.95	$[M-H]^-$	153.20	153.20>109.15 153.20>108.15	16.0 17.0	14.0 23.0	22.0 23.0	100.0 100.0
Shikimic acid	0.72	$[M-H]^-$	173.05	173.05>111.20 173.05>93.05 173.05>105.05	18.0 15.0 12.0	12.0 15.0 9.0	17.0 29.0 19.0	100.0 100.0 100.0
Syringic acid	9.39	$[M-H]^-$	197.0	197.0>182.10 197.0>123.10 197.0>167.20	27.0 16.0 15.0	6.0 25.0 180	20.0 26.0 18.0	100.0 100.0 100.0
Coniferyl alcohol	6.66	$[M-H]^-$	179.20	179.20>134.95 179.20>110.90 179.20>78.80	18.0 12.0 13.0	9.0 10.0 26.0	29.0 21.0 27.0	100.0 100.0 100.0

* Single ion monitoring (SIM) was used for quantification of these compounds because they did not fragment.

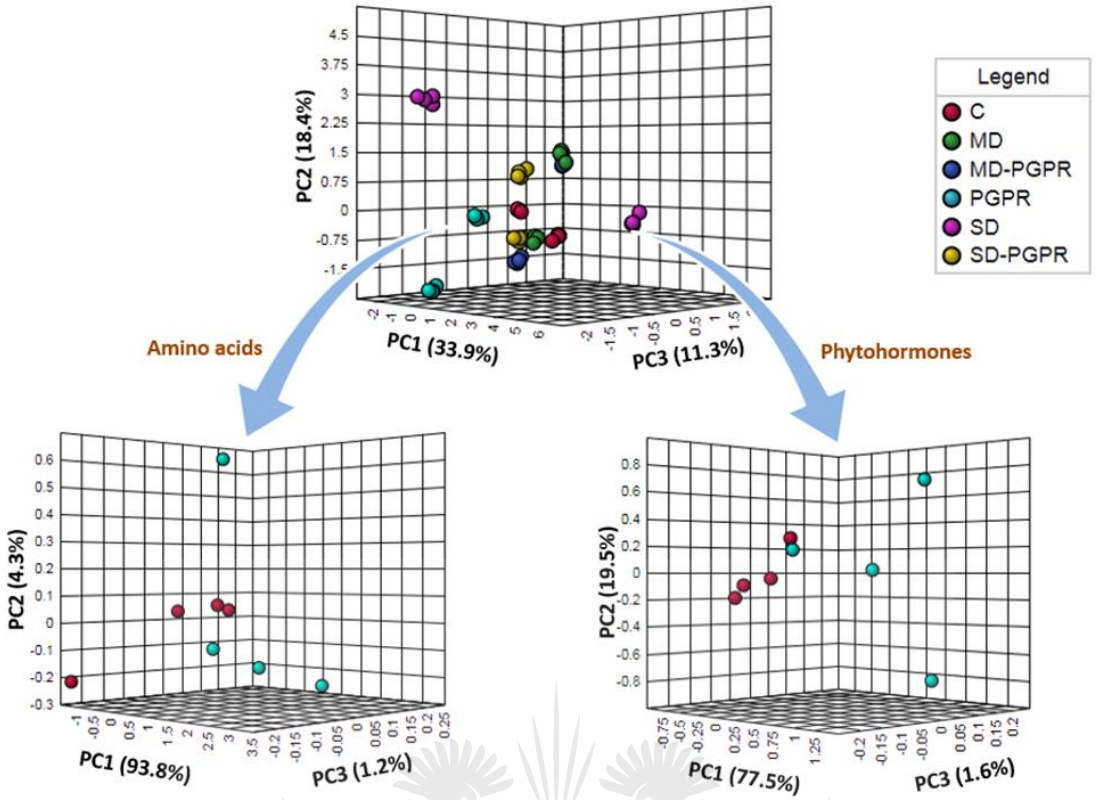

Figure S3.1: **PCA modelling showing the overall structure of data**. A 3D scores scatter plot of the PCA model of all the metabolite classes and treatments: model explains 92.4% (5-components) of the total variation in the log-transformed and Pareto scaled data at 4 and 6 weeks after emergence (WAE) leading to PCA modelling of well-watered plants (C, T2) and well-watered plants with PGRP (PGPR, T1) at 4 WAE for amino acids and phytohormones.

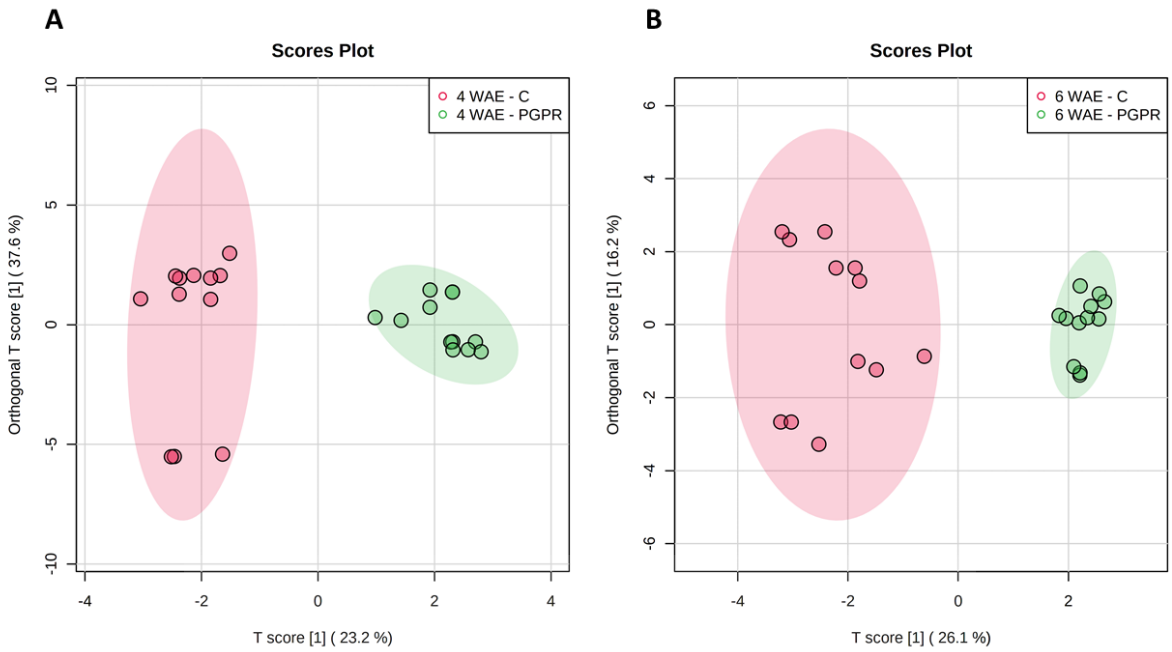

Figure S3.2: OPLS-DA modelling of phytohormones and amino acids under control conditions and PGPR treatment. (**A**) 4 WAE and (**B**) 6 WAE.

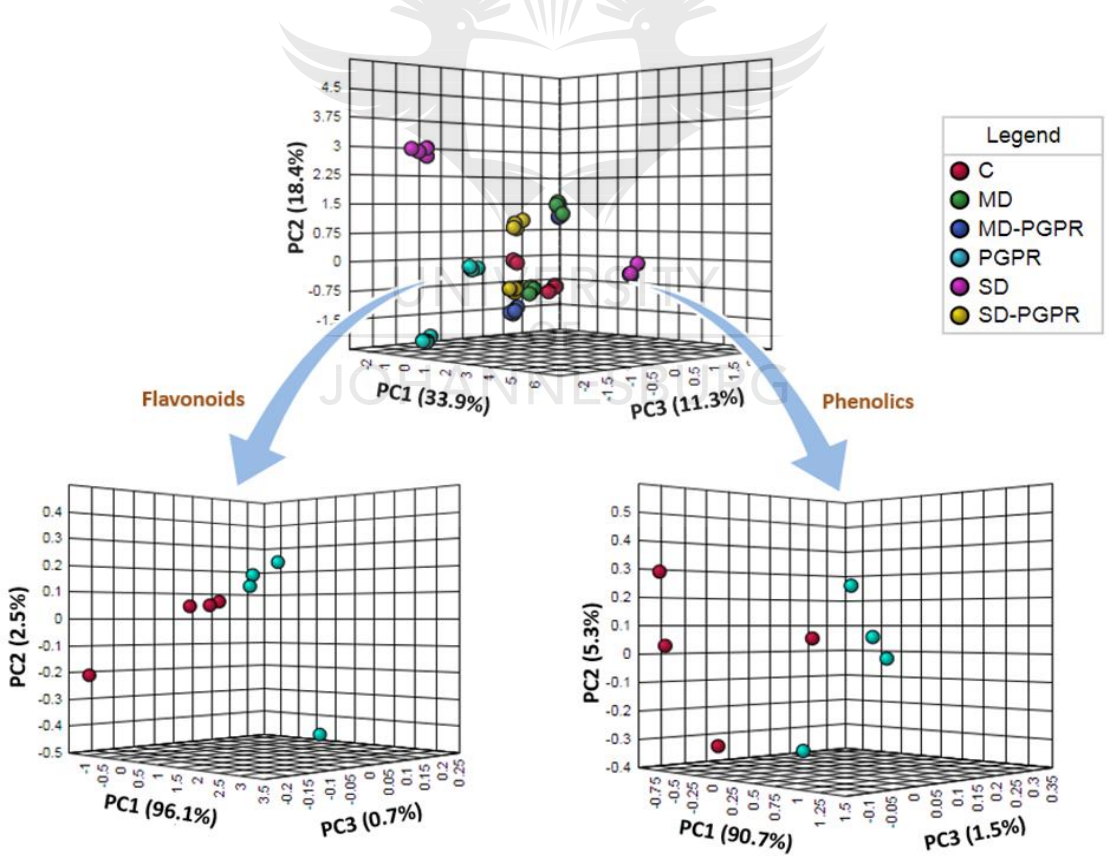

Figure S3.3: **PCA modelling showing the overall structure of data**. A 3D scores scatter plot of the PCA model of all the metabolite classes and treatments: model explains 92.4% (5-components) of the total variation in the log-transformed and Pareto scaled data at 4 and 6 WAE leading to PCA modelling of well-watered plants (C) and well-watered plants with PGRP (PGPR) at 4 WAE for flavonoids and phenolics.

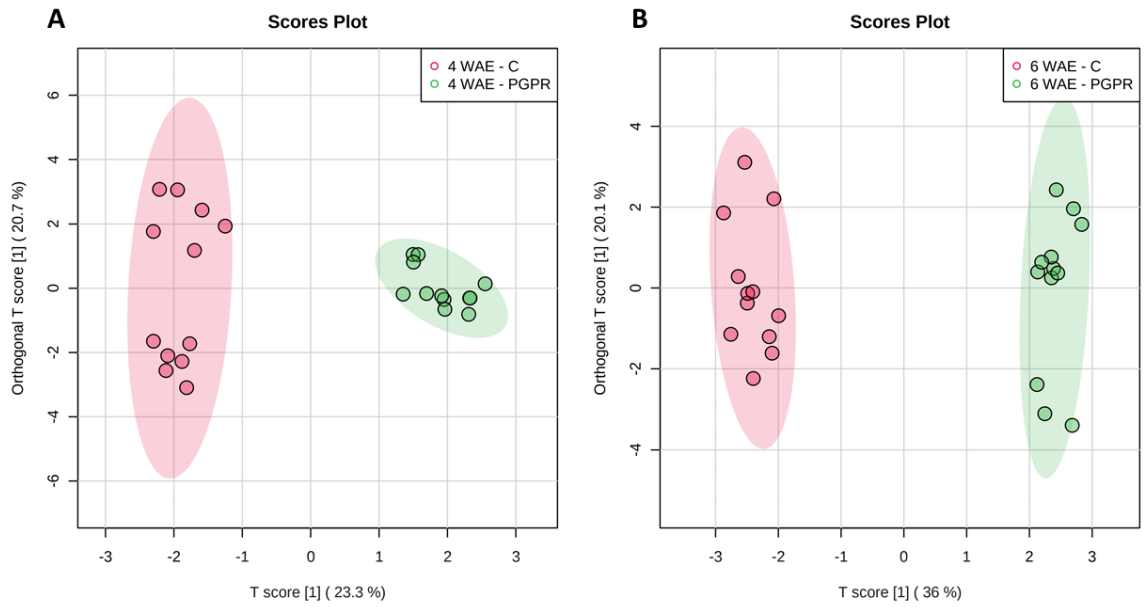

Figure S3.4: OPLS-DA modelling of flavonoids and phenolic acids under control conditions and PGPR treatment. (**A**) 4 WAE and (**B**) 6 WAE.

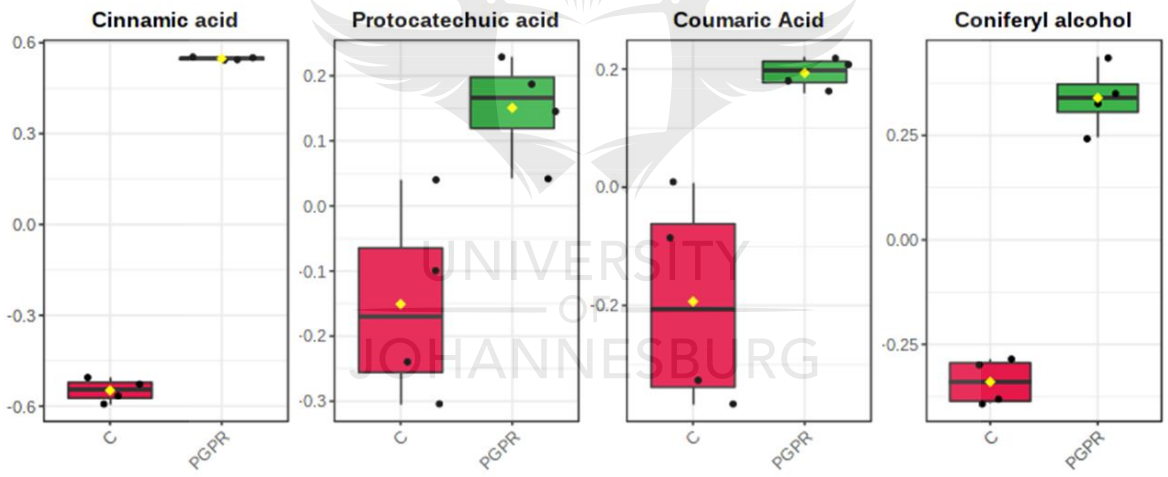

Figure S3.5: Box plots representing significant phenolic acids accumulated due to PGPR treatment in naïve maize plants.

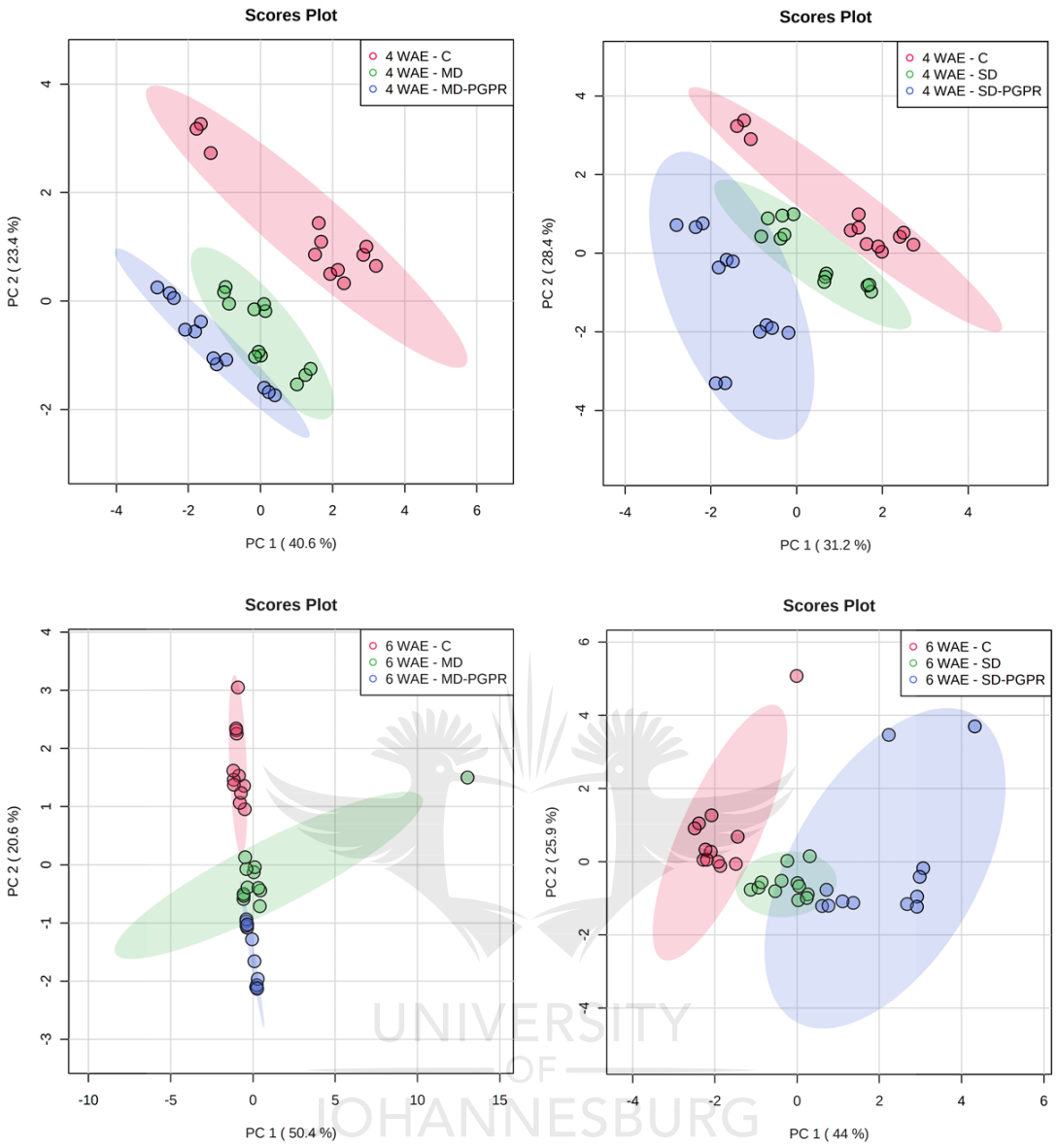

Figure S3.6: **PCA scores plot showing treatment related groupings**. PCA analysis of amino acids and phytohormones under control, mild drought, severe drought, mild drought with PGPR and severe drought with PGPR conditions at 4 and 6 WAE.

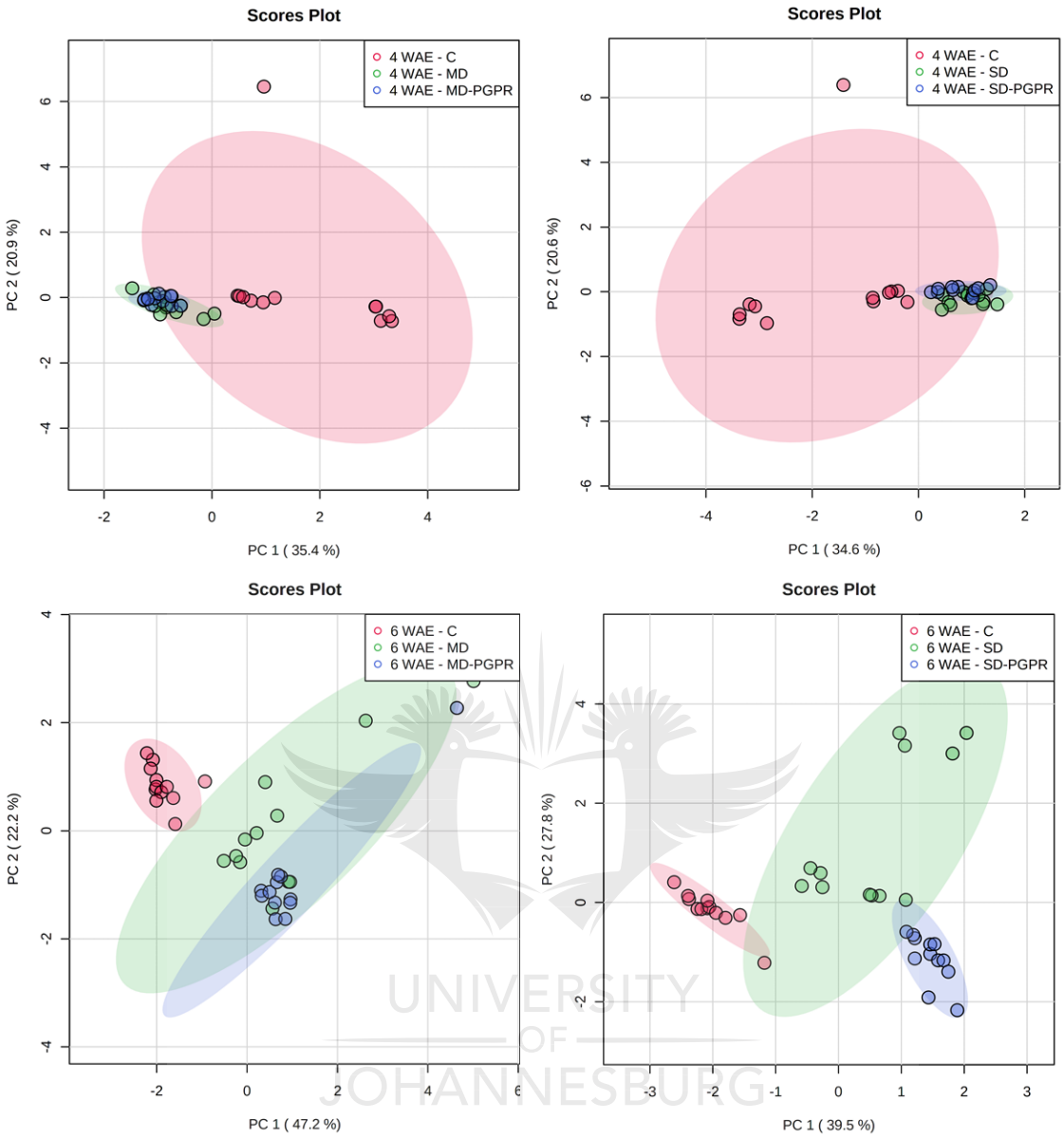

Figure S3.7: **PCA scores plot showing treatment related groupings**. PCA analysis of flavonoids and phenolic acids under control, mild drought, severe drought, mild drought with PGPR and severe drought with PGPR conditions at 4 and 6 WAE.

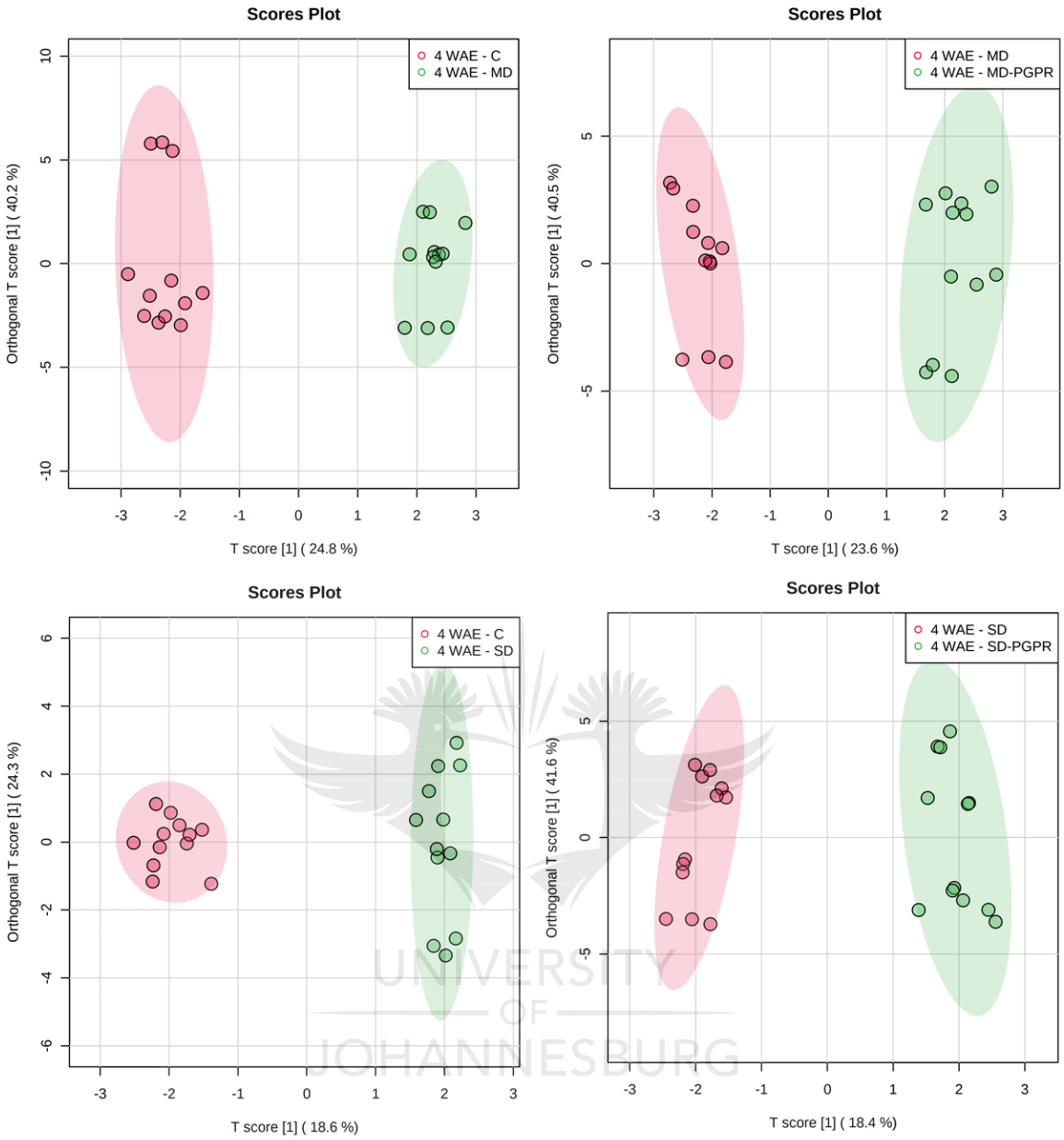

Figure S3.8: OPLS-DA modelling of amino acids and phytohormones under control conditions *versus* mild drought and severe drought stress together with PGPR treatment at 4 WAE.

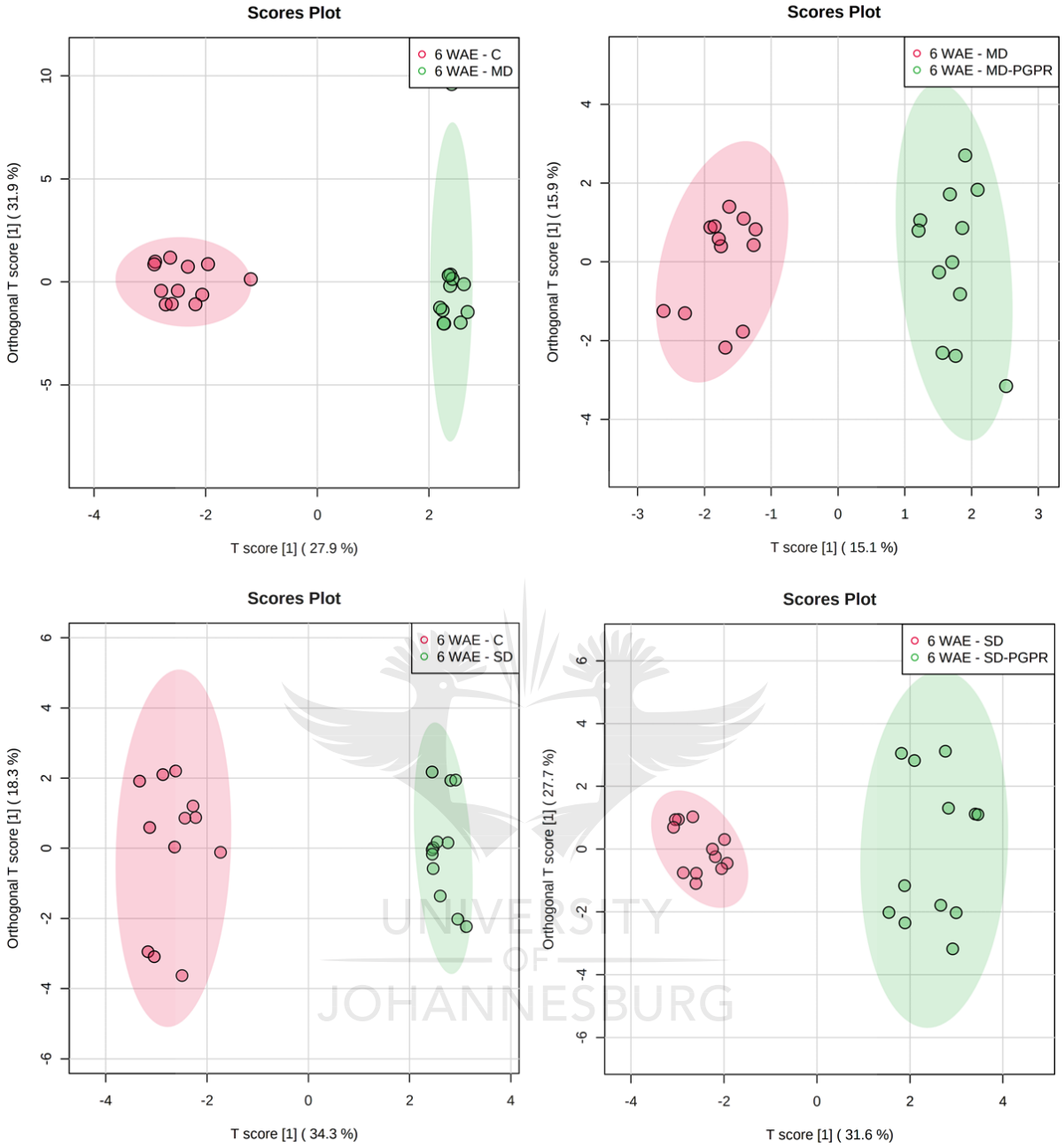

Figure S3.9: OPLS-DA modelling of amino acids and phytohormones under control conditions *versus* mild drought and severe drought stress together with PGPR treatment at 6 WAE.

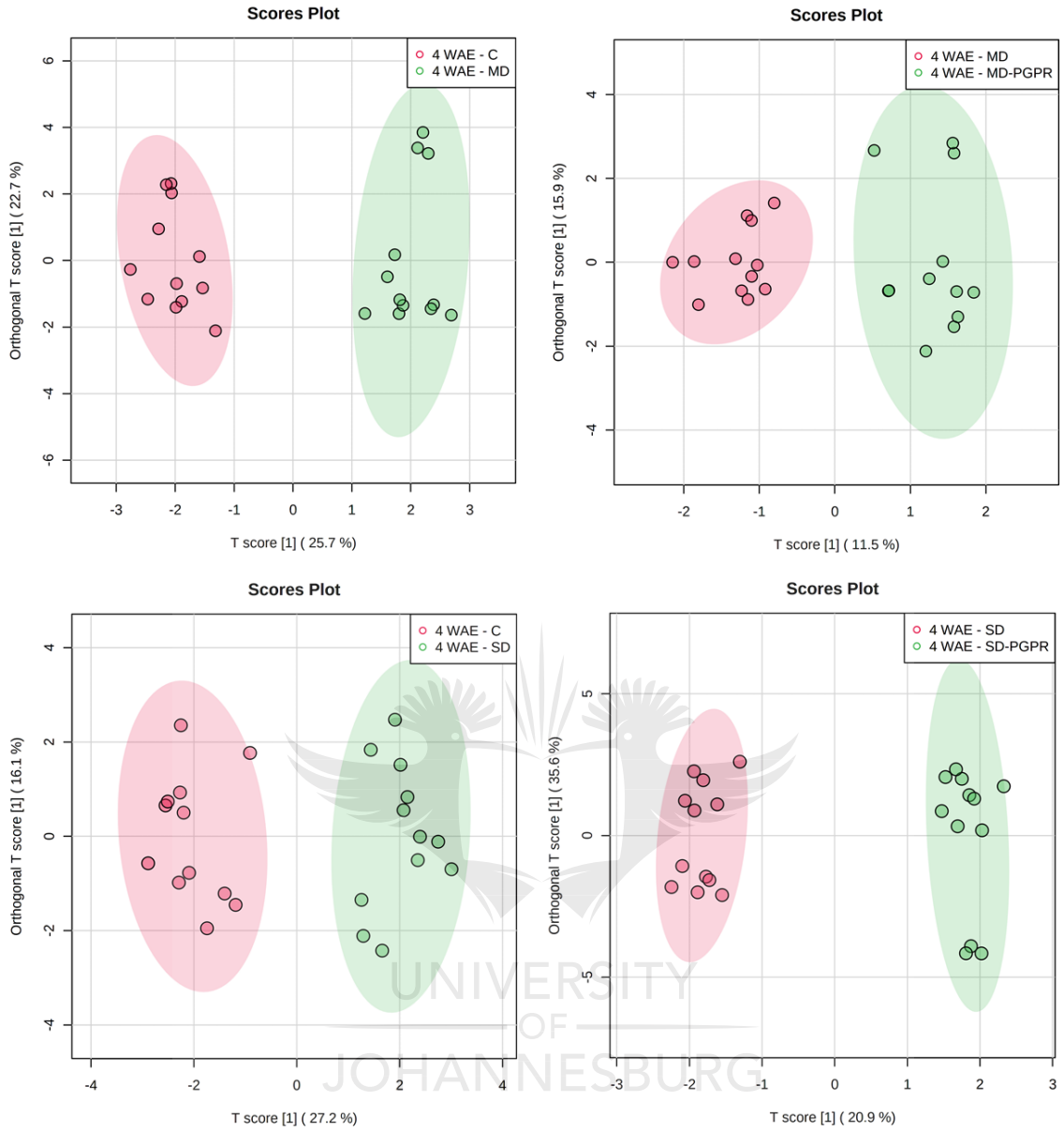

Figure S3.10: OPLS-DA modelling of flavonoids and phenolic acids under control conditions *versus* mild drought and severe drought stress together with PGPR treatment at 4 WAE.

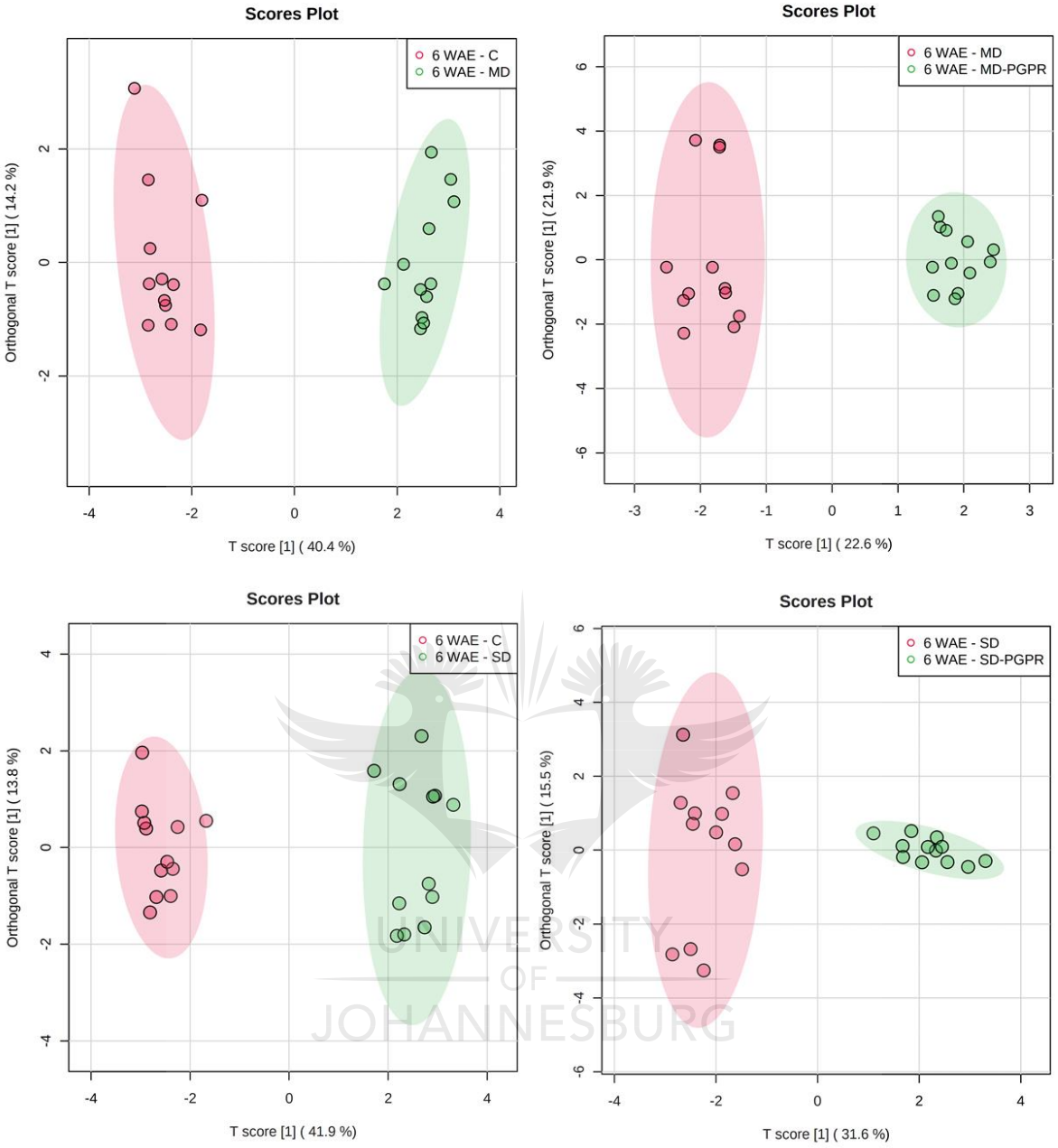

Figure S3.11: OPLS-DA modelling of flavonoids and phenolic acids under control conditions *versus* mild drought and severe drought stress together with PGPR treatment at 6 WAE.

Chapter 4

Global DNA Methylation Landscape and Targeted Gene Expression Profiles Associated with Microbial Biostimulant-Mediated Growth Enhancement and Drought Stress Tolerance in Maize Plants

Index

Chapter 4

Global DNA Methylation Landscape and Targeted Gene Expression Profiles Associated with Microbial Biostimulant-Mediated Growth Enhancement and Drought Stress Tolerance in Maize Plants

Summary

Metabolism carries imprints of genetic and environmental variations; hence investigating the gene space can provide a more detailed and integrated understanding of a metabolic landscape, such as that in **Chapter 3**. DNA methylation is an epigenetic modification in plants; playing a vital role in the gene regulation and genome maintenance. There is growing evidence suggesting that epigenetic changes may be implicated in gene regulation in response to abiotic stresses, such as drought. Spatial epigenetic variations may therefore contribute to variations in stress susceptibility and tolerance, but the scale and structure of epigenetic variation remains poorly defined. The present study sought to investigate epigenetic changes in microbial biostimulant-treated *Zea mays* plants under drought stress, which may point to a role in acclimation towards drought stress. To unravel molecular mechanisms underpinning biostimulant-induced drought stress tolerance and possible transgenerational epigenetic inheritance in *Zea mays*, global DNA methylation and gene expression (defence-responsive genes – *P5CS, DREB2A, PAL, FSNII*) analyses were carried out. The results showed that PGPR-treatment significantly increased DNA methylation levels in the pre-challenge and post-challenge phases, making DNA methylation an additional key mechanism through which PGPR-based biostimulant enhances plant growth, priming and drought stress tolerance. In addition, expression of targeted genes was upregulated, suggesting that PGPR not only influences the epigenome but also gene regulation to enhance the expression of drought-related defence-responsive genes. The findings of this study provide insight into PGPR-induced epigenome reprogramming and gene regulation dynamics, identifying them as part of the key mechanisms employed by PGPR-based biostimulant to enhance plant growth and drought tolerance. Although a transgenerational effect of these modifications on succeeding generations was not investigated in this study, it can be postulated that PGPR-induced epigenetic modifications may be transferred to the progeny resulting in a transgenerational enhanced drought tolerance.

Keywords: Biostimulants, *DREB2A*, drought stress, epigenetics, *FSNII*, global methylation, PGPR, *P5CS*, *PAL*, *Zea mays*

4.1 Introduction

The epigenetic events within the plant cell, such as DNA methylation, have been the focus of various studies, illuminating many mechanisms by which gene expression is regulated and patterns of expression inherited through cell divisions. DNA methylation, histone modifications and small non-coding RNAs have been shown to act in coordination to influence chromatin structure and gene expression (Chinnusamy & Zhu, 2009; Santos *et al.*, 2011). DNA methylation levels are modulated by an intricate interplay of enzymes (**Chapter 2**, **subsection 2.4.1**) such as DNA methyltransferases, and DNA demethylases, as well as other mechanisms, including the RNA-directed DNA methylation (RdDM) pathway mediated by small interfering RNA (siRNA). The functional significance of DNA methylation in plant responses to environmental stress conditions is still enigmatic; however, emerging studies have reported the involvement of DNA methylation (both hypo/hyper-methylation) in modulating abiotic stress responses and tolerance in plants. Changes in DNA methylation profiles are, hence, currently regarded as one of the general responses not only to drought stress, but also to other abiotic stresses that underlie stress memory and stress tolerance (Urano *et al.*, 2010; Kim *et al.*, 2015).

DNA methylation is known to regulate gene expression at the transcriptional as well as post-transcriptional levels, and such alterations are (or can be) contextually influenced by various abiotic factors such as drought conditions (Angers *et al.*, 2010; Kumar & Singh, 2016). Coordinated activation and expression of a repertoire of genes underlies plant development and shapes phenotypic plasticity in responses to the environmental and internal cues. Various studies have contributed to the current knowledge-base and understanding of the molecular mechanisms underlying plant responses to drought stress conditions. Several drought-responsive genes have been identified, which include membrane stabilising proteins, late embryogenic abundant proteins (LEA) and heat shock proteins (HSPs) which increase the plant cells' water binding capacity and stabilises protein structure (Wang *et al.*, 2004; Farooq *et al.*, 2009; Hanin *et al.*, 2011; Hasanuzzaman *et al.*, 2013; Kosová *et al.*, 2014; Zhang *et al.*, 2018). Furthermore, numerous transcription factors (TFs) involved in the regulation of drought stress responses have been identified (see **Chapter 2**, **subsection 2.2.1**), and include myeloblastosis (MYB), dehydration responsive element binding (DREB), C-repeat binding

factor (CBF), abscisic acid responsive elements binding factor (ABF), abscisic acid responsive elements binding (AREB) and NAC which is derived from no apical meristem (NAM) (for), Arabidopsis thaliana ACTIVATING FACTOR 1/2 (ATAF1/2), and cup-shaped cotyledon (CUC2) (Puranik *et al.*, 2012; Nuruzzaman *et al.*, 2013; Singh & Laxmi, 2015; Mun *et al.*, 2017).

The current knowledge and understanding of the gene network landscapes of drought stress responses are still limited. Furthermore, a systems biology view implies an appreciation of the multi-dimensionality of biochemical networks in a biological system, producing physiological and phenotypic coherence. Thus, a comprehensive understanding of the regulatory networks involving multi-layered molecular players (at epigenome, transcriptome, proteome and metabolome levels) that modulate the dynamic adaptive changes and response to abiotic stresses is an imperative for developing stress tolerant crop plants or agricultural strategies (Min *et al.*, 2016). **Chapter 3** of this dissertation revealed metabolic reprogramming events associated with microbial biostimulant-mediated growth enhancement and drought stress tolerance in maize plants. This metabolomics study (**Chapter 3**) points out that the PGPR-induced drought response phenomenology in maize involved remodelling of primary and secondary metabolism – alterations in amino acid-, phytohormone- and phenolic profiles – as pre-conditioning events. As echoed in **Chapter 2**, this priming memory is not only articulated at the metabolome level, it is also encrypted at the epigenome, and that the biochemical actions of metabolites are far-reaching, including regulation of epigenetic mechanisms and post-translational modifications.

Genome-wide epigenetic changes have been associated with variations in gene expression during plant developmental and environmental perturbations. These epigenetic changes, as well as the level of gene expression, may revert back to the pre-stress state (normal condition) once the stress is withdrawn; however, some of these epigenetic modifications are retained and may be carried forward to subsequent generations as stress memory (Grafi, 2013; Kumar, 2018). The accumulation of these epigenetic variations in response to environmental stimuli resulting in transgenerational epigenetic memory ensures plasticity and adaptability in the plant (**Figure 4.1**). For example, Zheng *et al.* (2017) identified a high proportion of stress-induced DNA methylation modifications under drought stress that were maintained in subsequent generations, suggesting that epigenetic mechanisms play a vital role in

transgenerational tolerance to environmental stresses. Mechanistic understanding of transgenerational stress memory is still fragmented. The current understanding proposes the involvement of DNA methylation, histone modifications and siRNA pathways in adaptation and stress memory in numerous plants (Lämke & Bäurle, 2017).

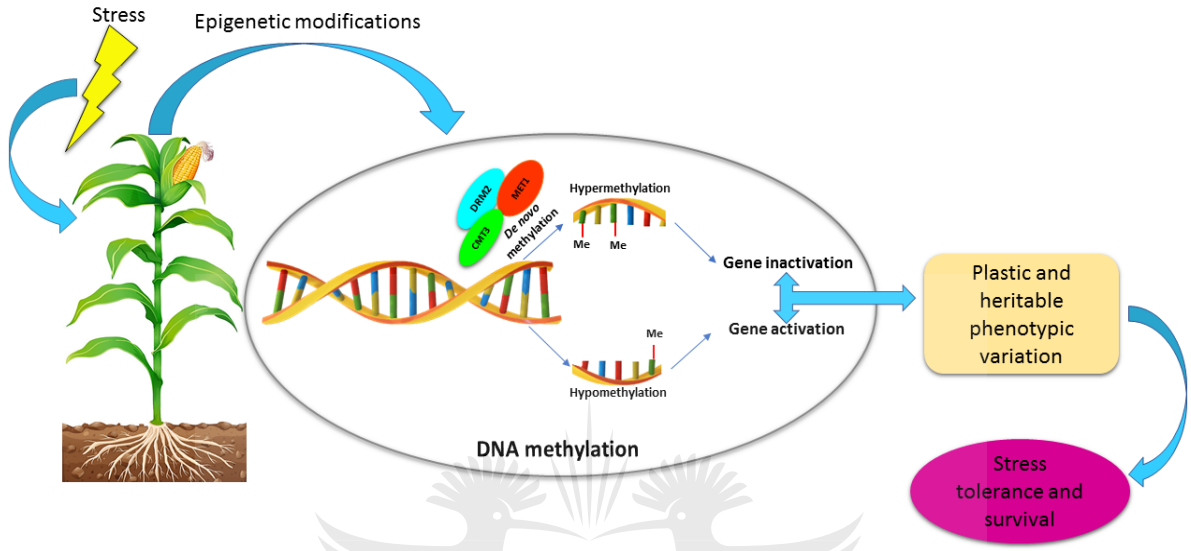

Figure 4.1: **The interaction between genetic and epigenetic variation in plant populations under abiotic stress**. Genetic and epigenetic variations enhance the stress responses mainly by altering the expression of genes involved in the development and physiology of the plant which, in turn, result in plastic phenotypic variation (short-term stress) or in heritable phenotypic variation (long-term stress) leading to stress tolerance and survival (Diagram created by the author of this dissertation)

How plants adapt to environmental changes *via* DNA methylation modifications and how this influences processes such as gene expression is still elusive. Knowledge gaps and contradictions in existing models point to the fact that the elucidation of epigenetic mechanisms underlying different cellular events is still an active research area. For example, hypermethylation of transposable elements (TEs) is often associated with increased gene expression of nearby genes (Secco *et al.*, 2015) but hypermethylation of TEs has also been found near downregulated genes (Eichten *et al.*, 2012). Additionally, increased DNA methylation in genes or near transcription start sites (TSS) is often believed to repress expression but there are also examples where methylated genes are moderately expressed and even cases where gene body methylation seems to stabilise expression (Suzuki & Bird, 2008).

Several techniques exist for the profiling of whole genome methylation which mostly rely on a methylation-dependent pre-treatment of genomic DNA in order to reveal the presence or absence of the methyl group at cytosine residues. Three main approaches to study DNA methylation include the endonuclease digestion, affinity enrichment of methylated regions and bisulfite conversion (Zuo *et al.*, 2009; Gupta *et al.*, 2010). Additional techniques available for the determination of the methylation status of DNA samples include mass spectrometry and Enzyme-Linked Immunosorbent Assay (ELISA). The method of choice for the detection of DNA methylation analysis depends on the biological questions and factors such as amount and quality of DNA samples, sensitivity, specificity, robustness and cost. In this study, ELISA was used for the determination of global genome methylation due to cost efficiency and identification of large changes in DNA methylation (Kurdyokov & Bullock, 2016). Briefly, the DNA sample is captured inside wells of an ELISA plate, and the methylated cytosines are detected through successive incubations steps with: (1) a primary antibody raised against 5-mC; (2) a labelled secondary antibody; and then (3) colourimetric/fluorometric detection reagents (**Figure 4.2)**.

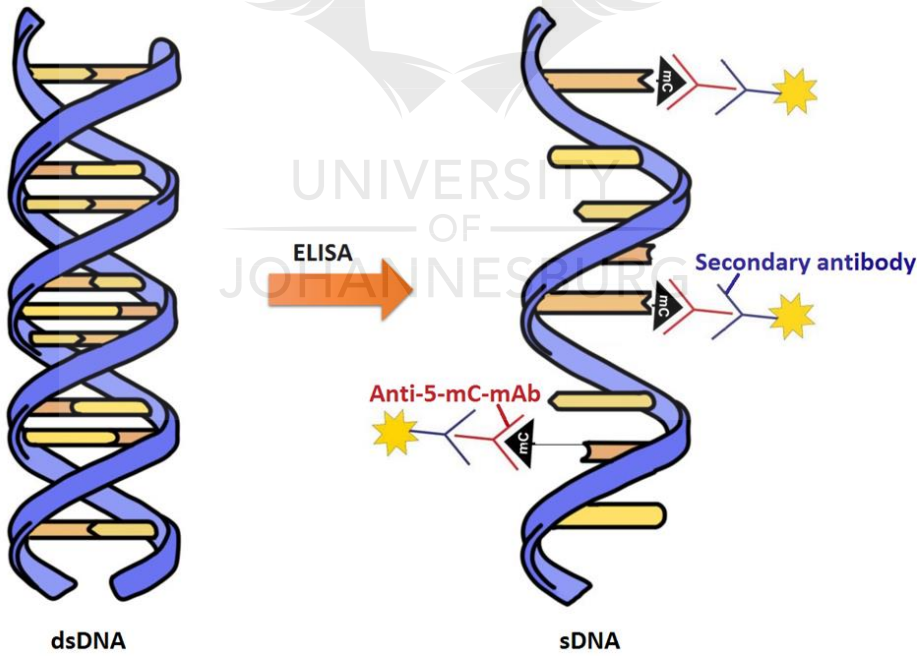

Figure 4.2: **ELISA-based DNA methylation profiling**. Global DNA methylation (5-methyl cytosine) detection using ELISA. The workflow for the 5-methylcytosine DNA ELISA technique utilises the indirect ELISA methodology where denatured, single-stranded DNA (ssDNA) samples are coated on the plate-well surfaces and an anti-5-mC-mAb and conjugate Horseradish peroxidase-secondary antibody are added to the wells (Diagram created by the author of this dissertation)

This work focuses in evaluating global DNA methylation levels, associated gene expression of selected genes in (i) key metabolic pathways: phenylalanine ammonia-lyase (*PAL*), flavone synthase II (*FNSII*); and (ii) drought responsive genes: pyrroline-5-carboxylate synthase (*P5CS*), dehydration-responsive element-binding protein 2A (*DREB2A*) in maize plants to complement the metabolic changes reported in **Chapter 3**. Thus, this work (reported in this **Chapter 4**) aims at evaluating global DNA methylation levels together with corresponding gene expression changes induced by the application of PGRP-based biostimulant in *Zea mays* plants under mild and severe drought stress conditions. The link between the epigenome, genome and metabolome in regard to abiotic stresses and priming is enigmatic, and therefore the integration and analysis of data of differential gene expression, epigenetic and metabolomic regulation can reveal a comprehensive picture of the dynamics of the stress-responsive genome in generating phenotypic diversity and could have significant implications in agriculture. Additionally, this will contribute to the current knowledge on the modes of action of biostimulants in promoting plant growth. The need to understand the underlying molecular mechanisms that define the benefit of biostimulants in plants is crucial for the formulation of novel biostimulants with optimal efficacy.

4.2 Materials and Methods

4.2.1 Plant material, growth conditions, drought stress and biostimulant application

The details regarding the experimental design, maize plant cultivation, treatment applications (both PGPR and drought conditions), harvesting of plant materials and sample preparation are provided in **Chapter 3**, **subsection 3.2.2 – 3.2.3**. It suffices to briefly mention that maize (*Zea mays*) plants, PAN 3Q-240, were cultivated in 10 L-pots, containing a sandy soil, placed in a randomised order on rotating tables in a greenhouse at Omnia facilities in Sasolburg, Free State, South Africa. A PGPR (*Bacilli* strains)-based biostimulant formulation (Omnia Group Ltd, South Africa) was applied at planting: a 5-strain formulation is referred to as PGPR (**Chapter 3**, **subsection 3.2.2**, **Table 3.1)**. The semantics used in regards to different groups referred to as ‘treatment’ (T) is again provided in **Table 4.1**; and the expressions microbial-based biostimulant or biostimulant or PGPR will be used interchangeably to simply refer to the biostimulant formulation (a consortium of *Bacilli* strains) used in this study.

Table 4.1: Description of treatment conditions used to study the effect of PGPR-based biostimulant application on mild and severe drought stressed plants.

Treatment	Treatment description
T1	Well-watered with biostimulant (**PGPR**)
T2	Well-watered without biostimulant (Control; **C**)
T3	Mild drought with biostimulant (**MD-PGPR**)
T4	Severe drought with biostimulant (**SD-PGPR**)
T5	Mild drought without biostimulant (**MD**)
T6	Severe drought without biostimulant (**SD**)

4.2.2 DNA extraction and quantification of global DNA methylation

Genomic DNA (gDNA) was extracted from well-watered plants, drought stressed plants and PGPR-treated plants, four and six weeks after emergence (4 and 6 WAE), each comprising of three biological- and two technical replicates, using a modified Cetyltrimethylammonium bromide (CTAB) method. gDNA extraction was performed using 500 mg of leaf tissue, which was ground into a fine powder using liquid nitrogen. This plant material was added to 500 µL of extraction buffer (2% w/v CTAB, 2% w/v polyvinylpyrrolidone (PVP), 0.5 M ethylenediaminetetraacetic acid (EDTA), 5 M sodium chloride (NaCl), 100 mM tris(hydroxymethyl)aminomethane-hydrochloride (Tris-HCl) pH 8.0 and 0.2% v/v β-mercaptoethanol) and incubated at 65 °C for 60 min. Following incubation, 500 µL of chloroform : isoamyl alcohol (24:1) was added to each sample and the mixture then centrifuged at 13 000 × *g* for 10 min at 4 °C. The aqueous phase was aspirated into a new microcentrifuge tube, to which an equal volume (500 µL) of isopropanol was added to induce DNA precipitation. The mixture was then centrifuged 13 000 × *g* for 10 min at 4 °C. The supernatant was discarded, and the precipitated pellet was washed in 1 mL ice cold 70% ethanol (v/v) and centrifuged at 12 000 × *g* for 5 min. DNA pellets were dried by heating at 55 °C for 5 min on a heating block and resuspended in TE buffer containing 20 µg/mL of RNase A. The extracted DNA quality and quantity was estimated using the NanoDrop 2000 (NanoDrop Technologies Inc., Rockland, DE, USA), followed by ethidium bromide staining on 1% agarose electrophoresis gels in 1X Tris-acetate-EDTA (TAE) buffer.

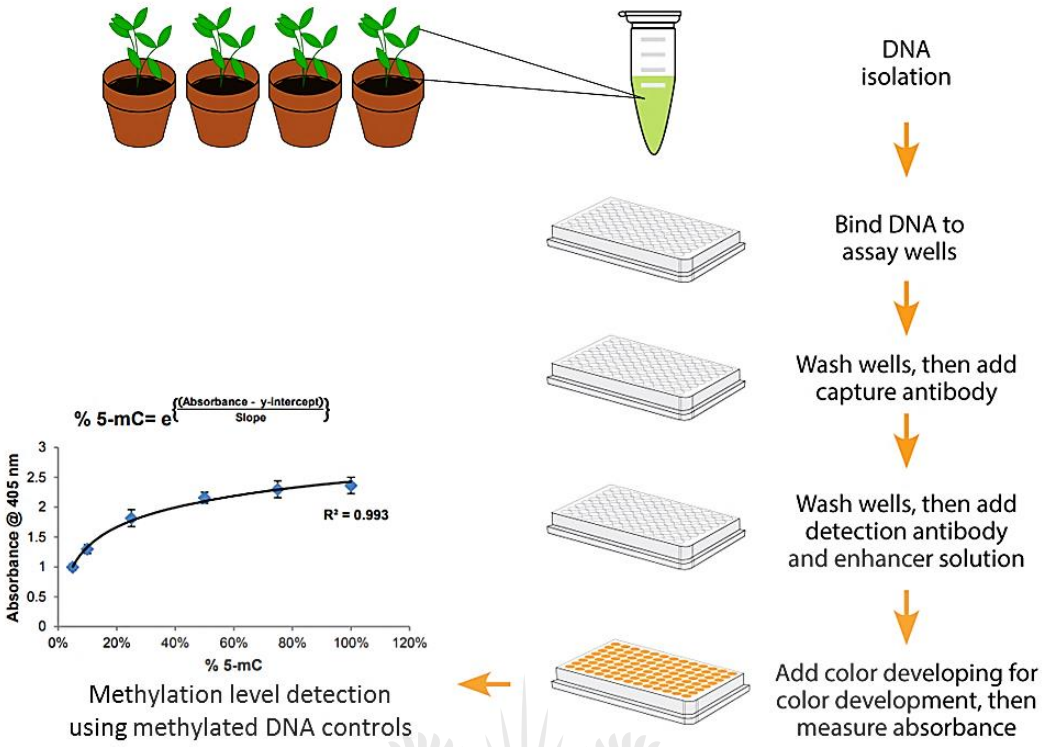

Figure 4.3. **Schematic procedure for global DNA methylation quantification**. DNA is extracted from plant tissue and coated onto DNA wells where methylation is measured through the colourimetric development arising from the antibody binding to methyl-cytosines. (Diagram created by the author of this dissertation).

Relative quantification of global DNA methylation levels was acquired with an ELISA-based colourimetric assay (**Figure 4.3**) using the 5-mC DNA ELISA Kit (Zymo Research, Irvine, CA) according to manufacturer's instructions. In brief, the DNA samples and methylation standards (0 %, 5 %, 10 %, 25 %, 50 %, 75 % and 100 % - provided in the kit) were diluted to 100 ng/µL in TE buffer and denatured at 98 °C for 5 min in a thermal cycler (Master cycler, Eppendorf AG, Hamburg, Germany), followed by immediate incubation on ice for 10 min. The denatured DNA was then added to the plate wells and incubated at 37 ºC for 1 h. To prevent non-specific binding to the wells, the buffer from the wells was discarded and each well washed 3 times with 200 µL of 5-mC ELISA buffer, discarding the buffer after each wash. Following this, 200 µL of 5-mC ELISA buffer was added to each well, and the plate was covered with foil and incubated at 37 ºC for 30 min. A prepared antibody mix (100 µL) consisting of anti-5-methylcytosine (1:2000) and secondary antibody (1:1000) in 5-mC ELISA buffer was added to each well. The plate was then covered with foil and incubated at 37 ºC for 1 h. The antibody mix was discarded from the wells and each well washed 3 times with 200 µL of 5-mC ELISA buffer. Colour development was performed by adding 100 µL

of HRP Developer to each well and allowed to develop for 10-60 min at room temperature. The absorbance was read on a microplate reader at 450 nm (BioTek Power Wave XS, Vermont, USA).

The readings obtained from the ELISA reader were exported into Microsoft Excel and a standard curve was constructed by plotting absorbance (*y*-axis) against the percentage methylation (*x*-axis) of controls. The percentage methylation (5-mC) each DNA sample was quantified relative to the standard negative and positive methylated control DNA by extrapolation from the standard curve, using the equation; $\%\,5\mathrm{mC} = e^{\{\frac{(\text{Absorbance} - \text{y-intercept})}{\text{Slope}}\}}$, derived from the logarithmic second order regression. All samples were assayed in duplicate according to the manufacturer's recommendation and to ensure accurate global DNA methylation detection and quantitation.

4.2.2.1 Statistical analysis of global DNA methylation

Statistical analysis was performed using IBM's Statistical Product and Services Solutions software version 26 (SPSS 26, IBM, NY, https://www.ibm.com/analytics/spss-statistics-software), following Pallant (2007, 2010) guidelines. To evaluate the distribution of the data, tests of normality (parametric – Shapiro-Wilk test; non-parametric – Kolmogorov-Smirnov test with Lilliefors significance correction) were employed. The distribution of the data sets was normal according to the Shapiro-Wilk test, and the *p*-values according to the Kolmogorov-Smirnov test could not be computed. Although the data distribution was found to follow a normal/Gaussian distribution in which parametric tests are employed, non-parametric tests were reported due to sample size ($n \leq 50$). Parametric analysis tests are used for large sample sizes ($n > 50$), whereas non-parametric analysis are typically used for small sample sizes which follow a skewed distribution (Pallant, 2007). The overall significant differences between the groups reported as $p \leq 0.05$*, $p \leq 0.01$** and $p \leq 0.001$*** were analysed using the Kruskal-Wallis test. The global methylation levels for the control, PGPR treatment, mild and severe drought stress and mild and severe drought stress together with PGPR treatment were analysed for 4 and 6 WAE.

4.2.3 RNA extraction and gene expression study by real-time quantitative PCR (qPCR)

RNA was extracted from 200 mg of *Zea mays* leaves (done for all the treatments - **Table 4.1**) using Direct-zol™ RNA miniprep plus (Zymo Research, Irvine, CA) according to the manufacturer's recommendation. Frozen leaf tissue (200 mg) was ground in liquid nitrogen to which 600 µL of TRI Reagent was added. The lysed samples were centrifuged at 16 000 × *g* for 1 min and an equal volume ethanol (95-100%) was added. The mixture was thoroughly mixed and transferred into a Zymo-Spin™ IIICG column placed in a collection tube. The samples were centrifuged at 16 000 × *g* for 30 sec and the column was transferred into a new collection tube. DNase I treatment was performed on the samples by first adding 400 µL RNA Wash Buffer to the column and centrifuging at 16 000 × *g* for 30 sec. Following this, a prepared mixture of 5 µL DNase I (6 U/µL) and 75 µL DNA digestion buffer was added directly to the column matrix in each tube and the samples were incubated at room temperature (20-30 °C) for 15 min. RNA bound to the column in each tube was washed by adding 400 µL Direct-zol™ RNA Prewash, followed by centrifugation at 16 000 × *g* for 30 sec, and this was performed twice. A wash step was performed by adding 700 µL RNA wash buffer to the column and centrifuging for 1 min at 16 000 × *g* to ensure complete removal of the wash buffer. The column was then transferred into an RNase-free tube and the RNA eluted by adding 100 µL of DNase/RNase-free water directly to the column matrix followed by centrifugation at 16000 × *g* for 30 sec. Concentrations of extracted RNA samples were determined using the NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific; Waltham, USA) and RNA integrity was assessed by electrophoresis on 1% agarose gel.

Five hundred nanograms of the total RNA extracted from each biological replicate per treatment was used for first strand cDNA synthesis (**Table 4.2**), which was performed using random hexamers and LunaScript® RT SuperMix Kit (E3010, New England Biolabs, Massachusetts, USA) in 20 µL reactions, per the manufacturer's recommendation. The synthesised cDNA (1 µL) was used in the second step PCR using LunaScript® Universal qPCR Master Mix (M3003, New England Biolabs, Massachusetts, USA), per the manufacturer's recommendations. Reactions (**Table 4.3**) were performed on a CFX-96 (BioRad, Johannesburg, SA) system, with the thermal cycling conditions as follows: initial denaturation 95 °C for 1 min followed by 40 cycles of 95 °C for 15 sec and 61.2 °C for 30 sec. The lyophilized primer sets (Integrated DNA Technologies, Coralville, IA) used in this

study, defined in **Table 4.5**, were dissolved in TE buffer (Integrated DNA Technologies, Coralville, IA) to a stock solution of 100 µM and aliquots of 10 µM were prepared in nuclease-free water. Following this, preparation, primer amplification efficiency was checked before any further analysis and the primers were stored at – 20 °C. Elongation factor 1 alpha (EF1α), and β-tubulin (β-TUB) primer sets were used for normalisation of gene expression which have been reported to be the most stability expressed reference genes under abiotic stress (Reddy *et al.*, 2018) and 'no template' and 'no RT' (**Table 4.4**) controls were included in each run.

Table 4.2: Reaction components for cDNA synthesis.

Component	20 µL Reaction	Final concentration
LunaScript RT SuperMix (5X)	4 µL	1X
RNA sample	Variable[a]	(up to 1 µg)
Nuclease-free Water	to 20 µL[b]	

[a]The concentration of each RNA sample determined the volume to be added to the reaction, ensuring that the final concentration of the cDNA consisted of 500ng RNA.
[b]The volume of nuclease-free water added was dependent on the volume of the RNA added to the mixture.

Table 4.3: Reaction components for qPCR analysis.

Component	20 µl Reaction	Final Concentration
Luna Universal qPCR Master Mix	5 µL	1X
10 µM forward primer	0.4 µL	0.25 µM
10 µM reverse primer	0.4 µL	0.25 µM
cDNA products	1 µL	< 4 µL
Nuclease-free water	3.2 µL	

Table 4.4: Reaction components for no -RT control reactions.

Component	20 µL Reaction	Final Concentration
No-RT Control Mix (5X)	4 µL	1X
RNA (up to 1 µg)*	variable	(up to 1 µg)
Nuclease-free water	to 20 µL	

[a]The concentration of each RNA sample determined the volume to be added to the reaction, ensuring that the final concentration of the cDNA consisted of 500ng RNA.
[b]The volume of nuclease-free water added was dependent on the volume of the RNA added to the mixture.

Table 4.5: Targeted key genes and primer sequences used in this study.

Gene	Primer sequences (5'-3')	Amplicon length (bp)	Reference
Δ1-Pyrroline-5-carboxylate synthetase (*P5CS*)	F: GCGAGGAAGTGGGCAAGTGGT R: TTGGGGAGGTGGGGTGGC	250	(Sun *et al.*, 2018)
Flavone synthase type 2 (*FSNII*)	F: CAAGATCGACATGTCGGAGTC R: GCATGGTATCCACATTCTTCG	115	(Righini *et al.*, 2019)
Phenylalanine ammonia-lyase (*PAL*)	F: CGAGGTCAACTCCGTGAACG R: GCTCTGCACGTGGTTGGTGA	318	(Farag *et al.*, 2005)
Dehydration-responsive element binding protein 2 (*DREB2A*)	F: GCAGCCCGGAAGGAAGAA R: GATGACAGCTGCCACTGACGTA	70	(Qin *et al.*, 2007)
Elongation factor 1-alpha (*EF1α* – Ref)	F: TGGGCCTACTGGTCTTACTACTGA R: ACATACCCACGCTTCAGATCCT	135	(Phillips *et al.*, 2018)
Beta-tubulin (*β-TUB* – Ref)	F: CTACCTCACGGCATCTGCTATGT R: GTCACACACACTCGACTTCACG	139	(Phillips *et al.*, 2018)

Relative quantity (ΔCq) (1) for each sample per gene of interest against control samples was calculated according to the CFX Maestro Software (BioRad, Johannesburg, SA) equations and guidelines. Following this, normalised expression (ΔΔCq) (2) of each target gene was calculated using the same software against the two reference genes. Normalised gene expression was expressed as logarithmic fold change and fold change.

$$(1)\ \text{Relative Quantity}_{\text{Sample (GOI)}} = E_{\text{GOI}}^{(C_{q(\text{control})} - C_{q(\text{sample})})}$$

$$(2)\ \text{Normalised Expression}_{\text{sample (GOI)}} = \frac{RQ_{\text{sample (GOI)}}}{(RQ_{\text{sample (Ref 1)}} \times RQ_{\text{sample(Ref 2)}})^{\frac{1}{2}}}$$

Where: E = Efficiency; RQ = relative quantity; GOI = gene of interest (one target); Ref = Reference gene.

Statistical analysis and graphical representations were carried out in GraphPad Prism 9.0.0. (Motulsky, 1999). The Mann-Whitney U test was employed to compare the differences between the different groups (C, PGPR, MD-PGPR and SD-PGPR). The Mann-Whitney U test is the non-parametric equivalent of the independent samples t-test and in this study, it was used to complement the non-parametric tests employed in the DNA methylation analysis.

4.3 Results and Discussion

In this study, to complement the findings reported in **Chapter 3** (a metabolomics study), informing microbial biostimulant-induced alterations in maize gene space, the global DNA methylation profiles of naïve and PGPR-treated maize leaves under normal and drought stress (mild and severe) conditions were generated using ELISA. Furthermore, gene expression levels of drought stress-inducible genes (*PAL*, *DREB*, *FSNII* and *P5CS*), in naïve and PGPR-treated maize plants, were assessed. Thus, this section is divided into three main subsections: (i) DNA methylation patterns in naïve and PGPR-treated plants, (ii) DNA methylation patterns in PGPR-treated plants under mild and severe drought conditions, (iii) differential expression profiles of stress-related genes in naïve and PGPR-treated maize under drought stress conditions.

Firstly, the quality of the extracted DNA was evaluated using two different techniques: (i) spectrophotometric measurements to estimate purity by using the absorbance ratio at 260 nm and 280 nm wavelengths (A260/A280); and (ii) to assess the integrity and size of the extracted DNA, agarose gel electrophoresis was performed (**Figure S4.1**). As previously highlighted in **sub-subsection 4.2.2.1**, tests of normality were performed to determine the distribution of the data. The Kolmogorov-Smirnov test could not be computed, and the Shapiro-Wilk Test resulted in non-significant results ($p > 0.05$) (**Table S4.1**). This therefore showed that the data in the present study was normally distributed since the assumption of normality was not violated except for *SD-PGPR where $p < 0.05$. Additionally, normality was assessed graphically using normality quantile-quantile (Q-Q) plots (**Figure S4.2**) which compare the quantiles of a data distribution with the quantiles of a standardised theoretic distribution from a specified family of distributions which follow a normal distribution (Rani Das, 2016).

4.3.1 Global DNA methylation profiles in naïve and PGPR-treated maize leaves under well-watered conditions

The quantification of global amounts of 5-mC was performed in leaf tissues of naïve and PGPR-treated maize plants to assess the effect of PGPR on the plant methylome. Differential methylation levels between the two test groups were observed, suggesting that PGPR-based

biostimulant alter the epigenome landscape of maize plants. Global DNA methylation levels were higher in PGPR-treated plants than in naïve plants (**Figure 4.4**). PGPR-treated plants showed an increase of 2.4-fold (33.3%) of global DNA methylation when compared to the naïve plants; however, these differences were not found to be statistically significant based on the Kruskal-Wallis test under the study's experimental conditions. However, these differences are considered biologically significant since DNA methylation (to such extent) influences gene regulation and phenotype. Similar to the findings of this study, Gagné-Bourque *et al.* (2015) reported an increase of 6-fold and 1.5-fold of global DNA methylation in model grass *Brachypodium distachyon* under normal plant growth conditions triggered by plant-growth promoting bacteria (PGB) *Bacillus subtilis* B26. The reported hypermethylation was correlated to an increase in the abundance of methyltransferases involved in the maintenance and regulation of DNA methylation. Additionally, the PGB-induced hypermethylation levels remained constant when compared to the naïve plants after five and eight days of drought treatment, suggesting that *Bacillus subtilis* B26 potentially acts at an epigenetic level to increase drought stress tolerance in *Brachypodium distachyon* by inducing DNA methylation changes under normal conditions that, in turn, increase drought resistance by allowing the expression of drought responsive genes.

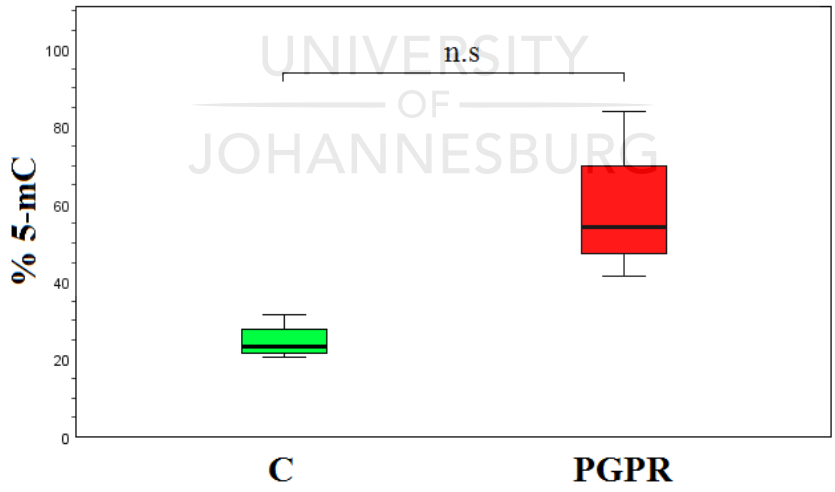

Figure 4.4: **Global DNA methylation levels in naïve and PGPR-treated plants under well-watered conditions**. Genomic DNA from maize leaves in control and PGPR conditions was used to determine the relative global DNA methylation using ELISA. The horizontal line within a box-plot represents the median. The lower and upper edges of boxes show the 25th and 75th percentiles, respectively. Whiskers represent the maximum and minimum values. **Abbreviations**: C, control; PGPR, plant growth-promoting rhizobacteria and n.s, non-significant. The Kruskal-Wallis test reported no statistical significance between the two groups; however, since methylation levels directly influence the genome, this change suggests that the reported difference may be biologically significant.

Studies have evidenced that DNA methylation plays a critical role in the regulation of defence priming since it regulates the transcription of defence-related genes through evolutionarily conserved functions (Espinas *et al.*, 2016). The observed hypermethylation in PGPR-treated maize plants under well-watered conditions (**Figure 4.4**) suggests that the molecular mechanisms activated by the PGPR consortium in maize plants include altering DNA methylation status. Supported by the results in **Chapter 3**, it can be postulated that this PGPR-induced epigenetic modification (hypermethylation), in maize plants under well-watered conditions, gravitates towards enhancement of growth and priming the plants against subsequent stress conditions. Evidence of epigenetic regulation as one of the key mechanisms in the priming phenomenology has been reported in various studies. A recent study by De Palma *et al.* (2019) reported a *Trichoderma harzianum* T22-induced modification of the epigenome in which hypermethylation was observed in tomato roots. Abiotic stress tolerance can be mediated by stimulating memory responses at the epigenetic level improved by pre-treatment with several other non-pathogenic bacterial strains such as PGPR, the focus of this study.

DNA methylation plays a crucial role in the orchestration of varying gene expression profiles during the plant life cycle, *i.e.* during seed development, shoot/root apical meristem regulation, floral transition (from vegetative phase to reproductive phase), gametogenesis and embryogenesis (Kaufmann *et al.*, 2010; Kawashima & Berger, 2014). This regulation results in enhanced plant growth and thus the observed increase in DNA methylation due to PGPR treatment implies an additional mechanism through which PGPR promotes plant growth and preconditioning of the plant for future stress encounters. Yang *et al.* (2018) reported that DNA methylation regulates the expression of key genes involved in the biosynthesis of phenolic acids in *Salvia miltiorrhiza*. The accumulation of these secondary metabolites can precondition the plant through various mechanisms such as the establishment of antioxidant machinery as reported in **Chapter 3**. From this finding, it is epistemologically logical to extrapolate that the observed DNA methylation changes, induced by PGPR-based biostimulant, would affect the expression of various genes including those involved in the biosynthesis of secondary metabolites, which subsequently can activate ROS detoxification under drought stress (**Figure 4.5**).

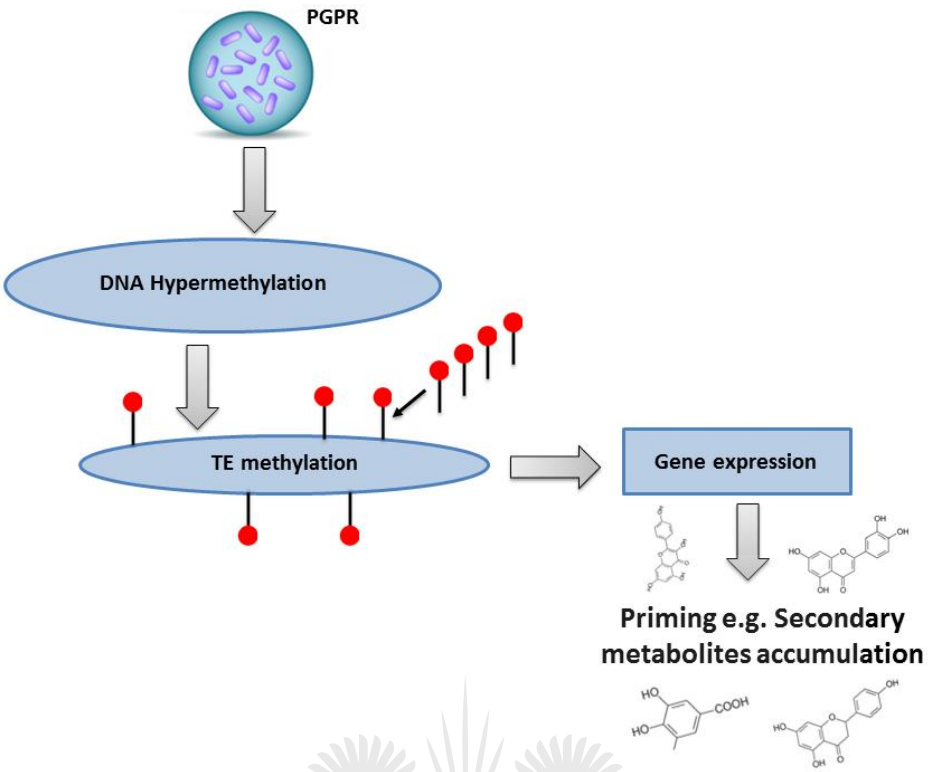

Figure 4.5. **Proposed model of PGPR-induced DNA methylation changes**. PGPR activates DNA hypermethylation in non-stressed plants, inducing the biosynthesis of secondary metabolites which may be a key mechanism in priming (the diagram was created by the author of this dissertation).

Plants possess multiple transposable elements (TEs) which represent a large portion of the genome with potential to modify gene expression and rearangements. TEs are DNA sequences that can move from one location to the other within the genome *via* different cut/copy and paste mechanisms (Vicient, 2010a). In maize, ~ 85% of the genome is composed by TEs of numerous families (Baucom *et al.*, 2009; Vicient, 2010b), making maize an ideal system for studying genome-wide influence of TEs on gene regulation. As highlighted above, PGPR treatment induced global hypermethylation in maize when compared to the control (**Figure 4.4**). Epigenetic regulation of TEs has been employed to inhibit unrestricted movement of TEs that would result in unfavourable effects due to insertion in essential defence-responsive genes. Additionally, under the absence of enviromental elicitors, TEs are highly methylated and remain silent, resulting in normal gene transcription (Galindo-González *et al.*, 2018). DNA methylation has been reported to silence TEs and repeats along with gene expression regulation (Finnegan *et al.*, 2000; Gehring & Henikoff, 2007; Bartels *et al.*, 2018). Although TE methylation and their genomic context is outside the scope of the present study, it can be suggested that priming by PGPR induces global hypermethylation to influence gene regulation towards enhanced genomic stability to

support targeted and regulated expression of key genes involved in the metabolic processes for plant growth and priming. Furthermore, the observed epigenome reprogramming (DNA methylation increase) induced by PGPR can be identified as a key mechanism through which this PGPR-based biostimulant enhances plant growth and preconditions the maize plant defence machinery.

4.3.2 Comparative analysis of global DNA methylation patterns in naïve and PGPR-primed maize plants under mild and severe drought stress conditions

The metabolomics study, reported in **Chapter 3**, revealed that the PGPR consortium (the microbial biostimulant, referred to as PGPR) conferred to maize plants enhanced responses to drought stress conditions *via* the priming phenomenology, involving reprogramming of metabolic landscapes of maize plants. Primed plants showed alterations in amino acids, hormonal signalling networks and phenolic profiles – manifestations of metabolic memory mediated by PGPR. This priming memory (towards enhanced defences and resistance mechanisms) is multi-layered and regulated at different levels including transcriptional reprogramming and epigenetic alterations. As evidenced in **section 4.3.1**, the microbial biostimulant induced differential global DNA methylation profiles in treated-maize plants compared to naïve plants under well-watered conditions (**Figure 4.4**). These PGPR-induced modifications in the methylome of maize plants were also reflected under (mild and severe) drought stress conditions (**Figure 4.6**). Shifts in global DNA methylation levels revealed a decrease in DNA methylation (hypomethylation) due to mild and severe drought imposition with a decrease of 0.23- and 0.1-fold change (18.9% and 23.2%) respectively when compared to naïve plants. It is worth noting that PGPR can restore methylation levels closer to the control levels irrespective of severity of drought stress as there is no difference between MD-PGPR and SD-PGPR which both display an increase in DNA methylation (**Figure 4.6**).

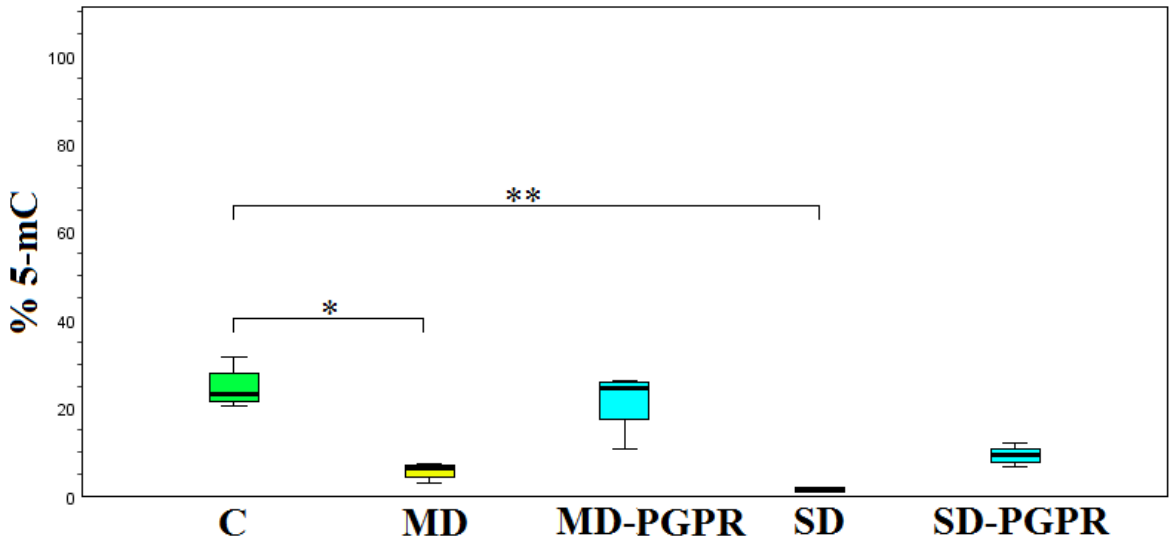

Figure 4.6. **Global DNA methylation levels in control, drought stress and drought-stress PGPR treatment conditions in maize leaves**. Genomic DNA from leaves in the different conditions were used to determine the relative global DNA methylation using a commercial ELISA-based kit. The methylation values represented in the plot correspond to the percentage methylation of the samples relative to the methylated control DNA samples supplied with the kit. The horizontal line within a box-plot represents the median. The lower and upper edges of boxes show the 25th and 75th percentiles, respectively. Whiskers represent the maximum and minimum values. **Abbreviations**: C, control; PGPR, plant growth-promoting rhizobacteria; MD, mild drought and SD, severe drought. Statistical significance findings were reported as $p \leq 0.05$* and $p \leq 0.01$**.

DNA methylation is an epigenetic mechanism which plays a fundamental role in tuning gene expression in response to enviromental perturbations resulting in phenotypic variations that do not stem from genetic variation in plants. DNA methylation is therefore considered a marker of plant stress reponses under abiotic stresses; and several studies have highlighted the involvement of DNA methylation in drought stress regulation (Yaish, 2013; Baulcombe & Dean, 2014). Under stress, plants exhibit dynamic DNA methylation patterns which are dependent on the plant species, tissues and the type of stress. For example, hypermethylation is observed under salt stress in alfafa (Al-Lawati *et al.*, 2016), however, hypomethylation is observed in salt-sensitive rapeseed (Marconi *et al.*, 2013). It has been reported that drought stress induces both a decrease/increase in the methylation of DNA throughout the genome of barley (Chwialkowska *et al.*, 2016) and ryegrass (Tang *et al.*, 2014). Under drought treatment, a decrease in the level of total DNA methylation was observed in leaf tissues of two faba bean genotypes (Abid *et al.*, 2017), rice (Wang *et al.*, 2011; Gayacharan & Joel, 2013) and rye grass (Tang *et al.*, 2014), which coincides with the findings of the current study.

Plant DNA methylation targets TEs and other repetitive sequences where it is associated with either transcriptional activation or repression. Long TEs are primarily located in heterochromatin and methylated by CMT2 and CMT3, however, gene-rich euchromatic regions contain shorter TEs or TE-derived sequences that are targeted by RdDM (Stroud *et al.*, 2014). The primary role of plant DNA demethylation is to maintain genome stability by counteracting DNA methylation, thus preventing the spread of methylation to neighbouring genes. Another major role of DNA demethylation in plants is to activate genes in response to abiotic stimuli, in many cases by targeting TE sequences located at their 5´ regions.

DNA methylation promotes transcriptional regulation by inhibiting transcription activators from binding to their respective target sites. This regulation serves as a form of stress adaptation towards different abiotic stresses such as drought. The degree of regulation is highly dependent upon the intensity and duration of the stress (Urano *et al.*, 2010), and the extent of epigenome modifications under mild and severe drought conditions differed (**Figure 4.6**). Under mild and severe drought stress, PGPR-primed plants showed a change in the global DNA methylation levels when compared to the corresponding controls. A positive increase in DNA methylation levels was observed under both mild and severe drought stress conditions (3.7- and 6.4-fold; 9.2 % and 21.5 % respectively) in PGPR-primed plants (**Figure 4.6**). Interestingly, under mild drought stress, the DNA methylation level was closer to that of the control suggesting that the primed plants may exhibit growth and development properties similar to the control plants. The observed DNA methylation increase due to PGPR treatment may suggest this epigenome modification as a mechanism for enhanced drought stress tolerance. DNA methylation has been widely implicated in physiological and developmental processes in plants and evidence that genome-wide DNA methylation changes are involved in abiotic stress responses in maize plants has been reported (Eichten & Springer, 2015).

DNA hypomethylation has been reported to often have negative effects on the plant's ability to tolerate environmental stresses. For example, under drought stress, reduction in DNA methylation decreased the ability of *Arabidopsis* to cope under salt stress (Baek *et al.*, 2011). However, a recent study by Sapna *et al.* (2020) assessed the DNA methylation patterns in rice plants under drought stress. In rice, increased DNA methylation seems to be an imperative mechanism associated with drought stress responses, which probably regulates methylation

sensitive gene expression. The drought-induced changes in DNA methylation were suggested to contribute for epigenetic mechanism and the study provided evidence to argue that drought-induced increased methylation might be one of the main mechanisms associated with acclimation responses in field crops like rice.

In the present study, PGPR-induced increase in DNA methylation in mild and severe drought stress (**Figure 4.6**) can be postulated to be a key mechanism through which PGPR augments drought stress tolerance by regulating gene expression and remodelling the methylation profiles. Additionally, as mentioned in **section 4.3.1**, in non-stressed plants, PGPR treatment induced an increase in DNA methylation when compared to the control. The observed increase may serve as a priming mechanism regulating gene expression directed towards drought stress tolerance. Gagné-Bourque *et al.* (2015) reported changes in DNA methylation levels induced by PGB *Brachypodium distachyon* under drought stress which coincides with the findings of the present study. Furthermore, *Branchypodium* colonised plants were reported to be more tolerant to drought stress when compared to the naïve plants.

4.3.3 Expression profiles of stress-related genes in naïve and PGPR-treated maize plants under drought conditions

In this study (**Chapter 4**) no direct link between observed global DNA methylation levels (**Figures 4.4** and **4.6**) and particular gene expression profiles can pragmatically be made. However, in an epistemological outlook of methylation-and-gene regulation framework, it is worth investigating the expression status of stress-related genes involved in key metabolic pathways revealed in **Chapter 3**. The targeted genes were pyrroline-5-carboxylate synthetase (*P5CS*), dehydration-responsive element-binding protein 2A (*DREB2A*), phenylalanine ammonia lyase (*PAL*) and flavone synthase (*FSNII*). Quantitative real-time PCR assays were carried out to monitor the transcript accumulation profiles of selected maize genes (**Table 4.2**) which have been previously characterised to play dynamic roles in drought-stress responses. Prior to analysis, gradient PCR analysis was conducted to determine the optimum annealing temperature (Ta) of the primers. Identical reactions, each containing a fixed primer concentration across a temperature range of 58.0 °C – 63.0 °C, were performed (**Figure 4.7A** and **S4.3**) and the primer sets were successfully annealed at all the temperatures. Additionally, melt curve analysis was performed to assess whether the PCR products produced single, specific products. The melt curves generated for each gene primer set all

gave rise to single distinct peaks at specific temperatures (**Figure 4.7B and S4.3**), indicating that the amplified DNA products are single discrete amplicons.

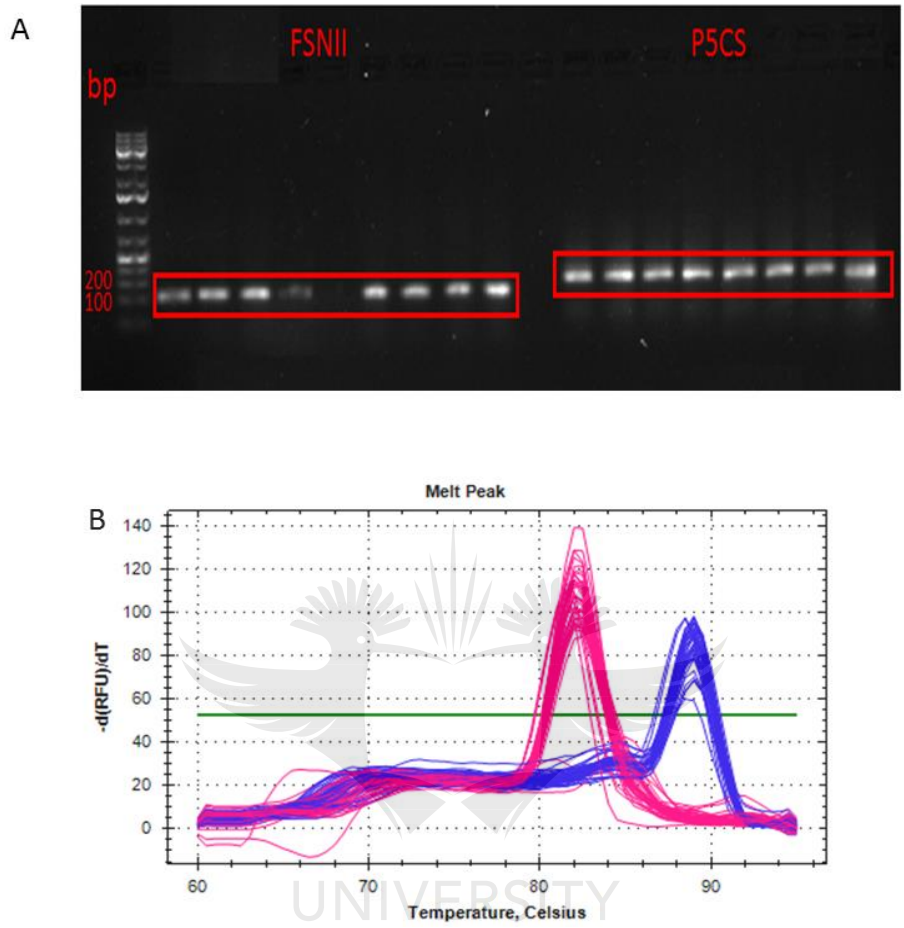

Figure 4.7. Primer efficiency check. Representative agarose gel (**A**) and melt curve analysis of qPCR amplicons for *FSNII* and *P5CS* genes (**B**). The melt curve plots the change in relative fluorescence units over time (-d(RFU)/dT) against temperature (°C) and shows single melt peaks for both genes, *FSNII* (pink) and *P5CS* (blue).

PGPR triggered differential gene expression levels of all the genes (**Figure 4.8**) under normal conditions (pre-challenge phase) and drought conditions (post-challenge phase). Under normal conditions (pre-challenge phase), PGPR treatment induced an upregulation of *P5CS* expression (1.8 fold) when compared to the control (**Figure 4.8A**). Plants synthesise proline from glutamic acid and ornithine. This reaction is catalysed by the P5CS enzyme, yielding pyrroline-5-carboxylate (P5C) which is further reduced to proline by Δ1-pyrroline-5-carboxylate reductase (Hu *et al*., 1992). In this proline biosynthesis pathway, P5C5 is a rate-limiting enzyme and owing to this, *P5CS* has a crucial role in the regulation of proline accumulation. The expression pattern of *P5CS* gene therefore is a direct indicator of proline

levels in plants. The overexpression of P5CS induced by PGPR can therefore be correlated to increased proline biosynthesis in plants during the pre-challenge phase of priming. In agreement to this, quantitative analysis revealed an accumulation of proline due to PGPR treatment in the pre-challenge phase (**Chapter 3, sub-subsection 3.3.2.1, Figure 3.9B**).

Proline is a compatible solute that affects numerous cellular and molecular aspects of a plant in both normal and stressful conditions. Proline forms a part of many proteins involved in osmotic regulation, cell wall and membranes and it is essential for maintaining their stability (Szabados & Savouré, 2010). Additionally, in the absence of stress, proline is transported to organelles, mainly vacuoles and plastids where it is stored and only distributed to the cytosol under water-deficit conditions (Lehmann *et al.*, 2010). Finally, proline can act as a source of nitrogen, carbon, and energy (Verbruggen & Hermans, 2008; An *et al.*, 2013). From this, PGPR therefore pre-conditions the plants by activating the overexpression of *P5CS* which, in turn, enhances proline production in order to maintain cellular structures, increase proline reserves and energy production which may be utilised upon stress encounters thus ensuring survival.

Mild and severe drought stress conditions caused a decrease and increase in the expression of *P5CS* respectively (0.69- and 1.14-fold). Proline accumulation under stressful conditions is a well-known defence response employed by plants. Under drought stress, proline retains osmotic potential, redox balance inside the cells, acts as an antioxidant by scavenging reactive oxygen species (ROS), protects macromolecules from denaturation by acting as a chemical chaperone and regulates cytosolic pH (Hare *et al.*, 1999; Hong *et al.*, 2000; Guerzoni *et al.*, 2014). Interestingly, in PGPR-primed drought stressed plants (post-challenge phase), the overexpression of *P5CS* was very evident and higher than all the treatments (8.2 and 2.6 fold), with MD-PGPR exhibiting the highest overexpression (8.2 fold). This suggests that PGPR-induced accumulation of proline observed in the pre-challenge phase become maintained throughout the post-challenge phase, enhancing drought stress tolerance. Recent studies (Maghsoudi *et al.*, 2018; Anton *et al.*, 2020) have reported on the accumulation of *P5CS* and proline under drought stress. In these studies, the overexpression of *P5CS* was correlated to the measured levels of proline, suggesting that this gene is involved in the regulation of proline biosynthesis and has the potential to be used for improvement of drought stress tolerance. Yoshiba *et al.* (1997) first reported an increase in proline content

attributed to the upregulation of *P5CS* due to *Bacillus* inoculation under drought stress. Similarly, a recent study by Ghosh *et al.* (2017) reported on how *Pseudomonas putida* alleviates the effects of drought stress in *Arabidopsis thaliana* by drastically changing proline gene expression profiles. In this study, an upregulation of the expression of genes involved in proline biosynthesis, *i.e*. ornithine-Δ-aminotransferase (OAT), P5CS was observed. These observations were positively correlated with morphophysiological evidences of water-stress mitigation in the plants inoculated with *Pseudomonas putida* that showed improved growth, increased fresh weight, enhanced plant water content, reduction in primary root length, enhanced chlorophyll content in leaves, and increased accumulation of proline.

Under normal conditions, PGPR induced a slight upregulation of *DREB2A* expression (1.5-fold) when compared to the control (**Figure 4.8B**). As previously mentioned in **Chapter 2** (**subsection 2.2.2**), TFs are involved in the regulation of stress-responsive gene expression through the abscisic acid (ABA)-dependent/independent pathways. DREB is an ABA-independent regulon which regulates the expression of numerous stress-responsive genes and plays a crucial role in improving abiotic stress tolerance by interacting with *cis*-elements present in the promoters of these abiotic stress-responsive genes. *DREB2A* is a DREB encoding gene which similarly induces the expression of various stress-responsive genes (*RD29/ERD1/RD22*) (Nakashima & Yamaguchi-Shinozaki, 2006; Nakashima *et al.*, 2009). In the pre-challenge phase, it is plausible to suggest that PGPR induces the expression of downstream stress-responsive genes through the overexpression of *DREB2A*, augmenting stress tolerance in stressful conditions.

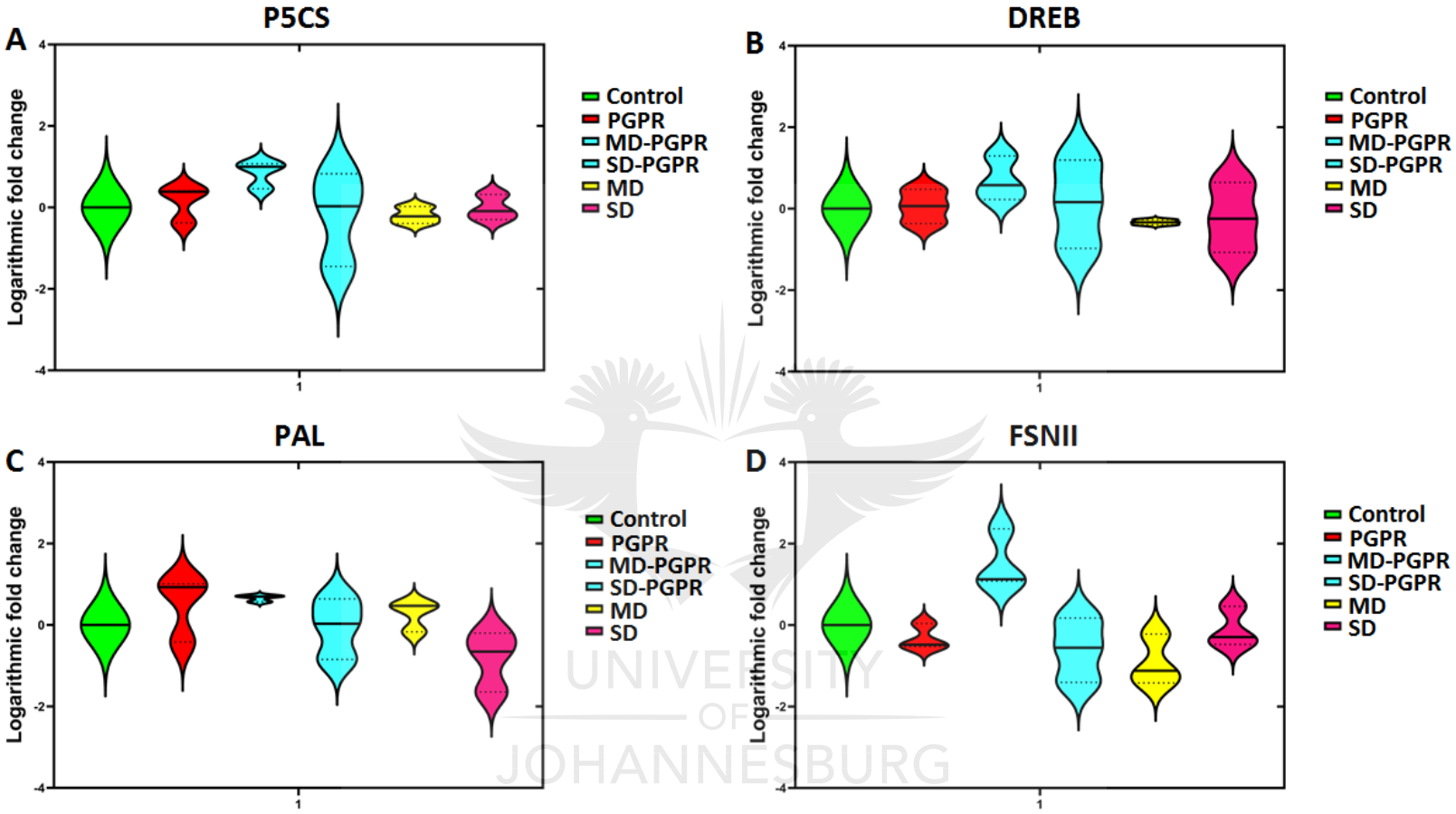

Figure 4.8. **Normalised gene expression expressed as logarithmic fold change**. Violin plots show the differential gene expression of (**A**) *P5CS*, (**B**) *DREB*, (**C**) *PAL* and (**D**) *FSNII* under control, PGPR treatment, mild and severe drought stress (MD and SD respectively), and PGPR-primed mild and severe drought stressed conditions. The relative quantification of each gene against reference genes (EF1α and β-TUB) was calculated, which was then used to calculate normalised gene expression. Violin plots depict the distribution of data using density curves, which are overlaid by boxplots. The horizontal line within a violin plot represents the median. The lower and upper dotted lines show the 25th and 75th percentiles, respectively. Abbreviations: C, control; PGPR, plant growth-promoting rhizobacteria; MD, mild drought and SD, severe drought.

On the other hand, *DREB2A* expression was downregulated under mild drought stress conditions (0.46-fold) (**Figure 4.8B**) in the absence of PGPR treatment. This defence response in naïve plants suggests a different drought stress defence mechanism in which the expression of *DREB2A* is repressed. Although *DREB2A* regulates the expression of many genes involved in stress response and tolerance as highlighted previously, it has been implicated in growth retardation and reduced reproduction rate in plants and its transcript and protein levels are therefore tightly regulated (Yoshida *et al.*, 2014). Under drought stress, plants can therefore supress *DREB2A* gene expression to allow for growth and development under adverse environmental conditions. This, however, poses a threat to non-primed plants since the induction of stress-responsive gene expression would be suppressed, resulting in enhanced susceptibility to drought stress.

PGPR-primed plants exhibited an upregulated expression of *DREB2A* under both mild and severe drought stress conditions (8.4- and 5.7-fold respectively) (**Figure 4.8B**). *DREB* genes have been reported as the best studied group of TFs involved in the activation of gene expression of many target genes responsible for controlling aspects such as osmoprotection under abiotic stress (Shinozaki & Yamaguchi-Shinozaki, 2007; Hussain *et al.*, 2011). For example, under drought stress, *DREB2A* induces the expression of various drought stress-responsive genes such as *RD29A*. Qin *et al.* (2007) first reported on the accumulation of *DREB2A* in maize plants under drought stress. Additionally, *DREB2A* was significantly induced by drought stress and this induction was directly correlated to the upregulation of *RD29A* gene expression. Furthermore, the stress-induced expression of *DREB2A* resulted in improved drought stress tolerance. The modulation of the expression of drought responsive genes by *Bacillus* has been reported by Gagné-Bourque *et al.* (2015), in which plants inoculated with *Bacillus subtilis* displayed a higher accumulation of *DREB* and enhanced drought tolerance when compared to the non-inoculated plants. This correlates with the findings of the current study, and therefore it can be postulated that PGPR-based biostimulant enhances drought stress tolerance by inducing DREB encoding gene, which consecutively activates the expression of downstream drought-responsive genes.

In the pre-challenge phase, PGPR induced the upregulated expression of *PAL* (6.3-fold) (**Figure 4.8C**) and a decrease in the expression level of *FSNII* (0.6-fold) (**Figure 4.8D**). The differential expression of *PAL* and *FSNII* indicates that PGPR activates the expression and

translation of these key enzymes involved in the phenylpropanoid pathway to drive the biosynthesis of secondary metabolites such as flavonoids and phenolics. As previously mentioned in **Chapter 3** (**subsection 3.3.2.2**), phenylpropanoids are grouped into flavonoids, phenolic acids, coumarins and monolignols depending on their chemical structures. PAL is a key gateway enzyme that links the primary and secondary metabolism, particularly *via* the phenylpropanoid pathway, which branches into a network of other pathways. This PAL enzyme catalyses the deamination of phenylalanine giving rise to cinnamic acid, which is then drives the biosynthesis of other phenylpropanoids (Huang *et al.*, 2010; Kong, 2015).

Phenylpropanoids play numerous roles in plant growth and development. For example, lignin, which is synthesised from the phenylpropanoid pathway, is a complex phenolic polymer which is indispensable for plant growth. Lignin enhances cell wall integrity and promotes mineral and photosynthetic products translocation through vascular bundles in plants (Barros *et al.*, 2015; Liu *et al.*, 2018). The induced overexpression of *PAL* by the microbial biostimulant, which leads to the accumulation of phenylpropanoids, is thus one of the mechanisms governing enhanced plant growth and development. The PGPR-primed plants exhibit enhanced growth and development through activation of the phenylpropanoid pathway and compounds such as lignin. Quantitative analysis of phenolic acids and flavonoids induced by PGPR in the pre-challenge phase (**Chapter 3**, **subsection 3.3.2.2**) revealed an augmented accumulation of these secondary metabolites linked to plant growth and pre-conditioning of defences. The observed overexpression of *PAL* in the current study therefore complements the reported metabolomics findings.

In the absence of PGPR, drought stress induced the downregulation of both *PAL* and *FSNII* (0.26- and 0.24-fold respectively) (**Figure 4.8 C-D**) when compared to the control. A rapid transient production of excessive amounts of ROS is one of the prime aspects of plant response to different types of environmental stresses including drought stress. ROS pose a major threat to plant growth and development due to their toxicity in plant system which causes membrane damages that often lead to the cell death (Blokhina *et al.*, 2003). In contrast, plants have evolved antioxidative mechanisms to combat the danger posed by ROS through the production of antioxidants. Plants normally employ the accumulation of the defensive phenylpropanoid pathway compounds (phenolics and flavonoids) which act as antioxidants through the induction of *PAL* to mitigate the deleterious effects of drought stress.

An increase in PAL activity under drought stress has been reported in numerous plants including maize (Gholizadeh, 2011). PAL and activities of other key enzymes of the phenylpropanoid pathway regulate the biosynthesis of phenolics under abiotic stress. Additionally, enhanced performance of these enzymes is accompanied by the up-regulation of the transcript levels of genes encoding key biosynthetic enzymes such as *PAL*, *C4H* (cinnamate 4-hydroxylase), *CHI* (chalcone isomerase), *F3′H* (flavonoid 3′-hydroxylase), *FLS* (flavonol synthase), *IFS* (isoflavone synthase), *IFR* (isoflavone reductase) and *FSNII* (Ma *et al.*, 2014; Gharibi *et al.*, 2019). The downregulation of *PAL* and *FSNII* therefore indicates supressed PAL and FNSII enzyme activity in non-primed plants, resulting in the decreased biosynthesis of phenylpropanoids and enhances drought susceptibility due to the absence of antioxidant compounds.

In contrast to the downregulation of *PAL* and *FSNII* gene expression in non-primed plants under drought stress, PGPR-primed plants induced an upregulated expression of these genes under mild drought stress conditions (4.6- and 12.7-fold) and a down-regulation of *FNSII* under severe drought stress conditions (0.6-fold) (**Figure 4.8**). The enhanced expression of these genes can be correlated to increased PAL and FSNII activity implying increased biosynthesis of phenylpropanoids. As echoed in the paragraphs above, PAL is the first enzyme in the phenylpropanoid pathway. Similarly, FSNII is a key enzyme in the phenylpropanoid pathway but only in the biosynthesis of flavones – a major class of flavonoids, stemming from the main phenylpropanoid pathway. The accumulation of flavonoids and phenolic compounds derived from the phenylpropanoid pathway has been linked to enhanced ROS scavenging through various mechanisms including inhibition of enzymes involved in ROS production and quenching. These antioxidants contribute to the mitigation of oxidative stress induced by drought stress. The suggested accumulation of phenolics due to *PAL* and *FSNII* overexpression induced by PGPR is supported by the downstream metabolic reprogramming in which phenolic acids and flavonoids were accumulated due to PGPR treatment as evidenced in **Chapter 3**, **section 3.3**, **Figures 3.16** and **3.20**.

Numerous studies have reported phenolic acids as indicators of drought tolerance through increased antioxidant capacity (Weidner *et al.*, 2009; Varela *et al.*, 2016; Laxa *et al.*, 2019). Coinciding with the findings of the current study, Singh *et al.* (2020) reported an

overexpression of *PAL*, *DREB* and antioxidant enzymes (e.g. superoxide dismutase, *SOD*), H_2O_2 peroxidation (*APX*, *PO*) and oxidative defence response (*CAT*) in rice plants inoculated with *Pseudomonas fluorescens*. This resulted in enhanced antioxidant capacity and contributed towards drought stress mitigation. In **Chapter 3** (**subsection 3.3.1**), enzymatic antioxidant machinery markers (CAT, SOD and APX) were measured under drought stress in PGPR-primed plants and all these markers were significantly increased. Thus, the postulated (hypothetical) model (**Figure 4.9**), emerging from these findings, is that the PGPR-based biostimulant triggers DNA hypermethylation, which (by an epistemological extrapolation) could be linked to the (up)regulation of the expression of key defensive genes such as *P5CS*, *DREB*, *PAL* and *FSNII*. The activation of these defence mechanisms amplifies drought inducible stress responses such as the accumulation of osmolytes and the activation of the phenylpropanoid pathway, resulting in enhanced drought stress tolerance when compared to naïve plants.

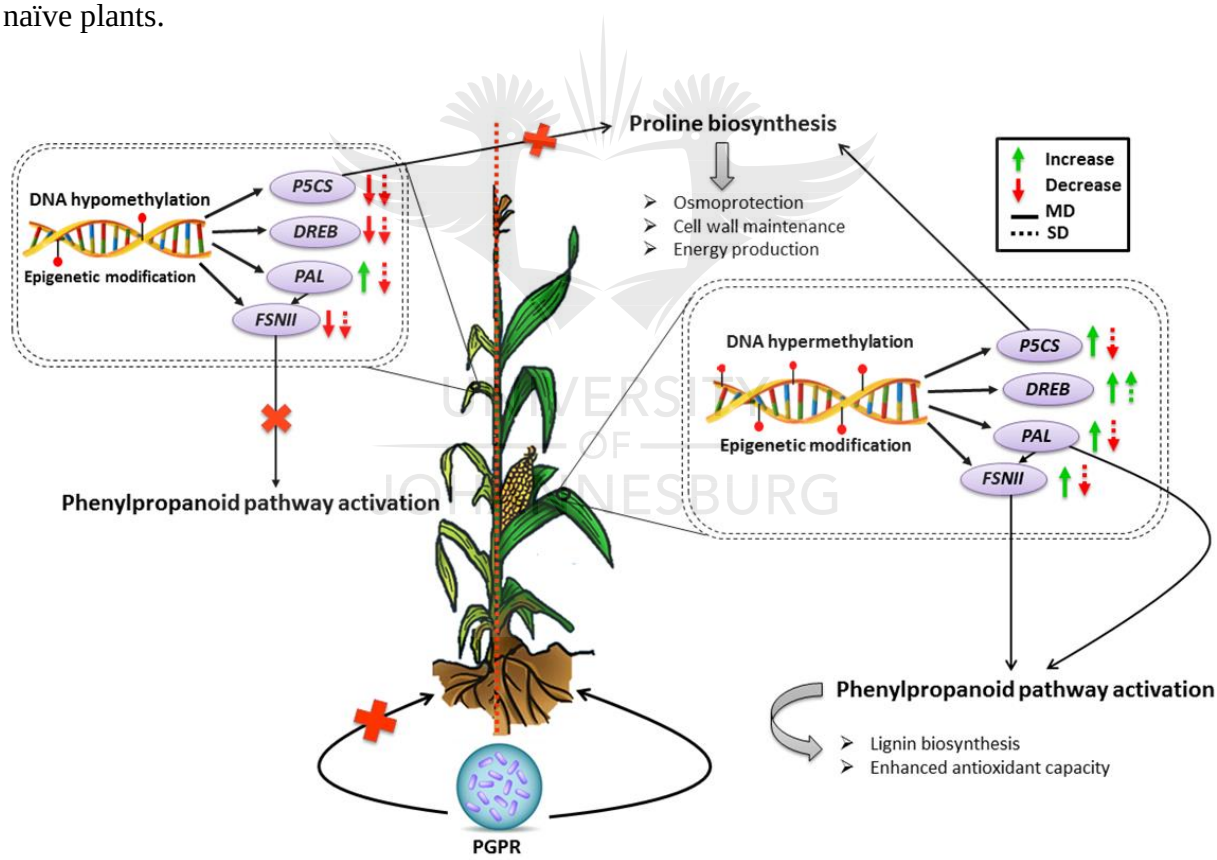

Figure 4.9. **Summary diagram of DNA methylation and gene expression alterations triggered by PGPR under drought stress**. PGPR-primed plants exhibit enhanced induction of drought stress responsive mechanisms such as increased DNA methylation and expression of key stress-responsive genes, resulting in stress tolerance (the diagram was created by the author of this dissertation).

The ability of plants to maintain their primed state throughout their life cycle and successfully passing the primed state modifications onto their progeny has been reported in numerous studies (Mauch-Mani *et al.*, 2017). As mentioned in the introduction section (**section 4.1**), the transfer of these epigenetic modifications onto successive generations serves as a transgenerational memory which can contribute to phenotypic plasticity and improved stress tolerance (Boyko & Kovalchuk, 2008; Chinnusamy & Zhu, 2009). In this case, transgenerational memory refers to observable and stable stress memory effects over multiple generations in the absence of the original stress (Molinier *et al.*, 2006). Bird (2002) and Mirouze & Paszkowski (2011) proposed that stress memory mechanisms in plants are encoded by epigenetic modifications.

Experimental evidence of stress-induced transgenerational memory, resulting from epigenetic modifications changes covering different abiotic stresses in various plant species has been reviewed in Crisp *et al.* (2016). In a recent study by Cong *et al.* (2019), heavy metal responsive genes were upregulated in response to heavy metal stress. Additionally, evidence of transgenerational memory *via* these changes in gene regulation even after the removal of heavy metals was reported. The study further reported on DNA methylation alterations in response to the heavy metal stress which showed transgenerational inheritance. In this study (reported in this **Chapter 4**), PGPR application led to the reprogramming of the epigenetic landscape through altered DNA methylation profiles. In spite of the fact that monitoring of transgenerational effects over subsequent generations was outside the scope of this study, based on the findings highlighted above, it can be suggested that these epigenetic modifications (**Figure 4.9**) can potentially be passed onto succeeding generations resulting in heritable phenotypic variation leading to stress tolerance and survival.

4.4 Conclusion

Herein, the PGPR-induced reprogramming of DNA methylation in maize plants under mild and severe drought stress conditions was investigated using ELISA. PGPR induced an increase in DNA methylation in maize, under both drought stress and non-stress conditions. This increase in DNA methylation was assumed to be one of the modes of action of microbial-based biostimulants in enhancing growth promotion, priming activation and drought stress tolerance. These findings correlate to the observed morphophysiological and metabolic changes reported in **Chapter 3**. Drought responsive genes were overexpressed due to PGPR treatment in both well-watered and drought stress conditions, suggesting that PGPR induces the expression of these key genes for priming activation and drought stress tolerance enhancement. To the best of our knowledge, this study is the first suggesting an involvement of epigenetic and transcriptional regulatory mechanisms (**Figure 4.9**) underlying the effects of PGPR-based biostimulants on crop plants (*e.g*. maize as in this case), under normal and stress conditions. However further investigation is required to elucidate PGPR-induced modifications in gene specific DNA methylation profiles and the downstream regulation of gene expression.

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4.6 Supplementary Materials

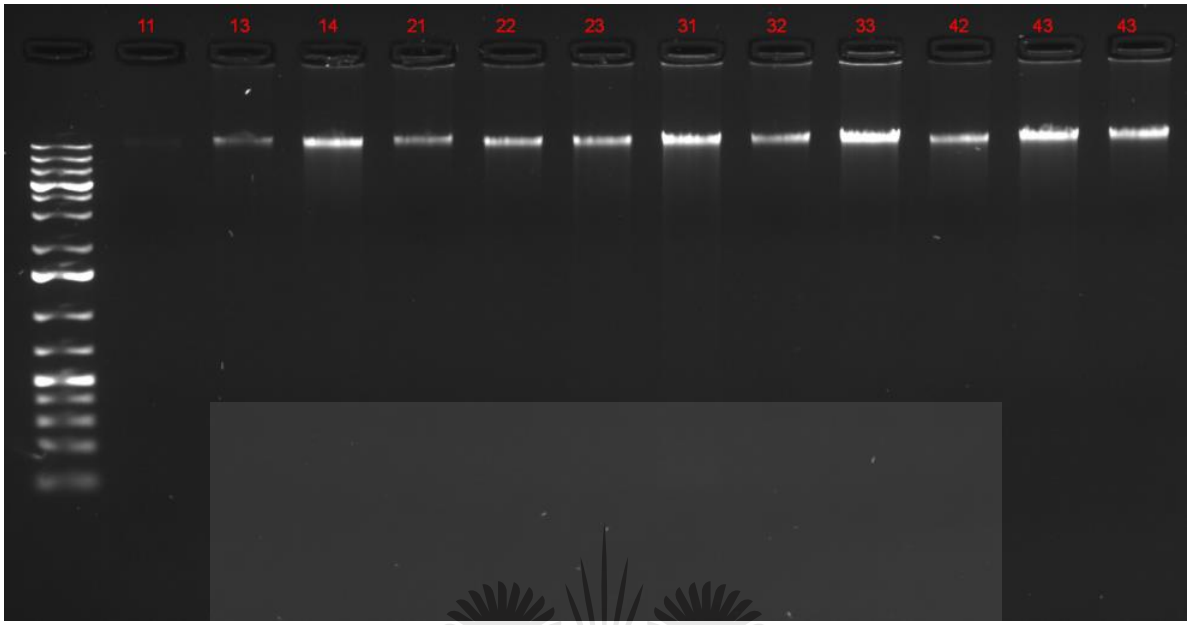

Figure S4.1: Representative gel for the DNA integrity check of the extracted DNA from samples using 1% agarose gel electrophoresis.

Table S4.1: Summary of the normality tests for DNA methylation levels in different treatments.

Tests of Normality							
Treatment		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	Degrees of freedom	Sig.	Statistic	df	Sig.
% 5-mC	PGPR	0.309	12	-	0.902	4	0.440
	C	0.312	12	-	0.878	4	0.331
	MD-PGPR	0.376	12	-	0.751	4	*0.040
	SD-PGPR	0.181	12	-	0.992	4	0.965
	MD	0.273	12	-	0.901	4	0.434
	SD	0.267	12	-	0.903	4	0.446
a. Lilliefors Significance Correction							

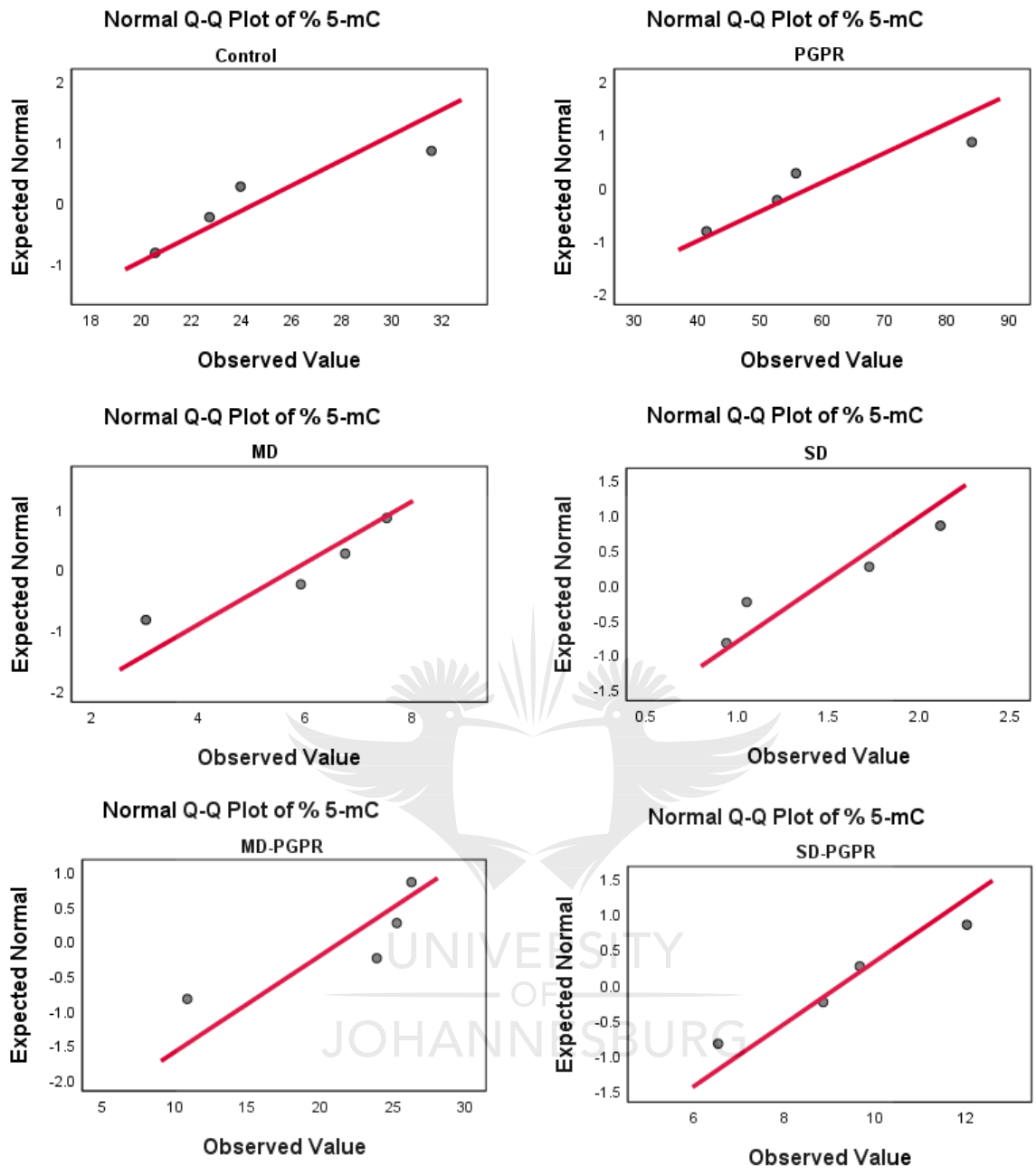

Figure S4.2: Normal Q-Q plots depicting the distribution of the DNA methylation levels in different treatments.

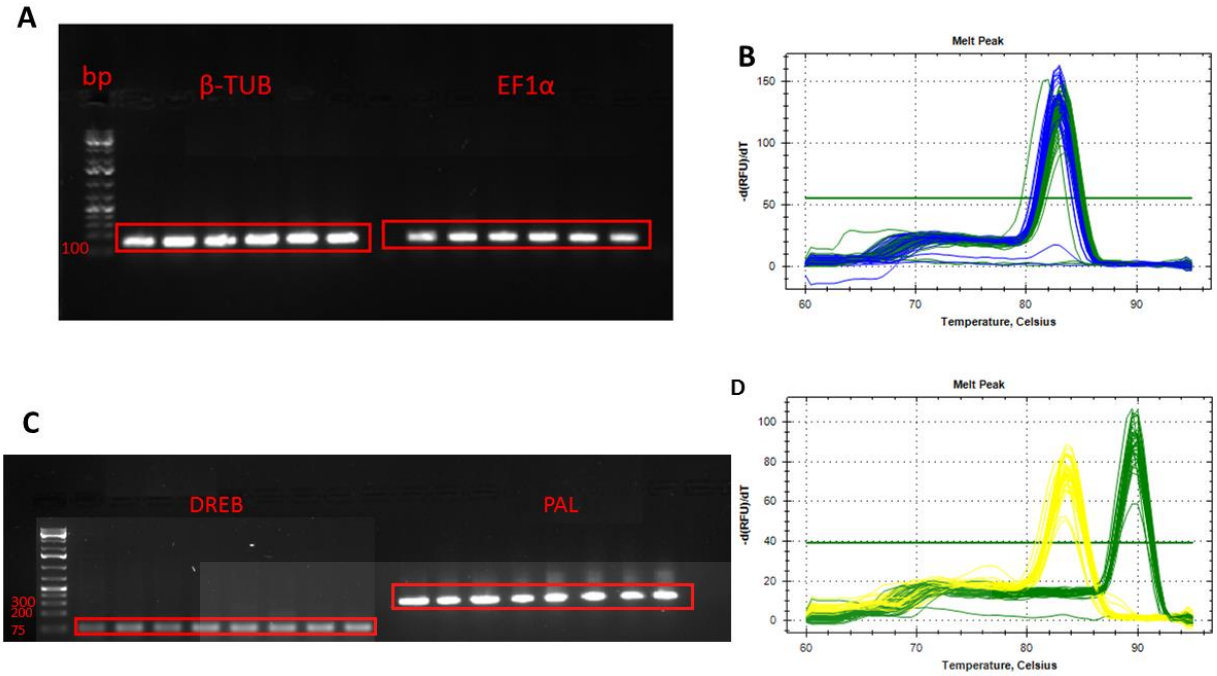

Figure S4.3: **Primer efficiency check**. Agarose gel electrophoresis of qPCR amplicons and melt curve analysis. (**A** and **B**) *β-TUB* and *EF1α1*, (**C** and **D**) *DREB* and *PAL*. (**B**) *β-TUB* (Blue) and *EF1α1* (green) and (**D**) *DREB* (yellow) and *PAL* (green).

Chapter 5

General Conclusions and Perspectives

Improving plant adaptation to abiotic stresses, for sustainable food production, has long been a pursuit of crop plant breeders. However, this is understandably difficult to achieve since abiotic stress resistance is a multigenic - and quantitative trait controlled by multi-layered cellular and molecular events. Hence, innovative and efficient strategies to improve crop quality and tolerance against abiotic stresses are imperatively required. As mentioned in **Chapters 1** and **2**, microbial biostimulants represent potentials to provide sustainable and economically favourable solutions that could introduce novel approaches to improve agricultural practices and crop productivity. However, to effectively establish and devise novel biostimulant-based agricultural strategies, there is a necessity to firstly understand the physiology and biochemistry governing the interactions between biostimulants and plants at both cellular and molecular levels. This knowledge gap – decoding molecular and physiological mechanisms underlying biostimulant action – is one of the main bottlenecks that hamper the biostimulant field and industries from implementing and maximising the value of (traditional and novel) such formulations in agronomic practices (**Chapter 1**). Thus, the current work, presented in this dissertation, contributes to on-going scientific efforts to elucidate biochemical and molecular frameworks as well as predictive models that describe the mechanisms activated/stimulated by biostimulants (in crop plants) towards growth promotion and enhanced adaptability to abiotic stress conditions. The work was designed to explore both the chemical and gene space of maize plants treated with a PGPR consortium (5 strains of *Bacilli*), under both mild- and severe drought stress conditions.

Chapter 3, an LC-MS-based targeted metabolomics study, revealed altered metabolic profiles associated with PGPR-mediated growth enhancement and drought stress tolerance in maize plants. The microbial biostimulant induced differential changes in the levels of amino acids, phytohormones, flavonoids and phenolic acids in maize plants under well-watered, mild and severe drought stress conditions. These quantitative (metabolic) alterations spanned several pathways in both primary and secondary metabolism, and reflect underlying

mechanisms employed by PGPR in promoting plant growth and defence priming for enhanced drought stress tolerance. These metabolic reconfigurations lead to biochemical and physiological events that include (i) growth promotion of roots, leaves and shoots; (ii) enhanced photosynthetic capacity; (iii) energy production through amino acid recycling; (iv) nutrient and water uptake; (v) ethylene inhibition through ACC degradation; (vi) production of osmolytes; (vii) protein biosynthesis; and (viii) augmented antioxidant capacity. Furthermore, this PGPR-induced metabolic reprograming was translated into morphophysiological alterations as confirmed by a significant increase in enzymatic regulators of oxidative stress and biomass accumulation in PGPR-treated plants compared to naïve plants. At the gene space level (**Chapter 4**), PGPR-treatment significantly increased DNA methylation levels in the pre-challenge and post-challenge phases, making DNA methylation an additional key mechanism through which PGPR-based biostimulant enhances plant growth, priming and drought stress tolerance. In addition, expression levels of targeted genes (defence-responsive genes – *P5CS, DREB2A, PAL, FSNII*) were upregulated, suggesting that PGPR not only influences the epigenome but also gene regulation to enhance the expression of drought defence-responsive genes.

Future studies could include targeted DNA methylation analyses to gain more insights into specific spatiotemporal methylation status, and correlating these methylations to downstream gene expression profiles. Furthermore, the interrogation of transgenerational effects of PGPR-induced changes in the maize methylome could shed light on stress memory, *i.e.* the extent of biostimulant-induced stress priming. These future studies (and alike), build on the knowledge generated from this work, and would be part of on-going scientific efforts to decode molecular mechanisms governing biostimulant effects in crop plants. Thus, the work presented in this dissertation revealed that microbial-based biostimulant (a consortium of *Bacilli* spp.) can enhance growth and improve maize responses to drought stress conditions by modulating cell metabolic pathways and gene regulation events. This mechanistic and predictive framework, explaining the modes of action of the microbial-based biostimulant, is an actionable (fundamental) knowledge necessary for the biostimulant industry, and can be used to derive novel biostimulant formulations and design biostimulant-based agricultural programs for sustainable food production.

The End