Metabolomic analysis of maize (*Zea mays* L.) seedlings treated with selected plant growth promoting rhizobacteria

by
Faith. O. Otukpa
13039042

Submitted in partial fulfilment of the degree *Magister Scientiae* Biochemistry in the Faculty of Natural and Agricultural Sciences
University of Pretoria
Pretoria

4th December 2019

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Denkleiers • Leading Minds • Dikgopolo tša Dihlalefi

Submission Declaration

I, Faith Obiye Otukpa, declare that the thesis/dissertation, which I hereby submit for the degree Magister Scientiae in the Department of Biochemistry, at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:…….

DATE……4 December 2019……

Declaration of Originality University of Pretoria

The Department of ……Biochemistry, Genetics & Microbiology…… places great emphasis upon integrity and ethical conduct in the preparation of all written work submitted for academic evaluation.

While academic staff teach you about referencing techniques and how to avoid plagiarism, you too have a responsibility in this regard. If you are at any stage uncertain as to what is required, you should speak to your lecturer before any written work is submitted.

You are guilty of plagiarism if you copy something from another author's work (e.g. a book, an article or a website) without acknowledging the source and pass it off as your own. In effect, you are stealing something that belongs to someone else. This is not only the case when you copy work word-for-word (verbatim), but also when you submit someone else's work in a slightly altered form (paraphrase) or use a line of argument without acknowledging it. You are not allowed to use work previously produced by another student. You are also not allowed to let anybody copy your work with the intention of passing if off as his/her work.

Students who commit plagiarism will not be given any credit for plagiarised work. The matter may also be referred to the Disciplinary Committee (Students) for a ruling. Plagiarism is regarded as a serious contravention of the University's rules and can lead to expulsion from the University.

The declaration which follows must accompany all written work submitted while you are a student of the Department of...Biochemistry..... No written work will be accepted unless the declaration has been completed and attached.

Full names of student: Faith Obiye Otukpa.....

Student number: 13039042.....

Topic of work: Metabolomic analysis of maize (*Zea mays* L.) seedlings treated with selected plant growth promoting rhizobacteria.....

Declaration

1. I understand what plagiarism is and am aware of the University's policy in this regard.
2. I declare that this …Dissertation……(eg essay, report, project, assignment, dissertation, thesis, etc) is my own original work. Where other people's work has been

used (either from a printed source, Internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.

3. I have not used work previously produced by another student or any other person to hand in as my own.

4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

SIGNATURE.....
.....

Acknowledgements

I would like to start the acknowledgements by thanking God almighty for the gift of life, the strength, courage and direction throughout this journey. It was not an easy journey, but it was a beautiful one nonetheless, and I am grateful to God for it.

"*I consider that our present sufferings are not worth comparing with the glory that will be revealed in us.*" (Romans 8:18)

I was able to make it through my MSc with the assistance and support of a number of individuals. I would like to first of all thank my supervisor Prof. Zeno Apostolides and co-supervisor Prof. Nico Labuschagne. Both of them for their invaluable expertise and guidance through this journey, and my co-supervisor for the funding in my first year.

From the PGPR research group in the Department of Plant and Soil Sciences, I would like to acknowledge Dr. René Carlson, Phumzile Siboza, Nicole Rudolph, Lizzy Marakalla and Mandla Sibiya for their assistance during the greenhouse trials.

From the Department of Biochemistry, Genetics & Microbiology, I would like to acknowledge Ms Sandra van Wyngaardt for all the technical assistance. Next, I would like to acknowledge my colleagues from the CAM research group and the Department for their support.

I would like to especially thank the people who mean the world to me, my parents Mr. Joseph Audu Otukpa and Mrs. Priscilla Otukpa; and my siblings Victoria, Victor, Emmanuel, Godswill, Regina, and Ainema, for their spiritual, emotional and financial support through this journey. Thank you for being my unconditional support through it all.

I would also like to thank my friends for being the distraction I needed when things got too hard.

I would like to acknowledge the National Research Foundation for funding.

Last but not least, I would like to acknowledge myself, for being brave and persevering through this experience. It was not easy and I am so very proud of me.

Abstract

Global food production has significantly increased mainly due to the use of high-yield crop varieties, fertilisers, fungicides and pesticides, and improved irrigation methods. Despite the increase in production, there has been a significant cost to the environment in the form of pollution, and to farmers in the form of rising fertiliser and pesticide costs. The impacts on the environment include but are not limited to groundwater contamination, declining soil health and increased pest and pathogen resistance, all which increase the financial cost to farmers. To reverse or rather salvage the situation, more sustainable agricultural practices need to be employed that will maintain high productivity with little to no damage to the environment, and will reduce agrochemical use, thus, reducing the financial strain on farmers. A potential solution would be to exploit soil dwelling rhizospheric microorganisms to improve plant growth with little to no application of agrochemicals.

The rhizosphere refers to the region of soil directly influenced by plant roots and is home to microorganisms known as plant growth promoting rhizobacteria (PGPR). These PGPR have been found to stimulate plant growth via numerous mechanisms, which directly affect the plant metabolome and in turn translates into observable effects in the plant phenotype. The plant metabolome has been described as the bridge between the genotype and phenotype, thus metabolomics acts as a useful tool to evaluate the contribution of external influences on the plant phenotype based on metabolic changes. The effect of PGPR on the plant metabolome is vital in understanding their mode of action, which will further validate their use in farming.

The overall aim of this project was to assess the effect of selected PGPR strains with known plant growth promoting activity on the metabolic profile of maize seedlings; and to evaluate if these changes in the metabolic profile directly correlate with the observable effects on the growth of the seedlings. To achieve this aim, firstly, the effect of *Lysinibacillus sphaericus* (T19), *Paenibacillus* sp. (T29) and *Bacillus megaterium* (A07) on early maize growth, i.e., the effects on dry root and shoot biomass, leaf chlorophyll content, stem diameter and shoot length, was assessed. Secondly, the effect of single strain PGPR inoculation on the metabolic profile of maize was evaluated. Finally, metabolomics analysis was conducted on the roots and shoots of the maize seedlings inoculated with strains T29 and T19 respectively. Statistical analysis of the metabolomics results was conducted to find significant pathways and discriminating metabolites between the control and inoculated plants.

To assess the effects of the PGPR strains on early maize growth, a greenhouse trial was first conducted. Non-invasive techniques were used to measure the growth parameters that could be recorded prior to harvest. Next an untargeted metabolomics approach was used to analyse the metabolome of harvested roots and shoots. Metabolomics data acquisition was achieved using ultra-performance liquid chromatography hyphenated to quadrupole time of flight mass spectrometry detection (UPLC/QTOF-MS). Finally, to evaluate the effect of single strain inoculation on the maize root and shoot metabolome, univariate and multivariate methods were applied.

The results of the greenhouse trial showed a tendency of strains T19 and T29 in stimulating shoot growth and root growth respectively in the maize seedlings. Pathway analysis using results from univariate analysis revealed a number of pathways affected by T19 and T29 in the shoots and roots respectively. Multivariate statistical analysis also showed that the inoculated samples differed from the control samples, albeit with varying trends, which indicates differing metabolic states. Some of the metabolic pathways deemed significant in the inoculated shoots and roots were amino acid, nucleotide metabolism or carbon fixation related. A number of discriminating features were found to be differentially regulated in the inoculated roots and shoots. Overall, the results showed that T19 and T29 inoculation stimulated metabolic responses in maize shoots and roots linked to plant growth and development.

Table of Contents

List of Figures

List of Tables

Abbreviations

ABA	Abscisic acid
ACC	Aminocyclopropanecarboxylic acid
AHL	Acyl-homoserine-lactones
BNF	Biological nitrogen fixation
CCI	Chlorophyll content index
ESI	Electrospray ionisation
GC	Gas chromatography
GR	Green revolution
GSEA	Gene set enrichment analysis
HPLC	High-performance liquid chromatography
IAA	Indole acetic acid
ISR	Induced systemic resistance
LC	Liquid chromatography
MS	Mass Spectrometry
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
PGPR	Plant growth promoting rhizobacteria
PLS	Partial least squares
QS	Quorum sensing
QTOF	Quadrupole time of flight
RF	Radio frequency
SAR	Systemic acquired resistance
UPLC	Ultra-performance liquid chromatography

Chapter 1: Introduction/Literature Review

1.1) Food production and sustainable agriculture

In a 50 year timespan from 1961-2010, the developing world has experienced an extraordinary period of food productivity despite increasing land scarcity (Pingali, 2012). The higher crop production was mostly due to a greater yield per unit area as a result of an amazing technological feat known as the Green Revolution (GR) (Zeng, Zhao, Collatz, Kalnay, Salawitch, West, & Guanter, 2014). The success of the GR can be attributed to: increased investments in crop research at the time, high-yield crop varieties (e.g., high-yield maize and hybrid dwarf rice), use of fertilisers and pesticides, and use of irrigation (Pingali, 2012; Zeng *et al.*, 2014).

The GR provoked a very significant increase in food production, thereby making food more available to the greater population. The improvements the GR brought about to food availability is indisputable. However, it brought along serious environmental problems. These environmental problems have become more evident over the decades. The environmental implications of the GR include the release of greenhouse gases, surface and groundwater contamination, increased soil degradation, increased pest and pathogen resistance and loss of biodiversity (Badgley, Moghtader, Quintero, Zakem, Chappell, Aviles-Vazquez, Samulon, & Perfecto, 2007). In addition to the environmental implications of the GR, other factors such as climate change and intensive agricultural practices all add up to threaten global food security.

It is estimated that the world's population will rise from 7.7 to 9-10 billion by 2050 (Badgley *et al.*, 2007), and this increase in population cannot be supported by the current rate of food production. There are currently two opposing solutions to this crisis. One side advocates for a more intensified GR, while, the other side notes that the practices of the GR have significantly incurred direct and indirect costs to the environment, thereby representing a Faustian bargain (Badgley *et al.*, 2007). Supporters of the latter argument advocate for more sustainable agricultural practices, which will be better in the long term (Badgley *et al.*, 2007).

There is now greater urgency to find sustainable agricultural methods that will maintain high productivity with little to no alterations to the environment (Pérez-Montaño, Alías-Villegas, Bellogín, Del Cerro, Espuny, Jiménez-Guerrero, López-Baena, Ollero, & Cubo, 2014). It is important, now more than ever, to find methods to produce food with little to

no damage caused to the soil, and reduced application of chemical fertilisers and pesticides. A potential solution would be to exploit soil dwelling rhizospheric microbes to improve plant growth with reduced or no application of fertilisers or pesticides. This solution may result in a reduction of surface or groundwater contamination due to reduced fertiliser and pesticide use, as well as an improvement in soil quality.

1.2) The rhizosphere and PGPR

The plant structure can be divided into three main regions, namely the phyllosphere, the endosphere and the rhizosphere. The phyllosphere refers to the parts of the plant aboveground while the endosphere refers to the internal transport system. The rhizosphere refers to the limited region of soil that is directly influenced by plant roots, is bounded by the plant roots and has a reach of only a few millimetres into the soil (Bringhurst, Cardon, & Gage, 2001). This region of soil is densely populated with a variety of microorganisms compared to the surrounding soil as a result of the root exudates, which serve as a nutrient source (Bringhurst *et al.*, 2001). The rhizosphere is home to a high density of bacteria, protists, fungi and nematodes that live off organic acids, sugars, amino acids and polysaccharides, thereby resulting in a greater microbial population in that region (Bringhurst *et al.*, 2001). All three regions have one thing in common; they are areas where plant-microbe interactions take place.

Plants face a number of challenges in the form of pathogenic microorganisms that threaten their overall health and ultimately their survival. However, not all plant-microbe interactions are detrimental as there are also beneficial ones that improve plant nutrition and the ability to overcome abiotic and biotic stresses (Planchamp, Glauser, & Mauch-Mani, 2015). The term ‘rhizobacteria’ was first coined by Kloepper and Schroth in 1978, who described PGPR as specific strains of rhizosphere bacteria that stimulate plant growth. In this pioneer study, these researchers provided evidence of the stimulatory effect of certain rhizosphere bacteria on radish growth, both in the greenhouse and in field trials. It was found that a number of rhizobacteria strains improved root growth and plant yield, hence leading to the term plant growth promoting rhizobacteria (Kloepper & Schroth, 1978).

PGPR are characterised by their inherent distinctive ability to (i) proficiently colonise root surfaces, (ii) survive, compete and multiply with other microbes and (iii) promote plant growth (Kloepper, 2003). However, the concept of PGPR now applies to bacterial strains that fulfil at least two out of the three characteristics mentioned (Bhattacharyya & Jha,

2012). Furthermore, PGPR can be divided into two broad groups based on the way they interact with plant root cells. These broad groups are termed intracellular PGPR (iPGPR) and extracellular PGPR (ePGPR) respectively (Gray & Smith, 2005).

Intracellular PGPR refers to the bacteria that can be found inside plant cells, produce nodules and are localised inside specialised structures (Gray & Smith, 2005). The majority of iPGPR belong to the *Rhizobiaceae* family and fall under the following genera; *Rhizobium, Allorhizobium, Azorhizobium, Bradyrhizobium and Sinorhizobium* (Gray & Smith, 2005). Bacteria that fall under the previously mentioned genera are collectively known as rhizobia and are known to invade plant root systems and form root nodules (Gray & Smith, 2005; Wang & Martinez-Romero, 2000). A great proportion of the endophytic PGPR are Gram-negative and rod shaped while a lesser portion are Gram-positive rods, cocci and are pleomorphic (Bhattacharyya & Jha, 2012).

Extracellular PGPR refers to the bacteria that reside outside plant cells, do not form nodules and produce compounds that have a direct effect on plant growth or resistance towards pathogens (Gray & Smith, 2005). ePGPR can be subdivided into three types based on their location: those that reside on the root surface (the rhizoplane), in the soil near the roots (the rhizosphere), and finally those that reside in the spaces between the cells of the root cortex (Bhattacharyya & Jha, 2012; Gray & Smith, 2005). The genera *Flavobacterium, Micrococcous, Pseudomonas, Bacillus, Agrobacterium, Erwinia, Serratia, Caulobacter, Hyphomicrobium, Chromabacterium, Athrobacter*, *Azotobacter, Azospirillum, Burkholderia* and other free-living nitrogen-fixing bacteria, all fall under the ePGPR group (Gray & Smith, 2005). It should be noted that the interaction between ePGPR and plants is the most common plant-microbe interaction in healthy plants (Picard & Bosco, 2005).

The convenient classification of PGPR into iPGPR and ePGPR is important as it highlights the differences or similarities in the way PGPR induces plant growth. The iPGPR group primarily induces plant growth by nitrogen fixation (Gray & Smith, 2005). On the other hand, ePGPR primarily induces plant growth by stimulating the production of phytohormones, improving plant resistance or improving the mobilisation of soil nutrients (Rodríguez-Díaz, Rodelas-Gonzalés, Pozo-Clemente, Martínez-Toledo, & González-López, 2008).

So far, there are two paradigms that have emerged from the study of PGPR. The first being that many of the best PGPR strains are multifunctional and secondly, that traits are

distributed among many different species and genera of bacteria, many of which are indigenous to the soil microbial community (Martínez-Viveros, Jorquera, Crowley, Gajardo, & Mora, 2010). It is evident from the various microbial genera previously mentioned, that PGPR strains are broadly distributed between many taxa which include but are not limited to *Actinobacteria, Firmicutes, Cyanobacteria, Proteobacteria, Betaproteobacteria, Alphaproteobacteria* and *Gammaproteobacteria* (Martínez-Viveros *et al.*, 2010; Rodríguez-Díaz *et al.*, 2008). Single strains often vary in performance considerably and there is no distinct relationship between PGPR function and taxonomy (Martínez-Viveros *et al.*, 2010).

1.3) PGPR in agriculture

The most intensively studied application of PGPR is in agriculture. Numerous studies have been conducted on the application of PGPR for the growth of major crops such as rice, wheat, sugarcane, barley, soybeans, maize, tomatoes, peppers, etc (Lucy, Reed, & Glick, 2004). The focus of this project will be on maize as it is one of the most important cereal crops in the world, and a staple crop in South Africa and many African countries. The most common PGPR strains used for these studies are *Azospirillum, Pseudomonas, Herbaspirillum, Bradyrhizobium, Arthrobacter* and *Azotobacter* (Canellas, Balmori, Médici, Aguiar, Campostrini, Rosa, Façanha, & Olivares, 2013; Glick, 2012; Hungria, Campo, Souza, & Pedrosa, 2010; Pérez-Montaño *et al.*, 2014; Planchamp *et al.*, 2015).

In most cases, farmers' biggest expenses are those required for the purchase of agrochemicals such as fertilisers and pesticides. The global increase in energy prices has led to the increase in price of energy products or products which are produced with high energy input, such as fertilisers (Fowowe, 2016). The high fertiliser cost has in turn resulted in an increase in price of agricultural commodities (Fowowe, 2016). Furthermore, farmers have to deal with soil degradation or declining soil health, which affects crop production. Soil degradation processes are as follows: accelerated erosion, depleted soil organic carbon pool, loss in biodiversity, loss of fertility, acidification and salinization (Lal, 2015). All of these processes occur due to extreme farming practices, chemical contamination and overuse of agrochemicals over several years (Lal, 2015). To provide crops to a growing population, farmers have to face high fertiliser costs, poor soil quality and environmental decline; all which pose as grave threats to food security in the 21st century.

In the face of the challenges mentioned in the previous paragraph, it is crucial that more sustainable farming practices are introduced to reverse soil degradation and reduce the input cost of agrochemicals to farmers. Furthermore, it is important that major crops produced are equipped with disease, salt and drought tolerance, and higher nutritional quality. As the movement towards sustainable agriculture intensifies, the use of PGPR to enhance plant growth, yield and health, becomes more promising; with the hope of eliminating the need or reducing the use of agrochemicals. However, for this to be accomplished, PGPR have to be intensely applied to agricultural practices.

The most frequently studied and commercially available PGPR are listed in Table 1. As seen in Table 1, a number of bacterial species have plant growth promoting abilities. However, the use of PGPR in agriculture represents a small fraction of worldwide agricultural practice (Glick, 2012). The poor use of PGPR in agriculture can mainly be attributed to the inconsistencies in results in laboratory, greenhouse and field trials (Ahemad & Kibret, 2014). The soil is an unpredictable environment and often results obtained in the greenhouse or laboratories are not observed in the field. Moreover, properties of the inoculated PGPR can be inconsistent. The successful use of PGPR is dependent on a number of factors such as survivability in the soil, crop compatibility, interaction with soil indigenous microflora and climatic conditions (Martínez-Viveros *et al.*, 2010). Additionally, as not all rhizobacteria possess the same mechanisms of action, the same PGPR can either act positively or not at all depending on the crop. Moreover, not all mechanisms of action are fully known. It is therefore, imperative that a full understanding of the mechanisms of action is achieved so that the application of PGPR in agriculture can be fully exploited.

Furthermore, PGPR are capable of affecting the host plants on a molecular level. However, the mode of action or the molecular effects are not always the same between strains or different crops. To further improve the understanding of how different PGPR works, it then becomes important to understand what happens on a systems biology level.

Table 1: Commercially available and most frequently studied PGPR according to phylum, class and specie (Glick, 2012; Pérez-Montaño *et al.*, 2014).

Phylum	Class	Specie
Proteobacteria	α-proteobacteria	*Agrobacterium radiobacter, Azospirillum* sp., *Azospirillum brasillense, Azospirillum amazonense, Azospirillum lipoferum, Bradyrhizobium japonicum, Brevundimonas* sp., *Sphingomonas* sp., *Rhizobium tropici*
	β-proteobacteria	*Paraburkholderia* sp., *Burkholderia vietnamiensis, Delftia acidovorans, Delftia tsuruhatensis, Herbaspirillum* sp., *Herbaspirillum seropedicae*
	γ-proteobacteria	*Azotobacter* sp., *Azotobacter chroococcum, Pseudomonas* sp., *Pseudomonas fluorescens, Pseudomonas solanacearum, Pseudomonas syringae, Serratia* sp., *Serratia entomophilia, Xanthomonas* sp.
Firmicutes	Bacilli	*Bacillus sp., Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus circulans, Bacillus megaterium, Bacillus subtilis, Paenibacillus* sp.
Actinobacteria	Actinobacteria	*Arthrobacter* sp.

1.4) Mechanisms of action of PGPR

Numerous studies have been conducted in the past on the use of microbial strains to improve growth and yield in different crops. Presently, there is a gradual shift from traditional chemicals to the use of microorganisms to improve plant growth and protection from biotic and abiotic stresses. Modern agriculture depends heavily on the use of chemicals to achieve high yields, which has been beneficial for the worlds growing population but detrimental to the environment. Copious amounts of chemical fertilisers are used to replenish soil nitrogen and phosphorus levels. However, the efficiency of chemical fertilisers is estimated to be approximately 50% or lower for nitrogen fertilisers, less than 10% for phosphorus, about 40% for potassium and much lower for manure (Turan, Ekinci, Yildirim, Güneş, Karagöz, Kotan, & Dursun, 2014). Additionally, fertiliser sources around the world are depleting and the chemical runoff is affecting groundwater quality, thereby making it unsustainable in the long term (Walker, Couillerot, Von Felten, Bellvert, Jansa, Maurhofer, Bally, Moënne-Loccoz, & Comte, 2012). In the article by Walker *et al.,* 2012, two strategies were mentioned to reduce the use and counter the negative effects of chemical fertilisers. The first strategy would be to reduce chemical input through the cultivation of more nutrient effective plant varieties thus maintaining

productivity (Walker *et al.*, 2012). The second strategy involves the use of beneficial plant microbes such as PGPR, arbuscular mycorrhizal fungi and symbiotic nitrogen-fixing bacteria (Pérez-Montaño *et al.*, 2014; Walker *et al.*, 2012).

PGPR have been shown to improve nutrient availability to plants, and as such they can be used to improve fertiliser efficiency. Improving fertiliser efficiency will in turn lead to reduced application of fertilisers, which will not only be beneficial for the environment but also to farmers. In a paper by Adesemoye *et al.,* 2009, it was hypothesised that PGPR and/or arbuscular mycorrhizal fungi combined with fertilisers would improve fertiliser efficiency and reduce the amount used (Adesemoye, Torbert, & Kloepper, 2009). The results from the investigation supported the hypothesis because it was found that reduced fertiliser application combined with PGPR inoculation had the same impact on tomato plant height, shoot and root dry weight, yield and nutrient uptake as the full rate of fertiliser application without inoculants (Adesemoye *et al.*, 2009). It was also found that N_2 use efficiency in response to inoculation was enhanced (Adesemoye *et al.*, 2009). The investigation demonstrated that PGPR shows promise of improving plant growth by improving nutrient use efficiency, while reducing the recommended amount of fertiliser applied.

For successful plant growth promotion, PGPR alter the rhizosphere niche through either direct or indirect mechanisms (Bhattacharyya & Jha, 2012). The difference between the two mechanisms is not always clear. As a rule of thumb, indirect mechanisms take place outside the plant while direct mechanisms occur inside the plant and directly alter the plant's metabolism (Solano, Maicas, & Mañero, 2008). Direct mechanisms involve adjusting plant hormone levels or facilitating resource acquisition by the plants such as nitrogen, phosphorus and minerals (Fig. 1) (Ahemad & Kibret, 2014). On the other hand, indirect mechanisms involve bio-control activities in which the inhibitory activity of pathogens or pests on plant growth is decreased through chemicals released by the PGPR (Fig. 1) (Ahemad & Kibret, 2014). PGPR possess genetically determined traits that can be used to classify the mode of action under direct or indirect mechanisms (Table 2). It is common for one strain to possess multiple traits, thereby having different mechanisms of action. It should also be noted that the positive effects of PGPR on plant growth are not just as a result of a single bacterial strain but involves molecular dialogue between soil and plant microorganisms and quorum sensing mechanisms (Solano *et al.*, 2008).

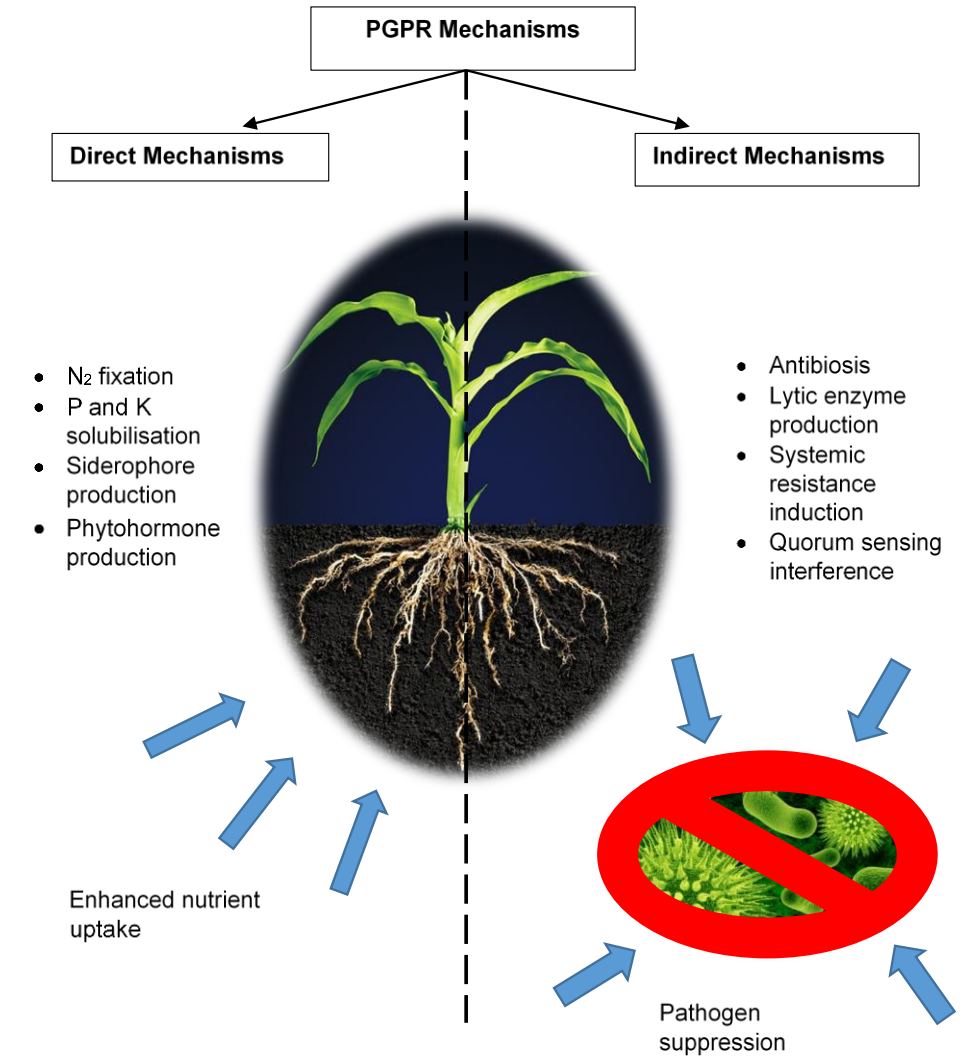


Figure 1: Mechanisms of plant growth promotion by PGPR. The mechanisms are classified as either direct or indirect. Direct mechanisms normally involve biofertilisation activities to enhance resource acquisition for the plants such as N_2 fixation, P and K solubilisation, siderophore and phytohormone production. Indirect mechanisms involve biopesticide or biocontrol activities to suppress pathogens and improve plant resistance such as antibiosis, lytic enzyme production, systemic resistance induction and quorum sensing interference (Ahemad & Kibret, 2014).

Table 2: Summary of PGPR traits and the corresponding mechanism of action.

Mechanism	Trait	Reference
Biofertilisation	N_2 fixation	(Kuan, Othman, Rahim, & Shamsuddin, 2016)
Biofertilisation	Phosphorus solubilisation	(Canbolat, Bilen, Çakmakçı, Şahin, & Aydın, 2006)
Biofertilisation	Potassium solubilisation	(Parmar & Sindhu, 2013)
Biofertilisation/Biocontrol	Siderophore production	(Sharma, Johri, Sharma, & Glick, 2003)
Phytostimulation	Phytohormone production: *IAA, ABA, GB, cytokinins, ethylene	(Kumar, Agarwal, Dheeman, & Maheshwari, 2015)
Phytostimulation	ACC deaminase activity	(Iqbal, Khalid, Shahzad, Ahmad, Soleman, & Akhtar, 2012)
Biocontrol	Antibiotics production	(Fernando, Nakkeeran, & Zhang, 2005)
Biocontrol	Salicylic acid production	(Zhang, Moyne, Reddy, & Kloepper, 2002)
Biocontrol	Jasmonic acid production	(Ryu, Murphy, Mysore, & Kloepper, 2004)
Biocontrol	Acylase and lactonase activity	(Ahmad, Aqil, Ahmad, Zahin, & Musarrat, 2008b)
Biocontrol	Hydrolytic enzyme production	(Raza, Yang, Wu, Wang, Xu, & Shen, 2009)

***Indole acetic acid (IAA), abscisic acid (ABA), gibberellic acid (GB)**

Based on functional activities, PGPR can be classified into four distinct categories: biofertilisers, phytostimulators, biopesticides and rhizoremediators (Somers, Vanderleyden, & Srinivasan, 2004). It is important to understand the mechanisms of action of different PGPR strains in order to improve the processes within the rhizosphere, the effects in the plant and to select the best strain for commercial purposes.

1.4.1) Biofertilisers

Biofertilisers can be defined as substances prepared from living microorganisms, that when applied to plants are capable of colonising the rhizosphere or the intracellular regions of the plant, leading to plant growth by improving the nutrient status of the plant (Vessey, 2003). As biofertilisers, PGPR increase the availability of nutrients to plants, thereby changing the nutritional status of the host plant. Therefore, biofertilsation using PGPR results in the promotion of the root surface area and enhancement of beneficial interaction between the soil microbes and host plants (Pérez-Montaño *et al.*, 2014).

Biofertilisers increase the availability of nutrients to plants, and this is achieved through activities such as biological N_2 fixation (BNF), phosphate solubilisation, potassium solubilisation and siderophore production, to name a few (Gupta, Parihar, Ahirwar, Snehi, & Singh, 2015). Biofertilisation currently accounts for approximately 65% of the nitrogen supply to crops globally (Bloemberg & Lugtenberg, 2001). Nitrogen makes up an essential part of plant nutrition, and is assimilated from soil in the form of nitrite, nitrate or ammonia; which is usually supplied chemically (Pérez-Montaño *et al.*, 2014). There is a low abundance of the different forms of nitrogen in the soil, and the chemical nitrogen fertiliser is normally lost due to rainfall and mineral leaching (Pérez-Montaño *et al.*, 2014). BNF is the process whereby atmospheric N_2 is converted to plant usable ammonia by nitrogen-fixing microorganisms, through the nitrogenase enzyme system (Ahemad & Kibret, 2014).

Bacteria from the genera under the iPGPR group are known to be the most efficient nitrogen fixers (Bloemberg & Lugtenberg, 2001). These bacteria symbiotically associate with the host plant and form root nodules where atmospheric N_2 is fixed (Pérez-Montaño *et al.*, 2014). BNF involving iPGPR is limited to leguminous plants such as peas, soybeans, peanuts, etc. (Pérez-Montaño *et al.*, 2014). A major practical challenge is therefore introduced; which is to widen the host range of symbiosis to major non-leguminous crops. On the other hand, a number of free-living rhizobacteria from the genera of the ePGPR group (*Bacillus, Athrobacter*, *Azotobacter, Azospirillum, Burkholderia*) are known to fix atmospheric N_2 (Bloemberg & Lugtenberg, 2001). The free-living N_2 fixing bacteria are capable of fertilising important non-leguminous crops such as maize, wheat, sorghum etc., without the need for symbiosis (Pérez-Montaño *et al.*, 2014). Inoculation of plants using the non-symbiotic N_2 fixing bacteria is known to increase flowering and grain production, and the plants' dry weight (Pérez-Montaño *et al.*, 2014). In a study by Rudolph *et al.,* (2015); Breedt *et al.,* (2017), maize growth trials were conducted with PGPR that showed free N_2 fixing ability. It was observed that some strains

caused a significant increase in plant dry weight and grain production more than the others (Breedt, Labuschagne, & Coutinho, 2017; Rudolph, Labuschagne, & Aveling, 2015). Most of the strains used in these studies tested positive for N_2 fixing ability (Breedt *et al.*, 2017). The results could be attributed to the N_2 fixing ability of those strains, as well as other traits such as phosphorus solubilisation, for which the strains also tested positive.

Phosphorus is another nutrient that is highly limited to plants after nitrogen. Despite the large phosphorus reserves in the soil, it is not in a form that is readily available to plants. The phosphorus in the soil is mostly in the non-soluble form which plants cannot absorb, thereby limiting plant growth (Pérez-Montaño *et al.*, 2014). A contributing factor to the low P levels in the soil is the high reactivity of soluble P with calcium, iron or aluminium, which leads to P precipitation (Gyaneshwar, Kumar, Parekh, & Poole, 2002). The non-soluble phosphorus in the soil can be mineralised by microorganisms into a soluble form that is readily available to plants (Solano *et al.*, 2008). PGPR from different genera such as *Bacillus, Pseudomonas, Burkholderia, Chryseobacterium, Achromobacter, Rhizobium, Flavobacterium, Agrobacterium, Erwinia, Micrococcus* and *Aerobacter* are capable of solubilising phosphate (Solano *et al.*, 2008).

There are two main mechanisms by which bacteria solubilise phosphorus. The first mechanism involves the releasing of phosphatases that can cleave off phosphate groups bound to organic matter (Solano *et al.*, 2008). The second mechanism involves the release of complexing or mineral dissolving compounds, such as organic acids that mobilise phosphorus through ionic interactions (Gupta *et al.*, 2015; Solano *et al.*, 2008). Bacteria that solubilise phosphorus are able to do so more efficiently in basic soil. Additionally, conditions such as soil composition (phosphorous deficit) and the presence of insoluble phosphorus have an effect on the results of phosphate solubilising PGPR (Solano *et al.*, 2008). As a result, if the aforementioned conditions are absent, then phosphate solubilising PGPR will behave erratically. Often, phosphate solubilising PGPR greatly improve plant growth; however, sometimes they are completely inefficient (Solano *et al.*, 2008). Hence, a knowledge of the working mechanisms of phosphate solubilising PGPR in plants and in the rhizosphere, would prove beneficial for sustainable agriculture use.

Potassium is another essential macronutrient for plant growth after phosphorus. Potassium plays essential roles in plant growth, metabolism and development; a deficiency leads to poor root development, slow growth, small seeds and low yield (Gupta *et al.*, 2015; Parmar & Sindhu, 2013). Usually, the concentration of soluble potassium in

the soil is very low, 90-98% of soil potassium is in the form of insoluble rocks and silicate unavailable for plant uptake (Gupta *et al.*, 2015). PGPR that are capable of solubilising potassium present as a viable solution to maintaining the potassium levels in soil, which can sustain crop production.

Bacteria such as *Burkholderia, Ferrooxidans, Bacillus mucilaginosus, B. circulans, B. edaphicus, Paenibacillus* and *Pseudomonas* are known to have potassium solubilising ability (Parmar & Sindhu, 2013). Potassium solubilising PGPR improve the availability of potassium to plants by producing and secreting organic acids that dissolve potassium, silicon and aluminium from insoluble potassium bearing minerals (Parmar & Sindhu, 2013). The use of potassium solubilising PGPR as a biofertiliser can therefore reduce the use of agrochemicals, thereby supporting eco-friendly crop production.

Iron is the fourth most abundant mineral on earth; however, despite its abundance, it is not readily assimilated by bacteria or plants. Iron is poorly assimilated because it is abundant in its oxidised state of ferric iron (Fe^{3+}), which is sparingly soluble and reacts to form insoluble oxides and hydroxides (Solano *et al.*, 2008). Plants that are deficient in iron experience alterations in their metabolism, as iron acts as a cofactor for several enzymes in respiration, photosynthesis and nitrogen fixation reactions (Solano *et al.*, 2008).

Some microorganisms have evolved specialised mechanisms to facilitate iron uptake. The specialised mechanism involves the production of low-molecular weight iron chelating compounds known as siderophores (Gupta *et al.*, 2015). The siderophores improve iron availability to plants by converting iron from an insoluble to a soluble form. Siderophore-producing PGPR fall under the genera of *Serratia, Pseudomonas, Bacillus, Burkholderia, Rhizobium, Aeromonas, Azotobacter, Azadirachta* and *Streptomyces spp* (Gupta *et al.*, 2015). Siderophore producing PGPR have been implicated in both direct and indirect enhancement of plant growth. This specific group of PGPR is capable of not only improving the plants' iron nutrition but also inhibiting the growth of other microorganisms and pathogens by releasing antibiotics and by limiting the iron available to the pathogen, respectively (Solano *et al.*, 2008).

1.4.2) Phytostimulators

One of the most important mechanisms by which PGPR promote plant growth is through the production of phytohormones. Phytohormones can be defined as organic compounds produced by both plants and bacteria, which are capable of influencing the biochemical,

physiological and morphological processes in plants (Fuentes-Ramirez & Caballero-Mellado, 2005). Phytohormones work at very low concentrations, and their synthesis is highly regulated (Fuentes-Ramirez & Caballero-Mellado, 2005). These compounds function as messengers to coordinate and regulate cellular activities in plants, including abiotic and biotic stress responses (Tsukanova, Meyer, & Bibikova, 2017).

Various PGPR are capable of altering root architecture and promote plant development by producing phytohormones such as auxins, gibberellins, cytokinins, ethylene and abscisic acid (Gupta *et al.*, 2015). PGPR capable of promoting plant growth by producing phytohormones are known as phytostimulators. The aforementioned hormones affect cell proliferation in the root architecture by overproduction of lateral roots and root hairs, which in turn increases the plants' water and nutrient uptake. Therefore, the PGPR stimulatory effect in this instance is as a result of manipulation of the balanced and complex network of plant hormones that are directly involved in growth or stimulation of root formation (Pérez-Montaño *et al.*, 2014).

IAA is the most common natural auxin found in plants (Gupta *et al.*, 2015). Auxin is a very important plant growth regulator as it is responsible for numerous processes in plants such as cell cycle progression (Martínez-De La Cruz, García-Ramírez, Vázquez-Ramos, De La Cruz, & López-Bucio, 2015), release of bud dormancy, gravitropism and phototropism of roots and shoots (Friml, Wiśniewska, Benková, Mendgen, & Palme, 2002), modulation of plant associations with pathogenic (Kazan & Manners, 2009) and symbiotic organisms, and coordination of plant responses associated with the establishment and maintenance of plant-microbe interactions, to name a few. The research on auxins in plant growth is extensive; however, new studies on its role in plant development are constantly emerging. The majority of physiological processes in plants are either directly or indirectly linked to auxins (Ahmed & Hasnain, 2014).

Over 80% of rhizosphere bacteria are capable of producing and releasing IAA as a secondary metabolite (Ahemad & Kibret, 2014). Rhizospheric bacteria from the genera *Bacillus, Azotobacter, Burkholderia, Azospirillum, Herbaspirillum, Acetobacter diazotrophicus, Paenibacillus, Bradyrhizobium* and *Rhizobium,* are known for their auxin producing abilities in plant growth promotion (Ahmad, Ahmad, Aqil, Khan, & Hayat, 2008a). The IAA released from the PGPR is proposed to work in conjunction with the plants endogenous IAA levels to stimulate cell proliferation and enhance the plants uptake of minerals and nutrients from the soil (Ahemad & Kibret, 2014). The principal target of auxins are the roots. Auxins released from PGPR affect root systems by increasing the

size and biomass, branching number and surface area, hence improving the plants' access to soil nutrients (Ahemad & Kibret, 2014; Solano *et al.*, 2008).The improved access to soil nutrients in turn leads to enhanced root exudation, which stimulates bacterial colonisation and amplifies the inoculation effect (Spaepen, Vanderleyden, & Remans, 2007). The effects of the bacteria produced auxin on plant growth promotion can vary considerably, depending on the concentration that reaches the root system. An excessive amount of auxin that reaches the root system can inhibit plant growth (Ahmad *et al.*, 2008a).

Gibberellins are the largest group of phytohormones responsible for processes such as stem elongation, seed germination, reproductive organ development, leaf expansion and root growth (Ayano, Kani, Kojima, Sakakibara, Kitaoka, Kuroha, Angeles-Shim, Kitano, Nagai, & Ashikari, 2014; Debeaujon & Koornneef, 2000; Gou, Strauss, Tsai, Fang, Chen, Jiang, & Busov, 2010; Plackett & Wilson, 2016; Tsavkelova, Klimova, Cherdyntseva, & Netrusov, 2006). PGPR are capable of influencing the concentration of endogenous gibberellins in plants similar to other phytohormones. PGPR strains capable of synthesising gibberellins include *Azotobacter, Azospirillum, Acetobacter, diazotrophicus, Herbaspirillum, Bacillus* and *Rhizobium* (Ahmad *et al.*, 2008a). After inoculating red pepper plug seedlings with three different gibberellin producing *Bacillus* species, a distinct increase in growth was observed (Joo, Kim, Lee, Song, & Rhee, 2004). In another experiment, the amount of endogenous gibberellins in red pepper shoots increased when inoculated with the gibberellin producing bacteria *Bacillus cereus* (Joo, Kim, Kim, Rhee, Kim, & Lee, 2005). Another study also demonstrated the ability of gibberellin producing bacteria *Leifsonia soli sp SE134* to stimulate shoot growth in gibberellin deficient mutant rice plants (Kang, Khan, You, Kim, Kamran, & Lee, 2014). All these findings indicate that these strains are capable of enhancing or compensating for gibberellin that is absent within the plant.

Cytokinins are a class of phytohormones represented as N^6-substituted adenine derivatives. The cytokinins are involved in many aspects of the plant’s life such as cell cycle progression, root system architecture, and chlorophyll and chloroplast synthesis (Aloni, Aloni, Langhans, & Ullrich, 2006; Cortleven & Schmülling, 2015; Riou-Khamlichi, Huntley, Jacqmard, & Murray, 1999). Additionally, cytokinins are involved in plants resistance to abiotic and biotic stresses (Großkinsky, Naseem, Abdelmohsen, Plickert, Engelke, Griebel, Zeier, Novák, Strnad, & Pfeifhofer, 2011). For instance, inoculation of *Platycladus orientalis* plants with cytokinin producing *Bacillus subtilis* improved the

drought resistance of the plants (Liu, Xing, Ma, Du, & Ma, 2013). Several PGPR capable of synthesising cytokinins include among others *Azotobacter, Azospirillum, Paenibacillus* and *Rhizobium* to mention a few (Ahmad *et al.*, 2008a). PGPR strains capable of cytokinin synthesis are also able to enhance shoot growth and fruit formation in host plants (Azcón & Barea, 1975). Cytokinin producing PGPR strains are clearly capable of affecting the plants cytokinin homeostasis. Thus, an understanding of the mechanisms by which these cytokinin producing bacteria work will give a better understanding of PGPR mediated plant growth stimulation and increased response to biotic and abiotic stresses.

Abscisic acid is an isoprenoid phytohormone that is produced in response to abiotic stresses such as drought, pollution, cold and salt stress (Sah, Reddy, & Li, 2016). Additionally, abscisic acid also activates the genes responsible for stress tolerance and as a result it is also known as a stress hormone (Sah *et al.*, 2016). The hormone also plays a role in plant growth and development under non-stressful conditions and is therefore responsible for a number of physiological processes. Some of the processes abscisic acid is involved in include seed dormancy and germination, plant senescence, leaves and fruit abscission, stomata regulation and modulation of root architecture (Harris, 2015; Xiong & Zhu, 2003).

Azospirillum, Bacillus and *Pseudomonas* are known PGPR capable of synthesising abscisic acid *(Tsukanova et al., 2017)*. These strains can affect plant abscisic acid content through different methods. Some of these strains are capable of increasing the internal abscisic acid content in plants. It was found that the inoculation of *in vitro* grown grapevines with *Bacillus licheniformis* Rt4M10 and *Pseudomonas fluorescens* Rt6M10 increased the abscisic acid content of the plants 76-fold and 40-fold respectively (Salomon, Bottini, De Souza Filho, Cohen, Moreno, Gil, & Piccoli, 2014). Furthermore, the plants showed diminished water loss with increasing abscisic acid content (Salomon *et al.*, 2014). PGPR, therefore, are capable of affecting plant abscisic acid content, in turn affecting plant growth and resistance to abiotic stress.

Ethylene is another phytohormone implicated in plant growth and stress response (Solano *et al.*, 2008). Ethylene plays a vital role in mediating stress response and adaptive processes, hence it is essential for plant survival (Solano *et al.*, 2008). The hormone is also involved in other physiological processes such as fruit ripening, seed germination, root growth, leaf senescence and response to biotic and abiotic stresses (Solano *et al.*, 2008; Tsukanova *et al.*, 2017). Ethylene is known as a multifunctional phytohormone as

it is capable of stimulating and inhibiting plant growth, depending on the plant species and its concentration (Iqbal, Khan, Ferrante, Trivellini, Francini, & Khan, 2017).

PGPR can alter the homeostasis of ethylene in plants by either increasing or decreasing its content in plant tissues. PGPR accomplishes this by affecting the genes encoding the enzymes aminocyclopropane-carboxylic acid (ACC) synthase and ACC-oxidase, which are responsible for ethylene synthesis (Tsukanova *et al.*, 2017). A decrease in ethylene levels is related to an increase in growth of root systems (Solano *et al.*, 2008), and as mentioned previously, this is highly beneficial to plant growth. A lot of emphasis has therefore been placed on PGPR research to reduce ethylene levels in plants, which could improve certain physiological processes.

There are many PGPR strains that can express the ACC deaminase enzyme, which can degrade plant ACC, the precursor of ethylene (Solano *et al.*, 2008). PGPR strains that exhibit ACC deaminase activity span a wide range of genera such as: *Bacillus, Burkholderia, Enterobacter, Acinetobacter, Achromobacter, Agrobacterium, Alcaligenes, Ralstonia, Serratia, Rhizobium, Azospirillum and Pseudomonas,* etc. (Gupta *et al.*, 2015). In a field trial, it was found that inoculation with ACC deaminase producing *Pseudomonas sp*. and *Rhizobium leguminosarum*, resulted in improved nodule dry weight, fresh biomass, grain yield and nitrogen content in lentil grains (Iqbal *et al.*, 2012). Another beneficial role of PGPR with ACC deaminase activity is the ability to boost plant growth under stressful environmental conditions (salinity, drought, pathogenicity, contaminants, etc.) (Bhattacharyya & Jha, 2012). In another instance, inoculation of canola plants with the wild-type *Pseudomonas putida UW4* improved plant growth and salt tolerance compared to the ACC deaminase absent mutant strain (Cheng, Park, & Glick, 2007).

1.4.3) Rhizoremediators

Contamination of soil and groundwater is becoming a serious problem worldwide and is one of the major reasons for soil degradation. The general methods to clean polluted areas tend to be very costly and cause additional pollution. Thus, there is a search for alternative methods to restore polluted sites, which are less expensive, safe and environmentally friendly. One such alternative method is rhizoremediation, which happens to be a combination of bioaugmentation and phytoremediation (Kuiper, Lagendijk, Bloemberg, & Lugtenberg, 2004). Bioaugmentation is a method to improve the breakdown or transformation of xenobiotics in soil using the indigenous microbial population (Kuiper *et al.*, 2004). A number of microbes have the genetic capability to

degrade certain xenobiotics such as long chain alkanes, aromatics, chlorinated aliphatics etc. (Kuiper *et al.*, 2004). Phytoremediation on the other hand, involves the use of plants to sequester, extract or detoxify pollutants (Kuiper *et al.*, 2004). Rhizoremediation is a combination of both processes as pollutants are degraded by rhizospheric bacteria (bioaugmentation) of the plants that emerge as natural vegetation on a polluted site (phytoremediation) (Kuiper *et al.*, 2004). The use of PGPR in rhizoremediation increases the microbial population and metabolic activity in the rhizosphere (Kuiper *et al.*, 2004). Additionally, the PGPR improves the chemical and physical properties of the soil, decreases the bioavailability of toxic compounds and in turn improves the agroclimatic conditions of the soil thereby supporting plant growth (Bhattacharyya & Jha, 2012).

1.4.4) Biopesticides/Biocontrol

As mentioned previously, one of the implications of the GR is the increase in pathogen resistance due to the heavy use of agrochemicals over the past decades. Additionally, other implications are pest resurgence, the outbreak of secondary pests and pesticide residues in water, air, soil and produce (Kumar & Singh, 2015). An alternative, cost effective and environmentally friendly strategy is the use of PGPR as biocontrol agents against phytopathogens. These PGPR produce various metabolites against pathogens; are capable of inducing systemic resistance in plants against pests and diseases and also improve plant growth. As a biopesticide, PGPR utilises different mechanisms to protect plants such as antagonism, induction of systemic response and interference with quorum sensing (Gupta *et al.*, 2015; Pérez-Montaño *et al.*, 2014).

Antagonism involves activities that inhibits pathogen growth such as production of antibiotics, toxins, biosurfactants and lytic enzymes (Gupta *et al.*, 2015; Pérez-Montaño *et al.*, 2014). Other activities also includes competition for nutrients and colonisation sites (Pérez-Montaño *et al.*, 2014). Bacteria from the genera *Pseudomonas, Bacillus, Serratia, Streptomyces* and *Stenotrophomonas,* have proven microbial effects on plant health (Pérez-Montaño *et al.*, 2014). Of all the antagonistic activities of PGPR, antibiosis (production of antibiotics) is one of the most powerful mechanisms. A range of antibiotics produced by PGPR have been reported, including compounds such as bacillomycin, zwittermycin A, plipastatins A and B, kanosamine, produced by *Bacillus* (Fernando *et al.*, 2005; Pérez-Montaño *et al.*, 2014). Other compounds such as 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin, oomycin A, phenazine, amphisin, pyoluteorin, tensin, cyclic lipopeptides and tropolone, were found to be produced by *Pseudomonads* (Gupta et al., 2015). These antibiotics were found to display antibacterial, antiviral, antihelminthic,

phytotoxic, cytotoxic, antioxidant, insect and mammalian antifeedant plant growth promoting and even antitumour properties (Fernando *et al.*, 2005). Certain PGPR also have the ability to produce enzymes such as phosphatases, lipases, chitinases, proteases, dehydrogenases, β-glucanases etc. (Gupta *et al.*, 2015). Collectively these enzymes have lytic capabilities and are able to suppress pathogens by cell wall degradation (Gupta *et al.*, 2015; Pérez-Montaño *et al.*, 2014).

Plants exhibit two types of systemic resistance namely systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Tsukanova *et al.*, 2017). Other than antagonism, PGPR can reduce pathogen activity by activating the plants own defence systems. The PGPR triggers a systemic response in the plant, which involves metabolic changes that are not always apparent (Solano *et al.*, 2008). Both ISR and SAR are mediated by distinct pathways; ISR is associated with the jasmonate/ethylene pathway while SAR is associated with the salicylic acid/ethylene pathway (Tsukanova *et al.*, 2017). SAR is the activated response to attack from biotrophic pathogens, and is characterised by a hypersensitive response at the attack site; accumulation of pathogenesis related proteins and defence compounds in other parts of the plant and cell wall remodelling (Tsukanova *et al.*, 2017). Contrarily, ISR is activated in response to necrotrophic pathogens, and is characterised by accumulation of specific proteins, increased phytoalexin synthesis, and increased cell wall callose and phenolic content (Tsukanova *et al.*, 2017; Van Loon, 2007). Once induced, resistance is conferred to the plant against a wide range of biotic pathogens and will last over a prolonged period (Van Loon, 2007).

Both ISR and SAR are induced by molecules known as elicitors that are either present or synthesised by PGPR. Elicitors can be defined as chemicals or bio-factors that can induce a physiological, morphological response and phytoalexin accumulation in plants (Bhattacharyya & Jha, 2012). Biotic elicitors from PGPR can be classified as proteins, polysaccharides, lipopolysaccharides, volatile compounds, antibiotics, siderophores and N-acyl-homoserine-lactones (AHLs) (Pérez-Montaño *et al.*, 2014; Solano *et al.*, 2008). A working knowledge of the elicitors produced by PGPR is important for practical use in agriculture and industry; some of the defence compounds produced in response by the plant may have pharmacological activity. In essence, PGPR is capable of inducing both ISR and SAR, sometimes even simultaneously (Niu, Liu, Jiang, Wang, Wang, Jin, & Guo, 2011; Tsukanova *et al.*, 2017). Therefore, the use of PGPR or PGPR mixes to trigger ISR and SAR will lead to a significant improvement in plant defence systems.

Finally, PGPR are capable of interfering with the quorum sensing (QS) systems of bacterial pathogens. QS is a form of communication in bacterial communities to regulate gene expression in response to population density and environmental cues (Ahmad *et al.*, 2008b). This type of communication is dependent on small, diffusible signal molecules known as autoinducers; the most widely known autoinducer being AHL (Ahmad *et al.*, 2008b; Pérez-Montaño *et al.*, 2014). AHLs are the most common autoinducers for genes necessary for the successful establishment of pathogenic microbes in plants (Ahmad *et al.*, 2008b). As such, several bacteria produce enzymes such as acylase and lactonase that degrade AHL molecules. As a biocontrol activity, specific *Bacillus* species produce lactonase (Dong, Gusti, Zhang, Xu, & Zhang, 2002). It was found that the QS controlled virulence of *Erwinia carotovora* was attenuated by lactonase from *Bacillus* (Dong *et al.*, 2002). Therefore, PGPR that have the ability to interfere with QS of plant microbial pathogens, have a great potential for use in biocontrol.

1.5) Impact of PGPR inoculation on plant metabolomics

1.5.1) Metabolomics

Metabolomics can be defined as the comprehensive qualitative and quantitative study of the metabolites present in an organism (Hall, 2006). Plants have an arsenal of very chemically diverse, small molecular weight compounds that have roles in plant growth, development and response to the environment (Hong, Yang, Zhang, & Shi, 2016). These metabolites form the chemical basis of crop yield, quality, performance in the field and nutrition (Hong *et al.*, 2016; Memelink, 2005). Metabolites have been classified into two main groups namely, primary and secondary metabolites. Primary metabolites are essential for the growth and development of the plant, while secondary metabolites are crucial for plant's survival under stressful conditions (Hong *et al.*, 2016). Furthermore, primary metabolites are highly conserved in structure and abundance, whereas secondary metabolites vary considerably among different plants (Scossa, Brotman, E Lima, Willmitzer, Nikoloski, Tohge, & Fernie, 2016).

The highly diverse nature of metabolites is one of the greatest limitations in metabolomics. Currently, there is no single platform to give a comprehensive overview of an organism's metabolic profile. As such there are numerous techniques to aid in identification and quantification, which are often at times combined to give the best 'metabolic picture'. The most common platforms employed in metabolomic studies are mass spectrometry (MS) and nuclear magnetic resonance (NMR).

NMR is a spectroscopic analytical technique that utilises the innate spin properties of the nucleus of atoms (Moco, Vervoort, Bino, De Vos, & Bino, 2007). In NMR, strong magnetic fields and radio frequency (RF) pulse are applied to the nuclei of specific atoms under observation (e.g. ^{1}H, ^{13}C, ^{15}N or ^{32}P) (Moco *et al.*, 2007; Sumner, Mendes, & Dixon, 2003). The RF energy promotes the nuclei from a low energy spin state to a high one, and the subsequent emission of radiation after relaxation is detected as a signal peak (Moco *et al.*, 2007). NMR is one of the oldest, most popular and reproducible analytical techniques for metabolomics. A diverse range of secondary metabolites and primary metabolites can be detected simultaneously, and sample preparation is relatively easy (Kim, Choi, & Verpoorte, 2010). Furthermore, calibration curves are unnecessary as signals for each compound are directly proportional to their molar concentration (Kim *et al.*, 2010). Therefore, NMR is able to reflect the real molar concentrations of metabolites in a sample. Additionally, NMR is the only stand-alone analytical technique that can provide full structure elucidation (Kim *et al.*, 2010). Despite all these benefits, NMR has poor sensitivity compared to MS techniques, which means a number of metabolites will escape detection.

MS is the most widely used detection technique in metabolomics due to its speed, sensitivity and broad application (Hall, 2006). Compounds are turned into ions, separated according to their mass to charge (*m/z*) ratio, and then detected (Dunn & Ellis, 2005). There are numerous separation techniques employed with MS to improve compound detection. However, depending on the type of extract or desired compounds under study, the two most common separation techniques are gas chromatography (GC) and liquid chromatography (LC) (Hall, 2006).

Gas Chromatography-MS (GC-MS) is a relatively low-cost, technique and the principal method for analysing volatile metabolites such as alcohols, esters and monoterpenes (Hall, 2006; Sumner *et al.*, 2003). GC-MS is also applicable to non-volatile polar compounds such as amino acids, sugars and organic acids (primary metabolites). However, the samples must be chemically derivatised to enhance detection (Hall, 2006). Chemical derivatisation converts the metabolites into volatile and thermostable compounds that can withstand GC separation (Hall, 2006). The main limitations with GC-MS lie in the additional sample preparation and derivatisation, and variance as a result. However, GC-MS remains one of the oldest analytical techniques with a wide range of applications in metabolomics. Moreover, as this technique has been around for many

years, there are already established protocols and large databases for compound identification (Lisec, Schauer, Kopka, Willmitzer, & Fernie, 2006).

Liquid Chromatography-MS (LC-MS) is a very important and versatile technique that is used to analyse high and low molecular weight secondary metabolites alike (Hall, 2006). Contrary to GC-MS, this technique has simpler sample preparation and can analyse non-volatile compounds (Dunn & Ellis, 2005). The use of different column chemistry and mobile elution phases allows for the separation and analysis of dissimilar and specific types of plant metabolites; hence the versatility. An advantage of LC-MS is its restriction to molecules that can be ionised as positively or negatively charged ions (Hall, 2006). This restriction gives greater analytical coverage as compounds that are susceptible to proton loss or gain can still be analysed.

There are different types of LC techniques with the two most popular being high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC). The major differences between the two techniques are that UPLC uses higher system operating pressures and smaller sized packing material in the columns compared to HPLC (Lu, Zhao, Bai, Zhao, Lu, & Xu, 2008). As such, UPLC has the benefits of a shorter analysis time and better chromatographic resolution (Moco *et al.*, 2007).

1.5.2) Metabolomics of beneficial microbe- plant interactions

From pharmaceuticals to diagnostics, agriculture and food, metabolomics has become a popular omics tool. Metabolomics has become especially important in plant research and breeding. The plant metabolome has been described as the bridge between the genotype and phenotype, revealing different biological endpoints as the final result of gene expression (Arbona, Iglesias, TalóN, & GóMez-Cadenas, 2009). The information gained from the biological endpoints is vital to understand how plants are able to exist, function and respond to their environments (Hall, 2006).

As plants are naturally sessile and obligate autotrophs, they developed a molecular response to survive in a continuously changing and hostile environment (Hall, 2006). A change in metabolism, therefore, compensates for environmental, temporal or seasonal changes plants go through, thereby enabling their viability. Hence, a study of the specific metabolic perturbations gives an insight into important nutritional or agronomic biomarkers, which can be used to optimise crop growth (Arbona *et al.*, 2009). In essence,

metabolomics acts as a useful tool to evaluate the contribution of external influences on the plant phenotype based on metabolic changes.

When plants interact with microbes, be it a pathogenic or beneficial interaction, the phenotype and metabolome often undergo drastic changes. These changes are complex, and metabolomics offers a way to link the phenotypic changes with metabolic changes. During plant-pathogen interactions, certain metabolites are produced, which define the resistant, tolerant or susceptible phenotype; these metabolites translate into the phenotypic responses (Heuberger, Robison, Lyons, Broeckling, & Prenni, 2014). The same applies to plant-PGPR interactions; metabolites are produced that translate into improvements in growth under varying conditions, or induced resistance against pathogens.

The majority of PGPR research focuses on the bacterial mechanisms of growth, while little emphasis is placed on the metabolic effects on the host plant (Bloemberg & Lugtenberg, 2001). The studies that have been done on the metabolomics of different plant-PGPR interactions, have shown that PGPR are capable of affecting both primary and secondary metabolite content in plants. For example, a study conducted on marigold showed that inoculated plants displayed a significant increase in phenolics (Del Rosario Cappellari, Santoro, Nievas, Giordano, & Banchio, 2013). In another study, the leaves of inoculated Arabidopsis plants showed PGPR-dependent changes in primary and secondary metabolites (Su, Gilard, Guérard, Citerne, Clément, Vaillant-Gaveau, & Dhondt-Cordelier, 2016). It was also found that long term inoculation triggered modifications on a larger set of metabolites compared to short term inoculation (Su *et al.*, 2016). Other studies included pathogens or abiotic stressors to demonstrate the role PGPR played in modifying plant metabolism under stressful conditions. For example, PGPR inoculation of tomato plants induced production of phenolics, proteins and chlorophyll, while alleviating the effects of *Spodoptera litura* infestation (Bano & Muqarab, 2017).

As the current project's main focus is on maize, it is worth mentioning that there have been studies on the effect of PGPR strains on the maize metabolome. A study conducted by Walker *et al.,* (2012), investigated the effect of inoculation and co-inoculation of PGPR strains on the secondary metabolite content in maize seedlings. The results of the study showed that inoculation of PGPR strains did, in fact, affect variation of secondary metabolites, specifically benzoxazinoids, cinnamic acids, xanthone like compounds and simple phenols (Walker *et al.*, 2012). Furthermore, the metabolic effects were inoculant

specific, which was not surprising as microorganisms from different genera were used; however, different strains from the same genus have also been known to elicit different effects on maize physiology (Walker, Bertrand, Bellvert, Moënne-Loccoz, Bally, & Comte, 2011; Walker *et al.*, 2012). The benzoxazinoids were of special interest in the study. Benzoxazinoids are constitutive defence molecules known as phytoanticipins, that are produced during root emergence (Ahmad, Veyrat, Gordon-Weeks, Zhang, Martin, Smart, Glauser, Erb, Flors, & Frey, 2011; Park, Hochholdinger, & Gierl, 2004). Benzoxazinoids show great importance in biotic interactions in maize as they contribute to basal resistance against aphids and pathogenic fungi (Ahmad *et al.*, 2011). It was concluded in the study by Walker *et al* (2012) that benzoxazinoids may play a role in establishing rhizosphere colonisation; however, this would require further work to be established (Walker *et al.*, 2012).

In a different study by Planchamp *et al.,* (2015), inoculation of maize plants led to the induction of jasmonic and abscisic acid signalling, variation of certain phospholipid and benzoxazinone levels. In that study, it was found that *Pseudomonas putida* KT2440 inoculated maize plants were more abundant in several phospholipids compared to the control (Planchamp *et al.*, 2015). Phospholipid-dependent mechanisms are known to be linked to the mechanisms of action of some defence-related plant hormones like ABA, auxins, cytokinins and JA (Cowan, 2006). Phospholipids were the most significantly induced compounds in the inoculated plants, and the observed changes correlated with the induction of JA and ABA signalling (Planchamp *et al.*, 2015). Therefore, the observed changes with regards to phospholipids and JA/ABA signalling corroborated with the hypothesis that KT2440 inoculation is capable of activating plant defence mechanisms as the plant initially regards the PGPR as a threat (Planchamp *et al.*, 2015). Additionally, the study included the fungal pathogen *Colletotrichum graminicola,* and the metabolic changes discussed above demonstrated the ability of KT2440 to induce maize systemic resistance against the pathogen.

In another instance where maize was inoculated with N_2 fixing PGPR strains, a variation in primary metabolites such as mannitol, trehalose, isocitrate, etc., was observed (Brusamarello-Santos, Gilard, Brulé, Quilleré, Gourion, Ratet, De Souza, Lea, & Hirel, 2017). Mannitol and trehalose are known to play significant roles in signalling during plant and microbe or fungi interactions, and in defence mechanisms (Brusamarello-Santos *et al.*, 2017). The results also showed a small but significant increase in the concentration of asparagine and alanine from the N_2 fixing PGPR inoculated plants (Brusamarello-

Santos *et al.*, 2017). This result suggested that ammonia assimilation was enhanced as a result of the bacterial N_2 fixation. It was concluded from the study that the differences in the type of metabolites were specific for the N_2 fixing capacity of the PGPR strains used. Furthermore, the significantly different metabolites could be used as markers for the plant-bacteria interaction (Brusamarello-Santos *et al.*, 2017).

1.6) Project justification

From the metabolomic plant-PGPR studies discussed, it has been established that PGPR can affect the metabolic profile of host plants; however, these results are often plant dependent or strain dependent, additive or not (Brusamarello-Santos *et al.*, 2017; Planchamp *et al.*, 2015; Walker *et al.*, 2012). The main point being that the effects of PGPR on the metabolome are not fixed.

As part of the PGPR research program within the Department of Plant and Soil Sciences, several PGPR strains have been shown to promote growth of maize plants. To be able to optimise plant growth promotion by these strains it is essential to understand the plant growth promoting mechanisms of action of the strains. Determining the effects of PGPR inoculation on the plants' metabolome will provide insight into the mode of action of the strains. Elucidation of the mode of action of the PGPR strains will facilitate the effective application of the strains as biofertilisers.

1.7) Aims/Objectives

The overall aim of this project was to assess the effect of selected PGPR strains with known plant growth promoting activity on the metabolic profile of maize seedlings, which should translate to the observable effects on the growth of the seedlings. To reach this aim, the following objectives were achieved:

- The effect of *Lysinibacillus sphaericus* (T19)*, Paenibacillus alvei* (T29) *and Bacillus megaterium* (A07) on early maize growth, i.e., the effects on root and shoot biomass, leaf chlorophyll content, shoot length and stem diameter were assessed through a greenhouse trial.
- The effect of single strain PGPR inoculation on the metabolic profile of maize was evaluated through:
    - Metabolomic analysis of extracts from inoculated and uninoculated samples using UPLC/QTOF-MS.
    - Finding metabolites exhibiting significant fold changes in the inoculated samples compared to the control and subjecting them to metabolic pathway

analysis.

- Finding discriminating metabolites between the control and inoculated plants, putatively annotating them and linking them to roles in plant growth promotion.

- Assessment on the best performing strains based on growth promotion and metabolic effects.

1.8) Hypotheses

The major null hypotheses are as follows:

$H1_0$: Inoculation of maize seedlings with PGPR does not result in any significant increase ($p=0.05$) in root and shoot biomass, chlorophyll content, shoot length and stem diameter.

$H2_0$: PGPR inoculation will not significantly ($p=0.05$) alter the metabolome of maize seedlings either similarly or differentially in the roots and shoots.

$H3_0$: Inoculation with PGPR will not result in any significant difference ($p=0.05$) in the relative concentration of specific metabolites in maize.

Chapter 2: Materials and methods

2.1) Overall methodology

The project can be broadly divided into two phases. Phase 1 involved growing PGPR inoculated maize seedlings in a greenhouse, followed by assessing the effect of PGPR inoculation on plant growth parameter. Phase 2 involved metabolomic analysis of extracts obtained from the treated and control maize seedlings' roots and shoots. Figure 2 is the graphical representation and flow of direction of the steps involved in this project. Both phases were equally important; however, a large part of this project is dedicated to phase 2.

An untargeted metabolomics approach was employed in this project. Untargeted metabolomics aims to globally profile all detectable metabolites. As such little to no *a priori* knowledge is needed for which metabolites may be affected. The biggest advantage of an untargeted approach is the possibility of collecting novel information that would otherwise be missed in a targeted situation. Thus, to get a more global view of the possible effect of the selected PGPR on the maize seedling metabolome, an untargeted approach was used.

Phase 1's purpose was to determine the effect of inoculation with the selected PGPR strains on seedling growth, whereas, the purpose of phase 2 was to determine the effect of PGPR inoculation on the maize seedlings metabolome. Finally, it was attempted to indicate a link between the phenotypic effects and the biochemical effects of PGPR inoculation. The steps to achieve this are discussed in the following sections of this chapter.

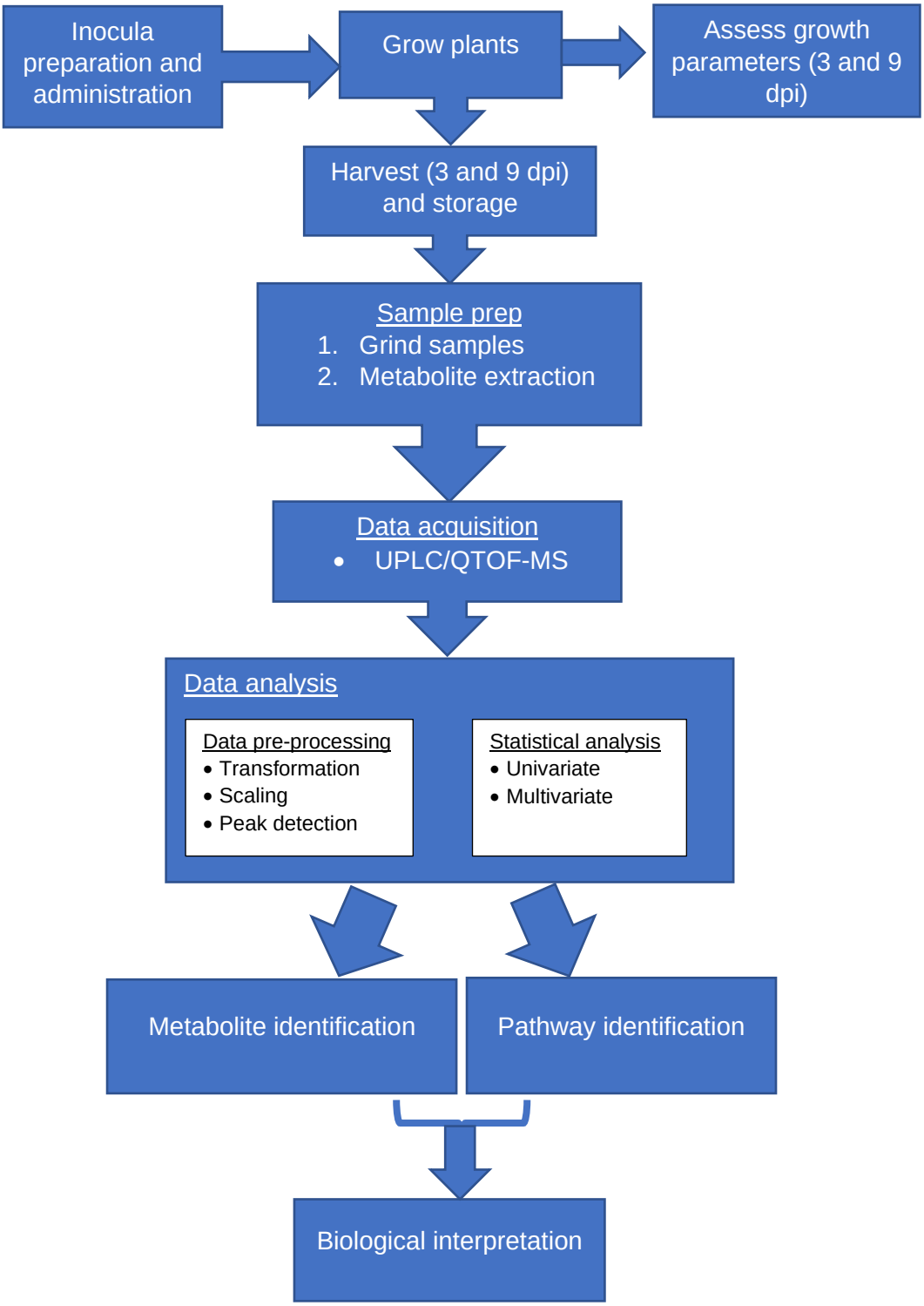


Figure 2: Flow diagram of the procedural steps employed in this project. The first step was inocula preparation followed by plant inoculation. Growth parameters were assessed 3 and 9 days post inoculation (dpi) respectively. Seedlings were harvested 3 and 9 dpi and freeze dried for 3 days. Grounded samples were extracted and the extracts analysed by UPLC/QTOF-MS. All acquired data was analysed by univariate and multivariate statisitcs to obtain discriminating metabolites and identify affected metabolic pathways.

2.2) Preparation of rhizobacterial inocula

PGPR strains (T19, T29 and A07) were obtained from the University of Pretoria's PGPR culture collection stored at -80°C in the Department of Plant and Soil Sciences. The bacterial isolates were streaked onto sterile nutrient agar (Biolab, Wadeville, South Africa) in 65mm petri dishes then incubated for 48 h at 25°C to obtain single colonies.

To prepare the liquid culture, sterile nutrient broth (Biolab, Wadeville, South Africa) was prepared and inoculated with a single colony of the respective strain. The inoculated broth was incubated in a shaker incubator at 25°C and 150 rpm for 48 h. Sterile nutrient broth was also dispensed and set aside for the control treatments. Next, serial dilutions were performed for each strain (three replicates per strain), to determine the number of viable bacterial cells in the prepared inocula. A volume of 0.1 ml was aliquoted and spread on nutrient agar plates then incubated for 48 h at 25°C. An average CFU/ml of 10^8 was obtained for each culture.

2.3) Greenhouse trial

Prior to germination, maize seeds (cultivar KKS 4250) were surface sterilised in 70% ethanol for 30 s, rinsed five times in autoclaved dH_2O, washed in 1% sodium hypochlorite solution and finally rinsed five times in autoclaved dH_2O. Sixty-four plastic pots, 12.5 cm in diameter, were filled with steam pasteurised soil. The soil was augmented with superphosphate (Efekto- Wonder Super Phosphate) at a rate of 1 g/4 kg soil prior to filling the pots. After the pots were filled, the soil was watered a day prior to sowing. Three seeds were planted per pot. The maize plants were grown in the University of Pretoria Greenhouse on the Hatfield Campus at an average temperature of 22 to 30°C. After 14 days of germination, 15 ml of the respective rhizobacterial cultures was directly applied to each seedling. To the control plants, 15 ml of nutrient broth was applied. The trial involved four different treatments (control, T19, T29 and A07), each replicated eight times (one pot containing three plants represented one replicate). The pots were arranged into two randomised groups, where one group represented the plants that would be harvested 3 dpi and the other 9 dpi.

2.4) Growth parameters assessments

Parameters that could be assessed using non-invasive methods (chlorophyll content and stem diameter) were recorded first. Chlorophyll content was measured using a chlorophyll content meter (CCM-200 plus, Opti-Sciences), and readings were taken from the first

most developed leaf, a third of the distance from the tip. Stem diameter was measured using Vernier callipers and was recorded on the day of harvest along with the chlorophyll content.

The plants were harvested 3 and 9 dpi respectively. The soil attached to the roots was washed off using tap water, then the roots were separated from the shoots using a scalpel. The shoot length was then measured with a measuring tape from the base of the shoot to the tip of the longest leaf, and immediately both roots and shoots were flash-frozen in liquid nitrogen and stored in zip lock bags at -20°C. Both the roots and shoots were freeze dried for 72 h, after which, the dry mass was weighed. The freeze lyophilised roots and shoots were stored at -80°C till future use.

2.5) Sample preparation for metabolomics analysis

The plant metabolome is highly complex, which is mostly due to the vast range of secondary metabolites that exceed the number and type of primary metabolites (Moco *et al.*, 2007). Due to the wide range of metabolites, there is no single extraction method capable of dissolving all the compounds. The extraction method, therefore, limits the type of metabolites that would be detected (Kim & Verpoorte, 2010).

A monophasic Bligh-Dyer type extraction was used to adequately extract both polar and nonpolar metabolites (Bligh & Dyer, 1959). Samples were ground and extracted using a solvent mixture of methanol (Microsep, South Africa), triple distilled water and chloroform (Merck, Germany) in a 1:3:1 ratio. For 50 mg of plant material (roots and shoots), 1 ml of extraction solvent was used. Steel beads were added to the tubes to facilitate homogenisation. Each sample was vortexed for 30 s followed by sonication for 20 min. Following sonication, samples were centrifuged for 15 min at 13 xg after which 500 µl of the supernatant was collected and transferred to 1.5 ml chromatography vials. Next, the samples were concentrated using a GeneVac EZ-2 Plus benchtop evaporator. Samples were then reconstituted with a mixture of triple distilled water and acetonitrile (Microsep, South Africa) (1:1) to a volume of 1 ml. To aid dissolution samples were vortexed for a few seconds. Next, a 10x dilution of the extracts was prepared. Finally, diluted samples were filtered into 1.5 ml chromatography vials using 0.2 µm syringe filters (Sartorius Stedim Biotech) for UPLC/QTOF-MS analysis.

2.6) Instrumentation and analysis

Metabolomic data was acquired using UPLC/QTOF-MS at the University of Pretoria Synapt Facility. A blank, which was a 1:1 mixture of distilled water and acetonitrile was injected first followed by quality control samples. Quality control samples were injected after every eight plant extract samples. The quality control samples were a mixture of all the extracts combined to a final volume of 1 ml. Samples were injected in a randomised order at a volume of 5 µl, and separated on an ACQUITY UPLC® HSS T3 (2.1 x 150 mm x 1.8 µm) column. Water with 0.1% formic acid was used as mobile phase A and mobile phase B was acetonitrile with 0.1% formic acid. Reagents for both mobile phases were supplied by Microsep, South Africa. A WATERS Synapt G2 QTOF detector was used for mass detection and the mass range was set to 50-1200 Da.

Samples were run for a total of 15 min, at a flow rate of 300 µl/min and a column temperature of 40°C; under gradient conditions. The gradient conditions are listed in the Table 3. The data was acquired in both positive and negative electrospray ionisation (ESI) modes in the continuum format. The source temperature for the ESI was kept at 120°C. The desolvation temperature was maintained at 400°C with a gas flow rate of 600 L/h. Both low and high energy data were acquired using a collision energy of 15 and 45 eV respectively.

Table 3: Gradient conditions specifying run time and % of solvent A (water + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid).

	Time (min)	Flow rate (µl/min)	% A	% B
1	Initial	300	95	5
2	2	300	95	5
3	10	300	0	100
4	12	300	0	100
5	13	300	95	5
6	15	300	95	5

2.7) Data analysis

2.7.1) Growth parameters

The data collected for the growth parameters were first subjected to the Shapiro-Wilk normality test to determine whether the data follows a normal distribution. Next, One-Way ANOVA was performed followed by an unpaired Student's t-test. All statistical tests were performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

The data collected for the growth parameters can be described as univariate data, as only one variable is studied at a time. Hence, univariate statistical analysis was applied to interpret it. To determine whether to apply parametric or nonparametric tests, a normality test was conducted. A normality test compares the scores in a sample to a normally distributed set of scores with the same mean and standard deviation (Ghasemi & Zahediasl, 2012). The Shapiro-Wilk test was selected for its good power properties compared to other normality tests (Shapiro & Wilk, 1965; Shapiro, Wilk, & Chen, 1968). Power is a common measure of the value of a normality test and refers to the ability of the test to detect whether a sample comes from a non-normal distribution (Ghasemi & Zahediasl, 2012).

Analysis of variance (ANOVA) was first performed on the data to establish whether there is a statistically significant difference between the groups, however, it fails to identify which groups cause the difference; this becomes problematic. There were only four groups that were analysed; thus, a Student's t-test was used to find which group(s) caused the significant difference. A one-tailed Student's t-test was used to compare the mean of the control group against the mean of each treatment group separately for all the growth parameters.

2.7.2) Metabolomics data

2.7.2.1) Data pre-processing

The first step in any metabolomics analysis is the pre-processing of data. This is necessary to reduce the complexity of the raw data so that meaningful information can be extracted from otherwise noisy data. Figure 3 shows the typical pipeline that is followed to clean up the data and turn it into a matrix that provides important metabolic information. As seen in Fig 3, filtering, feature detection, peak alignment and normalisation are the common modules of the pre- processing pipeline. Filtering aims to remove any interfering background from the main data either caused by instrumental or chemical distortions (Katajamaa & Orešič, 2007). Feature detection involves identification and quantification of signals corresponding to metabolites in a sample (Castillo, Gopalacharyulu, Yetukuri,

& Orešič, 2011). This step is important for downstream analysis and significantly reduces the complexity of the data (Katajamaa & Orešič, 2007). Once feature detection has been done, the next step is alignment, which is used to compare metabolites between samples (Castillo *et al.*, 2011). In this step, the same peaks are matched across all the samples and grouped prior to any statistical analysis (Castillo *et al.*, 2011). Finally, the data is normalised and this aims to remove unclear variation caused by experimental sources, while keeping important variation from the biological source (Castillo *et al.*, 2011). An additional step often associated with normalisation is scaling. There will be cases of metabolites present in very low concentrations compared to the rest; scaling makes it possible to compare metabolites present at widely differing concentrations (Yi, Dong, Yun, Deng, Ren, Liu, & Liang, 2016). All these steps need to be done before any multivariate analyses, as the downstream analyses are very susceptible to noise effects.

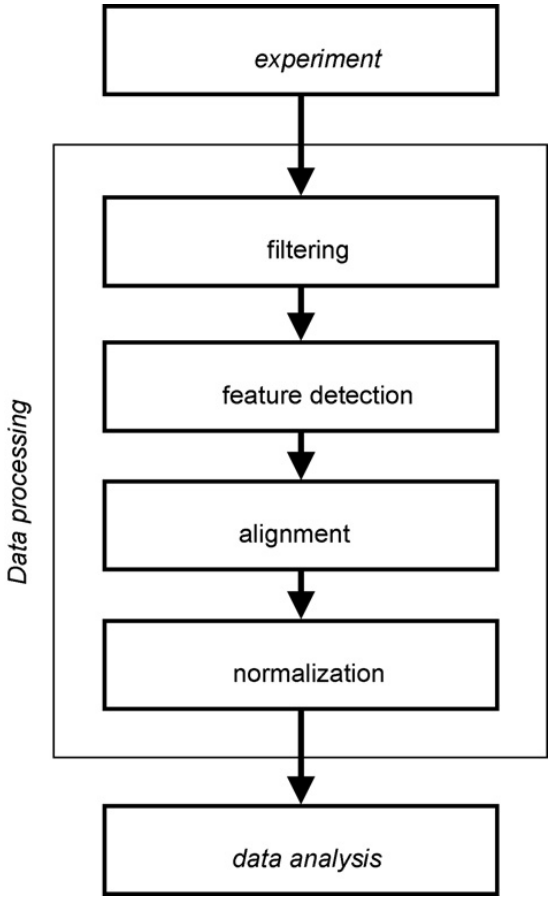


Figure 3: Typical workflow of metabolomics data analysis (Katajamaa & Orešič, 2007).

The acquired UPLC/QTOF-MS data was processed using the MarkerLynx^{TM} version 4.1 software. Processing involved filtering, peak detection, alignment and normalisation. The function one data for all the samples was processed; this is the data collected at low energy (no ion fragmentation data/ only the mother ions of the compounds are detected).

Processing was focused only on peaks within the retention time (rt) range of 0-11 min. A step known as smoothing is usually done at the feature detection stage to further reduce the noise in the data. For the data, a Savitzky-Golay smoothing function was applied. The result of processing was a table of detected markers with their corresponding normalised peak heights across all samples. Finally, the tables were exported to Microsoft Excel for missing value imputation. Ion features that had more than 50% missing values in both control and treatment groups were excluded/ deleted prior to statistical analysis.

2.7.2.2) Statistical analysis and metabolite identification

The last module in Fig 3 is data analysis, which is the step where useful information is modelled from the data. Data analysis in metabolomics serves a number of purposes such as identification of treatment-related similarities and differences in samples, classification of samples, quantification of metabolites, etc. Data analysis involves both univariate and multivariate statistical analysis. Univariate analysis is used to analyse one variable at a time and examples include the t-test and ANOVA (Saccenti, Hoefsloot, Smilde, Westerhuis, & Hendriks, 2014). Multivariate analysis, on the other hand, is more complex as it involves analysing multiple variables simultaneously, thereby reflecting the extent of the relationship among the variables (Saccenti *et al.*, 2014). When dealing with metabolomics data, which is inherently complex, it would make more sense to apply multivariate analysis methods. However, using both methods is beneficial as they may provide complementary results.

Multivariate analysis involves both unsupervised and supervised methods. Unsupervised methods such as principal component analysis (PCA) are usually the starting point for multivariate analysis of metabolomics data, as these methods provide an unbiased view of the data. Unsupervised methods are used for pattern or trend discovery, outlier detection and to visualise the overall data structure (Yi *et al.*, 2016). On the other hand, supervised methods are more commonly used to enhance separation between groups of observations as well as determining the basis of treatment differences. Due to the simplistic output from unsupervised methods, it is necessary to follow up with a supervised method such as partial least squares discriminant analysis (PLS-DA) or orthogonal projection to latent structures discriminant analysis (OPLS-DA), which can further improve data interpretation (Yi *et al.*, 2016).

To analyse the metabolomics data, both univariate and multivariate analysis methods were applied. For univariate analysis, a fold change analysis was performed between the

control and one of the inoculated groups. A value of 1.1 was used as a threshold value. A threshold of 1.1 indicates a 10% change in the relative concentration of an ion feature, where a value of 1 would indicate no change. The fold change analysis was done by comparing absolute value changes between the control and inoculated group means.

Prior to multivariate analysis, the data sets were log transformed and pareto scaled. For the multivariate analysis, both unsupervised (PCA) and supervised methods (PLS-DA and OPLS-DA) were used on the datasets. PLS-DA was applied to analyse all groups and OPLS-DA was used to analyse two groups at a time. A number of software and web-based platforms are available for metabolomics data analysis. A popular freely available web-based platform known as Metaboanalyst 4.0 (accessible at https://www.metaboanalyst.ca/) (Chong, Soufan, Li, Caraus, Li, Bourque, Wishart, & Xia, 2018) was used for all the univariate and multivariate statistical analysis conducted in this project.

2.8) Pathway analysis

A major bottleneck in untargeted metabolomics is the accurate annotation of MS acquired ion features. The annotation step is usually the most difficult and time consuming. Despite the use of high-resolution MS platforms, compound annotation is still a major issue in metabolomics. To circumvent the annotation bottleneck, a new concept has been introduced. The new concept shifts the focus from individual compounds to individual pathways or a group of functionally related metabolite sets (Chong, Yamamoto, & Xia, 2019). Therefore, the need to accurately annotate metabolites upfront is eliminated. The idea behind this concept is that it is more effective to understand the collective behaviour of a group of compounds compared to individual compounds for which identification is prone to random errors (Chong *et al.*, 2019).

Metaboanalyst 4.0 gives the option of two algorithms for the peaks to pathways module, which are the mummichog and the gene set enrichment analysis (GSEA) algorithms. The mummichog algorithm requires a user-specified pre-defined cut-off based on fold changes or t-statistics (Chong *et al.*, 2018; Chong *et al.*, 2019). The GSEA algorithm uses a similar approach; however, this method does not use a significance cut-off but rather considers the overall ranks of each peak. GSEA can detect subtle and consistent changes, which could otherwise be missed by the mummichog method.

To perform the peaks to pathways analysis, a two-column table of the *m/z* features and the fold change (univariate analysis previously conducted) values was uploaded to

Metaboanalyst in a txt format. Next, the instrument's mass accuracy was set to 5 ppm and the analytical mode was set to positive. Finally, the GSEA algorithm and metabolite library was selected. The metabolite library selected was the *Oryza sativa japonica* (Japanese rice) KEGG library. The peaks to pathways module provide only two plant metabolite libraries and the *Oryza sativa japonica* (Japanese rice) KEGG library was selected as both rice and maize are cereal crops.

2.9) Metabolite annotation

Once ion features have been identified as being discriminating factors between the groups, the next step is putative identification or annotation. Metabolite identification involves using specific features from the acquired metabolomics data and comparing it to established information or *in silico* data to confirm the identity of the supposed metabolites.

MS provides a range of tools that can be used for the structural elucidation and identification of metabolites, which are as follows: (i) the accurate *m/z* of molecular, fragment and associated ions, (ii) relative isotopic abundances of molecular and fragment ions, (iii) fragmentation patterns of molecular and fragment ions, and (iv) the comparison of experimentally obtained data to external databases containing physico-chemical information (such as molecular formulae and monoisotopic masses) or mass spectral libraries (Dunn, Erban, Weber, Creek, Brown, Breitling, Hankemeier, Goodacre, Neumann, & Kopka, 2013).

In the case of LC-MS data, the first step in the identification step would be to obtain an accurately measured *m/z* value. The *m/z* value can be used to match the feature to a number of possible molecular formulae (Moco *et al.*, 2007). The accuracy of the measured *m/z* value will determine the number of possible molecular formulae (Dunn *et al.*, 2013; Moco *et al.*, 2007). The molecular formulae are then matched to metabolites using online metabolomic databases. A single molecular formula can match with numerous metabolites, thus other chemical and biological knowledge can be applied. To reduce the list of possible metabolites, the fragmentation pattern of the mass signal can be used to elucidate the structure of the metabolite (Moco *et al.*, 2007). The fragmentation pattern can be compared to in silico fragmentation patterns of the metabolite in question using a database.

Pathway analysis utilised the results from univariate analysis of the acquired metabolomics data. However, it was also necessary to use the results of the multivariate

analysis to determine the putative identity of the metabolites that were affected by PGPR inoculation. Focus was placed on the plants harvested 9 dpi. The results of the OPLS-DA were used to identify discriminating features. Through MarkerLynx, the accurate *m/z* values were searched against a number of online databases and libraries, namely: AraCyc (http://www.arabidopsis.org), BioCyc (https://biocyc.org/), KEGG (https://www.genome.jp/kegg/), FoodDB (http://foodb.ca/) and Plant Metabolic Network (https://www.plantcyc.org/). The identity of the metabolites was putatively confirmed by comparing the experimentally obtained fragmentation pattern against *in silico* fragmentation patterns of possible metabolites. Function two data was used for this step, as this is the data collected at high energy (data with the ion fragmentation pattern). Fragmentation pattern comparisons were performed using the MassLynx in house software MassFragment and the online platform MetFrag (accessible at https://msbi.ipb-halle.de/MetFragBeta/) (Ruttkies, Schymanski, Wolf, Hollender, & Neumann, 2016).

Chapter 3: Results

3.1) Growth parameters

The first objective was to assess the effect of single strain PGPR inoculation on early maize growth. The following parameters were measured to be used as indicators of growth to compare the inoculated to the uninoculated control plants: chlorophyll content, stem diameter, shoot length, root and shoot dry biomass.

3.1.1) Three days post inoculation

The only growth parameter that showed statistically significant results was the root dry mass. However, with the other parameters, some trends could be observed. From the results in Fig 4B it can be observed that T29 inoculated plants had a greater average stem diameter compared to the control. In Fig 4C, the results of the T19 inoculated maize seedlings indicate a greater average shoot length compared to the control plants. The trends from Figs 4C and 4E indicate that A07 had no positive effect on the shoot length and shoot dry mass of the inoculated plants.

The root dry mass was the only parameter where there was a statistically significant difference between the inoculated and control plants. The results show that all strains had a statistically significant effect on the root dry mass (Fig 4D). Strain T29 had the most significant effect on the inoculated plants' root dry mass followed by strain T19 and lastly strain A07. The results show that maize inoculated with strain T29 had a 54% greater average root dry mass compared to the control. Maize seedlings inoculated with strains T19 and A07 showed a 35% and 31% greater average root dry mass compared to the control.

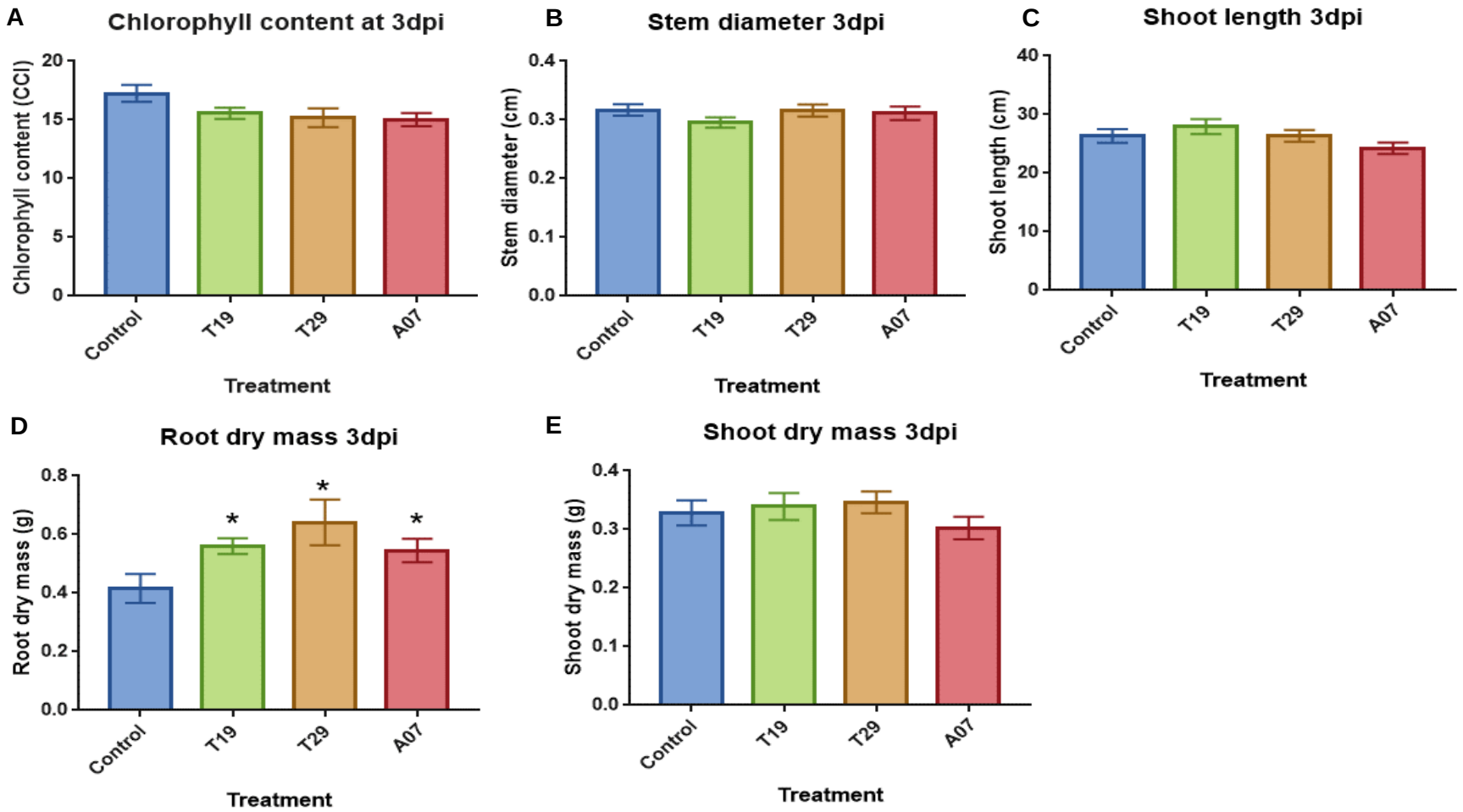


Figure 4: Effect of PGPR inoculation on growth of maize seedlings harvested 3 dpi. The y-axis for each parameter is as follow: (A) Chlorophyll Content Index, (B) Stem diameter (C) Shoot length, (D) Root dry mass and (E) Shoot dry mass. Each bar represents the average value for each sample group and the error bars represent the standard error of the mean. For each group n=8 and a star (*) indicates a mean value that is statisitically significantly greater than the control value at a p-value < 0.05 based on the Student's t-test.

3.1.2) Nine days post inoculation

The results showed a trend of enhanced growth in the maize seedlings inoculated with strains T29 and T19. In Fig 5A, the results indicate that the plants inoculated with strain T19 had the highest average chlorophyll content index. The average chlorophyll content index of the T19 treated plants was 18% significantly higher than that of the control. Additionally, T19 inoculated plants had an average stem diameter 15% significantly greater than that of the control plants (Fig 5B). The average stem diameter of the T29 and A07 treated plants were also significantly greater than the control plants by 9% and 12% respectively. The average shoot length of the T19 treated plants was 7% significantly greater than that of the control plants as seen in Fig 5C.

There were no statistically significant differences in the root and shoot dry mass of the control and treated plants. Nevertheless, the results in Fig 5D indicate the trend that strain T29 enhanced root dry mass the most in maize seedlings, followed by strain T19. In Fig 5E, the results show that strain T19 enhanced shoot dry mass the most, followed by strain T29.

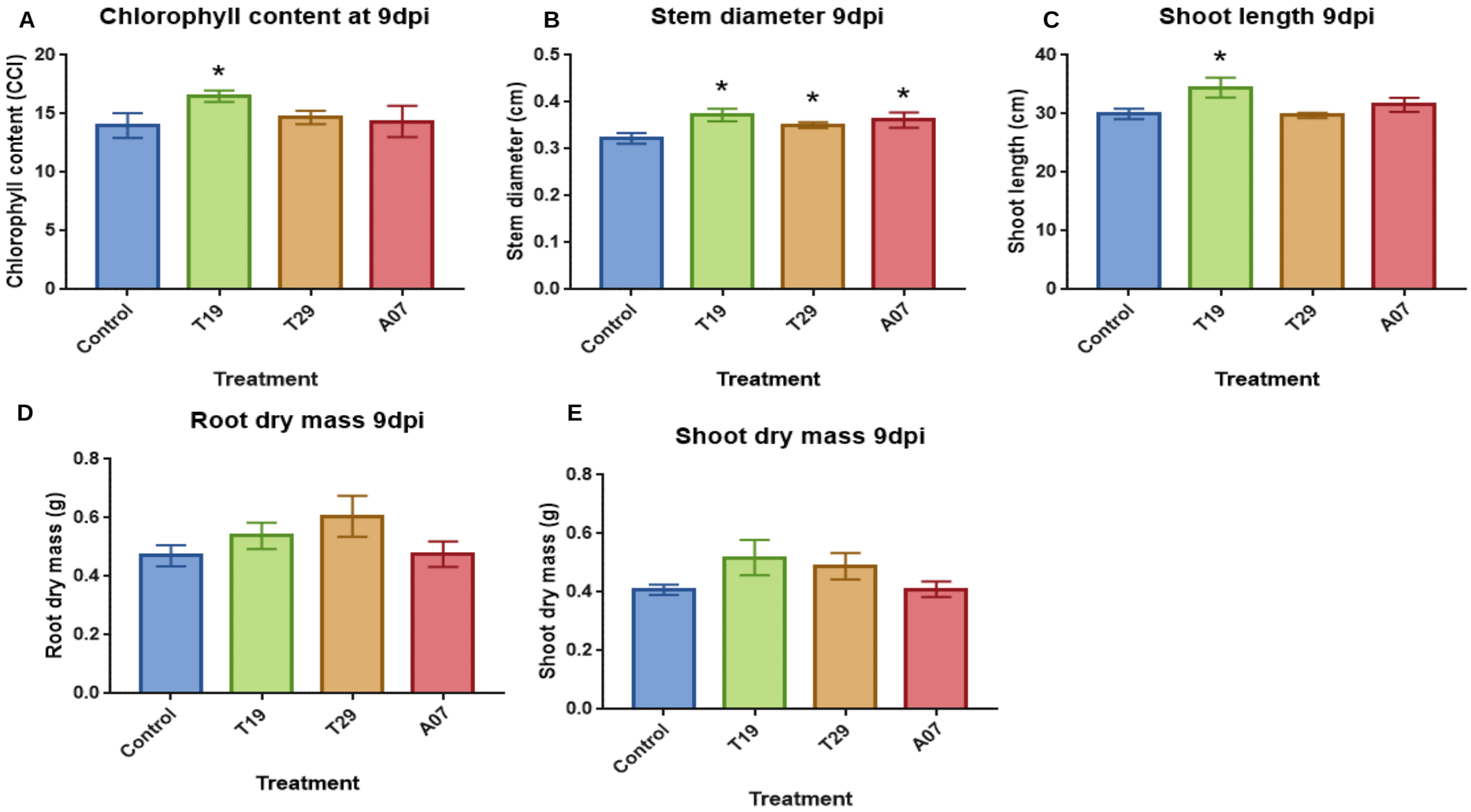


Figure 5: Effect of PGPR inoculation on growth of maize seedlings harvested at 9 dpi. The y-axis for each parameter is as follow: (A) Chlorophyll Content Index, (B) Stem diameter (C) Shoot length, (D) Root dry mass and (E) Shoot dry mass. Each bar represents the average value for each sample group and the error bars represent the standard error of the mean. For each group n=8 and a star (*) is indicative of a mean value that is statisitically significantly different from the control at a p-value < 0.05 based on the Student's t-test.

3.2) Metabolomics

The plants were harvested on different days to determine how early the effects of PGPR inoculation can be observed in the metabolome. The second and third objectives were to assess the effects of single strain PGPR inoculation on the maize metabolome and to find discriminating features between the control and inoculated samples respectively. To achieve this, metabolomic analysis was performed. It was established from the greenhouse experiments that strains T29 and T19 gave the best results. Further analysis was conducted on the T29 inoculated roots, and the T19 inoculated shoots. Both univariate and multivariate methods were applied for the metabolomics data analysis.

Mass spectra were collected in both positive and negative ionisation modes. For the purpose of this project, more focus was placed on the positive ionisation data as it provided results with the least amount of spectral noise. Both PCA and PLS-DA scores plots are displayed as 3D figures, as it shows the best separation between the groups. As such the 3D images are shown at angles that display the best clustering between groups.

3.2.1) Three days post inoculation

3.2.1.1) Roots

3.2.1.1.1) Multivariate analysis for all groups

The PCA scores plot (Fig 6A) represents an overview of the data, and the variation is explained by the three principal components that separate the data into various clusters. The three principal components explain 54.4% of the variation seen in the scores plot. It can be observed in Fig 6A that the inoculated samples (A07, T19 and T29), cluster to one side while the control samples are quite spread out. Figure 6A does not show perfect clustering; however, certain trends can be observed. Firstly, most of the inoculated samples cluster to the left. The control samples are, however, quite spread out and on the right. The A07 and T19 samples appear to be mixed together while the majority of the T29 samples group the most and the furthest from the other groups. One of the T29 samples is misgrouped and mixes with the control samples, in addition, two of the T19 samples appear to behave the same. However, none of those samples turned out to be outliers according to Hotelling's T^2 test. Groups

that are clustering together tend to show similar traits, while samples that are misclassified may share similar traits with the group they cluster with.

Figure 6B shows enhanced separation between the groups. As shown by the PCA scores plot in Fig 6A, there is a great degree of within group variation in the control samples and the two T19 samples are still misclassified as well as the one T29 sample. The same trends observed in Fig 6A can still be observed in Fig 6B despite the enhanced separation. As expected, T29 samples still showed the best clustering in comparison to the other groups.

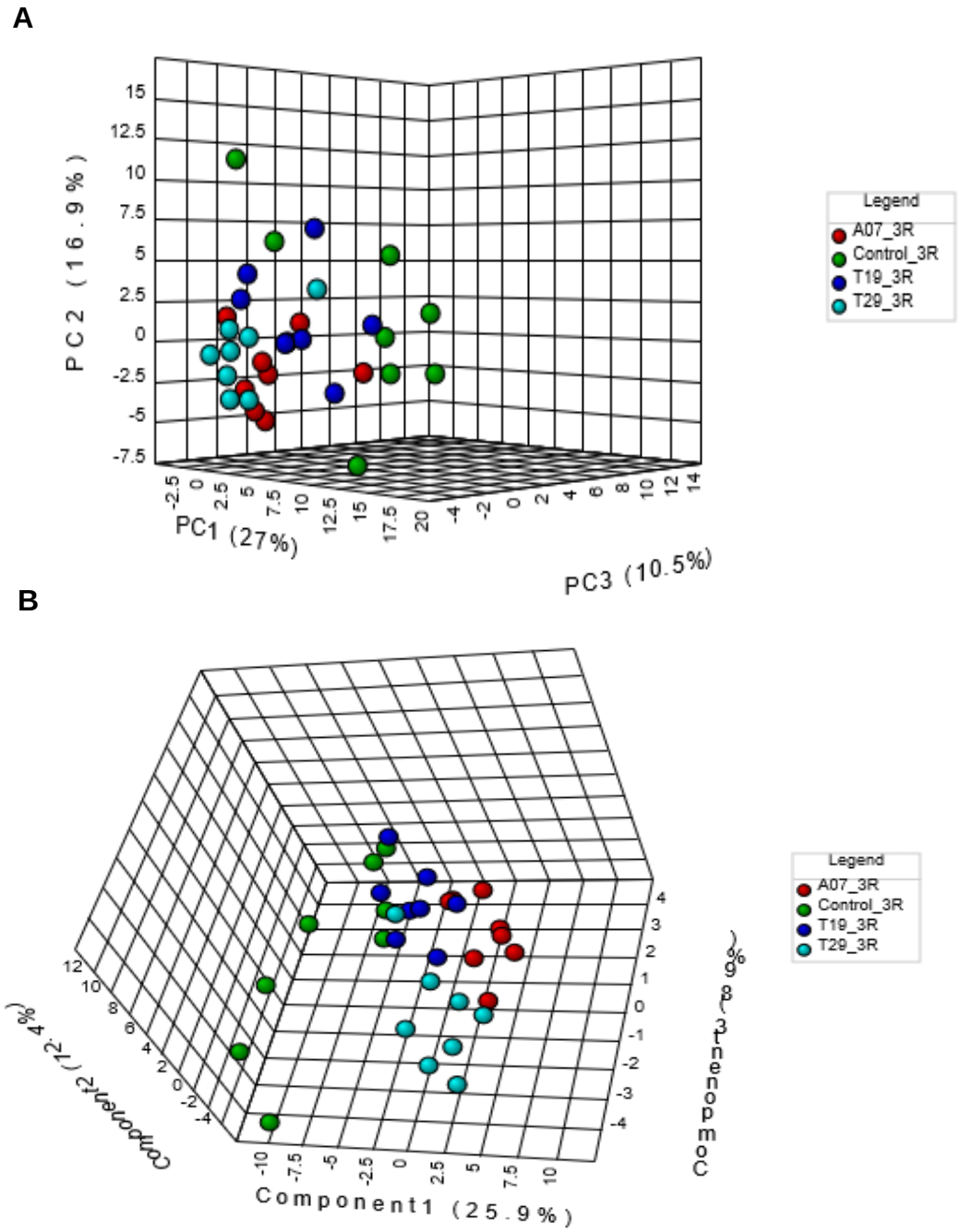


Figure 6: Unsupervised and supervised multivariate analysis of control and inoculated maize roots harvested 3 dpi. (A) Unsupervised analysis: 3-Dimensional Principal Component Analysis (PCA) scores plot showing distribution of the groups among the three major principal components along the x (PC1), y (PC2) and z (PC3) axes. PC 1, 2 and 3 contribute 27, 16.9 and 10.5% respectively to the variation in the data. (B) Supervised analysis: 3-Dimensional Partial Least Squares Discriminant Analysis (PLS-DA) plot showing distribution of the groups among the three major PLS components (Component 1, 2 and 3). Components 1, 2 and 3 contribute 25.9, 12.4 and 8.9% respectively to the model. For each group n=8.

3.2.1.1.2) Univariate analysis of the Control group versus the T29 group

Univariate analysis was performed on the two groups as a first step in the data exploration. A fold change analysis was performed to compare absolute value changes between the two group means. Therefore, the data before column normalisation was used instead. A log2 scale was used so that the same fold change (up/down-regulated) is the same distance to the zero baseline.

There are 57 significant ion features above the positive threshold while there are 49 below the negative threshold in Fig 7. The 57 features are up-regulated in the T29 samples compared to the control samples. On the other hand, the 49 features are down-regulated in the T29 samples compared to the control samples. The top 20 features that showed significant fold changes are listed in Table 4. The complete list of features can be found in the appendix (Table S 2). All the features within the dotted lines represent features that did not have a fold change greater than the set threshold.

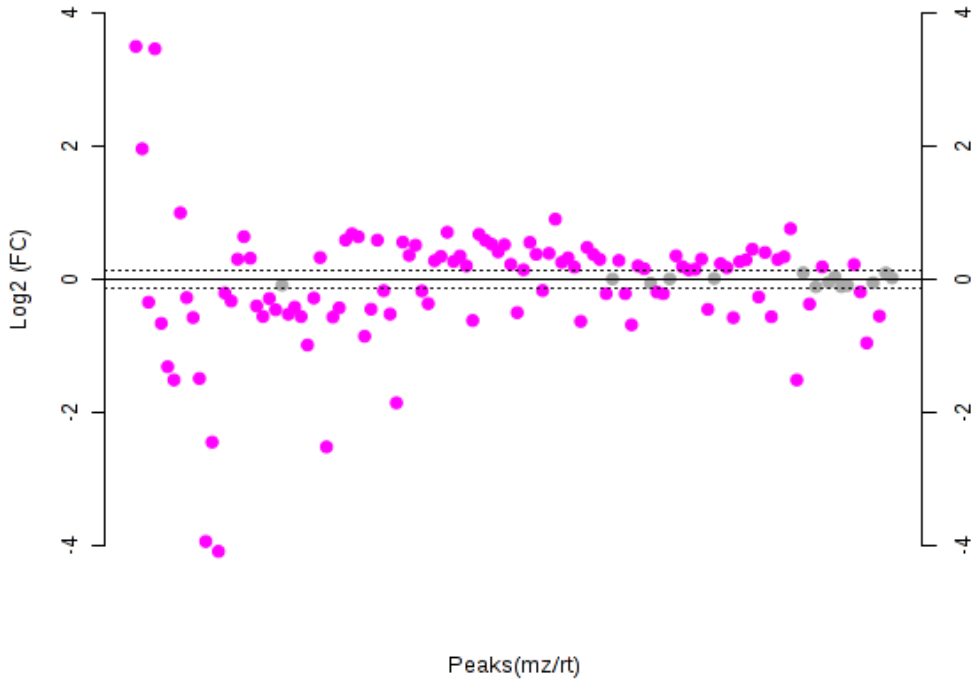


Figure 7: Fold change analysis between control and T29 inoculated maize roots harvested 3 dpi. A threshold of 1.1 was used to select important features from the fold change analysis. The pink circles represent the ion features. Note the values are on log scale, so that both up-regulated and down-regulated features can be plotted in a symmetrical way.

Table 4: Top 20 important features identified by fold change analysis between the control and T29 inoculated maize roots harvested 3 dpi.

Feature number	Retention time (min)	*m/z*	Fold change	log_2(FC)
1	7.92	290.2642	0.0591	-4.0803
2	8.63	318.2959	0.0655	-3.9319
3	9.15	453.1628	0.1750	-2.5148
4	7.89	334.2881	0.1839	-2.4429
5	9.15	437.1837	0.2766	-1.854
6	7.86	274.2677	0.3508	-1.5111
7	6.12	386.1782	0.3509	-1.5108
8	7.90	318.2942	0.3562	-1.4893
9	7.40	219.0601	0.4023	-1.3138
10	1.14	306.8466	0.5043	-0.98772
11	6.06	379.1705	0.5150	-0.95727
12	1.13	164.9167	0.5524	-0.85631
13	1.17	177.0205	0.6220	-0.68512
14	5.74	379.0803	1.6271	0.70229
15	4.79	588.1451	1.6895	0.7566
16	7.76	885.2319	1.8642	0.89855
17	9.69	335.2131	1.9916	0.99394
18	7.21	843.2214	3.8731	1.9535
19	5.81	373.1037	10.9380	3.4513
20	5.81	271.0365	11.2220	3.4883

3.2.1.1.3) Multivariate analysis of the Control group versus the T29 group

Multivariate techniques were applied to further investigate the effects of the T29 strains on the roots. A PCA (Fig 8A) and OPLS-DA (Figs 8B and 8C) were performed on just the control and T29 groups. The first three principal components in Fig 8A explains 68.2% of the total variation between the control and T29 groups. The control samples show a great deal of separation, whereas the T29 samples are mostly clustered together. The separation between the groups is better observed in Fig 8B, where the control group shows a bit of within group variation. Both groups are classified correctly and there is no misgrouping of samples. OPLS-DA provides information on the features that are responsible for class separation in the form of an S-plot (Fig 8C). In the S-plot, the features that are responsible for class separation are at the very ends, whereas features that are not significantly different between the groups are concentrated in the centre. The discriminating features are listed in Table 5.

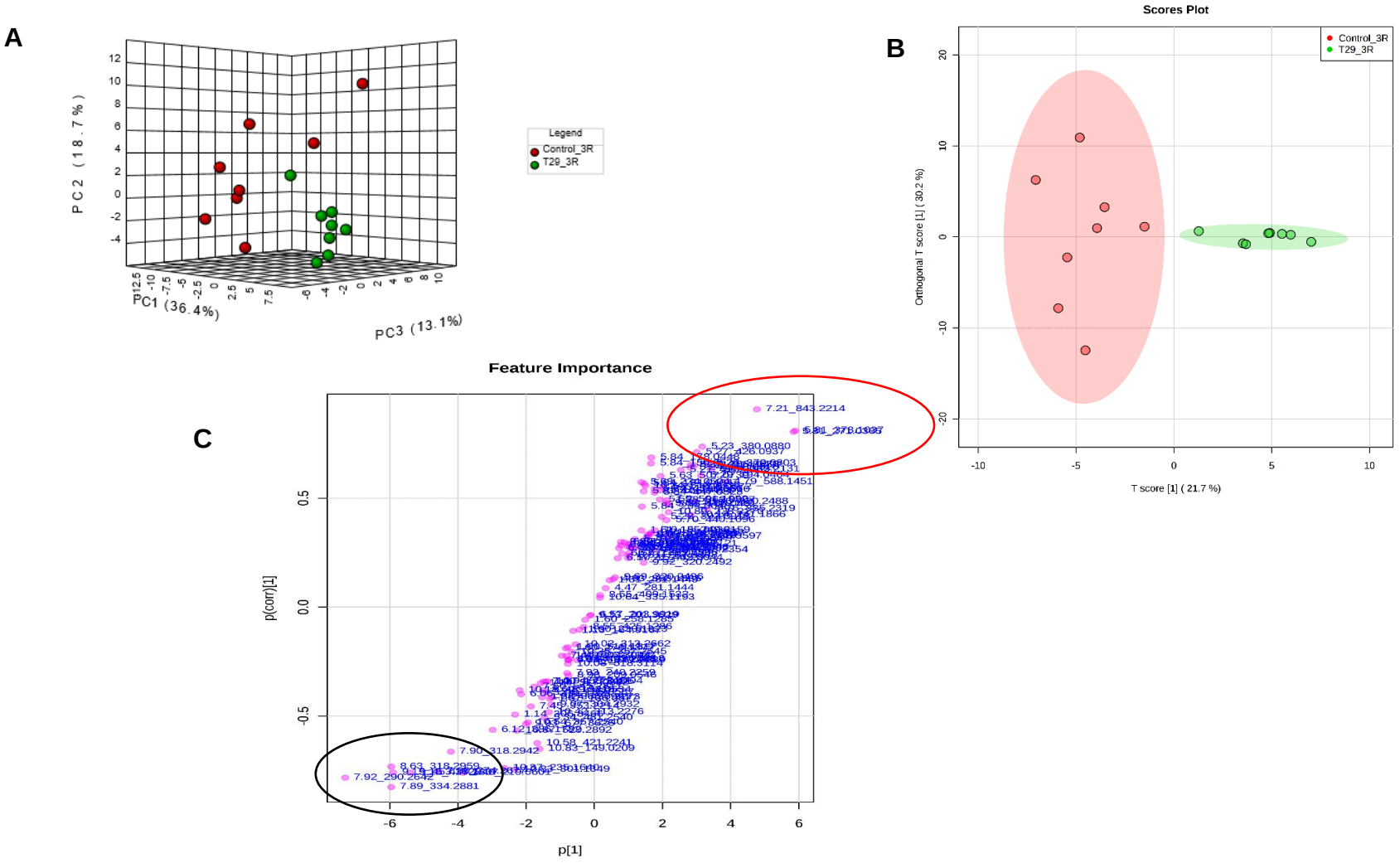


Figure 8: Multivariate analysis of control and T29 inoculated maize roots obtained 3 dpi. (A) 3D PCA plot showing clustering of the control and T29 groups. PC 1, 2 and 3 contribute 36.4, 18.7 and 13.1% respectively to the variation in the data. (B) OPLS-DA scores plot between the control and T29 roots. The predictive (x) axis explains 21.7% of the variation while the orthogonal (y) axis explains 30.2% of the variation. (C) OPLS-DA S-plot showing discriminating variables between the control (red circle) and T29 samples (black circle).

Table 5: Important features identified from the OPLS-DA S-plot.

Feature number	Retention time (min)	*m/z*
1	4.79	588.1451
2	5.81	271.0365
3	5.81	373.1037
4	7.21	843.2214
5	7.86	274.2677
6	7.89	334.2881
7	7.90	318.2942
8	7.92	290.2642
9	8.63	318.2959
10	9.15	437.1837
11	9.15	453.1628
12	9.69	335.2131

3.2.1.2) Shoots

3.2.1.2.1) Multivariate analysis of all groups

The PCA scores plot (Fig 9A) shows distinct separation between the control samples and the inoculated samples. It can immediately be seen that the control and T19 samples form separate clusters while the A07 and T29 samples cluster together. This differential clustering summarises the data as follows: the control and inoculated samples show different metabolic states, however, the A07 and T29 samples likely show a similar metabolic state. The three principal components explain a total of 47.4% of the variation seen in the PCA scores plot.

To further validate the overview of the data seen in Fig 9A, PLS-DA was conducted. The enhanced separation seen in Fig 9B confirms the classification of these groups. None of the samples from any of the groups is misclassified and the same clustering observed in Fig 9A is observed in Fig 9B. Thus, it can be concluded that there is a metabolic difference between the control and the inoculated samples, however, there is no metabolic difference between A07 and T29.

.

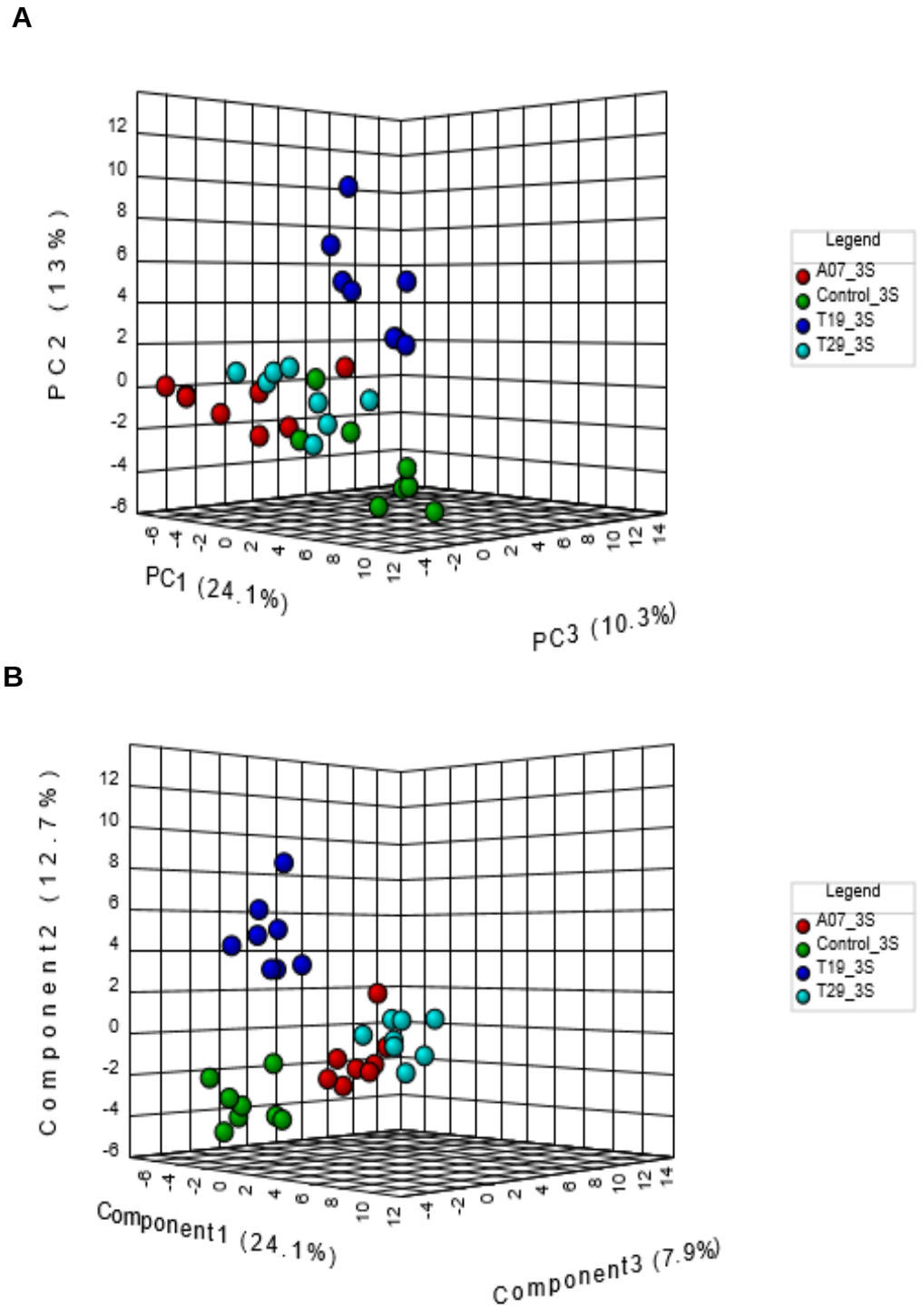


Figure 9: Unsupervised and supervised multivariate analysis of control and inoculated maize shoots harvested 3 dpi. (A) Unsupervised analysis: 3-Dimensional Principal Component Analysis (PCA) scores plot showing distribution of the groups among the three major principal components along the x (PC1), y (PC2) and z (PC3) axes. PC 1, 2 and 3 contribute 24.1, 13 and 10.3% respectively to the variation in the data. (B) Supervised analysis: 3-Dimensional Partial Least Squares Discriminant Analysis (PLS-DA) plot showing distribution of the groups among the three major PLS components (Component 1, 2 and 3). Components 1, 2 and 3 contribute 24.1, 12.7 and 7.9% respectively to the model. For each group n=8.

3.2.1.2.2) Univariate analysis of the Control group versus the T19 group

There are 56 significant ion features above the positive threshold, while there are 39 below the negative threshold in Fig 10. The 56 features are up-regulated in the T19 samples compared to the control samples. On the other hand, the 39 features are down-regulated in the T19 samples compared to the control. The top 20 features that showed significant fold changes are listed in Table 6. The full list of the features can be found in the appendix (Table S 3). All the features within the dotted lines represent features that did not have a fold change greater than the set threshold.

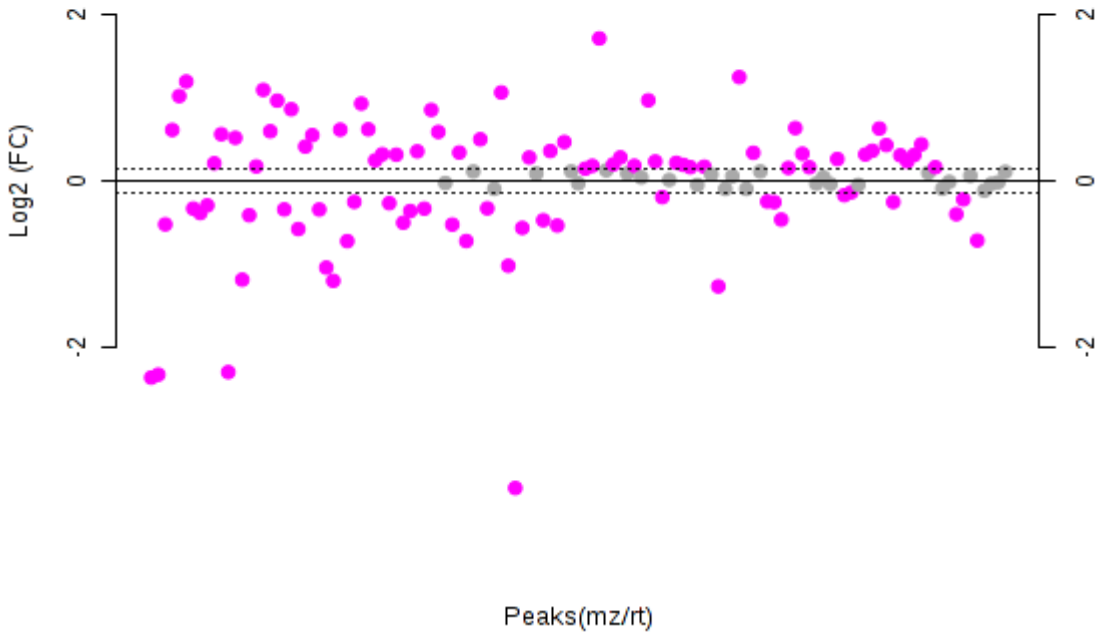


Figure 10: Fold change analysis between control and T19 inoculated maize shoots harvested 3 dpi. A threshold of 1.1 was used to select important features from the fold change analysis. The pink circles represent the ion features. Note the values are on log scale, so that both up-regulated and down-regulated features can be plotted in a symmetrical way.

Table 6: Top 20 important features identified by fold change analysis between the control and T19 inoculated maize roots harvested 3 dpi.

Feature number	Retention time (min)	*m/z*	Fold change	log_2(FC)
1	10.23	318.2349	0.0773	-3.6942
2	9.07	398.2365	0.1939	-2.3665
3	9.07	376.2534	0.1987	-2.3311
4	9.68	320.2518	0.2030	-2.3006
5	10.14	533.3585	0.4143	-1.2711
6	10.77	320.2508	0.4343	-1.2033
7	5.73	487.0781	0.4384	-1.1896
8	7.31	527.1505	0.4849	-1.0444
9	10.17	489.3329	0.4923	-1.0225
10	10.17	275.1973	1.8029	0.8503
11	7.70	212.0163	1.8126	0.8581
12	7.65	280.2002	1.9003	0.9262
13	7.70	167.9912	1.9484	0.9623
14	1.25	527.1536	1.9529	0.9656
15	5.71	409.1781	2.0239	1.0171
16	9.94	389.2634	2.0856	1.0605
17	10.44	577.3898	2.1301	1.0909
18	7.16	351.2090	2.2838	1.1914
19	8.96	518.3188	2.3690	1.2443
20	5.49	605.1098	3.2708	1.7096

3.2.1.2.3) Multivariate analysis of the Control group versus the T19 group

Further analysis was performed on the control and T19 groups. As seen in Fig 11A, the PCA shows separation between the control and T19 groups. The three principal components explain 51.5% of the total variation between the control and T19 group. The separation is further enhanced in the OPLS-DA scores plot (Fig 11B). Despite the clear separation between the control and T19 groups, there is also a bit of within group variation; however, none of the points are outliers. Discriminating features between the control and T19 groups can be seen in Fig 11C, and are listed in Table 7.

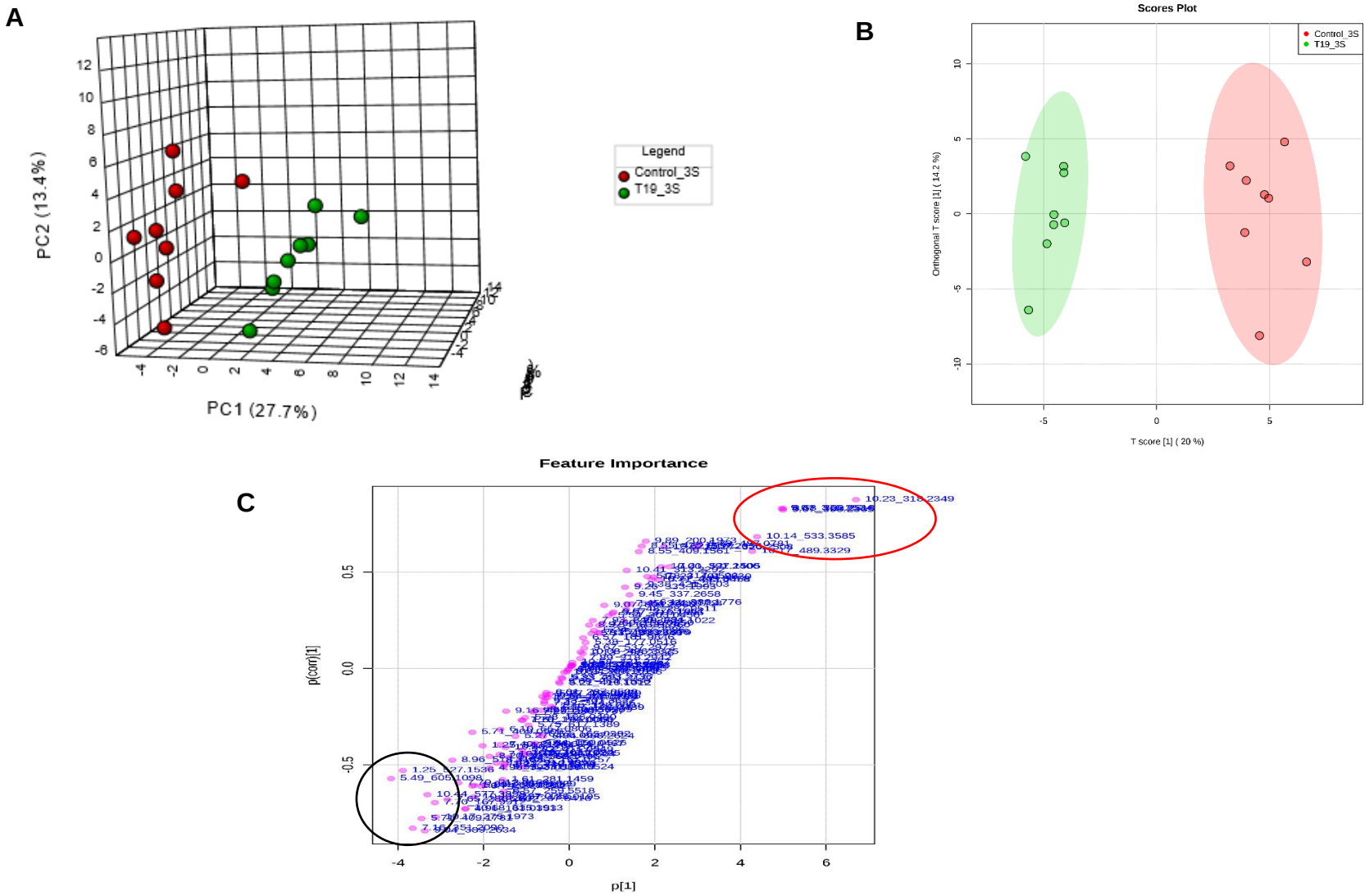


Figure 11: Multivariate analysis of control and T19 inoculated maize shoots harvested 3 dpi. (A) 3D PCA plot showing clustering of the control and T19 groups. PC 1, 2 and 3 contribute 27.7, 13.4 and 10.4% respectively to the variation in the data. (B) OPLS-DA scores plot between the control and T19 shoots. The predictive (x) axis explains 20% of the variation while the orthogonal (y) axis explains 14.2% of the variation. (C) OPLS-DA S-plot showing discriminating variables between the control (red circle) and T19 samples (black circle).

Table 7: Important features identified from the OPLS-DA S-plot.

Feature number	Retention time (min)	*m/z*
1	1.25	527.1536
2	5.49	605.1098
3	5.71	409.1781
4	7.16	351.2090
5	9.07	398.2365
6	9.07	376.2534
7	9.68	320.2518
8	9.94	389.2634
9	10.14	533.3585
10	10.17	489.3329
11	10.23	318.2349

3.2.2) Nine days post inoculation

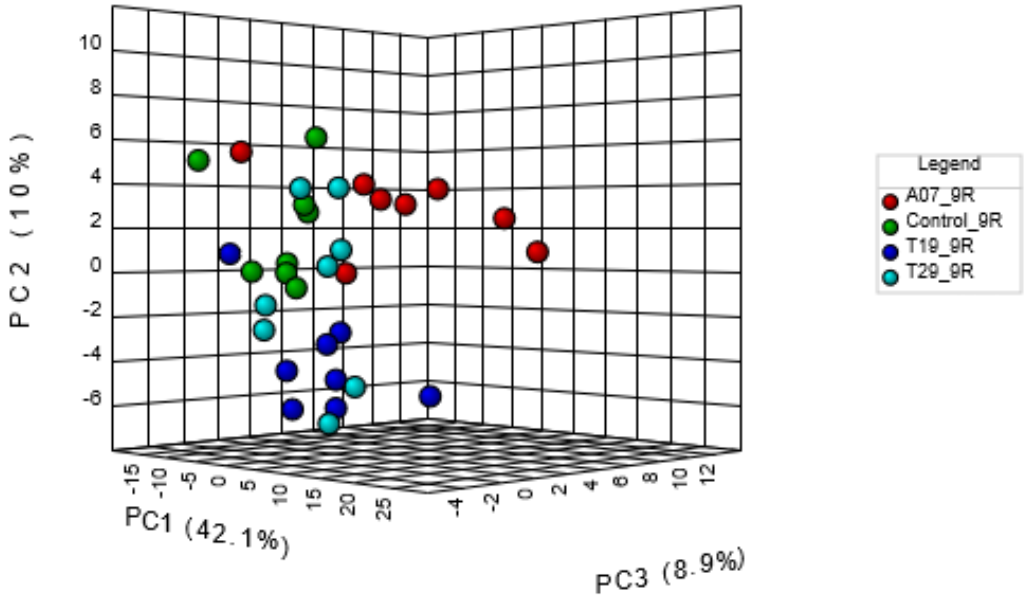
3.2.2.1) Roots

3.2.2.1.1) Multivariate analysis for all groups

The points in the PCA of the 9 dpi harvested roots (Fig 12A) are not as dispersed in comparison to the results in Fig 6A. The three principal components explain 61% of the variation seen in the PCA. Some of the samples are quite dispersed, which resulted in some samples being wrongly grouped. Despite the dispersion and wrong grouping of samples, there are still some clustering trends that can be observed. Samples from the T29 and A07 groups are quite dispersed, whereas samples from the control and T19 groups cluster more tightly than the former groups. The PLS-DA scores plot gives a more enhanced separation of the data (Fig 12B). The groups are appropriately clustered; however, of all the groups, the T29 samples have the most within group variation.

From the scores plots in Fig 12, none of the inoculated groups cluster with the control. It can be concluded that the differential clustering between all the groups represent different metabolic states. The T19 and T29 groups cluster very closely to one another and the control group clusters the furthest from all the other groups in Fig 12B, as expected.

A



B

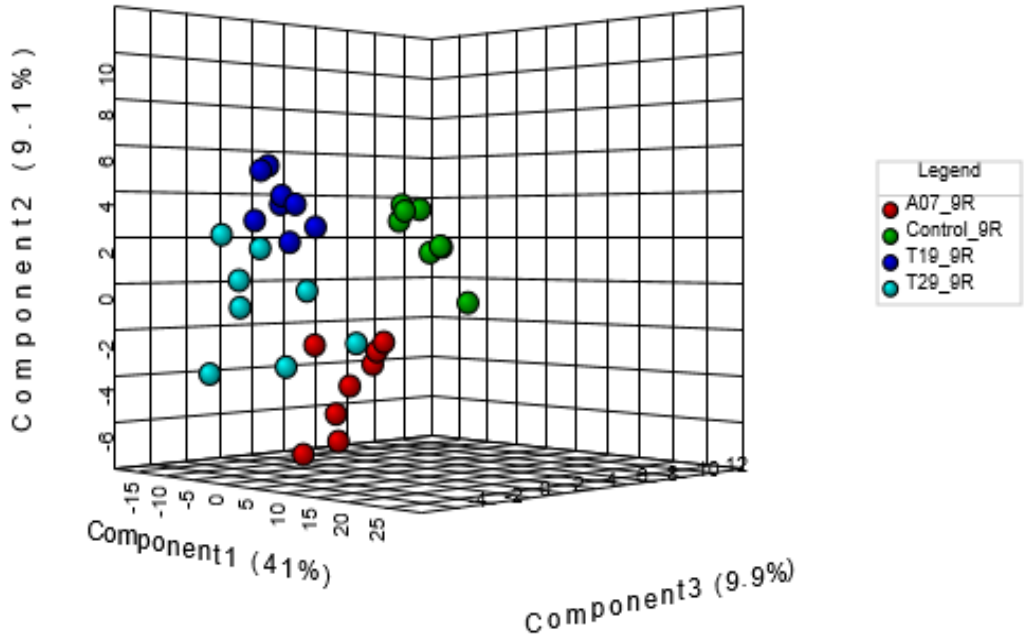


Figure 12: Unsupervised and supervised multivariate analysis of control and inoculated maize roots harvested 9 dpi. (A) Unsupervised analysis: 3-Dimensional Principal Component Analysis (PCA) scores plot showing distribution of the groups among the three major principal components along the x (PC1), y (PC2) and z (PC3) axes. PC 1, 2 and 3 contribute 42.1,10 and 8.9% respectively to the variation in the data. (B) Supervised analysis: 3-Dimensional Partial Least Squares Discriminant Analysis (PLS-DA) plot showing distribution of the groups among the three major PLS components (Component 1, 2 and 3). Components 1, 2 and 3 contribute 41, 9.1 and 9.9% respectively to the model. For each group n=8.

3.2.2.1.2) Univariate analysis of Control groups versus the T29 group

There are 61 significant ion features above the positive threshold, while there are 35 below the negative threshold in Fig 13. The 61 features are up-regulated in the T29 samples compared to the control samples. On the other hand, the 35 features are down-regulated in the T29 samples compared to the control samples. The top 20 features that showed significant fold changes are listed in Table 8. The full list of the features can be found in the appendix (Table S 4). All the features within the dotted lines represent features that did not have a fold change greater than the set threshold.

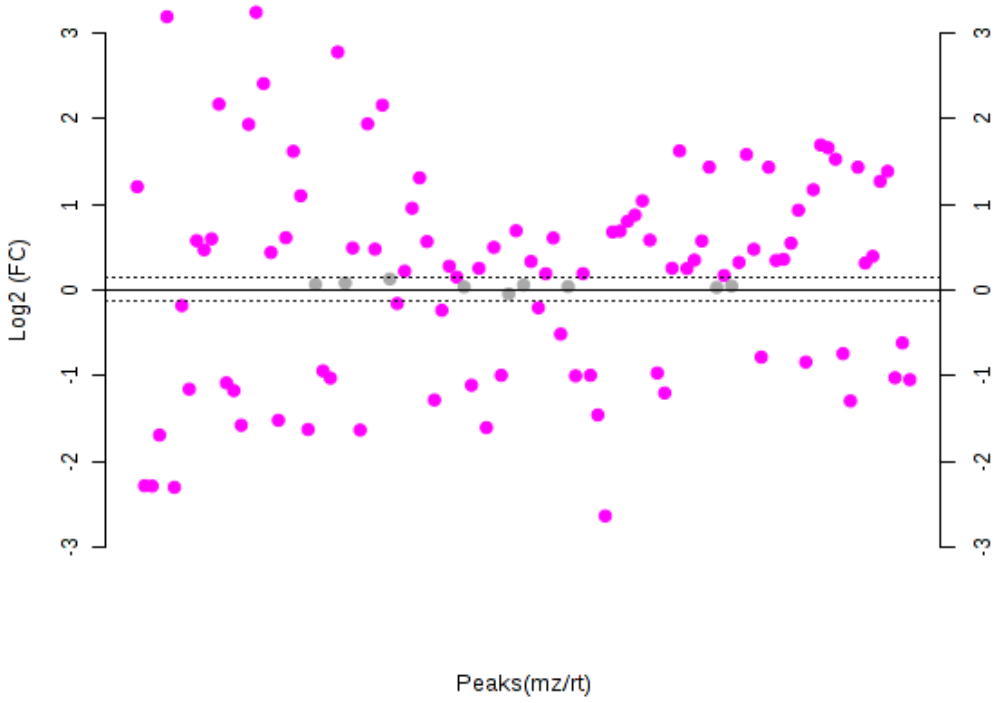


Figure 13: Fold change analysis between control and T29 inoculated maize roots harvested 9 dpi. A threshold of 1.1 was used to select important features from the fold change analysis. The pink circles represent the ion features up-regulated (above the threshold) and down-regulated (below the threshold) in T29 compared to the control. Note the values are on log scale, so that both up-regulated and down-regulated features can be plotted in a symmetrical way.

Table 8: Top 20 important features identified by fold change analysis between the control and T29 inoculated maize roots harvested 9 dpi.

Feature number	Retention time (min)	*m/z*	Fold change	log_2(FC)
1	7.00	602.2981	0.1606	-2.6388
2	9.48	184.0690	0.2024	-2.3048
3	9.48	542.3155	0.2045	-2.2896
4	9.48	520.3328	0.2050	-2.2864
5	7.58	329.1242	0.3090	-1.6945
6	5.70	440.1095	0.3216	-1.6366
7	5.70	196.0568	0.3232	-1.6296
8	9.65	542.3103	0.3282	-1.6074
9	1.50	262.1247	3.0678	1.6172
10	9.05	295.2235	3.0785	1.6222
11	5.92	264.0825	3.1568	1.6584
12	6.83	221.0766	3.2303	1.6917
13	7.45	353.2286	3.8144	1.9315
14	1.40	136.0607	3.8303	1.9375
15	9.07	398.2347	4.4576	2.1563
16	7.40	241.0441	4.4921	2.1674
17	1.13	266.1576	5.3085	2.4083
18	9.07	376.2529	6.8505	2.7762
19	5.80	373.1020	9.1169	3.1885
20	9.05	277.2125	9.4457	3.2397

3.2.2.1.2) Multivariate analysis of the Control group versus the T29 group

Multivariate analysis was performed on just the control and T29 groups. A PCA and OPLS-DA was performed on the data. Figure 14A shows the results of the PCA. The principal components in Fig 14A explains 72% of the variation observed in the data. Majority of the control samples are tightly clustered together except for one. On the other hand, the samples from the T29 group have equally separated into two clusters. Overall there is a distinct separation between the control and T29 samples. Supervised OPLS-DA in Fig 14B correctly classified the samples, however the same grouping trends can still be observed. There is a clear within group separation in the T29 group. The discriminating features circled in Fig 14C are listed in Table 9.

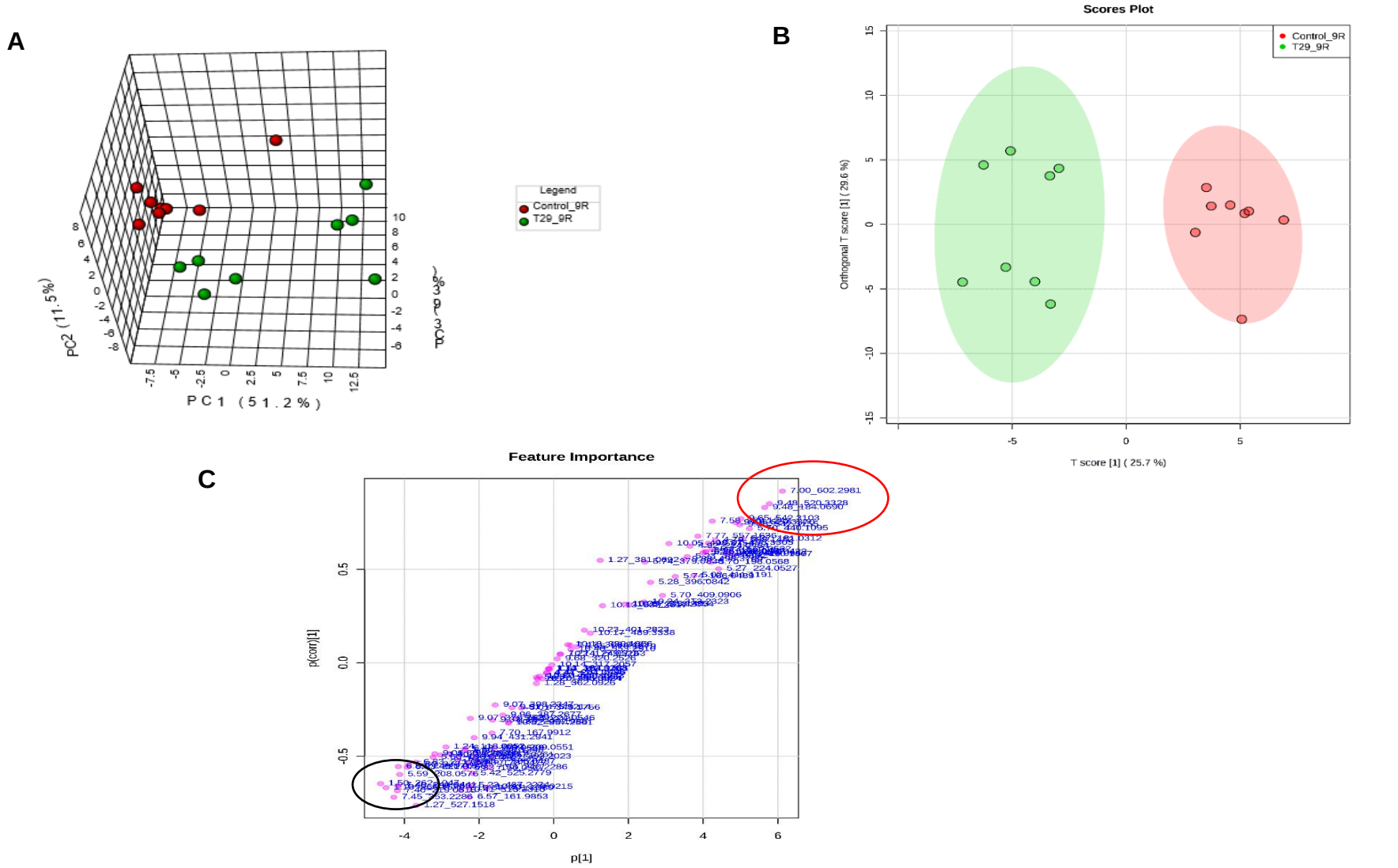


Figure 14: Multivariate analysis of control and T29 roots inoculated maize roots harvested 9 dpi. (A) 3D PCA plot showing clustering of the control and T29 groups. PC 1, 2 and 3 contribute 51.2, 11.5 and 9.3% respectively to the variation in the data. (B) OPLS-DA scores plot between the control and T29 roots. The predictive (x) axis explains 25.7% of the variation while the orthogonal (y) axis explains 29.6% of the variation. (C) OPLS-DA S-plot showing discriminating variables between the control (red circle) and T29 samples (black circle).

Table 9: Important features identified from the OPLS-DA S-plot.

Feature number	Retention time (min)	*m/z*
1	1.13	266.1576
2	1.50	262.1247
3	7.00	602.2981
4	7.40	241.0441
5	7.45	353.2286
6	9.05	277.2125
7	9.48	184.0690
8	9.48	520.3328

3.2.2.2) Shoots

3.2.2.2.1) Multivariate analysis of all groups

At 9 dpi, there is a greater degree of spread in the PCA (Fig 15A) compared to the results in Fig 9A of the shoots harvested 3 dpi. The principal components in Fig 9A explain about 60% of the variation in the data. The T29 samples appear to be spread between the T19 and A07 groups. The control, T19 and A07 groups show tighter clustering when compared to the T29 group. There are, however, two T19 samples that group closely with the control samples. Supervised analysis (Fig 15B) goes on to further show the samples to be quite spread out. The A07 samples are very close to the control samples, and of all the groups, T19 clusters the most tightly from the control.

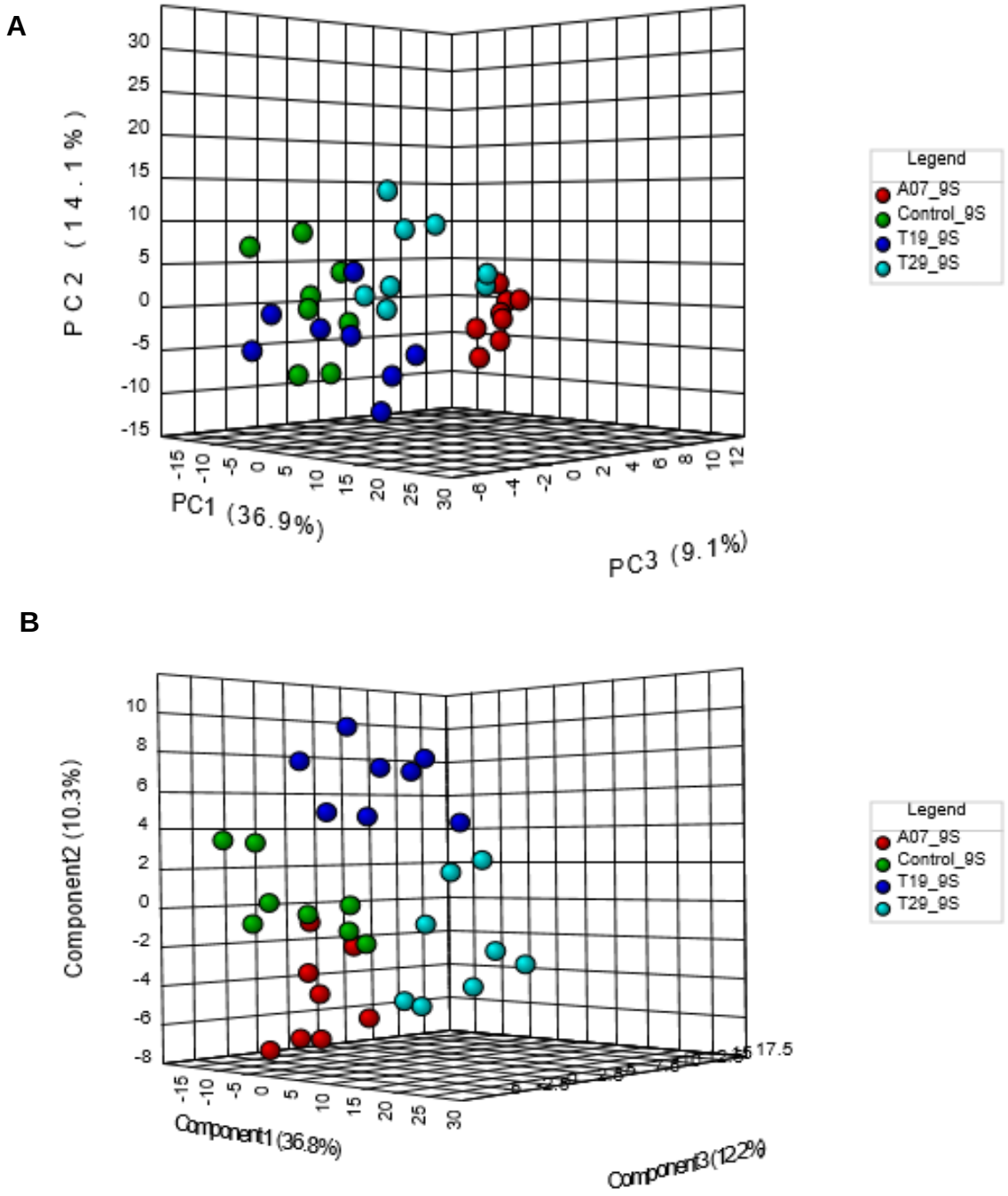


Figure 15: Unsupervised and supervised multivariate analysis of control and inoculated shoots obtained 9 dpi. (A) Unsupervised analysis: 3-Dimensional Principal Component Analysis (PCA) scores plot showing distribution of the groups among the three major principal components along the x (PC1), y (PC2) and z (PC3) axes. PC 1, 2 and 3 contribute 36.9, 14.1 and 9.1% respectively to the variation in the data. (B) Supervised analysis: 3-Dimensional Partial Least Squares Discriminant Analysis (PLS-DA) plot showing distribution of the groups among the three major PLS components (Component 1, 2 and 3). Components 1, 2 and 3 contribute 36.8, 10.3 and 12.2% respectively to the model. For each group n=8.

3.2.2.2.2) Univariate analysis of the Control group versus the T19 group

There are 154 significant ion features above the positive threshold, while there are 64 below the negative threshold in Fig 16. The 154 features are up-regulated in the T19 samples compared to the control samples while the 64 features are down-regulated in the T19 samples. The top 20 features that showed significant fold changes are listed in Table 10. The complete list of all the features can be found in the appendix (Table S 5). All the features within the dotted lines represent features that did not have a fold change greater than the set threshold.

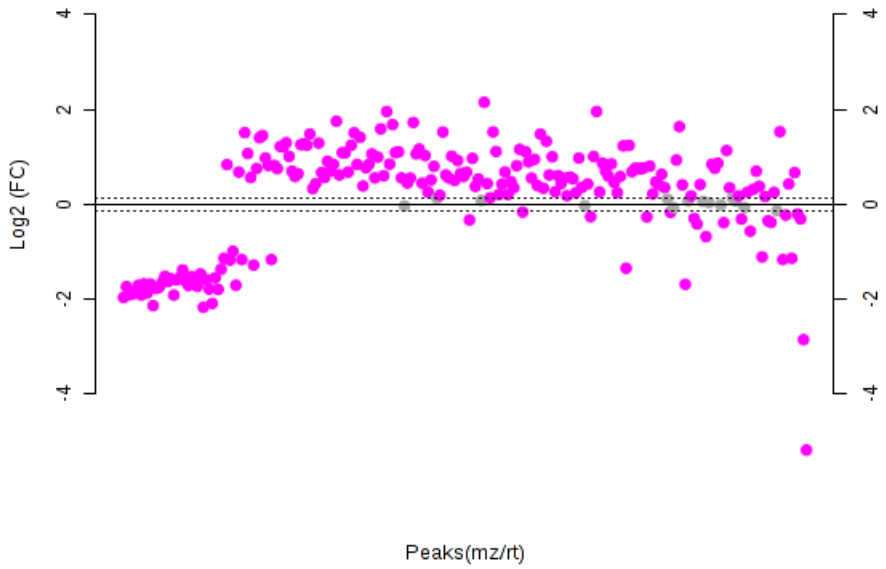


Figure 16: Fold change analysis between control and T19 shoots harvested 9 dpi. A threshold of 1.1 was used to select important features from the fold change analysis. The pink circles represent the ion features up-regulated (above the threshold) and down-regulated (below the threshold) in T19 compared to the control Note the values are on log scale, so that both up-regulated and down-regulated features can be plotted in a symmetrical way.

Table 10: Top 20 important features identified by fold change analysis between the control and T19 inoculated maize roots harvested 9 dpi.

Feature number	Retention time (min)	*m/z*	Fold change	log_2(FC)
1	10.49	388.3856	0.0277	-5.1724
2	9.04	376.2515	0.1388	-2.8491
3	9.79	651.4135	0.2226	-2.1672
4	9.81	602.4363	0.2281	-2.1320
5	10.29	748.5263	0.2355	-2.0861
6	9.81	607.3915	0.2568	-1.9613
7	9.95	387.2637	0.2660	-1.9105
8	10.37	665.4305	0.2664	-1.9082
9	10.47	511.3722	0.2672	-1.9039
10	9.90	475.3155	0.2696	-1.8910
11	9.93	431.2899	0.2745	-1.8649
12	10.96	679.4484	0.2837	-1.8178
13	9.84	563.3639	0.2890	-1.7910
14	9.87	519.3421	0.2904	-1.7838
15	10.47	528.3983	0.2932	-1.7698
16	10.40	621.4063	0.2977	-1.7479
17	4.96	393.0332	3.3654	1.7508
18	1.61	276.1375	3.8776	1.9552
19	1.18	247.9078	3.8780	1.9553
20	1.61	135.0278	4.4373	2.1497

3.2.2.2.3) Multivariate analysis of the Control group versus the T19 group

The separation between the control and T19 groups in Fig 17A is similar to the separation seen in Fig 15A. The PCA scores plot shows a distinction between the two groups despite two samples from the T19 group tending more to the control group. The principal components in Fig 15A explain 69.1% of the variation observed. Supervised OPLS-DA provides an enhanced view of the separation; samples are not misclassified in Fig 17B. The OPLS-DA S-plot in Fig 17C gives an indication of what features cause the separation between the control and T19 groups, which are listed in Table 11.

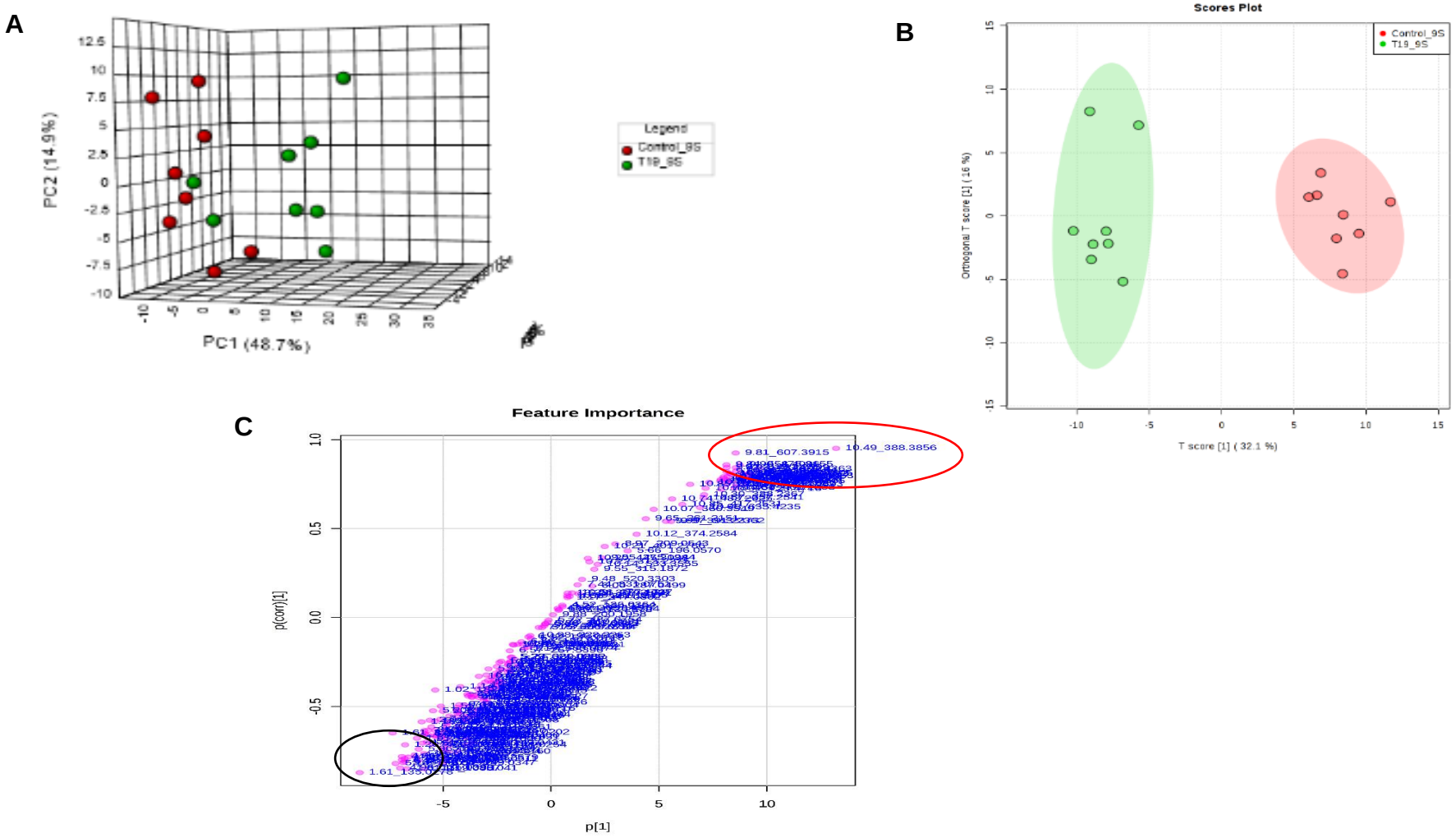


Figure 17: Multivariate analysis of control and T19 shoots obtained 9 dpi. (A) 3D PCA plot showing clustering of the control and T19 groups. PC 1, 2 and 3 contribute 48.7, 14.9 and 5.8% respectively to the variation in the data. (B) OPLS-DA scores plot between the control and T19 shoots. The predictive (x) axis explains 32.1% of the variation while the orthogonal (y) axis explains 16% of the variation. (C) OPLS-DA S-plot showing discriminating variables between the control (red circle) and T19 samples (black circle).

Table 11: Important features identified from the OPLS-DA S-plot.

Feature number	Retention time (min)	*m/z*
1	1.25	543.1219
2	1.61	135.0278
3	4.96	135.0399
4	5.38	177.0495
5	5.38	407.0499
6	9.81	602.4363
7	9.81	607.3915
8	9.84	558.4096
9	9.87	519.3421
10	9.90	475.3155
11	9.93	431.2899
12	10.49	388.3856

3.2.3) Metabolic pathways

The Metaboanalyst MS Peaks to Pathways module aims to predict biochemical pathways using MS data. The univariate results of the 9 dpi roots and shoots were used to find putative metabolic pathways that were affected by the T29 and T19 strains in the roots and shoots respectively. The output of the pathway analysis is graphically represented in a bubble chart and summarised in a ranked table, as seen in the next sections.

3.2.3.1) Pathways affected by T29 inoculation

The bubbles/circles in Fig 18 represent different metabolic pathways. Each circle is coloured according to the p-value, and its size is a reflection of the enrichment factor of that pathway based on the input data. The enrichment factor of a pathway is calculated as the ratio between the number of significant pathway hits and the expected number of compound hits within the pathway. The p-value refers to the significant changes of the metabolites in their corresponding pathways. A low p-value and high enrichment factor will be represented as a large and darkly coloured bubble/circle. The circles that are darkly coloured and larger in size represent the most significant and enriched pathways and would be of more interest for further analysis. All the pathways are listed in Table 12 and are ranked according to their p-value.

The MS peaks to pathways module only labelled the top five pathways in the summary plot. Fig 18 shows that alanine, aspartate and glutamate metabolism; inositol phosphate metabolism and carbon fixation in photosynthetic organisms were the most enriched and significant pathways in the roots from the maize seedlings inoculated with strain T29. On the other hand, Fig 18 shows that aminoacyl t-RNA biosynthesis, and amino sugar and nucleotide sugar metabolism, although significant, were not as enriched as the other three pathways; as represented by their small yet darkly coloured bubbles. Overall, Fig 18 shows that aminoacyl t-RNA biosynthesis is the least enriched out of all five top pathways (the ratio between the significant pathway hits and expected hits was very small). All predicted hits for the top pathways are listed in Table 13.

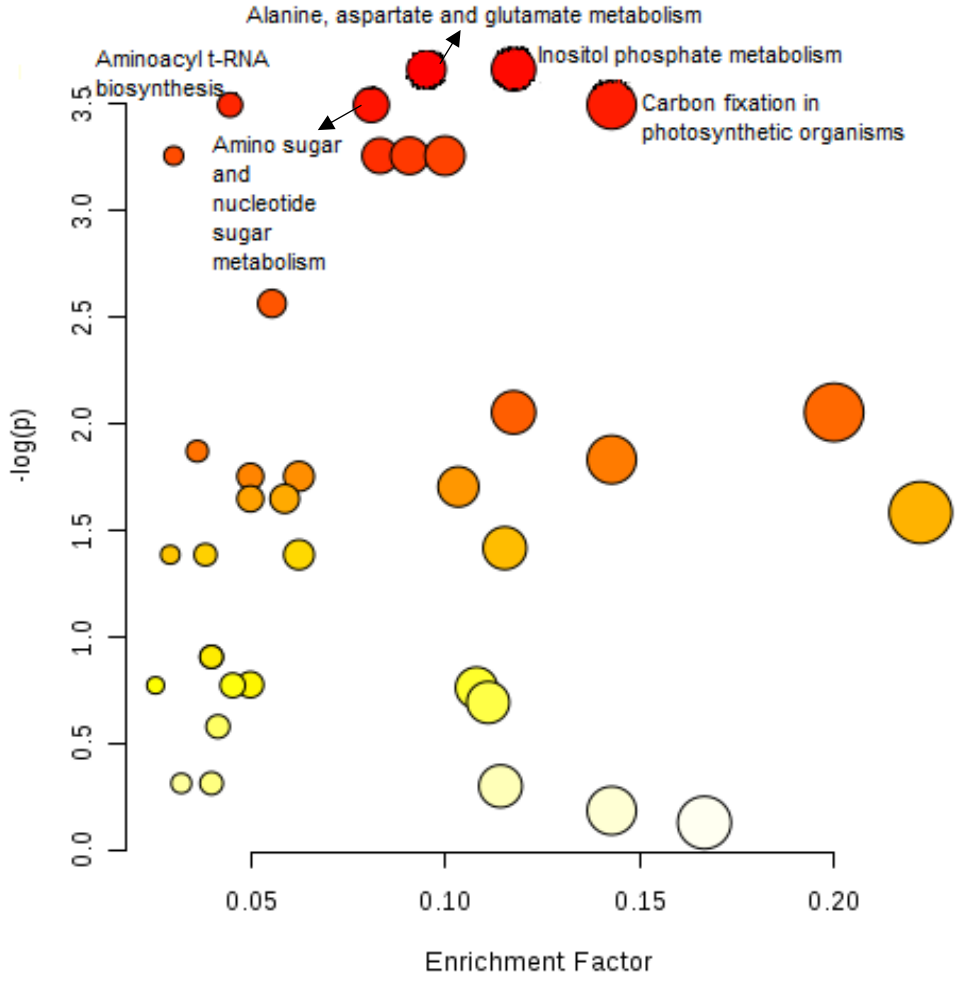


Figure 18: Summary of pathway analysis of features that showed a significant fold change in the T29 inoculated maize roots harvested 9 dpi. The pathway summary plot displays all matched pathways as bubbles/circles. The colour and size of each circle corresponds to its p-value and enrichment factor, respectively. The lower the p-value then the darker the colour. The higher the enrichment factor then the larger the circle.

Table 12: Results of the pathway analysis of the T29 inoculated maize roots harvested 9 dpi. The listed pathways are enriched in the T29 inoculated maize roots, and are ranked by p-value.

Pathway	Pathway total	Hits	P-value
Alanine, aspartate and glutamate metabolism	21	2	0.0256
Inositol phosphate metabolism	17	2	0.0256
Amino sugar and nucleotide sugar metabolism	37	3	0.0303
Carbon fixation in photosynthetic organisms	21	3	0.0303
Aminoacyl t-RNA biosynthesis	67	3	0.0303
beta-Alanine metabolism	12	1	0.0385
Cyanoamino acid metabolism	11	1	0.0385
Nicotinate and nicotinamide metabolism	10	1	0.0385
Porphyrin and chlorophyll metabolism	33	1	0.0385
Tyrosine metabolism	18	1	0.0769
Pentose phosphate pathway	17	2	0.1282
Pentose and glucuronate interconversions	10	2	0.1282
Purine metabolism	55	2	0.1538
Biotin metabolism	7	1	0.1600
Citrate cycle (TCA cycle)	20	1	0.1731
Zeatin biosynthesis	16	1	0.1731
Glycine, serine and threonine metabolism	29	3	0.1818
Pyruvate metabolism	20	1	0.1923
Glyoxylate and dicarboxylate metabolism	17	1	0.1923
Lysine biosynthesis	9	2	0.2051
Galactose metabolism	26	3	0.2424
Valine, leucine and isoleucine degradation	34	1	0.2500
Valine, leucine and isoleucine biosynthesis	26	1	0.2500
Pantothenate and CoA biosynthesis	16	1	0.2500
Glycolysis or Gluconeogenesis	25	1	0.4038
Starch and sucrose metabolism	25	1	0.4038
Butanoate metabolism	20	1	0.4600
Pyrimidine metabolism	39	1	0.4615
Phenylalanine, tyrosine and tryptophan biosynthesis	22	1	0.4615
Arginine and proline metabolism	37	4	0.4667
Riboflavin metabolism	9	1	0.5000
Terpenoid backbone biosynthesis	24	1	0.5600
Glycerophospholipid metabolism	25	1	0.7308
Phenylpropanoid biosynthesis	31	1	0.7308
Cysteine and methionine metabolism	35	4	0.7407
Ascorbate and aldarate metabolism	14	2	0.8308
Fructose and mannose metabolism	18	3	0.8788

Table 13: Pathway hits from the top five pathways affected by T29 inoculation in the maize roots.

Pathway	Pathway hits
Alanine, aspartate and glutamate metabolism	L-Aspartate* Fumarate
Inositol phosphate metabolism	Inositol Glucuronate
Amino sugar and nucleotide sugar metabolism	Galactose α-D-Glucose L-Arabinose D-Mannose
Carbon fixation in photosynthetic organisms	L-Malic acid L-Aspartate* D-Erythrose 4-phosphate
Aminoacyl t-RNA biosynthesis	L-Aspartate* L-Methionine L-Valine L-Proline

* The same hit.

3.2.3.2) Pathways affected by T19 inoculation

The largest and most darkly coloured circles in Fig 19 represent the most significant and enriched pathways in the shoots from the maize seedlings inoculated with strain T19. All the putative pathways affected by T19 are listed in Table 14. A number of these pathways were also observed in Table 12. The same pathways listed in tables 12 and 14 may indicate that both T19 and T29 stimulate similar pathways but to different degrees.

The most significant and enriched pathway as seen in Fig 19 is the citrate cycle (TCA cycle). Fig 19 shows that pyrimidine metabolism is the most enriched pathway, but it has a higher p-value compared to the citrate cycle. Out of all the top five pathways, alanine, aspartate and glutamate metabolism has the highest p-value. As seen in Fig 19, alanine, aspartate and glutamate metabolism is also quite enriched after the citrate cycle and pyrimidine metabolism. Vitamin B6 and nicotinate and nicotinamide metabolism are not the most enriched pathways among the top five pathways but are quite significant, as they have the lowest p-values after the citrate cycle as seen in Table 14. All predicted hits for the top pathways are listed in Table 15.

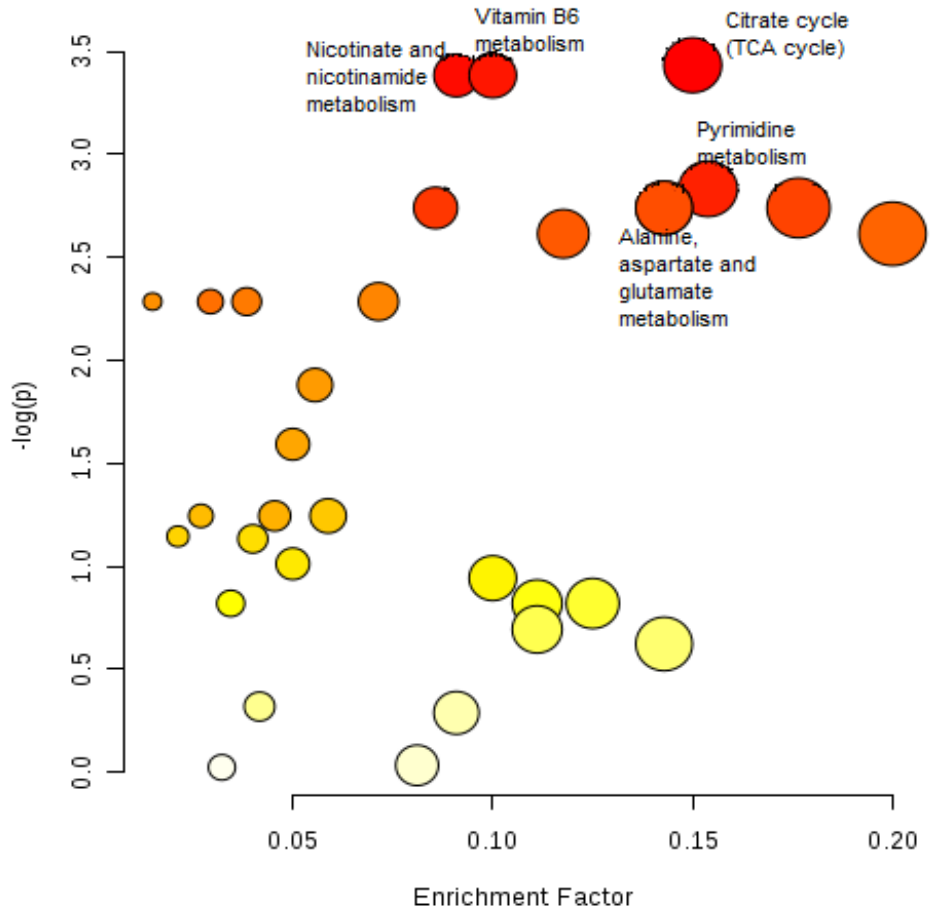


Figure 19: Summary of pathway analysis of features that showed a significant fold change in the T19 inoculated maize shoots harvested 9 dpi. The pathway summary plot displays all matched pathways as circles. The colour and size of each circle corresponds to its p-value and enrichment factor, respectively. Thus, a lower p-value means a darker colour. The higher the enrichment factor then the larger the circle.

Table 14: Results of the pathway analysis of the T19 inoculated maize shoots harvested 9 dpi. The listed pathways are enriched in the T19 inoculated maize shoots, and are ranked by p-value.

Pathway	Pathway total	Hits	P-value
Citrate cycle (TCA cycle)	20	3	0.0323
Vitamin B6 metabolism	11	1	0.0339
Nicotinate and nicotinamide metabolism	10	1	0.0339
Pyrimidine metabolism	39	6	0.0588
Alanine, aspartate and glutamate metabolism	21	3	0.0645
Cysteine and methionine metabolism	35	3	0.0645
Glyoxylate and dicarboxylate metabolism	17	3	0.0645
Carbon fixation in photosynthetic organisms	21	3	0.0645
Pentose phosphate pathway	17	2	0.0732
Pentose and glucuronate interconversions	10	2	0.0732
Valine, leucine and isoleucine degradation	34	1	0.1017
Glutathione metabolism	26	1	0.1017
Propanoate metabolism	14	1	0.1017
Aminoacyl-tRNA biosynthesis	67	1	0.1017
Tyrosine metabolism	18	1	0.1525
Pyruvate metabolism	20	1	0.2034
Phenylalanine, tyrosine and tryptophan biosynthesis	22	1	0.2881
Amino sugar and nucleotide sugar metabolism	37	1	0.2881
Inositol phosphate metabolism	17	1	0.2881
Fatty acid biosynthesis	47	1	0.3182
Glycerophospholipid metabolism	25	1	0.3220
Butanoate metabolism	20	1	0.3636
Thiamine metabolism	10	1	0.3898
Glycine, serine and threonine metabolism	29	1	0.4407
Lysine biosynthesis	9	1	0.4407
One carbon pool by folate	8	1	0.4407
Riboflavin metabolism	9	1	0.5000
Ascorbate and aldarate metabolism	14	2	0.5366
Terpenoid backbone biosynthesis	24	1	0.7273
Methane metabolism	11	1	0.7500
Arginine and proline metabolism	37	3	0.9677
Phenylpropanoid biosynthesis	31	1	0.9773

Table 15: Pathway hits from the top five pathways affected by T19 inoculation in the maize shoots.

Pathway	Pathway hits
Citrate cycle (TCA cycle)	2-Oxoglutarate* Isocitrate L-Malic acid/Malate Citrate Fumarate*
Vitamin B6 metabolism	Pyridoxal 5-phosphate
Nicotinate and nicotinamide metabolism	Nicotinamide adenine dinucleotide
Pyrimidine metabolism	(S)-Dihydroorotate Uridine 5'-monophosphate Cytidine-5'-monophosphate N-carbamoyl-L-aspartate* Orotate Uridine
Alanine, aspartate and glutamate metabolism	2-Oxoglutarate* Fumarate* N-carbamoyl-L-aspartate*

*The same hit.

3.2.4) Metabolites of interest

As with the metabolic pathways in section 3.2.3, more focus will be placed on the discriminatory features found 9 dpi in the roots and shoots. The metabolic features that showed discriminating ability between the control and T29 inoculated roots, and the control and T19 inoculated shoots are listed in tables 9 and 11 respectively. These features are the result of multivariate analysis, and their aim is to show the difference between the control and inoculated plants.

3.2.4.1) Discriminating metabolic features between the control and T29 inoculated roots

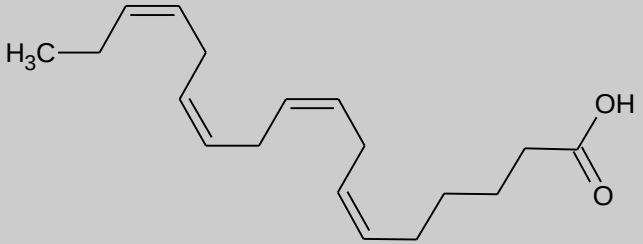
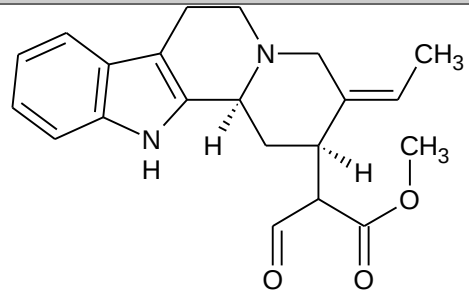
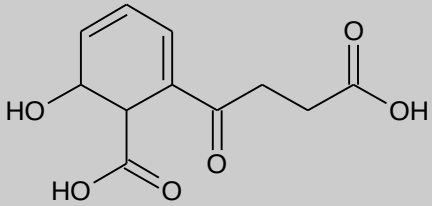
A total of seven ion features were putatively identified using different metabolite databases. The ion features that could not be identified did not have any successful matches across the databases that made metabolic sense with regards to the maize or plant metabolome. As seen in Table 16, four of the ion features are up-regulated in the T29 roots compared to the control, whereas, three of the features are down-regulated in the inoculated roots compared to the control. Another point to note would be that two of the ion features have the same retention time. This could be a clear indication of co-elution.

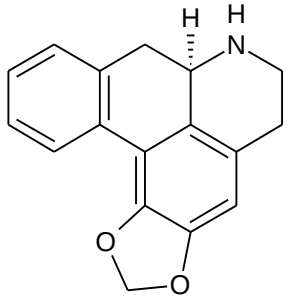
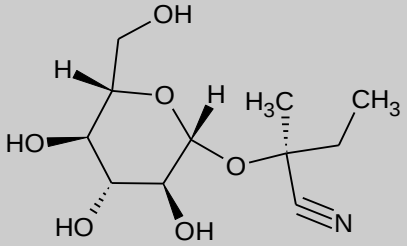
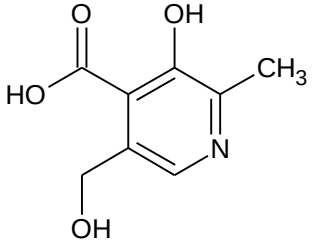
Information regarding the putatively identified metabolites can be seen in Table 17. The identified metabolites span a range of compound classes, and it should be noted that each metabolite can be involved in more than one metabolic pathway, which may not be listed in Table 15. Some of the discriminating metabolites fall under the category of secondary metabolites.

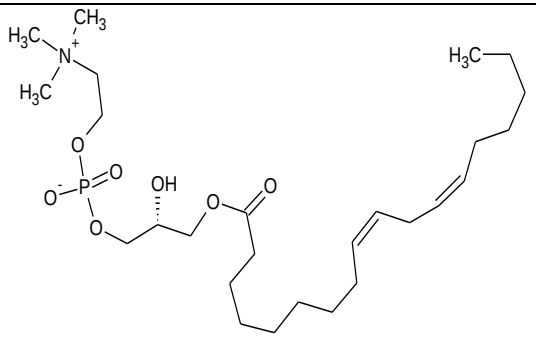
Table 16: List of discriminating metabolic features (*m/z* in the positive mode and retention time) found in the T29 inoculated roots along with their corresponding identity and regulated status.

Retention time (min)	*m/z*	ID	Regulation
9.05	277.2125	Stearidonic acid	Up
7.45	353.2286	Geissoschizine	Up
7.40	241.0441	2-succinyl-6-hydroxycyclohexa-2,4-diene-1-carboxylic acid	Up
1.13	266.1576	Anonaine	Up
1.50	262.1247	Lotaustralin	Up
9.48	184.0690	4-pyridoxic acid	Down
9.48	520.3328	1-linoleoyl-sn-glycero-3-phosphocholine	Down
7.00	602.2981	n/a	Down

Table 17: Compound information of the discriminating metabolites between the control and the T29 inoculated roots.

Compound	Accurate mass (Da)	Molecular formula	Compound class	Pathway(s) involved	Structure
Stearidonic acid	276.2089	$C_{18}H_{28}O_2$	Fatty acid	alpha-linolenic acid metabolism, Biosynthesis of secondary metabolites	
Geissoschizine	352.1787	$C_{21}H_{24}N_2O_3$	Alkaloid	Indole alkaloid biosynthesis, Biosynthesis of secondary metabolites	
2-succinyl-6-hydroxycyclohexa-2,4-diene-1-carboxylic acid	240.0634	$C_{11}H_{12}O_6$	Gamma-keto acid	Ubiquinone and other terpenoid-quinone biosynthesis, secondary metabolite biosynthesis	

Anonaine	265.1103	$C_{17}H_{15}NO_2$	Alkaloid	Alkaloids derived from tyrosine	
Lotaustralin	261.1212	$C_{11}H_{19}NO_6$	Cyanogenic glycoside	Cyanoamino acid metabolism. Biosynthesis of secondary metabolites	
4-pyridoxic acid	183.0532	$C_8H_9NO_4$	Methylpyridine	Vitamin B6 metabolism	

1-linoleoyl-sn-glycero-3-phosphocholine	519.3325	$C_{26}H_{50}NO_7P$	Lysophosphatidylcholine	Glycerophospholipid biosynthesis	

3.2.4.2) Discriminating metabolic features between the control and T19 inoculated shoots

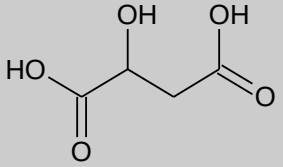
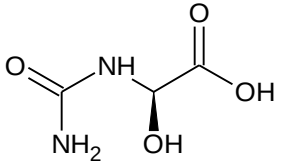
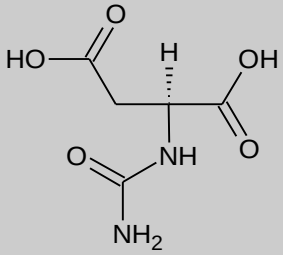
From the list of discriminating ion features (Table 11), only eight could be putatively identified (Table 18). The same reason mentioned in section 3.2.4.1 as to why a feature could not be identified also applies here. Five of the features were found to be up-regulated in the T19 shoots compared to the control, and seven were found to be down-regulated in the inoculated shoots compared to the control. The first two features in Table 18 appear to have very similar *m/z* values; however, they were found to be different features based on their different elution times and fragmentation patterns.

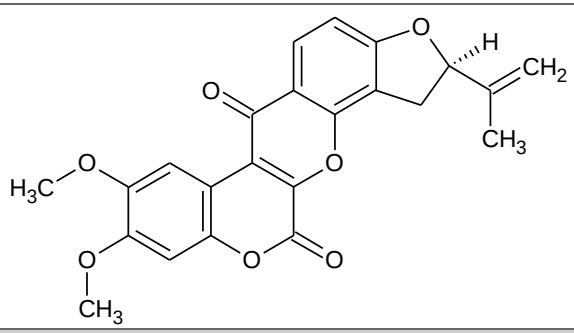
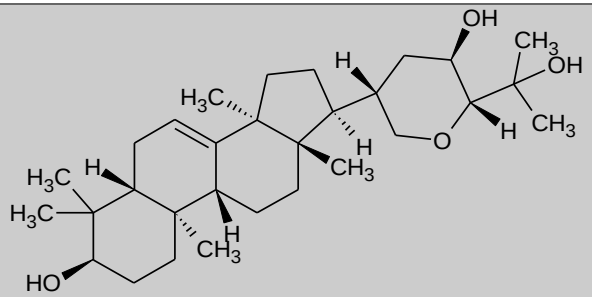
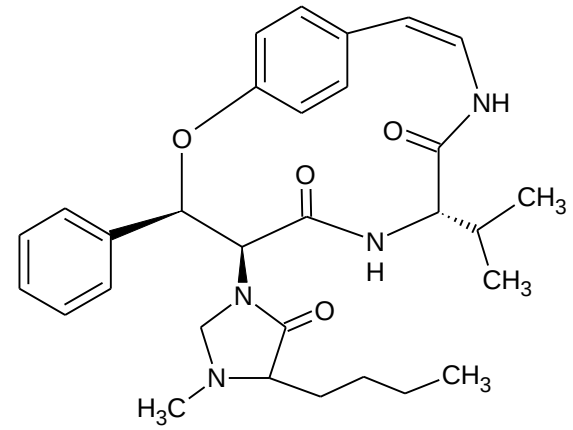
Additional information on the identified metabolites is recorded in Table 19. As with the metabolites identified in the roots, the metabolites in the shoots also span a wide range of compound classes. The first three metabolites can be grouped as primary metabolites, while the rest of the metabolites can be grouped together as secondary metabolites.

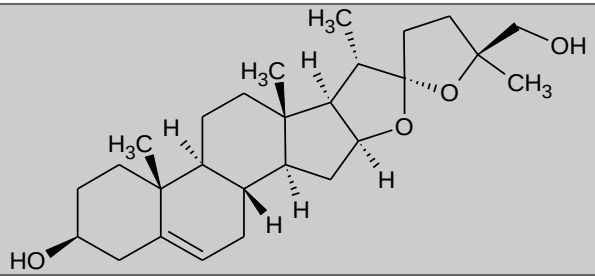
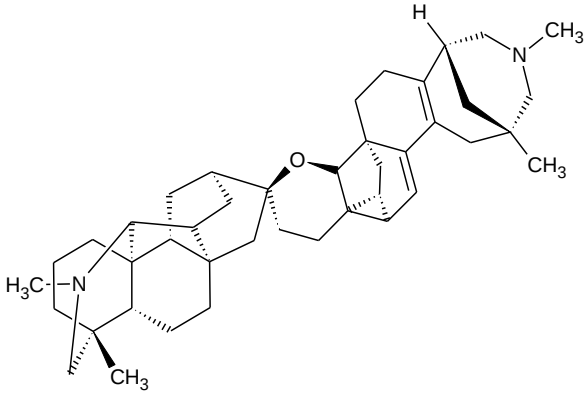
Table 18: List of discriminating metabolic features (*m/z* in the positive mode and retention time) found in the T19 inoculated shoots along with their corresponding identity and regulated status.

Retention time (min)	*m/z*	ID	Regulation
1.61	135.0278	Malic Acid	Up
4.96	135.0399	(S)-Ureidoglycolic acid	Up
5.38	177.0495	N-Carbamoyl-L-aspartic acid	Up
1.25	543.1219	n/a	Up
5.38	407.0499	Rotenonone	Up
9.90	475.3155	Sapelin A	Down
9.84	558.4096	n/a	Down
9.87	519.3421	Sativanine B	Down
9.81	602.4363	n/a	Down
9.93	431.2899	Nuatigenin	Down
10.49	388.3856	n/a	Down
9.81	607.3915	Staphidine	Down

Table 19: Compound information of the discriminating metabolites between the control and the T19 inoculated shoots.

Compound	Accurate mass (Da)	Molecular formula	Compound class	Pathway(s) involved	Structure
Malic acid	134.0215	$C_4H_6O_5$	Organic acid	Citrate cycle (TCA cycle), Carbon fixation in photosynthetic organisms, Biosynthesis of plant secondary metabolites	
(S)-Ureidoglycolic acid	134.0328	$C_3H_6N_2O_4$	Ureidocarboxylic acid	Purine metabolism	
N-Carbamoyl-L-aspartic acid	176.0433	$C_5H_8N_2O_5$	Aspartic acid derivative	Pyrimidine metabolism, Alanine, aspartate and glutamate metabolism	

Rotenonone	406.1052	$C_{23}H_{18}O_7$	Polyketide	Rotenoid flavonoid biosynthesis	
Sapelin A	474.3709	$C_{30}H_{50}O_4$	Terpenoid	Triterpenoid biosynthesis	
Sativanine B	518.2893	$C_{30}H_{38}N_4O_4$	Cyclopepetide alkaloid	Cyclopeptide alkaloid biosynthesis	

Nuatigenin	430.3083	$C_{27}H_{42}O_4$	Terpenoid	Terpenoid biosynthesis	
Staphidine	606.4549	$C_{42}H_{58}N_2O$	Alkaloid	Terpenoid alkaloid biosynthesis	

Chapter 4: Discussion

4.1) Effect of selected PGPR strains on the growth of maize seedlings

The first null hypothesis of this project stated that inoculation of maize seedlings with PGPR does not result in any significant increase (p=0.05) in root and shoot biomass, chlorophyll content, shoot length and stem diameter. Based on the results of the greenhouse trial, the null hypothesis cannot be fully rejected. As seen in Fig 4 (chapter 3), the root dry mass was the only growth parameter that was significantly affected by PGPR treatment, especially by treatment with strain T29. Likewise, statistically significant differences were not observed in all the growth parameters for the plants harvested 9 dpi; except in the chlorophyll content, stem diameter and shoot length (Fig 5). However, the results showed a tendency for strains T19 and T29 to be the most effective strains in stimulating growth. Harvesting the maize plants 3 and 9 dpi was too early to expect any significant results in the growth parameters. Despite the early harvest times, PGPR inoculation demonstrated a tendency to affect certain parameters in the early stages of maize growth. The results of the greenhouse trial do raise two major questions though, which shall be discussed in the subsequent paragraphs.

Why was the observed trend of increased growth after treatment with strains T19 and T29 not statistically significant? To answer this question, attention must be directed towards the sample size. The sample size for each group was selected as it was sufficient to perform statistical analysis, and it was a practical size to work with. A practical sample size in this context refers to a sample size that can be worked on without introducing significant error(s) in the experiment(s). The sample size becomes an area of interest as it is known that the larger a sample size is then the more likely it is for a significant result to be found in a study, if it exists (Thiese, Ronna, & Ott, 2016). This concept can explain the results of the greenhouse trial. One can expect more statistically significant results from a similar trial if the sample size for each group was significantly increased. On the other hand, in spite of the small sample size, it was observed that the T19 and T29 strains produced some promising results with regards to early maize growth.

Results of the greenhouse trial showed a trend of strain T19 being more effective in increasing the shoot length and shoot dry mass. Alternatively; the results showed that

strain T29 had a tendency of increasing the root dry mass of inoculated plants. Strain A07 was not as effective as strains T19 and T29. It was expected that strains T19 and T29 would be more effective promoting growth in the maize seedlings compared to A07. However, the manner in which T19 and T29 were effective is contrary to the results obtained in a previous study. In a study done by Rudolph *et al.* (2015), it was found that strain T19 promoted root length and had a tendency to enhance root dry mass, while strain T29 significantly enhanced shoot dry mass and shoot length (Rudolph *et al.*, 2015). The contradictory results may likely be due to the difference in PGPR inoculum application and the stage at which the inoculum was applied. In this study, the PGPR inoculum was applied as a drench treatment after the maize seedlings were pre-germinated. In the study by Rudolph *et al.,* 2015, the PGPR was applied as a seed treatment prior to germination. Despite the differences in application, the results from both studies showed that strains T19 and T29 have potential growth promoting effects on maize.

The second major question would be why did strain A07 underperform compared to strains T19 and T29? The most probable answer would be that the PGPR strains used in this study demonstrated a degree of plant-microbe specificity. Strains T19 and T29 appeared to show a preference to the maize, whereas A07 did not. There have been reports that the growth promoting effect of some PGPR may be highly specific to certain plant species, cultivar and genotype (Figueiredo, Seldin, De Araujo, & Mariano, 2010; Lucy *et al.*, 2004). For example, in a study by Kloepper *et al.,* 1980, it was found that four *Pseudomonas* strains promoted the growth of radish. However, only one of those strains could promote potato growth (Kloepper, Schroth, & Miller, 1980). A similar situation was observed in this study. As the maize growth was assessed under greenhouse conditions, most variables were controlled, to a certain extent, so a possible reason for the plant-PGPR specificity could be root exudate content. Root exudates play a key role in plant-microbe interactions and are capable of modifying the microbial community in the rhizosphere (Chaparro, Badri, Bakker, Sugiyama, Manter, & Vivanco, 2013). The phytochemical content of a plant's root exudate is capable of either supporting or repressing the growth of specific PGPR, which in turn either enhances or suppresses the growth promoting ability of that PGPR. It is likely that the root exudate of maize does not support the growth of strain A07 to the same extent as it does for strains T19 and T29.

The results of the greenhouse trial showed that strains T19 and T29 have a tendency to promote growth in maize seedlings. It is also possible that both T19 and T29 may have a synergistic growth promoting effect, even so, further study is still required to substantiate this. As the growth promoting abilities of both T19 and T29 had been established, it raised the question of how the maize seedlings responded to the PGPR on a metabolic level.

4.2) Effect of best performing PGPR strains on the metabolome of maize seedlings

The discussion on the metabolomics analysis will focus on the plants harvested 9 dpi. The second null hypothesis stated that PGPR inoculation would not alter the metabolome of maize seedlings either similarly or differentially in the roots and shoots. The second null hypothesis was rejected as it was observed from the results of the pathway analysis, different and similar pathways were influenced by strains T19 and T29 in the shoots and roots respectively. Additionally, the results of the multivariate analysis showed that different metabolites were affected by the two strains. Furthermore, null hypothesis 3 stated that inoculation with PGPR will not result in any significant difference (p=0.05) in the relative concentration of specific metabolites in maize. Null hypothesis 3 was rejected as metabolites were found between the control and treatment groups that were differentially regulated based on their relative concentration. The subsequent sections will answer the following questions:

- What do the pathways and metabolites have to do with plant growth?
- What do the pathways and metabolites indicate about the traits of strains T29 and T19?
- What does the differential regulation of the metabolites mean?
- What do the univariate and multivariate results mean together in a metabolomics context?

4.2.1) General pathways

From the results of the pathway analysis, there were a number of amino acid-related pathways affected in the roots from the T29 treated maize plants. In this section, the focus of the discussion will be on the top pathways affected in the roots and shoots of the T29 and T19 treated maize plants respectively.

In the roots from the T29 treated maize plants, three out of the five most significant pathways were amino acid-related. On the other hand, there was only one amino acid-related pathway out of the top five significant pathways in the shoots from the T19 treated maize plants. The number of amino acid-related pathways is interesting as it could signify the increased availability of assimilated nitrogen in the maize seedlings. The greater availability of nitrogen could be indicative of the nitrogen-fixing ability of the PGPR strains. Studies have shown that both T19 and T29 are capable of nitrogen fixation (Breedt *et al.*, 2017), as such, it is very likely that these PGPR make soil nitrogen more available to the maize seedlings, ergo supporting these amino acid-related pathways.

Amino acids play numerous roles in plants. As a final indicator of nitrate assimilation, amino acids function as protein constituents, are involved in plant growth and development, abiotic and biotic stress resistance and as signalling molecules (Häusler, Ludewig, & Krueger, 2014; Heldt & Piechulla, 2010; Pratelli & Pilot, 2014). Furthermore, amino acids also serve as precursors for secondary metabolites, such as alkaloids (Heldt & Piechulla, 2010). Alanine, aspartate and glutamate metabolism was found to be enriched in both the T29 roots, and the T19 shoots.

The predicted pathway hits in the T29 roots for alanine, aspartate and glutamate metabolism were aspartate and fumarate. While in the T19 shoots, the predicted pathway hits were 2-oxoglutarate, fumarate and N-carbamoyl-L-aspartate. Aspartate gets its carbon skeleton from oxaloacetate, and can be catabolised back to the TCA intermediate (Heldt & Piechulla, 2010; Hildebrandt, Nesi, Araújo, & Braun, 2015). In addition, aspartate is also the precursor for other amino acids such as asparagine, lysine, threonine and methionine (Heldt & Piechulla, 2010). Aspartate can be converted to fumarate and vice versa by the formation of oxaloacetate through the TCA cycle, thus the upregulation of fumarate supports aspartate synthesis. Oxogluterate is a key metabolite in carbon/nitrogen metabolism (Araújo, Martins, Fernie, & Tohge, 2014). It is an intermediate in the TCA cycle which can be used to synthesise glutamate and vice versa. Aside from being a substrate for amino acid synthesis, 2-oxogluterate is also involved in alkaloid, glucosinolate, gibberellin and flavonoid metabolism (Araújo *et al.*, 2014). Finally, N-carbamoyl-L-aspartate is synthesised from aspartate, and it links alanine, aspartate and glutamate metabolism to pyrimidine metabolism, which supports cell division and growth. It is evident that

alanine, aspartate and glutamate metabolism feeds into numerous metabolic pathways (Fig S 7), which produce metabolites involved in plant growth and development, thus it is very important.

Aminoacyl t-RNA biosynthesis was found to be a significant pathway in the T29 inoculated maize plants roots. It was the least enriched pathway among the top pathways due to the small number of hits that could be predicted. However, all the hits have significantly positive fold changes in the inoculated roots compared to the control. Aminoacyl t-RNA biosynthesis involves the covalent binding of an amino acid to its corresponding t-RNA (Heldt & Piechulla, 2010). This pathway provides the building blocks for protein synthesis as aminoacyl t-RNA translates the nucleotide sequence of m-RNA into an amino acid sequence. The significance of aminoacyl t-RNA biosynthesis in the inoculated roots suggests that T29 may support protein synthesis. This suggestion is not far-fetched as the result of improved nitrogen assimilation would be enhanced protein synthesis; thus, further reflecting T29's nitrogen-fixing ability.

Amino sugar and nucleotide sugar metabolism are responsible for the production of sugar derivatives of the same name. This metabolic pathway was found to be significant in the T29 inoculated maize roots. Amino and nucleotide sugars are sugar donors for the synthesis of polysaccharides, glycoproteins, glycolipids and proteoglycans (Bar-Peled & O'neill, 2011). The polysaccharides usually have a storage function, whereas the other compounds are present at the cell surface acting as mechanical support for cells and regulating plant growth and development (Bar-Peled & O'neill, 2011). All the predicted hits for this pathway were sugars, which were up-regulated. The significance of this pathway in the inoculated roots may indicate that T29 can stimulate carbohydrate metabolism, which is crucial for supporting plant growth and development.

Inositol or myo-Inositol phosphate metabolism plays a central role in plant growth and development. Products from this metabolic pathway are involved in numerous growth and development roles from signal transduction, to membrane biogenesis and response to stress (Loewus & Murthy, 2000). Inositol phosphate metabolism was found to be significant in the T29 roots, and the predicted hits were inositol and glucuronate. Inositol is central to this metabolic pathway and can be converted to glucuronate, which will be used in reactions to produce cell wall structures (Loewus &

Murthy, 2000). It is likely that T29 enhances pathways linked to cell structural integrity, which in turn supports plant development.

Carbon fixation in a C4 plant such as maize occurs through the Calvin cycle. The Calvin cycle supplies the carbohydrate substrates needed for ATP synthesis, which in turn drives the ion pumps in the roots needed for mineral uptake from the soil (Heldt & Piechulla, 2010). The Calvin cycle takes place in the bundle sheath cells of leaves; however, this pathway was found to be up-regulated in the roots. It is likely that the products of carbon fixation were transported to the roots. It is also very likely that carbon fixation is up-regulated in the T29 shoots. In that regard, the products of carbon fixation are showing significant fold changes in the roots, which may be why the results indicated carbon fixation as a significant pathway in the roots.

Nicotinate and nicotinamide metabolism was found to be significant in the T19 shoots, though not very enriched. Nicotinate and nicotinamide metabolism provide the necessary precursors for the synthesis of the coenzymes nicotinamide adenine dinucleotide (NAD) and its phosphate derivative (NADP) (Hashida, Takahashi, & Uchimiya, 2009). This is an essential metabolic pathway for plant development as the resulting coenzymes play crucial roles in driving other important metabolic pathways, e.g., the Calvin cycle, amino acid and lipid biosynthesis, TCA cycle, etc. (Hashida *et al.*, 2009). As there were not many pathway hits for nicotinate and nicotinamide metabolism, it was poorly enriched in the shoots. However, the pathway hit which was NAD showed a significant fold change in the shoots compared to the control. The other top pathways in the roots and shoots require NAD and NADP in their various reactions. Thus, the significance of the nicotinate and nicotinamide metabolism may be closely linked to the enrichment of other metabolic pathways necessary for plant growth.

The citrate cycle is a key metabolic pathway in plant growth and development, and was found to be significant in the T19 inoculated shoots. All the predicted hits are intermediates of the citrate cycle and have a significantly greater fold change in the inoculated shoots compared to the control. This may be indicative of strain T19's ability to stimulate this energy yielding metabolic pathway. The intermediates of the citrate cycle act as precursors for other biosynthetic pathways like gluconeogenesis, fatty acid and amino acid synthesis. In addition, reducing equivalents (NADH and $FADH_2$)

are produced through the citrate cycle, which are used in ATP synthesis via oxidative phosphorylation. Ultimately, ATP is involved in supporting growth processes in plants.

Finally, vitamin B6 and pyrimidine metabolism were found to be enriched in the shoots. The vitamin B6 metabolic pathway is crucial as it provides coenzymes for numerous metabolic reactions such as amino acid, sugar and fatty acid metabolism (Colinas, Eisenhut, Tohge, Pesquera, Fernie, Weber, & Fitzpatrick, 2016). Additionally, vitamin B6 has antioxidant properties, thus, it has been hypothesised that enriching vitamin B6 metabolism will reinforce plants' resistance against biotic and abiotic stress (Colinas *et al.*, 2016). Pyrimidine metabolism is one of two metabolic pathways that produce the nucleotides requisite for numerous biochemical pathways in plants. Nucleotides are essential constituents for nucleic acid synthesis necessary for cell replication, precursors for the biosynthesis of amino acids, phospholipids, sucrose, polysaccharides and secondary metabolites (Stasolla, Katahira, Thorpe, & Ashihara, 2003). Pyrimidine metabolism is therefore, fundamental in plant growth and development.

It was expected that the PGPR would affect the metabolic pathways in the roots and shoots. In a previous study, metabolomic analysis was conducted on maize plants grown in *Pseudomonas fluorescens* inoculated soil (Dhawi & Hess, 2017). The results of the study revealed that *Pseudomonas fluorescens* induced metabolites related to the aminoacyl t-RNA biosynthesis and alanine, aspartate and glutamate metabolism (Dhawi & Hess, 2017). Despite using different PGPRs, both metabolic pathways were also significant in the current study.

All the metabolic pathways discussed in the preceding paragraphs support plant growth and development in one way or another. Furthermore, these pathways are interconnected with one another in such a way that one feeds into the other. However, these results are akin to painting a picture with a broad brush. As such, it was necessary to get a more specific view of what metabolites separate the control from the inoculated seedlings, both in the roots and shoots.

4.2.2) Putatively identified metabolites that discriminate between the control and T29 inoculated roots

There are no known reports of any of the following putatively identified metabolites being found in maize, however, they have been reported in other plants. The lack of

reports of these metabolites in maize may simply indicate that studies have not been focused on them specifically. The first discriminating metabolite in the T29 inoculated roots was stearidonic acid. Stearidonic acid is a fatty acid and was found to be up-regulated in the inoculated roots compared to the control. Stearidonic acid is an omega-3 fatty acid that acts as a precursor for other omega-3 fatty acids (Lee, Kim, Kim, Hong, Jeon, Kim, Lee, & Kim, 2019). It has been reported in only a few plant species, such as *Boraginaceae* and *Primulaceae* (Ruiz-López, Haslam, Venegas-Calerón, Larson, Graham, Napier, & Sayanova, 2009). In addition to serving as a carbon store, fatty acids also play a role in providing structural integrity in plants, as well as acting as both intracellular and extracellular signal transduction mediators (Heldt & Piechulla, 2010; Lim, Singhal, Kachroo, & Kachroo, 2017). The upregulation of stearidonic acid in the T29 inoculated roots may be indicative of a carbon storage role. Stearidonic acid may also have a role in signal transduction in maize; however, that would require further study.

Another discriminating metabolite, still within the domain of fatty acids is 1-linoleoyl-sn-glycero-3-phosphocholine. This metabolite plays a role in glycerophospholipid biosynthesis and was found to be down-regulated in the inoculated roots compared to the control. The fact that this metabolite was found to be down-regulated in the inoculated roots does not necessarily mean that T29 suppresses the production of 1-linoleoyl-sn-glycero-3-phosphocholine. On the other hand, being down-regulated could equally be an indicator of a depleted metabolite pool. Glycerophospholipids play a role as membrane constituents (Heldt & Piechulla, 2010), as such it is likely that the 1-linoleoyl-sn-glycero-3-phosphocholine pool was depleted due to its role in glycerophospholipid biosynthesis caused by rapid growth.

Geissoschizine and anonaine are alkaloids, both found to be up-regulated in the inoculated roots. PGPR are capable of affecting alkaloid content in plants, and this is especially well studied in medicinal plants (Karthikeyan, Joe, Jaleel, & Deiveekasundaram, 2010). Ergo, the ability of T29 to stimulate alkaloid content in maize roots falls in line with what one would expect of a beneficial PGPR-plant relationship. As one of the largest groups of secondary metabolites, alkaloids support plant survival by improving plant defence or by attracting or repelling pollinators, depending on the concentration and type of alkaloid (Matsuura & Fett-Neto, 2017). As both alkaloids were found in the roots, their role may be that of defence, especially

against pathogens or predators in the soil. There are more studies on geissoschizine as a medicinal compound in comparison to the role it plays in plants. Anonaine on the other hand, has insecticidal activity (Boulogne, Petit, Ozier-Lafontaine, Desfontaines, & Loranger-Merciris, 2012). The growth trial did not involve any abiotic or biotic stressors; thus, this indicates that these alkaloids were constitutively produced. The presence of these alkaloids may indicate T29's ability to prime the maize plants' defence against any future abiotic or biotic stressors, thereby enhancing its survival and ultimately its development.

Penultimately, the other two putatively identified metabolites, lotaustralin and 2-succinyl-6 hydroxycyclohexa-2,4-diene-1-carboxylic acid (SHCHC) were found to be up-regulated. The former is a cyanogenic glycoside while the latter is a gamma-keto acid. Both act as intermediates in their respective pathways. Lotaustralin is involved in cyanoamino acid metabolism, and its upregulation may suggest the idea that strain T29 stimulates cyanoamino acid metabolism. SHCHC is involved in ubiquinone and terpenoid-quinone biosynthesis, and these end products are known for their roles as electron and proton carriers in the photosynthetic and respiratory electron transport chain, as well as antioxidants (Heldt & Piechulla, 2010).

Finally, 4-pyridoxic acid is involved in vitamin B6 metabolism and was found to be down-regulated in the inoculated roots. This result was unexpected and would require further confirmation as this form of vitamin B6 is not usually found or been reported in plants (Gerdes, Lerma-Ortiz, Frelin, Seaver, Henry, De Crécy-Lagard, & Hanson, 2012; Sampson, Eoff, Yan, & Lorenz, 1995). The origin and fate of 4-pyridoxic acid in plants is still a great mystery, and as such, there will be no further discussion on it as it is an area that requires further study.

4.2.3) Putatively identified metabolites that discriminate between the control and T19 inoculated shoots

Most of the putative discriminating metabolites found in the shoots can be classified as secondary metabolites. There are numerous studies that report different PGPR ability to affect secondary metabolite content in plants (Mañero, Algar, Martín Gómez, Saco Sierra, & Solano, 2012; Walker *et al.*, 2012). Secondary metabolites are not directly involved in plant growth but play other roles that supports plant growth. For

example, some secondary metabolites act as attractants of insect pollinators or as defence compounds against biotic and abiotic stressors (Heldt & Piechulla, 2010).

The first three putatively identified metabolites in the shoots were the only primary metabolites in the list of discriminating metabolites, and were all up-regulated in the inoculated shoots. Malic acid is involved in the citrate cycle and in carbon fixation. According to the pathway analysis, both metabolic pathways were found to be affected in the inoculated shoots, with the former being significantly affected. The next metabolite is (S)-ureidoglycolic acid, which is involved in purine metabolism or more specifically, purine catabolism. Purine catabolism is a means by which nitrogen is remobilised in plants, which supplies other parts of the plant with nitrogen to support growth (Werner, Sparkes, Romeis, & Witte, 2008). (S)-ureidoglycolic acid plays a key role in this process and as such acts as a nitrogen store. Finally, N-Carbamoyl-L-aspartic acid is involved in alanine, aspartate and glutamate metabolism and pyrimidine biosynthesis. According to pathway analysis, these pathways were found to be significant in the inoculated shoots. Two of the discussed metabolites link back to results in the pathway analysis, and this reinforces the idea that T19 plays a role in influencing those pathways to promote growth. Furthermore, it is possible that T19 plays a role in supporting nitrogen storage and remobilisation through (S)-ureidoglycolic acid.

The remaining discriminating metabolites can be classified as secondary metabolites, all belonging to different groups. Rotenonone has not been reported in maize but it has been reported in the roots of *Amorpha canescens* (Adesemoye *et al.*, 2009). Aside from being a polyketide, rotenonone belongs to a group of similar compounds known as rotenoids. Rotenoids have been reported to have insecticidal properties, and even used as a crop insecticide e.g., rotenone (Gupta, 2012). The upregulation of this metabolite may suggest a constitutive defence role in the maize plants. However, rotenoids are also known for being quite toxic, so further confirmation of its presence in maize shoots is needed so as to establish its role in maize shoots and the effect T19 has on its presence.

The remaining putatively identified metabolites have not been reported in maize either; nonetheless, belong to phytochemical classes that have numerous beneficial roles in plants. Sapelin A and nuatigenin are both terpenoids and down-regulated in the

inoculated shoots. Terpenoids are often reported as having pharmacological importance (Schwab, Fischer, & Wüst, 2015). Aside from a medicinal role, terpenoids play significant roles in cellular membranes and in cell physiology (Gershenzon & Dudareva, 2007). They are involved in light energy harvesting during photosynthesis, electron transfer, growth regulation and maintaining cell integrity (Gershenzon & Dudareva, 2007).

Sapelin A was reported in extracts from *Entandrophragma cylindricum* (Chan, Taylor, & Yee, 1970) and *Bursera klugii* for anti-cancer activity (Jolad, Wiedhopf, & Cole, 1977). There are not many reports about its role in plants. Nuatigenin was reported in *Avena sativa* for having anti-parasitic activity against intestinal nematodes (Doligalska, Jóźwicka, Donskow-Łysoniewska, & Kalinowska, 2017). The study concluded that nuatigenin may act as a deformation inducer in parasites (Doligalska *et al.*, 2017). At this point, the functional role of sapelin A and nuatigenin in maize can only be inferred from the compound class to which they belong. However, their downregulation in the inoculated shoots may be as a result of their use in their class functional roles mentioned in the previous paragraph, hence resulting in depleted levels.

Lastly, both the putatively identified metabolites sativanine B and staphidine are alkaloids and were down-regulated in the inoculated shoots. Sativanine B was reported in the bark of *Zizyphus sativa* (Tschesche, Shah, & Eckhardt, 1979) and staphidine in *Delphinium staphisagria* (Pelletier, Mody, Djarmati, Mićović, & Thakkar, 1976). Once again, there are not many reports on the role these alkaloids play in plants. The role of alkaloids in plants was mentioned in the previous section. The downregulation of these metabolites cannot be fully explained in this context, as their role in plants is unknown and their presence in maize needs confirmation. Nevertheless, the downregulation could be an indication that T19 suppresses different secondary metabolites. Additionally, the downregulation may be a sign that the alkaloids are being funnelled into other reactions. However, these are all speculations and would need further analysis. It cannot be expected that the secondary metabolites would only be up-regulated as a result of T19 inoculation. One can only make postulations on the differential regulation of these metabolites, especially as they belong to phytochemical groups with known functional roles in plants.

4.3) Linking the metabolomic and phenotypic effects of PGPR inoculation of maize seedlings

The results of the greenhouse trial revealed that strains T29 and T19 are capable of promoting growth in maize; T29 in the roots and T19 in the shoots. The results suggest that T29 and T19 are capable of promoting early maize growth by acting as biofertilisers; as they enhanced nutrient availability and root and shoot biomass. All of the metabolic pathways and metabolites found to be significant in the roots and shoots as a result of PGPR treatment, play a role in supporting plant growth. The metabolites support growth by either acting as substrates for the biosynthesis of cellular components, by acting as signals to drive growth or by reinforcing the defence system of the plants (Meyer, Steinfath, Lisec, Becher, Witucka-Wall, Törjék, Fiehn, Eckardt, Willmitzer, & Selbig, 2007). The results showed that a combination of metabolites and metabolic pathways are involved in stimulating the different growth parameters assessed in the greenhouse, especially root and shoot biomass increase.

In a study by Meyer *et al.*, it was found that a combination of a number of metabolites rather than a few, show a close correlation with growth and biomass increase (Meyer *et al.*, 2007). Furthermore, variation in growth can be linked with certain combinatorial changes in metabolite levels (Meyer *et al.*, 2007). In this project, some metabolites that were expected to be up-regulated as a result of PGPR inoculation were down-regulated. In the study by Meyer *et al.*, it was suggested that down regulation or negative correlation between metabolites and enhanced growth indicates reduced metabolic pool sizes as a result of strong growth (Meyer *et al.*, 2007). The same line of thought was also suggested in this project based on the differential regulation of the metabolites in the roots and shoots. The authors concluded from their observations that growth drives metabolism and not vice versa (Meyer *et al.*, 2007). However, the relationship between growth and metabolism may not only be unidirectional but rather reversible, i.e., growth drives metabolism and vice versa. Therefore, based on the results of the current study, one could say that PGPR drives growth, which in turn drives metabolism and, which then drives growth.

With regards to the mechanism of action of the selected PGPR's in this project, the results showed that strain T29 improved nitrogen assimilation in the maize roots due to most of the significant pathways being amino acid-related. Strain T19 also

stimulated alanine, aspartate and glutamate metabolism. Both strains T29 and T19 have the ability to fix nitrogen (Breedt *et al.*, 2017) and as such would be beneficial in improving nitrogen use efficiency in maize. Out of all the putatively identified metabolites in the roots, only 1-linoleoyl-sn-glycero-3-phosphocholine had a phosphorus incorporated though it was down-regulated. In addition to having nitrogen-fixing ability, T29 can also solubilise phosphate (Breedt *et al.*, 2017), which could explain the phosphate in 1-linoleoyl-sn-glycero-3-phosphocholine. This phosphate solubilising ability would prove very helpful in soils with low levels of available phosphate. A number of secondary metabolites were putatively identified in the shoots, which may suggest that one of T19's mode of operation is to stimulate important phytochemicals. An area of future study should be on the ability of T19 to stimulate phytohormone production in maize.

Results in the current project came from both univariate and multivariate analysis, and one may question which to use. The answer would be both. Despite there not being full correlation between the two sets of results, they do show some similarities. Using both sets of results deepens the understanding of how both selected PGPR affects the maize metabolome.

Finally, the most important question that needs to be answered is how will all this information benefit farmers and on a larger scale, food production? Metabolomic analysis provided a screenshot of the metabolic response of maize affected by a certain external factor, i.e., the PGPR. In general, a metabolic response in a biological system serves as an indicator of the effect of external factors or conditions on that system. The selected PGPR used in the current study are already commercially available. Therefore, understanding the metabolic effects of the PGPR inoculation in maize seedlings enhances our understanding of plant-PGPR or more specifically, maize-PGPR interactions and its benefits. This knowledge provides a molecular basis as to why the selected PGPR can promote maize growth. Furthermore, it provides a scientific reason to promote its use among maize farmers. Thus, sustainable agricultural practices can be reinforced by promoting the use of the selected PGPR.

Chapter 5: Conclusion

5.1) Summary

Null hypothesis 1 was partially rejected because not all growth parameter results showed statistically significant differences between the treatment and control samples. Null hypothesis 2 was rejected mainly because of the results of the pathway analysis. Null hypothesis 3 was also rejected because of the univariate and multivariate results. The results showed that the PGPR strains T19 and T29 were capable of stimulating early maize growth. Moreover, the results showed that strain T19 had a tendency to stimulate shoot growth more while strain T29 stimulated root growth more. As such these strains affected the shoot and root metabolome differentially, though there were a few similarities in the pathway analysis. Different metabolites were found to be differentially regulated in the inoculated roots' and shoots' metabolome. These metabolites were putatively identified and found to support plant growth in one way or another. Overall, the results showed that both these PGPR strains work very well with maize, are capable of stimulating maize growth and affecting metabolic pathways and metabolite content. The results shed some light on the effects of the PGPR strains on the metabolome of young maize plants, which were used to infer their possible mechanisms of action. The results further validate the use of T19 and T29 in commercial maize farming. In addition, the results may lead to future studies on the application of these strains (either individually or in combination) to maize farming, specifically in South Africa.

5.2) Project limitations

One of the biggest limitations of the project was the sample number per group. In the greenhouse trial, most of the growth parameters showed no statistically significant difference between the inoculated and control plants. This occurred despite the fact that the inoculated samples showed an increased effect in the respective parameters. At a larger sample size, results that were deemed insignificant may become significant. Plant systems are also very prone to variability, thus increasing the sampling size per group may assist in reducing variability in downstream analysis, such as metabolomic analysis.

The use of UPLC/QTOF-MS for metabolomics data acquisition was a limitation. The use of UPLC/QTOF-MS limits the classes of metabolites that can be detected as well as downstream metabolite identification. The number of metabolite libraries based on UPLC data is quite limited in comparison to libraries based on GC and NMR data. GC and NMR are widely used platforms and as such have much more reproducible sample preparation and analysis techniques. One data acquisition platform alone cannot offer the most comprehensive view of the metabolome, especially in an untargeted study, such as this one. Therefore, using two platforms would prove much more beneficial in exploratory projects, such as in this study.

Metabolite identification proved to limit the project as not all discriminating metabolic features could be putatively identified. Metabolite identification is one of the most difficult steps as a number of factors tend to be stumbling blocks. Firstly, identification is limited by the metabolite libraries as there is still a large part of the plant metabolome that is unknown. Secondly, the data acquisition platform limits the available libraries that can be used for identification. Thirdly, the incomplete knowledge of the maize metabolome, which is why some of the putatively matched metabolites could not be linked back to maize. The only way to circumvent this issue is to either use an acquisition platform with a well curated compound library or to conduct targeted metabolomic analysis in future studies.

5.3) Future research

A number of metabolic pathways and metabolites were highlighted as being significantly affected by strains T19 and T29. This project took an untargeted approach to get a global view of how the selected PGPR strains affect the maize metabolome. Thus, for future studies a more targeted metabolomics approach should be used. Studies can be conducted on the pathways that were found to be significant or on specific metabolite classes to which the compounds in tables 17 and 19 belong. Along with a targeted study, commercial standards need to be employed to confirm the presence of specific metabolites in the maize metabolome. An untargeted approach usually raises more questions than answers, as it is a hypothesis generating method. Therefore, a targeted approach would be highly beneficial as a future undertaking, as it will provide more specific answers to the questions raised in this study.

A biotic or abiotic challenge was not included in this study. A study that involves a biotic or abiotic challenge would further validate the use of the selected PGPR in maize farming and as a move towards sustainable agriculture. It is important to understand on a metabolic level how the selected PGPR would fare in protecting maize plants against challenges that threaten growth and maize production. In addition to including a pathogen, studies need to be conducted on nutrient challenged and contaminated soils. The availability of uncontaminated and fertile soil is a major challenge to farmers. A good PGPR needs to be able to promote maize growth in the face of contaminated and/or nutrient challenged soil.

As both T19 and T29 were the best performing strains individually, it is also worth studying their effects on maize growth and the metabolome when combined. It is likely that both PGPR will have a synergistic effect on maize growth. As the rhizosphere is populated with various PGPR that work together to promote plant growth, it would not be unexpected if strains T19 and T29 would work better in combination.

PGPR application in agriculture is still not very popular. The use of PGPR in agriculture needs to be increased so as to fully move towards sustainable agriculture practices. To enhance the use of PGPR in agriculture, more studies like this need to be conducted. The results of PGPR studies tend to be inconsistent between the greenhouse and the field, so to bypass this issue more field trials need to be performed. Furthermore, conducting metabolomics studies coupled to other omics techniques like genomics and transcriptomics would provide a stronger scientific basis to using PGPR in agriculture. Conducting studies using multiple omics techniques will provide a clearer picture on the effects and benefits of PGPR use on the gene, transcript and metabolic content in plants. In closing, thorough omics studies on the effect of PGPR inoculation on crops will surely drive current agricultural practices towards a more sustainable one as it will be better understood.

References

Adesemoye, A., Torbert, H., & Kloepper, J. (2009). Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. Microbial Ecology*, 58*(4), 921-929.

Ahemad, M., & Kibret, M. (2014). Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. Journal of King Saud University-Science*, 26*(1), 1-20.

Ahmad, F., Ahmad, I., Aqil, F., Khan, M. S., & Hayat, S. (2008a). Diversity and potential of nonsymbiotic diazotrophic bacteria in promoting plant growth. Plant-Bacteria Interactions: Strategies and Techniques to Promote Plant Growth, 81-109.

Ahmad, I., Aqil, F., Ahmad, F., Zahin, M., & Musarrat, J. (2008b). Quorum sensing in bacteria: Potential in plant health protection. Plant-Bacteria Interactions: Strategies and Techniques to Promote Plant Growth, 129-153.

Ahmad, S., Veyrat, N., Gordon-Weeks, R., Zhang, Y., Martin, J., Smart, L., Glauser, G., Erb, M., Flors, V., & Frey, M. (2011). Benzoxazinoid metabolites regulate innate immunity against aphids and fungi in maize. Plant Physiology, pp. 111.180224.

Ahmed, A., & Hasnain, S. (2014). Auxins as one of the factors of plant growth improvement by plant growth promoting rhizobacteria. Polish Journal of Microbiology*, 63*(3), 261-266.

Aloni, R., Aloni, E., Langhans, M., & Ullrich, C. (2006). Role of cytokinin and auxin in shaping root architecture: Regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. Annals of Botany*, 97*(5), 883-893.

Araújo, W. L., Martins, A. O., Fernie, A. R., & Tohge, T. (2014). 2-oxoglutarate: Linking tca cycle function with amino acid, glucosinolate, flavonoid, alkaloid, and gibberellin biosynthesis. Frontiers in Plant Science*, 5*, 552.

Arbona, V., Iglesias, D. J., Talón, M., & Gómez-Cadenas, A. (2009). Plant phenotype demarcation using nontargeted lc-ms and gc-ms metabolite profiling. Journal Of Agricultural And Food Chemistry*, 57*(16), 7338-7347.

Ayano, M., Kani, T., Kojima, M., Sakakibara, H., Kitaoka, T., Kuroha, T., Angeles-Shim, R. B., Kitano, H., Nagai, K., & Ashikari, M. (2014). Gibberellin biosynthesis and signal transduction is essential for internode elongation in deepwater rice. Plant, Cell & Environment*, 37*(10), 2313-2324.

Azcón, R., & Barea, J. (1975). Synthesis of auxins, gibberellins and cytokinins by *azotobacter vinelandii* and *azotobacter beijerinckii* related to effects produced on tomato plants. Plant and Soil*, 43*(1-3), 609-619.

Badgley, C., Moghtader, J., Quintero, E., Zakem, E., Chappell, M. J., Aviles-Vazquez, K., Samulon, A., & Perfecto, I. (2007). Organic agriculture and the global food supply. Renewable Agriculture and Food Systems*, 22*(2), 86-108.

Bano, A., & Muqarab, R. (2017). Plant defence induced by pgpr against *spodoptera litura* in tomato (*solanum lycopersicum* l.). Plant Biology*, 19*(3), 406-412.

Bar-Peled, M., & O'Neill, M. A. (2011). Plant nucleotide sugar formation, interconversion, and salvage by sugar recycling. Annual Review of Plant Biology*, 62*, 127-155.

Bhattacharyya, P., & Jha, D. (2012). Plant growth-promoting rhizobacteria (pgpr): Emergence in agriculture. World Journal of Microbiology and Biotechnology*, 28*(4), 1327-1350.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology*, 37*(8), 911-917.

Bloemberg, G. V., & Lugtenberg, B. J. (2001). Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Current Opinion in Plant Biology*, 4*(4), 343-350.

Boulogne, I., Petit, P., Ozier-Lafontaine, H., Desfontaines, L., & Loranger-Merciris, G. (2012). Insecticidal and antifungal chemicals produced by plants: A review. Environmental Chemistry Letters*, 10*(4), 325-347.

Breedt, G., Labuschagne, N., & Coutinho, T. A. (2017). Seed treatment with selected plant growth-promoting rhizobacteria increases maize yield in the field. Annals of Applied Biology*, 171*(2), 229-236.

Bringhurst, R. M., Cardon, Z. G., & Gage, D. J. (2001). Galactosides in the rhizosphere: Utilization by *sinorhizobium meliloti* and development of a biosensor. Proceedings of the National Academy of Sciences*, 98*(8), 4540-4545.

Brusamarello-Santos, L. C., Gilard, F., Brulé, L., Quilleré, I., Gourion, B., Ratet, P., De Souza, E. M., Lea, P. J., & Hirel, B. (2017). Metabolic profiling of two maize (*zea mays* l.) inbred lines inoculated with the nitrogen fixing plant-interacting bacteria *herbaspirillum seropedicae* and *azospirillum brasilense*. PloS One*, 12*(3), e0174576.

Canbolat, M. Y., Bilen, S., Çakmakçı, R., Şahin, F., & Aydın, A. (2006). Effect of plant growth-promoting bacteria and soil compaction on barley seedling growth, nutrient uptake, soil properties and rhizosphere microflora. Biology and Fertility of Soils*, 42*(4), 350-357.

Canellas, L. P., Balmori, D. M., Médici, L. O., Aguiar, N. O., Campostrini, E., Rosa, R. C., Façanha, A. R., & Olivares, F. L. (2013). A combination of humic substances and

herbaspirillum seropedicae inoculation enhances the growth of maize (*zea mays* l.). Plant and Soil*, 366*(1-2), 119-132.

Castillo, S., Gopalacharyulu, P., Yetukuri, L., & Orešič, M. (2011). Algorithms and tools for the preprocessing of lc–ms metabolomics data. Chemometrics and Intelligent Laboratory Systems*, 108*(1), 23-32.

Chan, W., Taylor, D., & Yee, T. (1970). Triterpenoids from *entandrophragma cylindricum sprague*. Part i. Structures of sapelins a and b. Journal of the Chemical Society C: Organic(2), 311-314.

Chaparro, J. M., Badri, D. V., Bakker, M. G., Sugiyama, A., Manter, D. K., & Vivanco, J. M. (2013). Root exudation of phytochemicals in arabidopsis follows specific patterns that are developmentally programmed and correlate with soil microbial functions. PloS One*, 8*(2), e55731.

Cheng, Z., Park, E., & Glick, B. R. (2007). 1-aminocyclopropane-1-carboxylate deaminase from *pseudomonas putida* uw4 facilitates the growth of canola in the presence of salt. Canadian Journal of Microbiology*, 53*(7), 912-918.

Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., Wishart, D. S., & Xia, J. (2018). Metaboanalyst 4.0: Towards more transparent and integrative metabolomics analysis. Nucleic Acids Research*, 46*(W1), W486-W494.

Chong, J., Yamamoto, M., & Xia, J. (2019). Metaboanalystr 2.0: From raw spectra to biological insights. Metabolites*, 9*(3), 57.

Colinas, M., Eisenhut, M., Tohge, T., Pesquera, M., Fernie, A. R., Weber, A. P., & Fitzpatrick, T. B. (2016). Balancing of b6 vitamers is essential for plant development and metabolism in arabidopsis. The Plant Cell*, 28*(2), 439-453.

Cortleven, A., & Schmülling, T. (2015). Regulation of chloroplast development and function by cytokinin. Journal of Experimental Botany*, 66*(16), 4999-5013.

Cowan, A. K. (2006). Phospholipids as plant growth regulators. Plant Growth Regulation*, 48*(2), 97-109.

Debeaujon, I., & Koornneef, M. (2000). Gibberellin requirement for arabidopsis seed germination is determined both by testa characteristics and embryonic abscisic acid. Plant Physiology*, 122*(2), 415-424.

del Rosario Cappellari, L., Santoro, M. V., Nievas, F., Giordano, W., & Banchio, E. (2013). Increase of secondary metabolite content in marigold by inoculation with plant growth-promoting rhizobacteria. Applied Soil Ecology*, 70*, 16-22.

Dhawi, F., & Hess, A. (2017). Plant growth-prompting bacteria influenced metabolites of *zea mays* var. Amylacea and *pennisetum americanum* p. In a species-specific manner. Advances in Biological Chemistry*, 7*(05), 161.

Doligalska, M., Jóźwicka, K., Donskow-Łysoniewska, K., & Kalinowska, M. (2017). The antiparasitic activity of avenacosides against intestinal nematodes. Veterinary Parasitology*, 241*, 5-13.

Dong, Y.-H., Gusti, A. R., Zhang, Q., Xu, J.-L., & Zhang, L.-H. (2002). Identification of quorum-quenching n-acyl homoserine lactonases from *bacillus* species. Applied and Environmental Microbiology*, 68*(4), 1754-1759.

Dunn, W. B., & Ellis, D. I. (2005). Metabolomics: Current analytical platforms and methodologies. TrAC Trends in Analytical Chemistry*, 24*(4), 285-294.

Dunn, W. B., Erban, A., Weber, R. J., Creek, D. J., Brown, M., Breitling, R., Hankemeier, T., Goodacre, R., Neumann, S., & Kopka, J. (2013). Mass appeal: Metabolite identification in mass spectrometry-focused untargeted metabolomics. Metabolomics*, 9*(1), 44-66.

Fernando, W. D., Nakkeeran, S., & Zhang, Y. (2005). Biosynthesis of antibiotics by pgpr and its relation in biocontrol of plant diseases Pgpr: Biocontrol and biofertilization (pp. 67-109): Springer.

Figueiredo, M. d. V. B., Seldin, L., de Araujo, F. F., & Mariano, R. d. L. R. (2010). Plant growth promoting rhizobacteria: Fundamentals and applications Plant growth and health promoting bacteria (pp. 21-43): Springer.

Fowowe, B. (2016). Do oil prices drive agricultural commodity prices? Evidence from south africa. Energy*, 104*, 149-157.

Friml, J., Wiśniewska, J., Benková, E., Mendgen, K., & Palme, K. (2002). Lateral relocation of auxin efflux regulator pin3 mediates tropism in arabidopsis. Nature*, 415*(6873), 806.

Fuentes-Ramirez, L. E., & Caballero-Mellado, J. (2005). Bacterial biofertilizers Pgpr: Biocontrol and biofertilization (pp. 143-172): Springer.

Gerdes, S., Lerma-Ortiz, C., Frelin, O., Seaver, S. M. D., Henry, C. S., de Crécy-Lagard, V., & Hanson, A. D. (2012). Plant b vitamin pathways and their compartmentation: A guide for the perplexed. Journal of Experimental Botany*, 63*(15), 5379-5395. doi:10.1093/jxb/ers208

Gershenzon, J., & Dudareva, N. (2007). The function of terpene natural products in the natural world. Nature Chemical Biology*, 3*(7), 408.

Ghasemi, A., & Zahediasl, S. (2012). Normality tests for statistical analysis: A guide for non-statisticians. International Journal of Endocrinology and Metabolism*, 10*(2), 486.

Glick, B. R. (2012). Plant growth-promoting bacteria: Mechanisms and applications. Scientifica*, 2012*.

Gou, J., Strauss, S. H., Tsai, C. J., Fang, K., Chen, Y., Jiang, X., & Busov, V. B. (2010). Gibberellins regulate lateral root formation in populus through interactions with auxin and other hormones. The Plant Cell*, 22*(3), 623-639.

Gray, E., & Smith, D. (2005). Intracellular and extracellular pgpr: Commonalities and distinctions in the plant–bacterium signaling processes. Soil Biology and Biochemistry*, 37*(3), 395-412.

Großkinsky, D. K., Naseem, M., Abdelmohsen, U. R., Plickert, N., Engelke, T., Griebel, T., Zeier, J., Novák, O., Strnad, M., & Pfeifhofer, H. (2011). Cytokinins mediate resistance against *pseudomonas syringae* in tobacco through increased antimicrobial phytoalexin synthesis independent of salicylic acid signaling. Plant Physiology*, 157*(2), 815-830.

Gupta, G., Parihar, S. S., Ahirwar, N. K., Snehi, S. K., & Singh, V. (2015). Plant growth promoting rhizobacteria (pgpr): Current and future prospects for development of sustainable agriculture. Journal of Microbial & Biochemical Technology*, 7*(2), 096-102.

Gupta, R. C. (2012). Rotenone Veterinary toxicology (pp. 620-623): Elsevier.

Gyaneshwar, P., Kumar, G. N., Parekh, L., & Poole, P. (2002). Role of soil microorganisms in improving p nutrition of plants. Plant and Soil*, 245*(1), 83-93.

Hall, R. D. (2006). Plant metabolomics: From holistic hope, to hype, to hot topic. New Phytologist*, 169*(3), 453-468.

Harris, J. M. (2015). Abscisic acid: Hidden architect of root system structure. Plants*, 4*(3), 548-572.

Hashida, S.-n., Takahashi, H., & Uchimiya, H. (2009). The role of nad biosynthesis in plant development and stress responses. Annals of Botany*, 103*(6), 819-824.

Häusler, R. E., Ludewig, F., & Krueger, S. (2014). Amino acids–a life between metabolism and signaling. Plant Science*, 229*, 225-237.

Heldt, H.-W., & Piechulla, B. (2010). Plant biochemistry: Academic Press.

Heuberger, A. L., Robison, F. M., Lyons, S. M. A., Broeckling, C. D., & Prenni, J. E. (2014). Evaluating plant immunity using mass spectrometry-based metabolomics workflows. Frontiers in Plant Science*, 5*, 291.

Hildebrandt, T. M., Nesi, A. N., Araújo, W. L., & Braun, H.-P. (2015). Amino acid catabolism in plants. Molecular Plant*, 8*(11), 1563-1579.

Hong, J., Yang, L., Zhang, D., & Shi, J. (2016). Plant metabolomics: An indispensable system biology tool for plant science. International Journal of Molecular Sciences*, 17*(6), 767.

Hungria, M., Campo, R. J., Souza, E. M., & Pedrosa, F. O. (2010). Inoculation with selected strains of *azospirillum brasilense* and *a. Lipoferum* improves yields of maize and wheat in brazil. Plant and Soil*, 331*(1-2), 413-425.

Iqbal, M. A., Khalid, M., Shahzad, S. M., Ahmad, M., Soleman, N., & Akhtar, N. (2012). Integrated use of *rhizobium leguminosarum*, plant growth promoting rhizobacteria and enriched compost for improving growth, nodulation and yield of lentil (*lens culinaris* medik.). Chilean Journal of Agricultural Research*, 72*(1), 104.

Iqbal, N., Khan, N. A., Ferrante, A., Trivellini, A., Francini, A., & Khan, M. (2017). Ethylene role in plant growth, development and senescence: Interaction with other phytohormones. Frontiers in Plant Science*, 8*, 475.

Jolad, S., Wiedhopf, R., & Cole, J. R. (1977). Cytotoxic agents from *bursera klugii* (*burseraceae*) i: Isolation of sapelins a and b. Journal of Pharmaceutical Sciences*, 66*(6), 889-890.

Joo, G.-J., Kim, Y.-M., Kim, J.-T., Rhee, I.-K., Kim, J.-H., & Lee, I.-J. (2005). Gibberellins-producing rhizobacteria increase endogenous gibberellins content and promote growth of red peppers. The Journal of Microbiology*, 43*(6), 510-515.

Joo, G.-J., Kim, Y.-M., Lee, I.-J., Song, K.-S., & Rhee, I.-K. (2004). Growth promotion of red pepper plug seedlings and the production of gibberellins by *bacillus cereus, bacillus macroides* and *bacillus pumilus*. Biotechnology Letters*, 26*(6), 487-491.

Kang, S.-M., Khan, A. L., You, Y.-H., Kim, J.-G., Kamran, M., & Lee, I.-J. (2014). Gibberellin production by newly isolated strain *leifsonia soli* se134 and its potential to promote plant growth. Journal of Microbiology Biotechnology*, 24*, 106-112.

Karthikeyan, B., Joe, M. M., Jaleel, C. A., & Deiveekasundaram, M. (2010). Effect of root inoculation with plant growth promoting rhizobacteria (pgpr) on plant growth, alkaloid content and nutrient control of *catharanthus roseus* (l.) g. Don. Natura Croatica*, 19*(1), 205.

Katajamaa, M., & Orešič, M. (2007). Data processing for mass spectrometry-based metabolomics. Journal of Chromatography A*, 1158*(1-2), 318-328.

Kazan, K., & Manners, J. M. (2009). Linking development to defense: Auxin in plant–pathogen interactions. Trends in Plant Science*, 14*(7), 373-382.

Kim, H. K., Choi, Y. H., & Verpoorte, R. (2010). Nmr-based metabolomic analysis of plants. Nature Protocols*, 5*(3), 536.

Kim, H. K., & Verpoorte, R. (2010). Sample preparation for plant metabolomics. Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques*, 21*(1), 4-13.

Kloepper, J. (2003). A review of mechanisms for plant growth promotion by pgpr. Paper presented at the 6th international PGPR workshop.

Kloepper, J., & Schroth, M. (1978). *Plant growth-promoting rhizobacteria on radishes.* Paper presented at the Proceedings of the 4th international conference on plant pathogenic bacteria.

Kloepper, J., Schroth, M., & Miller, T. (1980). Effects of rhizosphere colonization by plant growth-promoting rhizobacteria on potato plant development and yield. Phytopathology*, 70*(11), 1078-1082.

Kuan, K. B., Othman, R., Rahim, K. A., & Shamsuddin, Z. H. (2016). Plant growth-promoting rhizobacteria inoculation to enhance vegetative growth, nitrogen fixation and nitrogen remobilisation of maize under greenhouse conditions. PLoS One*, 11*(3), e0152478.

Kuiper, I., Lagendijk, E. L., Bloemberg, G. V., & Lugtenberg, B. J. (2004). Rhizoremediation: A beneficial plant-microbe interaction. Molecular Plant-Microbe Interactions*, 17*(1), 6-15.

Kumar, S., Agarwal, M., Dheeman, S., & Maheshwari, D. K. (2015). Exploitation of phytohormone-producing pgpr in development of multispecies bioinoculant formulation Bacterial metabolites in sustainable agroecosystem (pp. 297-317): Springer.

Kumar, S., & Singh, A. (2015). Biopesticides: Present status and the future prospects. Journal of Fertilizers & Pesticides*, 6*(2).

Lal, R. (2015). Restoring soil quality to mitigate soil degradation. Sustainability*, 7*(5), 5875-5895.

Lee, K.-R., Kim, K.-H., Kim, J. B., Hong, S.-B., Jeon, I., Kim, H. U., Lee, M. H., & Kim, J. K. (2019). High accumulation of γ-linolenic acid and stearidonic acid in transgenic perilla (*perilla frutescens* var. Frutescens) seeds. BMC Plant Biology*, 19*(1), 120.

Lim, G.-H., Singhal, R., Kachroo, A., & Kachroo, P. (2017). Fatty acid–and lipid-mediated signaling in plant defense. Annual Review of Phytopathology*, 55*, 505-536.

Lisec, J., Schauer, N., Kopka, J., Willmitzer, L., & Fernie, A. R. (2006). Gas chromatography mass spectrometry–based metabolite profiling in plants. Nature Protocols*, 1*(1), 387.

Liu, F., Xing, S., Ma, H., Du, Z., & Ma, B. (2013). Cytokinin-producing, plant growth-promoting rhizobacteria that confer resistance to drought stress in *platycladus orientalis* container seedlings. Applied Microbiology and Biotechnology*, 97*(20), 9155-9164.

Loewus, F. A., & Murthy, P. P. (2000). Myo-inositol metabolism in plants. Plant Science*, 150*(1), 1-19.

Lu, X., Zhao, X., Bai, C., Zhao, C., Lu, G., & Xu, G. (2008). Lc–ms-based metabonomics analysis. Journal of Chromatography B*, 866*(1-2), 64-76.

Lucy, M., Reed, E., & Glick, B. R. (2004). Applications of free living plant growth-promoting rhizobacteria. Antonie van Leeuwenhoek*, 86*(1), 1-25.

Mañero, F. J. G., Algar, E., Martín Gómez, M. S., Saco Sierra, M. D., & Solano, B. R. (2012). Elicitation of secondary metabolism in *hypericum perforatum* by rhizosphere bacteria and derived elicitors in seedlings and shoot cultures. Pharmaceutical Biology*, 50*(10), 1201-1209.

Martínez-de la Cruz, E., García-Ramírez, E., Vázquez-Ramos, J. M., de la Cruz, H. R., & López-Bucio, J. (2015). Auxins differentially regulate root system architecture and cell cycle protein levels in maize seedlings. Journal of Plant Physiology*, 176*, 147-156.

Martínez-Viveros, O., Jorquera, M., Crowley, D., Gajardo, G., & Mora, M. (2010). Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. Journal of Soil Science and Plant Nutrition*, 10*(3), 293-319.

Matsuura, H. N., & Fett-Neto, A. G. (2017). Plant alkaloids: Main features, toxicity, and mechanisms of action. Plant Toxins, 243-261.

Memelink, J. (2005). Tailoring the plant metabolome without a loose stitch. Trends in Plant Science*, 10*(7), 305-307.

Meyer, R. C., Steinfath, M., Lisec, J., Becher, M., Witucka-Wall, H., Törjék, O., Fiehn, O., Eckardt, Ä., Willmitzer, L., & Selbig, J. (2007). The metabolic signature related to high plant growth rate in *arabidopsis thaliana*. Proceedings of the National Academy of Sciences*, 104*(11), 4759-4764.

Moco, S., Vervoort, J., Bino, R. J., De Vos, R. C., & Bino, R. (2007). Metabolomics technologies and metabolite identification. TrAC Trends in Analytical Chemistry, *26*(9), 855-866.

Niu, D.-D., Liu, H.-X., Jiang, C.-H., Wang, Y.-P., Wang, Q.-Y., Jin, H.-L., & Guo, J.-H. (2011). The plant growth–promoting rhizobacterium *bacillus cereus* ar156 induces systemic resistance in *arabidopsis thaliana* by simultaneously activating salicylate-and jasmonate/ethylene-dependent signaling pathways. Molecular Plant-Microbe Interactions, *24*(5), 533-542.

Park, W. J., Hochholdinger, F., & Gierl, A. (2004). Release of the benzoxazinoids defense molecules during lateral-and crown root emergence in *zea mays*. Journal of Plant Physiology, *161*(8), 981-985.

Parmar, P., & Sindhu, S. (2013). Potassium solubilization by rhizosphere bacteria: Influence of nutritional and environmental conditions. Journal of Microbiology Research, *3*(1), 25-31.

Pelletier, S., Mody, N., Djarmati, Z., Mićović, I., & Thakkar, J. (1976). The structures of staphidine, staphinine, and staphimine, three novel bis-diterpene alkaloids from *delphinium staphisagria*. Tetrahedron Letters, *17*(14), 1055-1058.

Pérez-Montaño, F., Alías-Villegas, C., Bellogín, R., Del Cerro, P., Espuny, M., Jiménez-Guerrero, I., López-Baena, F. J., Ollero, F., & Cubo, T. (2014). Plant growth promotion in cereal and leguminous agricultural important plants: From microorganism capacities to crop production. Microbiological Research, *169*(5), 325-336.

Picard, C., & Bosco, M. (2005). Maize heterosis affects the structure and dynamics of indigenous rhizospheric auxins-producing *pseudomonas* populations. FEMS Microbiology Ecology, *53*(3), 349-357. doi:10.1016/j.femsec.2005.01.007

Pingali, P. L. (2012). Green revolution: Impacts, limits, and the path ahead. Proceedings of the National Academy of Sciences, *109*(31), 12302-12308.

Plackett, A. R., & Wilson, Z. A. (2016). Gibberellins and plant reproduction. Annual Plant Reviews.

Planchamp, C., Glauser, G., & Mauch-Mani, B. (2015). Root inoculation with *pseudomonas putida* kt2440 induces transcriptional and metabolic changes and systemic resistance in maize plants. Frontiers in Plant Science, *5*, 719.

Pratelli, R., & Pilot, G. (2014). Regulation of amino acid metabolic enzymes and transporters in plants. Journal of Experimental Botany, *65*(19), 5535-5556.

Raza, W., Yang, X., Wu, H., Wang, Y., Xu, Y., & Shen, Q. (2009). Isolation and characterisation of fusaricidin-type compound-producing strain of *paenibacillus*

polymyxa sqr-21 active against *fusarium oxysporum* f. Sp. *Nevium*. European Journal of Plant Pathology*, 125*(3), 471-483.

Riou-Khamlichi, C., Huntley, R., Jacqmard, A., & Murray, J. A. (1999). Cytokinin activation of arabidopsis cell division through a d-type cyclin. Science*, 283*(5407), 1541-1544.

Rodríguez-Díaz, M., Rodelas-Gonzalés, B., Pozo-Clemente, C., Martínez-Toledo, M. V., & González-López, J. (2008). A review on the taxonomy and possible screening traits of plant growth promoting rhizobacteria. Plant-Bacteria Interactions: Strategies and Techniques to Promote Plant Growth, 55-80.

Rudolph, N., Labuschagne, N., & Aveling, T. (2015). The effect of plant growth promoting rhizobacteria on seed germination and seedling growth of maize. Seed Science and Technology*, 43*(3), 507-518.

Ruiz-López, N., Haslam, R. P., Venegas-Calerón, M., Larson, T. R., Graham, I. A., Napier, J. A., & Sayanova, O. (2009). The synthesis and accumulation of stearidonic acid in transgenic plants: A novel source of 'heart-healthy'omega-3 fatty acids. Plant Biotechnology Journal*, 7*(7), 704-716.

Ruttkies, C., Schymanski, E. L., Wolf, S., Hollender, J., & Neumann, S. (2016). Metfrag relaunched: Incorporating strategies beyond in silico fragmentation. Journal of Cheminformatics*, 8*(1), 3.

Ryu, C. M., Murphy, J. F., Mysore, K. S., & Kloepper, J. W. (2004). Plant growth-promoting rhizobacteria systemically protect *arabidopsis thaliana* against cucumber mosaic virus by a salicylic acid and npr1-independent and jasmonic acid-dependent signaling pathway. The Plant Journal*, 39*(3), 381-392.

Saccenti, E., Hoefsloot, H. C., Smilde, A. K., Westerhuis, J. A., & Hendriks, M. M. (2014). Reflections on univariate and multivariate analysis of metabolomics data. Metabolomics*, 10*(3), 361-374.

Sah, S. K., Reddy, K. R., & Li, J. (2016). Abscisic acid and abiotic stress tolerance in crop plants. Frontiers in Plant Science*, 7*, 571.

Salomon, M. V., Bottini, R., de Souza Filho, G. A., Cohen, A. C., Moreno, D., Gil, M., & Piccoli, P. (2014). Bacteria isolated from roots and rhizosphere of *vitis vinifera* retard water losses, induce abscisic acid accumulation and synthesis of defense-related terpenes in in vitro cultured grapevine. Physiologia Plantarum*, 151*(4), 359-374.

Sampson, D., Eoff, L., Yan, X., & Lorenz, K. (1995). Analysis of free and glycosylated vitamin b6 in wheat by high-performance liquid chromatography. Cereal Chemistry*, 72*(2), 217-220.

Schwab, W., Fischer, T., & Wüst, M. (2015). Terpene glucoside production: Improved biocatalytic processes using glycosyltransferases. Engineering in Life Sciences*, 15*(4), 376-386. doi:10.1002/elsc.201400156

Scossa, F., Brotman, Y., e Lima, F. d. A., Willmitzer, L., Nikoloski, Z., Tohge, T., & Fernie, A. R. (2016). Genomics-based strategies for the use of natural variation in the improvement of crop metabolism. Plant Science*, 242*, 47-64.

Shapiro, S. S., & Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). Biometrika*, 52*(3/4), 591-611.

Shapiro, S. S., Wilk, M. B., & Chen, H. J. (1968). A comparative study of various tests for normality. Journal of the American Statistical Association*, 63*(324), 1343-1372.

Sharma, A., Johri, B., Sharma, A., & Glick, B. (2003). Plant growth-promoting bacterium *pseudomonas* sp. Strain grp3 influences iron acquisition in mung bean (*vigna radiata* l. Wilzeck). Soil Biology and Biochemistry*, 35*(7), 887-894.

Solano, B. R., Maicas, J. B., & Mañero, F. G. (2008). Physiological and molecular mechanisms of plant growth promoting rhizobacteria (pgpr). Plant-Bacteria Interactions: Strategies and Techniques to Promote Plant Growth. Wiley, Weinheim, Germany, 41-52.

Somers, E., Vanderleyden, J., & Srinivasan, M. (2004). Rhizosphere bacterial signalling: A love parade beneath our feet. Critical Reviews in Microbiology*, 30*(4), 205-240.

Spaepen, S., Vanderleyden, J., & Remans, R. (2007). Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiology Reviews*, 31*(4), 425-448.

Stasolla, C., Katahira, R., Thorpe, T. A., & Ashihara, H. (2003). Purine and pyrimidine nucleotide metabolism in higher plants. Journal of Plant Physiology*, 160*(11), 1271-1295.

Su, F., Gilard, F., Guérard, F., Citerne, S., Clément, C., Vaillant-Gaveau, N., & Dhondt-Cordelier, S. (2016). Spatio-temporal responses of arabidopsis leaves in photosynthetic performance and metabolite contents to *burkholderia phytofirmans* psjn. Frontiers in Plant Science*, 7*, 403.

Sumner, L. W., Mendes, P., & Dixon, R. A. (2003). Plant metabolomics: Large-scale phytochemistry in the functional genomics era. Phytochemistry*, 62*(6), 817-836.

Thiese, M. S., Ronna, B., & Ott, U. (2016). P value interpretations and considerations. Journal of Thoracic Disease*, 8*(9), E928.

Tsavkelova, E., Klimova, S. Y., Cherdyntseva, T., & Netrusov, A. (2006). Microbial producers of plant growth stimulators and their practical use: A review. Applied Biochemistry and Microbiology*, 42*(2), 117-126.

Tschesche, R., Shah, A. H., & Eckhardt, G. (1979). Sativanine-a and sativanine-b, two new cyclopeptide alkaloids from the bark of *zizyphus sativa*. Phytochemistry*, 18*(4), 702-704.

Tsukanova, K., Meyer, J., & Bibikova, T. (2017). Effect of plant growth-promoting rhizobacteria on plant hormone homeostasis. South African Journal of Botany*, 113*, 91-102.

Turan, M., Ekinci, M., Yildirim, E., Güneş, A., Karagöz, K., Kotan, R., & Dursun, A. (2014). Plant growth-promoting rhizobacteria improved growth, nutrient, and hormone content of cabbage (*brassica oleracea*) seedlings. Turkish Journal of Agriculture and Forestry*, 38*(3), 327-333.

Van Loon, L. (2007). Plant responses to plant growth-promoting rhizobacteria. European Journal of Plant Pathology*, 119*(3), 243-254.

Vessey, J. K. (2003). Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil*, 255*(2), 571-586.

Walker, V., Bertrand, C., Bellvert, F., Moënne-Loccoz, Y., Bally, R., & Comte, G. (2011). Host plant secondary metabolite profiling shows a complex, strain-dependent response of maize to plant growth-promoting rhizobacteria of the genus *azospirillum*. New Phytologist*, 189*(2), 494-506.

Walker, V., Couillerot, O., Von Felten, A., Bellvert, F., Jansa, J., Maurhofer, M., Bally, R., Moënne-Loccoz, Y., & Comte, G. (2012). Variation of secondary metabolite levels in maize seedling roots induced by inoculation with *azospirillum*, *pseudomonas* and *glomus* consortium under field conditions. Plant and Soil*, 356*(1-2), 151-163.

Wang, E., & Martinez-Romero, E. (2000). *Sesbania herbacea–rhizobium huautlense* nodulation in flooded soils and comparative characterization of *s. Herbacea*-nodulating rhizobia in different environments. Microbial Ecology*, 40*(1), 25-32.

Werner, A. K., Sparkes, I. A., Romeis, T., & Witte, C.-P. (2008). Identification, biochemical characterization, and subcellular localization of allantoate amidohydrolases from arabidopsis and soybean. Plant Physiology*, 146*(2), 418-430.

Xiong, L., & Zhu, J.-K. (2003). Regulation of abscisic acid biosynthesis. Plant physiology*, 133*(1), 29-36.

Yi, L., Dong, N., Yun, Y., Deng, B., Ren, D., Liu, S., & Liang, Y. (2016). Chemometric methods in data processing of mass spectrometry-based metabolomics: A review. Analytica Chimica Acta*, 914*, 17-34.

Zeng, N., Zhao, F., Collatz, G. J., Kalnay, E., Salawitch, R. J., West, T. O., & Guanter, L. (2014). Agricultural green revolution as a driver of increasing atmospheric co_2 seasonal amplitude. Nature*, 515*(7527), 394.

Zhang, S., Moyne, A.-L., Reddy, M., & Kloepper, J. W. (2002). The role of salicylic acid in induced systemic resistance elicited by plant growth-promoting rhizobacteria against blue mold of tobacco. Biological Control*, 25*(3), 288-296.

Appendix

Table S 1: Effect of PGPR treatment on mass of seedlings harvested 3 and 9 dpi. The total dry mass column is the sum of the average shoot and root dry mass. The average shoot and root mass are from eight replicates. A negative percentage change in mass indicates decreased or repressed growth.

Seedling mass												
Treatment	3 dpi						9 dpi					
	Dry mass (g)			Percentage change in mass			Dry mass (g)			Percentage change in mass		
	Shoot	Root	Total	Shoot	Root	Total	Shoot	Root	Total	Shoot	Root	Total
Control	0.33	0.42	0.74	0.00	0.00	0.00	0.41	0.47	0.88	0.00	0.00	0.00
T19	0.34	0.56	0.90	3.27	34.83	20.90	0.52	0.54	1.06	26.89	14.45	20.23
T29	0.35	0.64	0.99	5.41	54.35	32.75	0.49	0.61	1.09	19.54	28.75	24.47
A07	0.30	0.55	0.85	-7.84	31.22	13.98	0.41	0.48	0.89	0.43	1.12	0.80

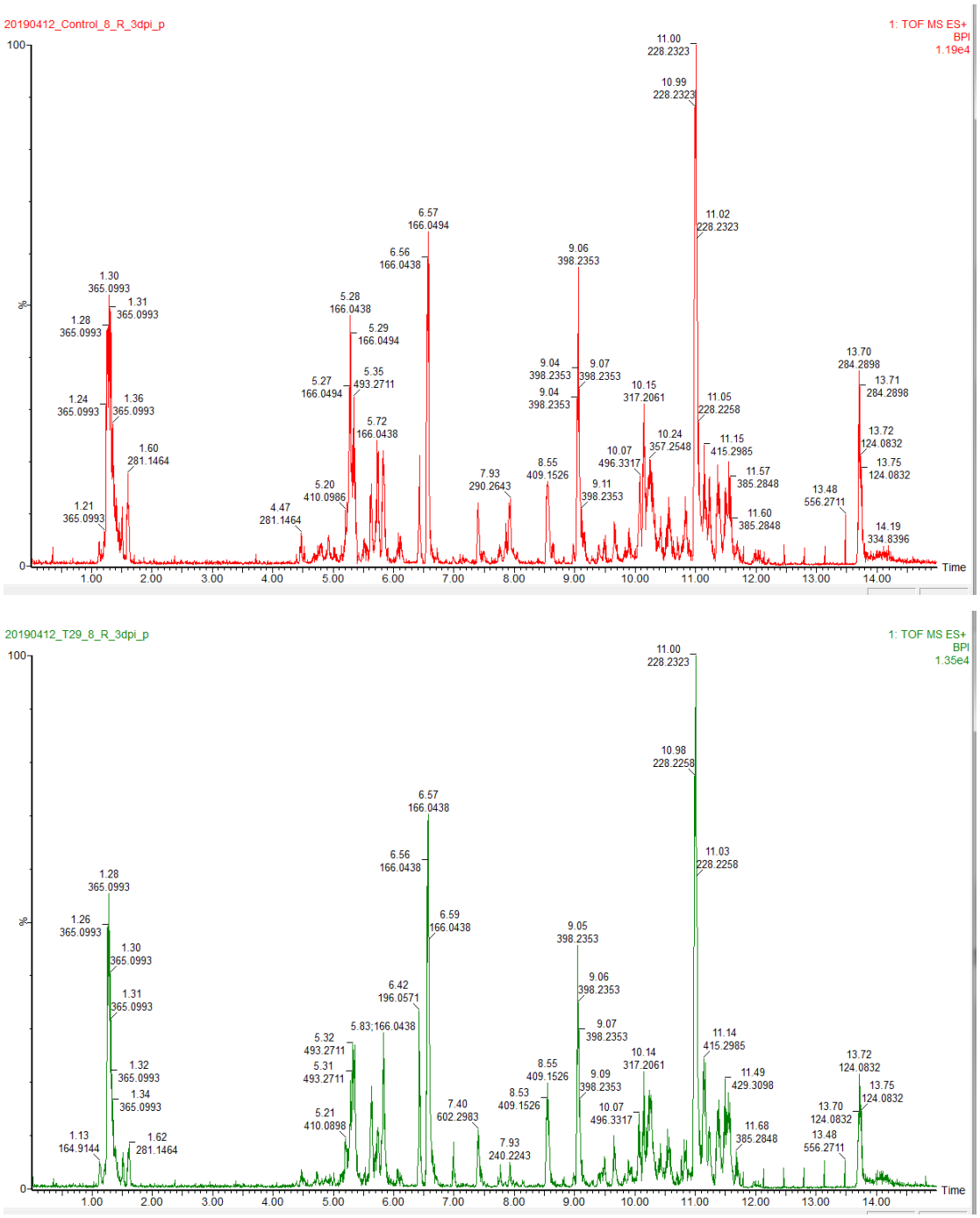


Figure S 1: Chromatogram of one of the controls (above) and T29 (below) inoculated root samples harvested 3 dpi, with a total run time of 15 min.

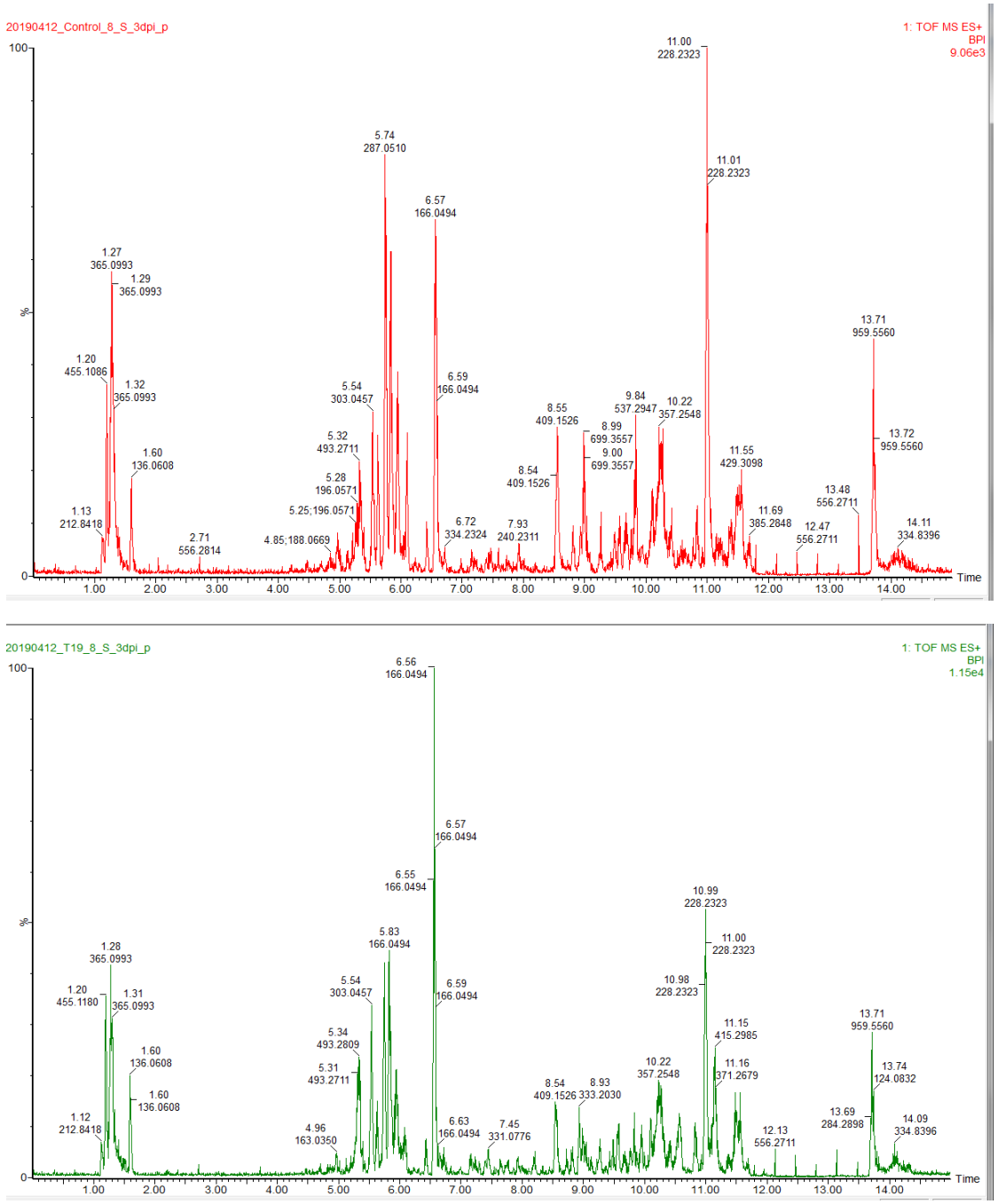


Figure S 2: Chromatogram of one of the controls (above) and T19 (below) inoculated shoot samples harvested 3 dpi, with a total run time of 15 min.

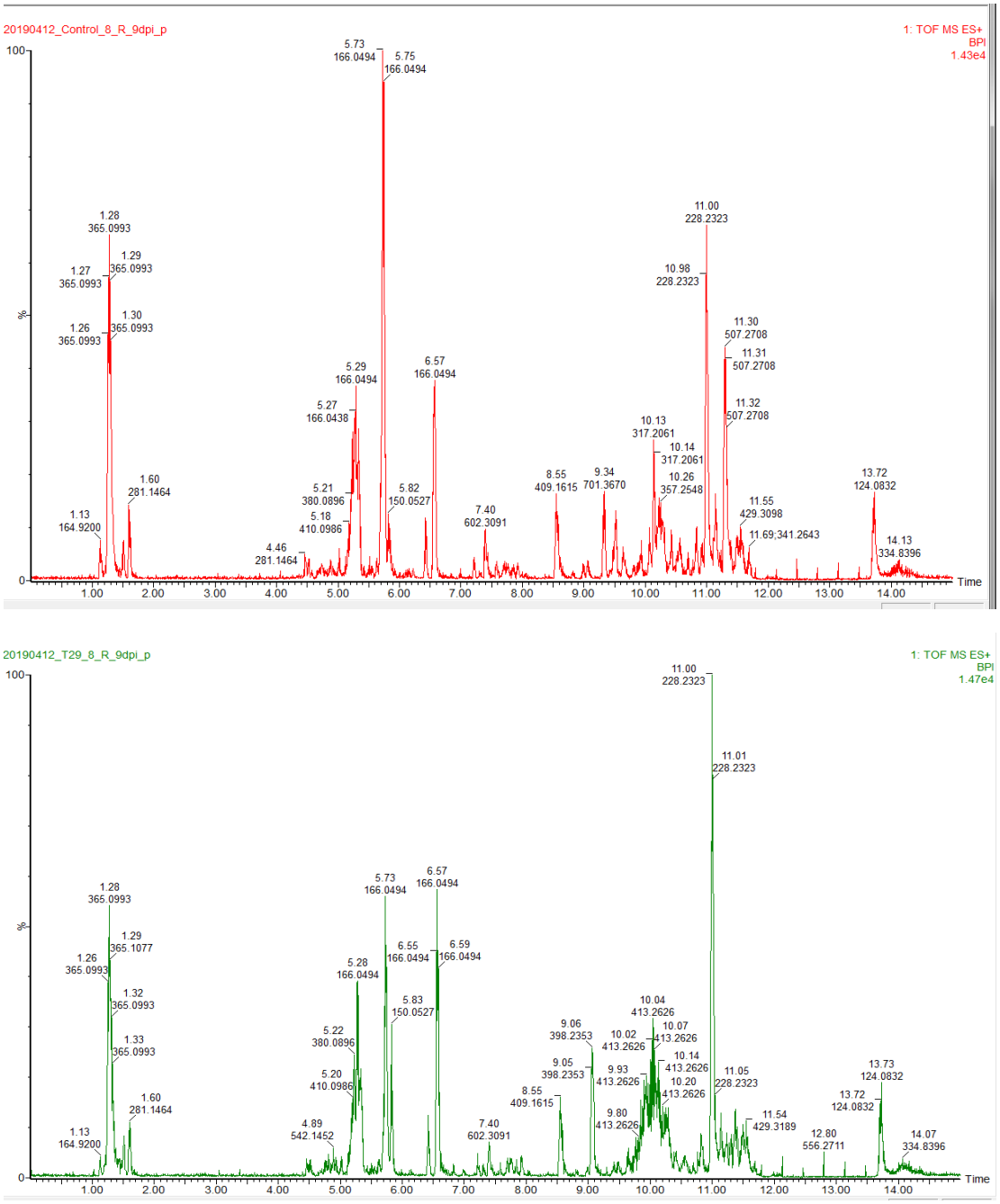


Figure S 3: Chromatogram of one of the controls (above) and T29 (below) inoculated root samples harvested 9 dpi, with a total run time of 15 min.

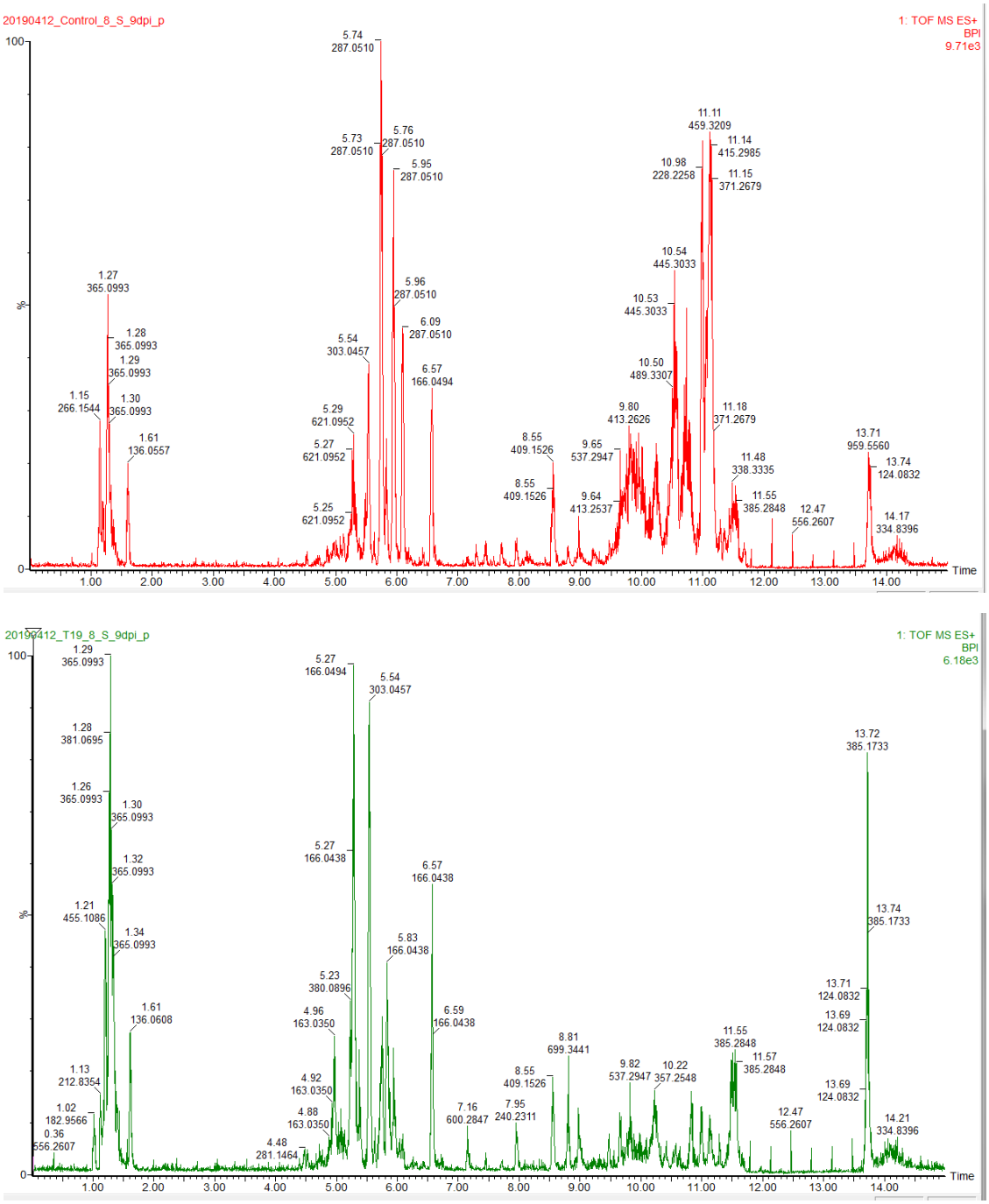


Figure S 4: Chromatogram of one of the controls (above) and T19 (below) inoculated shoot sample harvested 9 dpi, with a total run time of 15 min.

Table S 2: Significant features identified by fold change analysis between the control and T29 roots harvested 3 dpi.

Feature number	Retention time (min)	*m/z*	Fold change	log_2(FC)
1	5.81	271.0365	11.2220	3.4883
2	5.81	373.1037	10.9380	3.4513
3	7.21	843.2214	3.8731	1.9535
4	9.69	335.2131	1.9916	0.9939
5	7.76	885.2319	1.8642	0.8986
6	4.79	588.1451	1.6895	0.7566
7	5.74	379.0803	1.6271	0.7023
8	5.27	426.0937	1.6001	0.6782
9	5.70	440.1096	1.5933	0.6720
10	5.23	380.0880	1.5550	0.6369
11	5.27	224.0497	1.5533	0.6353
12	10.76	320.2488	1.5001	0.5851
13	5.27	196.0570	1.4996	0.5846
14	9.71	315.1868	1.4959	0.5810
15	9.82	335.2119	1.4688	0.5547
16	5.63	507.0739	1.4643	0.5502
17	5.24	150.0526	1.4360	0.5220
18	5.29	194.0404	1.4303	0.5163
19	5.21	410.0982	1.4240	0.5100
20	1.28	362.0907	1.3900	0.4750
21	9.04	335.2119	1.3645	0.4483
22	10.89	228.2270	1.3278	0.4091
23	9.04	295.2197	1.3196	0.4001
24	10.15	249.2159	1.3069	0.3861
25	5.21	208.0572	1.2951	0.3730
26	5.84	447.0528	1.2928	0.3706
27	10.14	241.1866	1.2803	0.3564
28	5.28	166.0451	1.2743	0.3497
29	5.21	180.0610	1.2717	0.3467
30	5.84	431.0566	1.2656	0.3398
31	5.70	409.0904	1.2610	0.3346
32	5.84	234.0048	1.2516	0.3238
33	10.15	317.2037	1.2470	0.3184
34	5.84	150.0526	1.2431	0.3139
35	5.55	591.1952	1.2319	0.3009
36	5.84	178.0448	1.2300	0.2986
37	10.15	277.2121	1.2281	0.2965
38	10.24	318.2336	1.2264	0.2944
39	5.63	224.0504	1.2216	0.2888
40	10.15	339.1848	1.2138	0.2796
41	5.84	194.0404	1.2107	0.2758
42	5.81	166.0454	1.2017	0.2651
43	9.05	277.2100	1.1996	0.2625
44	6.57	185.0185	1.1907	0.2518
45	10.15	259.2002	1.1741	0.2316
46	9.45	337.2667	1.1656	0.2211
47	5.28	393.0918	1.1645	0.2198
48	6.57	259.5506	1.1481	0.1992

49	6.57	149.0360	1.1474	0.1983
50	6.57	267.5388	1.1384	0.1871
51	1.51	185.0036	1.1357	0.1836
52	9.49	520.3306	1.1336	0.1809
53	6.57	171.0223	1.1266	0.1720
54	9.66	520.3308	1.1143	0.1561
55	6.57	157.0237	1.1076	0.1475
56	6.57	166.0462	1.1026	0.1409
57	6.57	165.0376	1.1014	0.1393
58	5.13	393.2041	0.8884	-0.1707
59	7.93	240.2259	0.8867	-0.1734
60	8.55	425.1286	0.8847	-0.1768
61	10.02	313.2662	0.8757	-0.1916
62	10.25	357.2545	0.8734	-0.1953
63	9.07	304.2932	0.8655	-0.2084
64	10.08	496.3318	0.8598	-0.2179
65	10.18	489.3310	0.8597	-0.2181
66	10.08	518.3114	0.8596	-0.2183
67	4.96	493.2718	0.8307	-0.2677
68	10.58	421.2241	0.8249	-0.2778
69	5.34	481.2540	0.8218	-0.2831
70	5.24	437.2273	0.8173	-0.2911
71	10.83	149.0209	0.7962	-0.3288
72	10.42	313.2276	0.7866	-0.3463
73	1.49	262.1222	0.7756	-0.3667
74	9.92	320.2492	0.7720	-0.3734
75	1.57	136.0577	0.7568	-0.4020
76	7.45	353.2214	0.7476	-0.4198
77	1.60	230.1323	0.7422	-0.4302
78	1.60	258.1285	0.7318	-0.4506
79	7.40	602.3004	0.7305	-0.4530
80	1.40	136.0587	0.7275	-0.4590
81	10.83	301.1349	0.7063	-0.5017
82	1.60	519.1317	0.6954	-0.5240
83	1.28	365.0991	0.6950	-0.5250
84	10.94	723.4704	0.6815	-0.5531
85	1.60	152.0534	0.6783	-0.5600
86	1.60	276.1377	0.6777	-0.5613
87	10.54	357.2540	0.6764	-0.5640
88	10.87	529.2892	0.6741	-0.5690
89	9.93	677.3626	0.6707	-0.5762
90	1.19	317.0892	0.6700	-0.5778
91	7.50	545.2811	0.6507	-0.6200
92	7.40	455.2481	0.6449	-0.6329
93	10.37	235.1640	0.6316	-0.6628
94	1.17	177.0205	0.6220	-0.6851
95	1.13	164.9167	0.5524	-0.8563
96	6.06	379.1705	0.5150	-0.9573
97	1.14	306.8466	0.5043	-0.9877
98	7.40	219.0601	0.4023	-1.3138
99	7.90	318.2942	0.3562	-1.4893
100	6.12	386.1782	0.3509	-1.5108
101	7.86	274.2677	0.3508	-1.5111

102	9.15	437.1837	0.2766	-1.8540
103	7.89	334.2881	0.1839	-2.4429
104	9.15	453.1628	0.1750	-2.5148
105	8.63	318.2959	0.0655	-3.9319
106	7.92	290.2642	0.0591	-4.0803

Table S 3: Significant features identified by fold change analysis between the control and T19 shoots harvested 3 dpi.

Feature number	Retention time (min)	*m/z*	Fold change	log_2(FC)
1	10.23	318.2349	0.0773	-3.6942
2	9.07	398.2365	0.1939	-2.3665
3	9.07	376.2534	0.1987	-2.3311
4	9.68	320.2518	0.2030	-2.3006
5	5.49	605.1098	3.2708	1.7096
6	10.14	533.3585	0.4143	-1.2711
7	8.96	518.3188	2.3690	1.2443
8	10.77	320.2508	0.4343	-1.2033
9	7.16	351.2090	2.2838	1.1914
10	5.73	487.0781	0.4384	-1.1896
11	10.44	577.3898	2.1301	1.0909
12	9.94	389.2634	2.0856	1.0605
13	7.31	527.1505	0.4849	-1.0444
14	10.17	489.3329	0.4923	-1.0225
15	5.71	409.1781	2.0239	1.0171
16	1.25	527.1536	1.9529	0.9656
17	7.70	167.9912	1.9484	0.9623
18	7.65	280.2002	1.9003	0.9262
19	7.70	212.0163	1.8126	0.8581
20	10.17	275.1973	1.8029	0.8503
21	10.00	391.2406	0.6043	-0.7268
22	5.77	303.0458	0.6049	-0.7253
23	5.29	621.1022	0.6074	-0.7192
24	9.16	437.1919	1.5498	0.6321
25	10.18	315.1913	1.5439	0.6266
26	1.14	266.1552	1.5339	0.6172
27	9.74	277.2127	1.5290	0.6126
28	4.96	163.0353	1.5256	0.6094
29	7.40	219.0614	1.5091	0.5937
30	1.25	543.1264	1.5007	0.5856
31	7.45	331.0772	0.6683	-0.5814
32	5.84	573.0758	0.6746	-0.5679
33	6.09	220.1665	1.4725	0.5583
34	4.96	393.0389	1.4611	0.5471
35	10.62	507.2635	0.6881	-0.5393
36	10.23	401.2830	0.6936	-0.5279
37	9.38	421.2503	0.6949	-0.5252
38	10.11	327.0755	1.4291	0.5151
39	5.78	317.0593	0.7047	-0.5050
40	8.97	333.1989	1.4117	0.4974
41	10.21	445.3078	0.7193	-0.4753

42	6.11	386.1776	0.7229	-0.4681
43	9.43	291.1905	1.3796	0.4643
44	8.20	331.1823	1.3540	0.4373
45	1.51	185.0060	1.3447	0.4273
46	7.48	287.0511	0.7496	-0.4158
47	7.70	194.0066	1.3289	0.4103
48	5.71	409.0906	0.7564	-0.4028
49	9.89	200.1973	0.7637	-0.3889
50	9.57	315.1886	0.7766	-0.3648
51	6.10	557.0806	1.2830	0.3596
52	7.16	275.1943	1.2803	0.3565
53	5.27	426.0947	1.2762	0.3519
54	8.55	425.1297	0.7864	-0.3467
55	8.55	409.1561	0.7872	-0.3451
56	1.13	212.8390	1.2644	0.3385
57	9.57	275.1961	0.7922	-0.3360
58	9.45	337.2658	0.7927	-0.3351
59	9.65	520.3325	1.2615	0.3351
60	9.13	295.2236	0.7942	-0.3324
61	9.57	331.1819	1.2519	0.3241
62	6.57	267.5416	1.2462	0.3176
63	1.61	281.1459	1.2442	0.3152
64	6.57	259.5518	1.2416	0.3122
65	1.21	439.1374	1.2398	0.3101
66	10.40	317.2050	1.2352	0.3047
67	9.26	333.1993	0.8137	-0.2974
68	5.28	166.0480	1.2139	0.2797
69	5.26	565.1496	1.2131	0.2787
70	8.72	333.1979	0.8288	-0.2709
71	5.75	617.1389	1.1973	0.2598
72	10.24	357.2558	0.8360	-0.2584
73	5.54	303.0456	0.8373	-0.2562
74	10.41	313.2292	0.8382	-0.2546
75	10.24	313.2298	0.8403	-0.2511
76	6.57	185.0195	1.1845	0.2443
77	10.54	445.3085	1.1739	0.2313
78	6.57	157.0257	1.1728	0.2300
79	5.54	633.1350	0.8554	-0.2254
80	5.83	411.0549	1.1603	0.2144
81	1.18	104.1054	1.1555	0.2085
82	7.15	600.2868	0.8718	-0.1979
83	5.27	224.0524	1.1454	0.1959
84	7.42	344.2536	1.1409	0.1902
85	7.25	380.2737	1.1320	0.1789

86	5.27	196.0570	1.1316	0.1784
87	1.60	152.0550	0.8859	-0.1748
88	9.33	701.3627	1.1260	0.1712
89	5.95	471.0845	1.1234	0.1679
90	1.27	138.0524	1.1188	0.1619
91	6.01	287.0509	1.1187	0.1618
92	5.75	287.0509	1.1180	0.1609
93	1.60	136.0604	1.1118	0.1530
94	6.57	171.0225	1.1054	0.1446
95	9.07	304.2960	0.9077	-0.1397

Table S 4: Significant features identified by fold change analysis between the control and T29 roots harvested 9 dpi

Feature number	Retention time (min)	*m/z*	Fold change	log_2(FC)
1	9.05	277.2125	9.4457	3.2397
2	5.80	373.1020	9.1169	3.1885
3	9.07	376.2529	6.8505	2.7762
4	7.00	602.2981	0.1606	-2.6388
5	1.13	266.1576	5.3085	2.4083
6	9.48	184.0690	0.2024	-2.3048
7	9.48	542.3155	0.2045	-2.2896
8	9.48	520.3328	0.2050	-2.2864
9	7.40	241.0441	4.4921	2.1674
10	9.07	398.2347	4.4576	2.1563
11	1.40	136.0607	3.8303	1.9375
12	7.45	353.2286	3.8144	1.9315
13	7.58	329.1242	0.3090	-1.6945
14	6.83	221.0766	3.2303	1.6917
15	5.92	264.0825	3.1568	1.6584
16	5.70	440.1095	0.3216	-1.6366
17	5.70	196.0568	0.3232	-1.6296
18	9.05	295.2235	3.0785	1.6222
19	1.50	262.1247	3.0678	1.6172
20	9.65	542.3103	0.3282	-1.6074
21	10.05	498.3754	0.3345	-1.5800
22	6.83	249.0715	2.9898	1.5800
23	1.60	135.0292	2.8795	1.5258
24	9.65	520.3370	0.3481	-1.5224
25	5.70	409.0906	0.3636	-1.4598
26	1.24	118.0852	2.6992	1.4325
27	1.60	152.0550	2.6981	1.4319
28	5.59	208.0576	2.6963	1.4310
29	6.83	271.0567	2.6099	1.3840
30	7.40	219.0615	2.4757	1.3078
31	5.27	181.0312	0.4071	-1.2966
32	4.79	588.1453	0.4104	-1.2850
33	5.60	180.0624	2.4063	1.2668
34	4.92	224.0484	0.4341	-1.2040
35	1.27	527.1518	2.3036	1.2039
36	5.27	426.0957	0.4427	-1.1756
37	1.60	276.1419	2.2494	1.1695
38	7.77	557.1636	0.4480	-1.1585
39	5.28	194.0422	0.4624	-1.1129
40	10.23	318.2357	2.1416	1.0987
41	9.88	498.3760	0.4712	-1.0856

42	5.73	410.1007	0.4835	-1.0483
43	1.60	230.1348	2.0563	1.0400
44	5.26	196.0567	0.4901	-1.0289
45	5.74	166.0489	0.4912	-1.0257
46	5.23	150.0522	0.4987	-1.0038
47	5.28	166.0479	0.5010	-0.9972
48	5.23	178.0472	0.5010	-0.9971
49	5.27	224.0527	0.5103	-0.9706
50	9.94	431.2941	1.9367	0.9536
51	10.07	496.3305	0.5193	-0.9453
52	6.43	196.0569	1.9057	0.9304
53	9.96	387.2677	1.8320	0.8735
54	5.28	396.0842	0.5577	-0.8424
55	7.85	274.2701	1.7420	0.8008
56	5.02	411.1191	0.5803	-0.7851
57	5.23	380.0892	0.5969	-0.7445
58	5.83	150.0527	1.6131	0.6898
59	10.41	313.2310	1.6090	0.6862
60	5.83	178.0477	1.5980	0.6763
61	5.74	379.0836	0.6513	-0.6186
62	5.42	525.2779	1.5249	0.6087
63	10.29	233.1483	1.5216	0.6056
64	10.83	301.1380	1.5110	0.5955
65	6.57	166.0487	1.4954	0.5805
66	5.23	437.2274	1.4896	0.5749
67	6.57	157.0261	1.4858	0.5712
68	7.70	167.9912	1.4777	0.5634
69	9.91	475.3214	1.4589	0.5449
70	5.65	619.1879	0.6992	-0.5163
71	8.99	209.0551	1.4104	0.4961
72	6.57	161.9853	1.4037	0.4892
73	1.14	322.8203	1.3902	0.4753
74	9.89	200.1986	1.3889	0.4740
75	1.28	362.0926	1.3832	0.4680
76	7.93	240.2286	1.3530	0.4362
77	5.13	393.2023	1.3118	0.3916
78	9.68	320.2526	1.2801	0.3563
79	10.83	149.0215	1.2725	0.3477
80	10.52	357.2561	1.2689	0.3436
81	9.81	326.3734	1.2584	0.3316
82	9.33	263.2314	1.2484	0.3201
83	10.07	318.2354	1.2424	0.3132
84	5.01	349.1756	1.2116	0.2770
85	5.63	224.0546	1.1912	0.2524

86	1.13	306.8483	1.1903	0.2514
87	1.27	138.0524	1.1883	0.2489
88	10.23	401.2823	0.8479	-0.2380
89	1.13	164.9188	1.1629	0.2177
90	1.27	381.0692	0.8649	-0.2094
91	10.86	529.2943	1.1408	0.1901
92	4.47	281.1446	1.1399	0.1889
93	10.13	533.3617	0.8806	-0.1835
94	10.14	317.2057	1.1235	0.1680
95	10.24	313.2323	0.8956	-0.1591
96	9.07	304.2961	1.1088	0.1489

Table S 5: Top features identified by fold change analysis between the control and T19 shoots harvested 9 dpi.

Feature number	Retention time (min)	*m/z*	Fold change	log_2(FC)
1	10.49	388.3856	0.0277	-5.1724
2	9.04	376.2515	0.1388	-2.8491
3	9.79	651.4135	0.2226	-2.1672
4	1.61	135.0278	4.4373	2.1497
5	9.81	602.4363	0.2281	-2.1320
6	10.29	748.5263	0.2355	-2.0861
7	9.81	607.3915	0.2568	-1.9613
8	1.18	247.9078	3.8780	1.9553
9	1.61	276.1375	3.8776	1.9552
10	9.95	387.2637	0.2660	-1.9105
11	10.37	665.4305	0.2664	-1.9082
12	10.47	511.3722	0.2672	-1.9039
13	9.90	475.3155	0.2696	-1.8910
14	9.93	431.2899	0.2745	-1.8649
15	10.96	679.4484	0.2837	-1.8178
16	9.84	563.3639	0.2890	-1.7910
17	9.87	519.3421	0.2904	-1.7838
18	10.47	528.3983	0.2932	-1.7698
19	4.96	393.0332	3.3654	1.7508
20	10.40	621.4063	0.2977	-1.7479
21	10.50	489.3304	0.3009	-1.7327
22	9.84	558.4096	0.3023	-1.7261
23	5.14	177.0495	3.3054	1.7248
24	10.30	753.4851	0.3059	-1.7088
25	10.50	467.3493	0.3069	-1.7044
26	10.99	630.4693	0.3071	-1.7033
27	10.85	388.3086	0.3116	-1.6821
28	1.20	455.1076	3.2040	1.6799
29	10.96	674.4935	0.3127	-1.6771
30	10.53	423.3217	0.3137	-1.6728
31	10.33	704.5025	0.3194	-1.6468
32	5.20	195.0839	3.1070	1.6355
33	10.91	718.5170	0.3228	-1.6312
34	10.47	533.3558	0.3238	-1.6269
35	10.33	709.4566	0.3266	-1.6143
36	10.44	577.3805	0.3316	-1.5927
37	1.25	293.0575	3.0088	1.5892
38	10.37	660.4736	0.3338	-1.5831
39	10.56	401.2808	0.3379	-1.5655
40	10.92	723.4716	0.3425	-1.5458
41	10.89	767.4995	0.3435	-1.5415
42	10.75	393.2906	2.8879	1.5300

43	7.16	351.2063	2.8802	1.5261
44	1.25	543.1219	2.8724	1.5223
45	10.53	445.3037	0.3488	-1.5196
46	10.26	410.2470	0.3503	-1.5132
47	4.96	135.0399	2.8488	1.5104
48	5.32	439.1491	2.8418	1.5068
49	5.38	407.0499	2.7946	1.4827
50	5.30	264.1535	2.7893	1.4799
51	10.88	762.5459	0.3624	-1.4642
52	5.38	177.0495	2.7312	1.4495
53	6.72	334.2321	2.6616	1.4123
54	4.97	163.0350	2.6447	1.4031
55	10.78	461.2797	0.3841	-1.3804
56	10.44	572.4273	0.3882	-1.3652
57	9.99	391.2373	0.3937	-1.3448
58	5.27	147.0405	2.5122	1.3290
59	4.96	231.0060	2.4583	1.2977
60	8.79	699.3415	2.4467	1.2909
61	10.98	635.4235	0.4124	-1.2780
62	2.04	556.2606	2.4196	1.2748
63	5.53	683.1323	2.3930	1.2588
64	5.36	149.0533	2.3711	1.2456
65	1.61	119.0367	2.3708	1.2453
66	5.03	377.0751	2.3644	1.2415
67	1.55	152.0537	2.3492	1.2322
68	1.17	378.9750	2.3296	1.2201
69	8.92	699.3435	2.3174	1.2125
70	10.85	417.2531	0.4435	-1.1730
71	1.61	136.0592	2.2422	1.1649
72	10.57	357.2541	0.4465	-1.1633
73	9.97	343.2362	0.4469	-1.1621
74	9.21	364.3132	0.4469	-1.1621
75	5.27	179.0517	2.2329	1.1589
76	10.07	360.3549	0.4551	-1.1357
77	10.30	388.2367	0.4552	-1.1353
78	1.02	182.9566	2.1936	1.1333
79	5.33	493.2713	2.1642	1.1139
80	1.61	519.1308	2.1552	1.1079
81	10.12	374.2584	0.4645	-1.1061
82	5.14	391.0935	2.1520	1.1057
83	5.25	547.1364	2.1314	1.0918
84	1.17	208.9668	2.1265	1.0885
85	5.25	565.1471	2.1243	1.0870
86	1.19	104.1041	2.1003	1.0706
87	1.26	381.0712	2.0889	1.0627
88	4.06	556.2664	2.0866	1.0611

89	2.71	556.2624	2.0381	1.0272
90	5.71	409.1747	2.0215	1.0154
91	10.29	333.1413	2.0144	1.0103
92	5.82	821.3006	2.0100	1.0072
93	1.20	439.1314	2.0031	1.0023
94	5.41	319.1240	1.9912	0.9936
95	10.74	483.2925	0.5046	-0.9867
96	10.57	421.2250	1.9676	0.9764
97	4.91	166.0449	1.9660	0.9752
98	1.61	91.0530	1.9577	0.9691
99	4.85	118.0615	1.9309	0.9493
100	5.28	110.0567	1.9097	0.9333
101	1.39	321.1073	1.8986	0.9249
102	1.37	556.2684	1.8761	0.9078
103	5.53	163.0356	1.8581	0.8939
104	5.27	541.1283	1.8254	0.8682
105	5.27	224.0497	1.8247	0.8676
106	4.39	556.2663	1.8070	0.8536
107	1.13	212.8361	1.8008	0.8486
108	1.14	266.1543	1.7963	0.8451
109	5.82	423.1504	1.7947	0.8438
110	5.41	301.1095	1.7912	0.8410
111	1.60	241.1483	1.7912	0.8409
112	7.95	240.2257	1.7874	0.8379
113	1.60	229.1489	1.7658	0.8203
114	8.80	268.2579	1.7627	0.8178
115	2.38	556.2673	1.7586	0.8144
116	5.27	196.0566	1.7476	0.8054
117	1.60	268.0994	1.7426	0.8012
118	9.44	556.2641	1.7075	0.7719
119	5.29	409.1742	1.7061	0.7707
120	7.15	600.2839	1.6941	0.7605
121	1.26	277.0824	1.6935	0.7601
122	5.75	549.1600	1.6921	0.7588
123	1.27	138.0511	1.6882	0.7555
124	6.57	149.0347	1.6868	0.7543
125	4.47	281.1426	1.6771	0.7460
126	10.04	413.2579	1.6279	0.7030
127	5.77	317.0578	1.6276	0.7028
128	5.21	208.0543	1.6235	0.6991
129	5.29	771.1559	1.6234	0.6990
130	5.27	426.0936	1.6104	0.6874
131	5.79	331.0750	1.6045	0.6822
132	8.91	261.2160	1.6025	0.6803
133	10.63	335.1175	1.6021	0.6799
134	9.65	261.2151	0.6243	-0.6797

135	5.63	224.0498	1.5991	0.6772
136	1.26	527.1508	1.5900	0.6690
137	5.53	633.1298	1.5899	0.6689
138	3.72	556.2708	1.5684	0.6493
139	5.83	178.0453	1.5640	0.6452
140	5.05	350.0780	1.5512	0.6334
141	3.05	556.2704	1.5365	0.6196
142	5.83	194.0400	1.5356	0.6188
143	3.39	556.2641	1.5351	0.6184
144	7.30	549.1265	1.5219	0.6059
145	10.83	121.0254	1.5187	0.6028
146	5.27	181.0317	1.5093	0.5939
147	6.57	161.9837	1.5033	0.5881
148	1.46	136.0583	1.5028	0.5877
149	5.21	410.0987	1.5021	0.5869
150	5.96	431.0885	1.4905	0.5758
151	5.83	150.0516	1.4811	0.5667
152	10.82	149.0202	1.4801	0.5657
153	8.97	209.0543	0.6767	-0.5633
154	9.00	304.2931	1.4716	0.5574
155	9.80	326.3716	1.4714	0.5572
156	1.17	363.0004	1.4713	0.5571
157	10.74	326.3304	1.4684	0.5542
158	10.78	556.2708	1.4678	0.5537
159	5.23	178.0453	1.4668	0.5527
160	10.45	556.2659	1.4508	0.5369
161	10.11	556.2651	1.4491	0.5351
162	5.92	449.0993	1.4272	0.5132
163	5.83	149.0431	1.4177	0.5035
164	6.57	157.0250	1.3961	0.4815
165	5.50	569.3029	1.3864	0.4714
166	5.23	150.0523	1.3748	0.4592
167	10.82	301.1336	1.3652	0.4491
168	6.57	166.0441	1.3635	0.4473
169	6.57	171.0206	1.3556	0.4389
170	6.08	220.1630	1.3553	0.4386
171	5.53	303.0453	1.3533	0.4364
172	7.87	274.2677	1.3469	0.4297
173	5.83	447.0518	1.3455	0.4282
174	8.79	261.2160	1.3420	0.4244
175	10.42	539.3074	1.3360	0.4179
176	1.27	365.0992	1.3351	0.4169
177	10.21	401.2786	0.7506	-0.4139
178	5.06	535.0993	1.3299	0.4113
179	9.85	413.2578	1.3149	0.3950
180	5.83	234.0063	1.3078	0.3872

181	9.55	275.1944	0.7654	-0.3857
182	9.82	261.2166	1.3028	0.3817
183	6.00	287.0499	0.7683	-0.3802
184	4.53	366.0715	1.3003	0.3789
185	5.23	380.0882	1.2807	0.3570
186	5.75	617.1338	1.2777	0.3535
187	6.57	203.9907	1.2750	0.3504
188	8.95	518.3158	1.2739	0.3493
189	9.55	315.1872	0.7875	-0.3446
190	5.82	573.0715	1.2655	0.3398
191	10.09	577.3822	1.2587	0.3319
192	10.56	277.1737	0.7968	-0.3277
193	5.33	481.2527	1.2467	0.3181
194	9.48	520.3303	0.8068	-0.3098
195	5.66	196.0570	0.8084	-0.3068
196	10.22	313.2275	0.8143	-0.2964
197	5.72	487.0754	1.2018	0.2652
198	10.20	445.3038	0.8330	-0.2636
199	1.17	533.0074	1.1971	0.2595
200	10.98	228.2263	0.8358	-0.2588
201	8.55	425.1285	1.1958	0.2579
202	9.64	520.3301	1.1932	0.2548
203	5.95	413.0782	1.1914	0.2527
204	5.29	621.0985	1.1873	0.2477
205	6.56	165.0384	1.1856	0.2456
206	7.44	331.0751	0.8547	-0.2265
207	10.60	507.2608	1.1639	0.2190
208	6.05	395.1970	1.1557	0.2088
209	4.52	136.0364	1.1555	0.2086
210	10.39	317.2035	0.8697	-0.2015
211	5.79	166.0444	1.1421	0.1916
212	5.76	303.0443	1.1339	0.1813
213	9.82	537.2952	1.1317	0.1785
214	9.74	277.2111	1.1310	0.1776
215	10.23	357.2538	0.8898	-0.1685
216	5.00	349.1743	1.1203	0.1639
217	10.14	533.3555	0.8935	-0.1624
218	5.83	411.0542	1.1008	0.1385

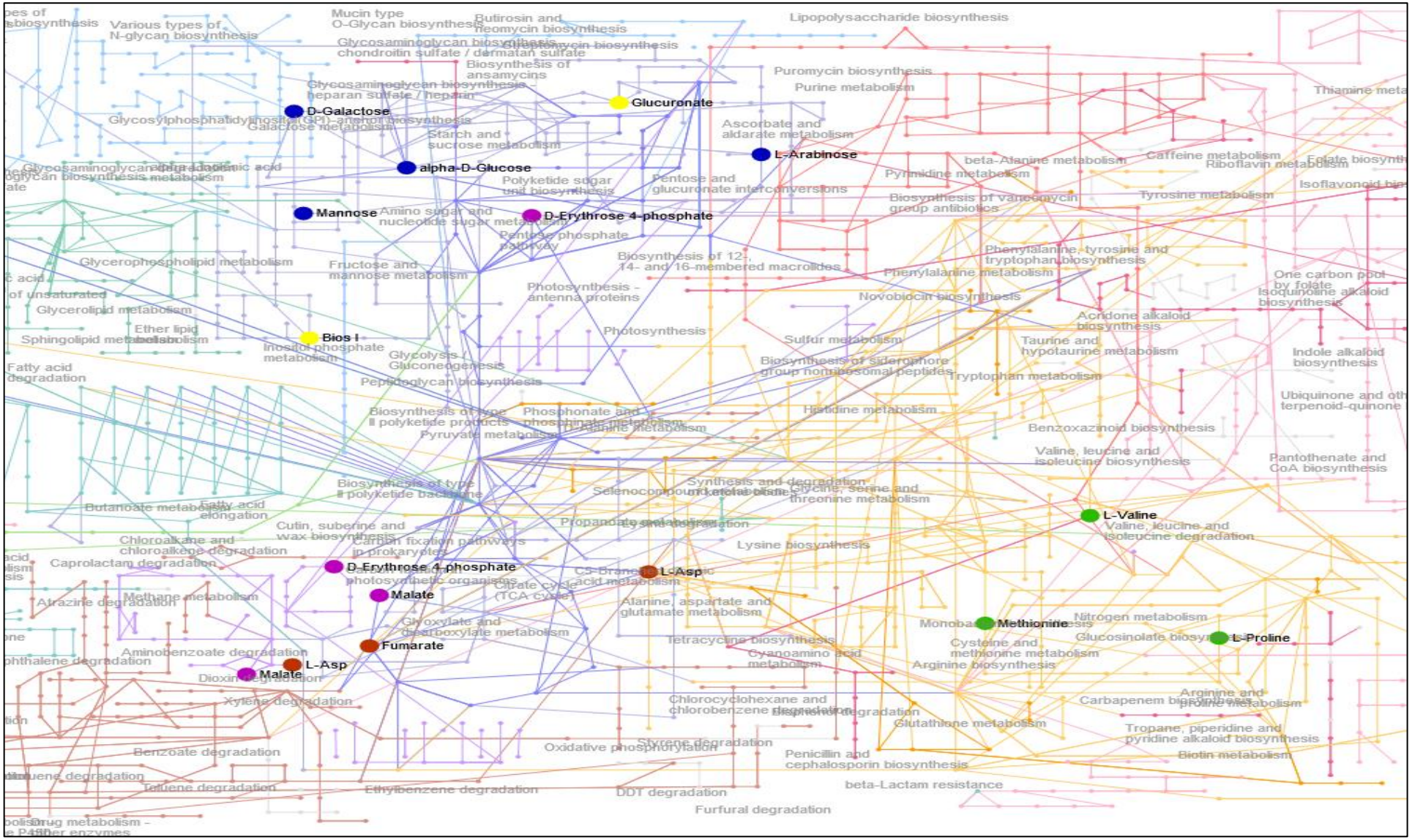


Figure S 5: Network mapping of pathway hits from the top five significant pathways in the T29 inoculated roots. Green points represent hits from aminoacyl t-RNA biosynthesis. Purple points represent hits from carbon fixation. Yellow points are hits from inositol phosphate metabolism. Red points are hits from alanine, aspartate and glutamate metabolism. Blue points are hits from amino sugar and nucleotide sugar metabolism.

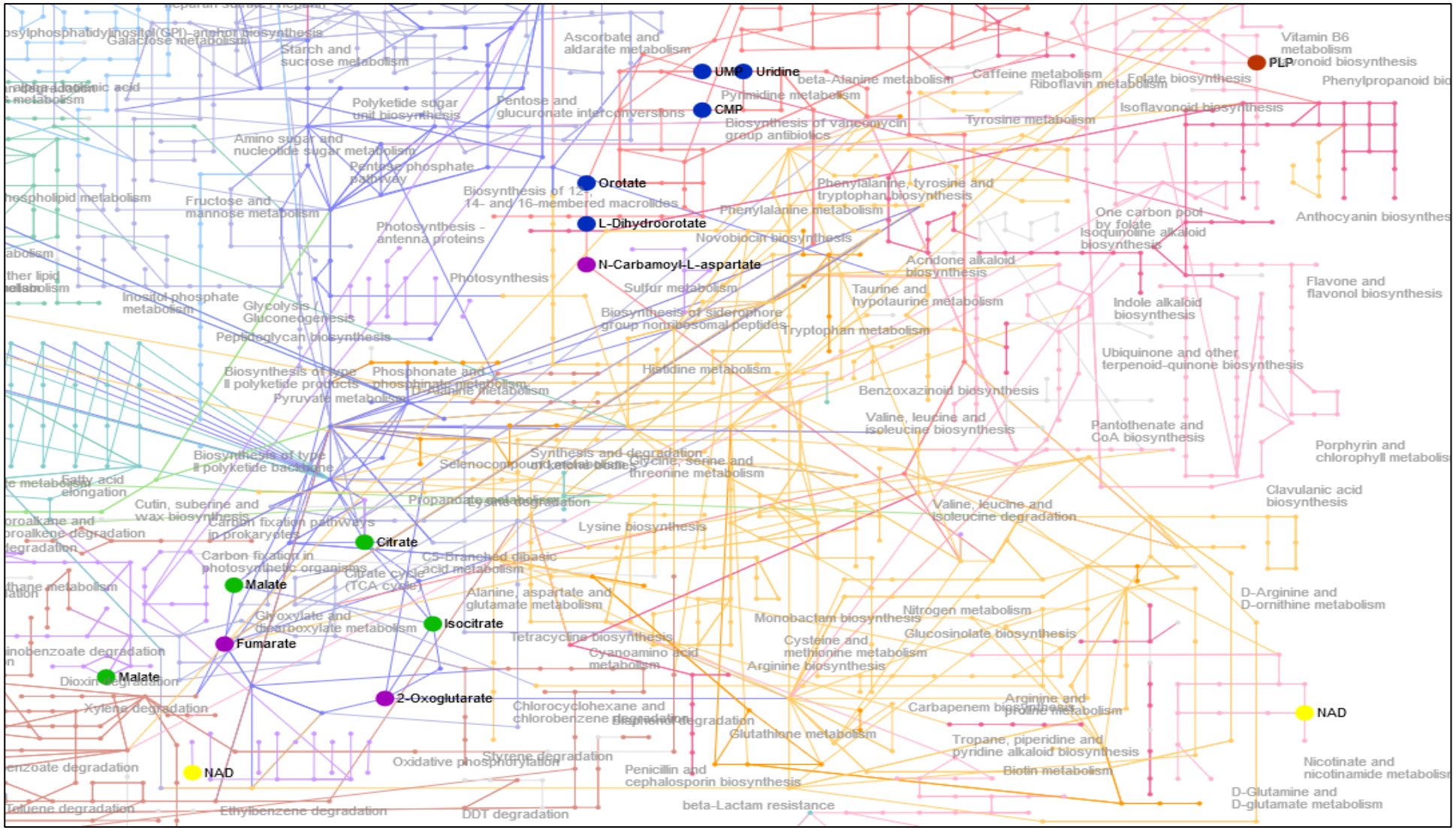


Figure S 6: Network mapping of pathway hits from the top five significant pathways in the T19 inoculated shoots. Yellow points are hits from nicotinate and nicotinamide metabolism. Green points are hits from the citrate cycle. Purple points are hits from alanine, aspartate and glutamate metabolism. Blue points are hits from pyrimidine metabolism. The red point is a hit from vitamin B6 metabolism.

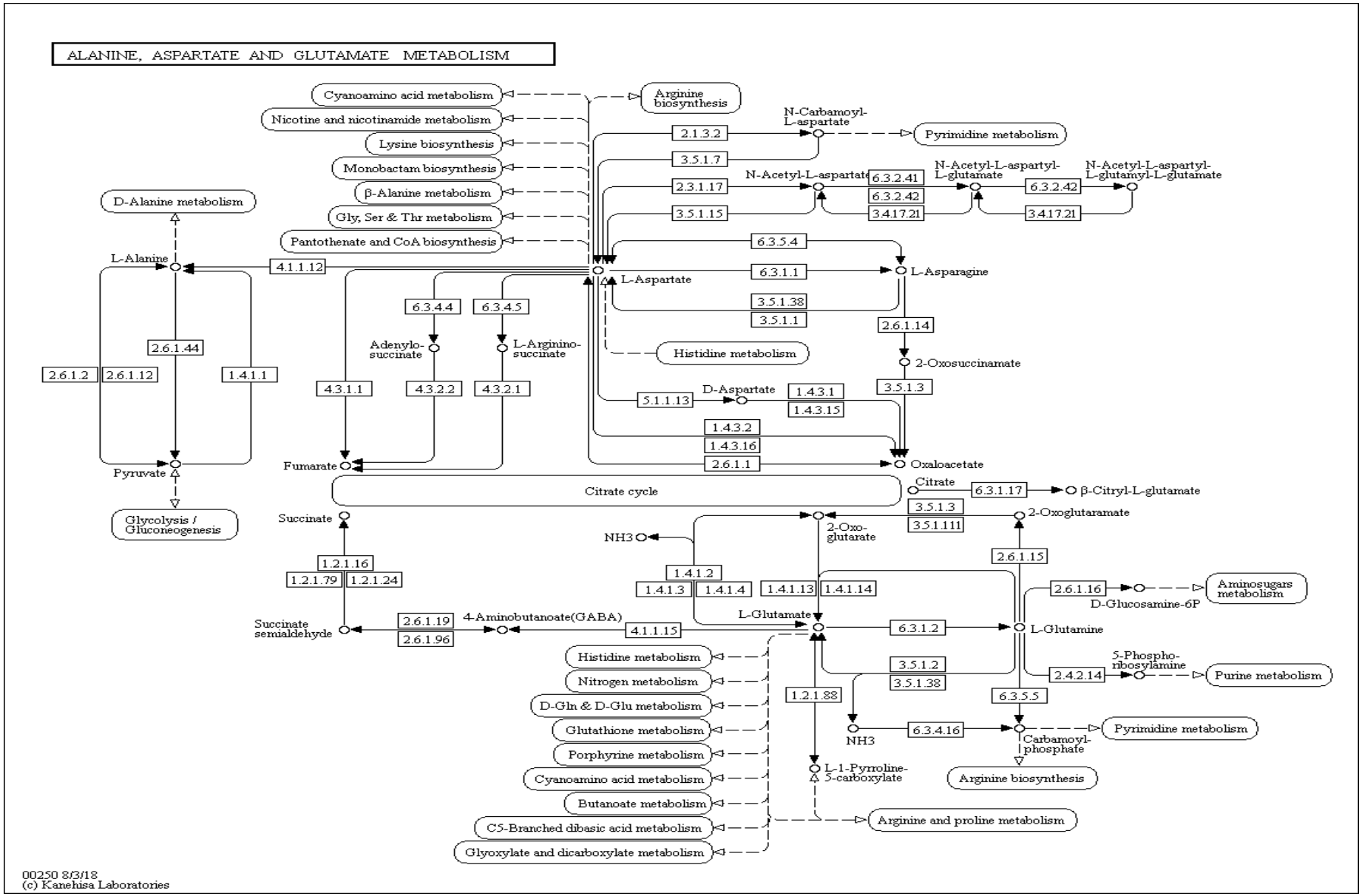


Figure S 7: Metabolic reactions and pathways that make up alanine, aspartate and glutamate metabolism obtained from the KEGG website (https://www.genome.jp/kegg/kegg3a.html).