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The Arbuscular Mycorrhizosphere

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I hereby declare that I have written this thesis independently without help from others and without the use of documents or aids, other than those stated. I have mentioned all sources used and cited them correctly according to established academic citation rules.

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0.2 Table of Contents

0.1 Acknowledgments	3
0.2 Table of Contents	4
0.3 List of Abbreviations Used in the Text	7
0.4 Introduction	9
0.4.1 The Impact of Long-Term Usage of Fertilisers and Pesticides Upon Sustainable Agriculture	9
0.4.2 The Root Extension – the Mycorrhizosphere	10
0.4.3 Arbuscular Mycorrhizal Fungi (AMF)	11
0.4.4 The Roles of Beneficial Bacteria in Promoting Plant Performance	13
0.4.5 Interactions of AMF with Rhizobacteria	15
0.4.6 Thesis Overview	16
1.0 Chapter 1: The Effects of Arbuscular Mycorrhiza Hyphosphere Bacteria on <i>Prunella vulgaris</i>	18
1.1 Abstract	18
1.2 Background	18
1.3 Materials and Methods	20
1.3.1 Seed Preparation and Germination	20
1.3.2 Soil Sterilisation Procedure	20
1.3.3 Bacterial Inoculum Preparation	20
1.3.4 Mycorrhizal Inoculum Preparation	21
1.3.5 Plant Growth Conditions and Harvesting	21
1.3.6 Quantitative Assessment of AMF Colonisation	22
1.4 Results and Discussion	23
1.4.1 The Biomass of <i>Prunella Vulgaris</i> Under Gnotobiotic Conditions using Eco2Boxes with Different Bacterial Treatments	23

1.4.2	The Biomass of <i>Prunella Vulgaris</i> in Greenhouse Conditions with Different Bacterial Treatments	24
1.4.3	Effect of Bacterial Treatment Upon Mycorrhization in Greenhouse Conditions	28
1.4.4	Further Steps	31
2.0 Chapter 2: Designing a qPCR Method for Quantification of AMF Colonisation in <i>P. vulgaris</i> Roots		33
2.1	Abstract	33
2.2	Background	33
2.3	Materials and Methods	34
2.3.1	DNA Extraction	34
2.3.2	AMF Primer Design	34
2.3.3	Plant Primer Design	34
2.3.4	Testing Primers	34
2.3.5	qPCR Efficacy Test	35
2.3.6	qPCR Method	35
2.4	Results and Discussion	35
2.4.1	Testing the Designed <i>P. vulgaris</i> Primer Pairs	35
2.4.2	qPCR Efficacy	36
2.4.3	Efficiency of Plant Primers	37
3.0 Chapter 3: Mycorrhizal fungal interactions with potential plant promoting <i>Pseudomonas</i> (WCS417, WCS374, WCS358) strains		40
3.1	Abstract	40
3.2	Background	40
3.3	Materials and Methods	41
3.3.1	Seed Preparation and Germination	41
3.3.2	Bacterial Inoculum Preparation	41
3.3.3	Soil Preparation	41
3.3.4	Plant Growth Conditions and Harvesting	41
3.4	Results	42

3.4.1	Mycorrhizal Interactions with <i>Pseudomonas</i> (WCS417, WCS374, WCS358) strains	42
3.4.2	Effect of bacterial treatment upon mycorrhization	43
3.5	Discussion	43
4.0	Chapter 4: Effects of WCS Strains Upon Immunity Against Pathogens in <i>Prunella vulgaris</i>	47
4.1	Abstract	47
4.2	Background	47
4.3	Materials and Methods	47
4.3.1	Seed Preparation and Germination, Soil Sterilisation and Bacterial Inoculum Preparation	47
4.3.2	Preparation of <i>Botrytis cinerea</i> Inoculum	48
4.3.3	Inoculation of <i>Prunella vulgaris</i> with <i>Botrytis cinerea</i>	48
4.4	Results	48
4.4.1	Lesion Size	48
4.4.2	Scoring System	49
4.5	Discussion	51
4.5.1	Pre-trial Experiment	51
4.5.2	Development of the Scoring System	51
4.5.3	Do the <i>Pseudomonas</i> strains protect <i>P. vulgaris</i> against <i>B. cinerea</i> infection?	51
4.5.4	Further Work	52
5.0	References	54
6.0	Supplementary Data	63

0.3 List of abbreviations used in the text

AM: Arbuscular mycorrhiza

ACS: Autoclaved conventional soil

AMF: arbuscular mycorrhizal fungi

APS: Autoclaved potting soil

ARS: Autoclaved river sand

AS: Autoclaved soil

BioCliVE: Biodiversity and Climate Variability Experiment

BLAST: Basic Local Alignment Search Tool

CS: Non-autoclaved conventional soil

DON: deoxynivalenol

ET: ethylene

HB: Hyphosphere bacteria

IDT: Integrated DNA Technologies

ISR: Induced Systemic Resistance

JA: jasmonic acid

KBA: King's B Agar

KOH: Potassium hydroxide

MHB: Mycorrhiza helper bacteria

MS: Murashige and Skoog agar

MSR: Modified Srullu and Romand medium

NCBI: National Center for Biotechnology Information

NPK: Nitrogen, phosphorus, potassium

NS: Natural soil

NTC: No template control

PCR: Polymerase Chain Reaction

PDA: Potato Dextrose Agar

PDB: Potato Dextrose Broth

PGPF: Plant-growth promoting fungi

PGPR: Plant-growth promoting rhizobacteria

PMI: Plant-Microbe Interactions Department at Universiteit Utrecht

PSB: phosphate-solubilizing bacteria

SA: Salicylic Acid

SB: Soil bacteria

SIR: systemic immune response

SOM: Soil organic matter

UU: Universiteit Utrecht

WCS: Willie Commelin Scholten (refers to all strains from the Willie Commelin Scholten Phytopathological Laboratory)

0.4 Introduction

Soil is a dynamic, living ecosystem in which a large variety of different microorganisms can be found. Of these microorganisms, bacteria and fungi dominate the soil, existing in concentrations of around 10^8 - 10^9 cfu/g and 10^5 - 10^6 cfu/g, respectively (Bhattarai, 2015). Microbial communities play important roles in ecosystem functioning, for example in nutrient cycling, primary production, litter decomposition and climate regulation (Delgado-Baquerizo et al., 2016), and evidence even suggests that a higher microbial diversity improves ecosystem functioning linearly (Delgado-Baquerizo et al., 2016). Thus, it is becoming ever more important that we understand the complex interactions taking place within the soil and rhizosphere, so that we may: reduce our reliance upon synthetic fertilisers and pesticides; improve biodiversity in terrestrial ecosystems; be better equipped for climate change adaptation.

In this introduction, we will discuss: the impact of synthetic fertilisers and pesticides in agriculture and the importance of finding sustainable alternatives; the role and importance of the mycorrhizosphere, including the roles of beneficial bacteria and AMF and their interactions; and the main research questions this thesis asks and its implications.

0.4.1 The impact of long-term usage of fertilisers and pesticides upon sustainable agriculture

Since the invention of the first synthetic fertiliser in 1861 (Russel & Williams, 1977), great advancements have been made in agriculture which have allowed us to feed a growing global population. This intensive use of chemicals as fertilisers and pesticides has massively increased yield and crops, but at a great cost. Most modern pesticides are today polluting water, soil and the atmosphere, and are damaging to human health (Riah et al., 2014). Pesticides may be effective at killing harmful pathogens and pests, but also kill the beneficial microbes in the soil, such as arbuscular mycorrhizal fungi (AMF) and rhizobacteria, and damage soil enzymes. This affects nutrient cycles and therefore fertilisation (Riah et al., 2014), as well as biodiversity, which has been identified by Rockström as one of the biggest threats that may overthrow the stability of the Holocene (Rockström et al., 2009).

It is common that everyday food and beverages contain pesticide residues (Nicolopoulou-Stamati et al., 2016), and that washing is not effective at completely removing these residues (Reiler et al., 2015). Although the concentrations are generally below the legislatively determined safe levels (Lorenzin, 2007; Witczak & Abdel-Gawad, 2014), the long term effects of the exposure of multiple pesticides has been understudied and they may act in a synergistic manner to negatively affect human health (Nicolopoulou-Stamati et al., 2016).

4.1 million tons of pesticides are used globally every year, creating a surplus of nitrate in the soil (Riedo et al., 2021). In fact, 30-50% of applied pesticides do not reach the intended targets (Riedo et al., 2021), which can lead to nitrate leaching into water and soil, and has a destructive effect on the environment. This can be the eutrophication of freshwater and marine ecosystems (Morelli et al., 2018), the pollution and degradation of the soil (Savci, 2012), and air pollution via nitrous oxide emissions (Savci, 2012). In terrestrial systems, pesticide use has been directly and indirectly linked to decreasing bird, insect and pollinator populations (Riedo et al., 2021), as well as negatively affecting microbial populations.

Thus, there is an urgent need to reduce our reliance on chemicals in agriculture, whilst still achieving high yields to feed a growing population. Of growing interest over recent decades, is the utilisation of certain biological components in the soil, which includes AMF, PGPR and rhizobacteria to reduce or replace synthetic chemicals (Raklami et al., 2019). These microorganisms can directly facilitate plant growth and can also act in a protective manner against pests and pathogens. When used in combination, the effects of rhizobacteria, PGPR and rhizobacteria induce a stronger effect upon plant yield, than when used by themselves (Raklami et al., 2019).

0.4.2 The root extension – the mycorrhizosphere

For ease of understanding, the soil can be separated into three sections: the rhizosphere, the hyphosphere and the remaining bulk soil (Figure 1). The rhizosphere contains the plant roots and microorganisms in the vicinity of the roots. The hyphosphere does not include the plant roots, but it does include the hyphae of the AMF associated with those roots, alongside other microorganisms. Together the rhizosphere and the hyphosphere make up the mycorrhizosphere (Audet, 2012), i.e., the total area colonised by AMF. The bulk soil exists outside of the mycorrhizosphere, and still contains a high microbial concentration.

The plant microbiota is mostly located within the rhizosphere and is defined as the total microorganisms that are hosted by the plant (in this case in the root system), that can act in a commensal or mutualistic manner with the plant (Pieterse et al., 2014). These microorganisms are often referred to as plant-growth promoting rhizobacteria (PGPR) and plant-growth promoting fungi (PGPF), and they can provide the plant with enhanced mineral uptake, nitrogen fixation, growth promotion and protection from pathogens (Pieterse et al., 2014). In return, microorganisms may receive up to 20% (Thirkell et al., 2020) to 40% (Bais et al., 2006) of the plants photosynthetically derived carbon, in a term known as rhizodeposition. Rhizodeposition can cause a 10-100 fold increase in the microbial density in the mycorrhizosphere, leaving a microbial community distinct from the remaining bulk soil (Bakker et al., 2013). This is also beneficial for the host plant, as it preserves its own microbiota.

However, the terms PGPR and PGPF can be misleading as they imply that these microorganisms will always assist in plant growth, which is not always the case: they may also play roles in protecting the plant from pathogen invasion via direct competition or by stimulating the plants induced systemic resistance (ISR) (Pieterse et al., 2014). In the latter case, the priming of the ISR may actually hinder plant growth, in what is known as the growth-defense trade off (Huot et al., 2014). Rhizodeposition may also hinder plant growth. Additionally, the term PGPR implies that it is only the bacteria that may play beneficial roles in plant health, when it is also bacteria in the hyphosphere that can do this. Thus, fungi and bacteria that provide benefit to its host will now be referred to as beneficial bacteria or beneficial fungi.

Although much of the plant microbiota is in the rhizosphere, the function of the hyphosphere should not be underestimated. The microbes associated with the AM mycelium are instrumental in determining the beneficial roles of AMF toward plant (Cabral et al., 2019). In return, the AM mycelium may also increase the presence of beneficial bacteria (Albertsen et al., 2006), but other evidence suggests it may have a suppressive effect on the hyphosphere microbial community (Welc et al., 2010). Contrasting evidence states that the AM mycelium

in soil has little influence on the microflora composition in the hyphosphere, but rather it is the colonisation of roots by AMF that influences the hyphosphere bacterial communities (Andrade et al., 1997), suggesting that the role of plant root exudates is more important in determining the hyphosphere microbial composition.

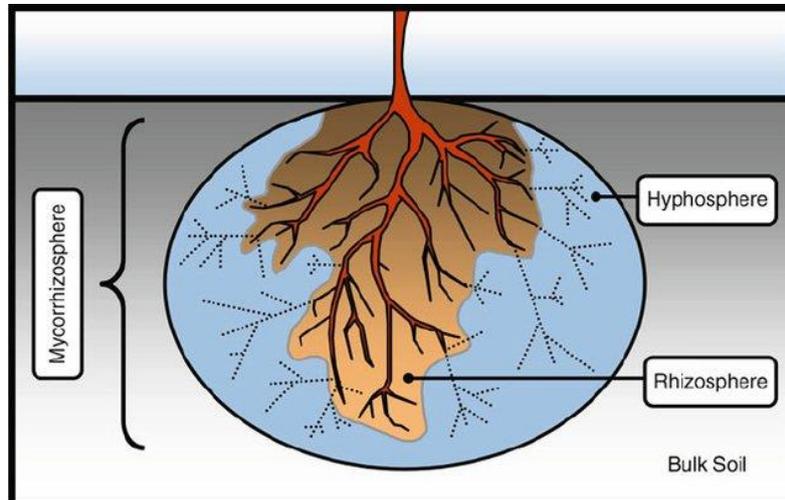


Figure 1: Defining the mycorrhizosphere and its zone of influence (Audet, 2012)

0.4.3 Arbuscular Mycorrhizal Fungi (AMF)

AMF have co-evolved with plants and have allowed plants to colonise terrestrial ecosystems for the past 400 million years (Bonfante & Genre, 2008). Approximately 70-90% of terrestrial plants have associations with AMF (Amalia et al., 1968) and in such plants, AMF can be thought of as an extension to the plants own root system, by increasing both surface area and area covered. AMF enhance the uptake of mineral nutrients, phosphorous, influences plant-plant interactions (in what is known as the wood wide web) and the structure of plant communities (Amalia et al., 1968). AMF may also provide enhanced adaptability to plants under stressful conditions, such as heat (Mathur et al., 2021), salinity (Dastogeer et al., 2020), drought (Mathur et al., 2019), heavy metals (Dhalaria et al., 2020) and other biotic and abiotic factors. It may achieve this via the up-regulation of tolerance mechanisms and by preventing the down-regulation of key metabolic pathways (Begum, Qin, Abass Ahanger, et al., 2019), as well as by improving plant health status via the acquisition of nutrients.

Fungal structures of AMF

In order to achieve a successful symbiosis with plants, AMF form several structures within plant roots, that include hyphae, vesicles and arbuscules. Outside of the root in the rhizosphere, hyphae and spores are also present. The hyphae are used for transport of nutrients from the soil to the root and for the transport of plant-derived carbon. AMF vesicles in plant roots are the storage organs of the AMF and may serve as propagules in certain species (Müller et al., 2017). The role of the arbuscule is to facilitate nutrient exchange in the plant-AMF symbiosis, this will be discussed below.

Formation of the arbuscule

Once in contact with a plant root, hyphae in the soil may enter and colonise the root via the exchange of signalling molecules between the symbionts (Luginbuehl & Oldroyd, 2017).

Plant-derived strigolactone(s) are perceived by the hyphae (K et al., 2005), which then triggers the formation of chitooligosaccharides and lipochitooligosaccharides which act as fungal signalling molecules to the plant; this activates the symbiosis signalling pathway and establishes the AMF-plant symbiosis (Sun et al., n.d.). Following attachment of hyphae on root epidermal cells, hyphae may then grow intercellularly through the plant root as intraradical hyphae (Figure 2) until they reach the inner cortex: this is where arbuscules are formed (Luginbuehl & Oldroyd, 2017). Intraradical hyphae enter the cortical cells and undergo extensive branching (Figure 2). A periarbuscular membrane containing phosphate transporters and other membrane localised proteins is formed and thus the arbuscule is created (Alexander et al., 2011). Now, the host-symbiont relationship is formed, and nutrient exchange between the partners can commence. For a more detailed review on the formation of arbuscules, please refer to the review by (Luginbuehl & Oldroyd, 2017).

Role of the arbuscule

The primary role of the arbuscule is to facilitate nutrient exchange between the two symbiotic partners. The highly branched hyphal structures in the arbuscule allow efficient exchange via a large surface area. AMF facilitates the uptake of water and essential mineral nutrients from the soil, such as phosphate and ammonium (Luginbuehl & Oldroyd, 2017). The hyphae in the rhizosphere attached to the arbuscule can also be thought of as an extension to the plant root surface area. The mycelium is able to mineralise nutrients in the soil much more effectively than the plant root, significantly improving the plant nutrient status (Smith & Smith, 2011). In return, fungi can receive up to 20% of the plants photosynthetically generated carbon to support their growth and survival as obligate biotrophs (Bago et al., 2000). This can in turn affect the CO₂ fixation by host plants by increasing the “sink effect” through the movement of photoassimilates from the aerial parts to the roots, i.e. plants can exhibit increased rates of photosynthesis (Begum, Qin, Abass Ahanger, et al., 2019).

Degeneration of the arbuscule

The average life span of an arbuscule is short and lasts between two to eight days before they rapidly collapse (Luginbuehl & Oldroyd, 2017). The degeneration of arbuscules may be followed by re-colonisation of the root, and oftentimes the formation of new arbuscules can take place in the same cells as previously (Kobae & Hata, 2010). Degenerating arbuscules show rapid shrinkage of the branched hyphae and the degeneration of the periarbuscular membrane and associated proteins (Kobae & Hata, 2010).

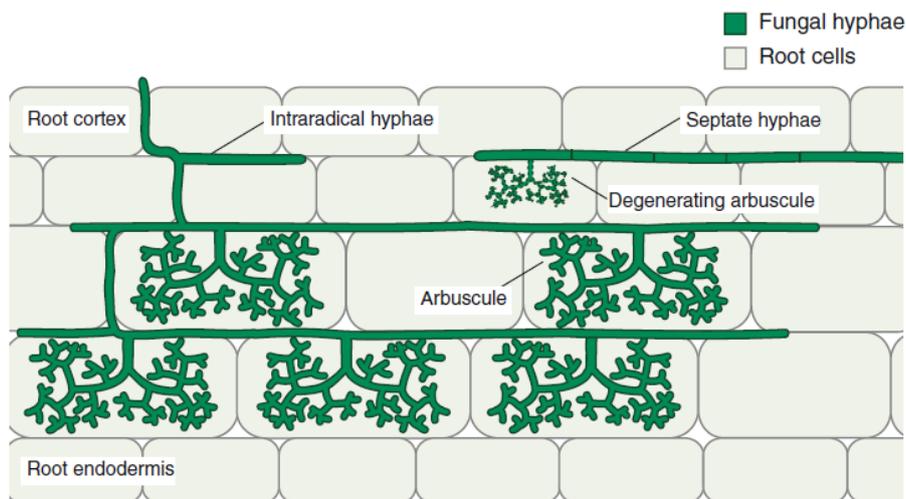


Figure 2: The formation of AMF arbuscules in plant roots, such as *Prunella vulgaris*. Intraradical hyphae grow intracellularly until they reach the inner root cortex, where they may enter root cortical cells to form an arbuscule. The host plasma membrane does not rupture, instead it expands to accommodate the hyphal branches, forming a periarbuscular membrane to separate the fungal hyphae and plant cytoplasm. Figure taken from (Luginbuehl & Oldroyd, 2017).

0.4.4 The roles of beneficial bacteria in promoting plant performance

The soil hosts a wide range of different bacterial genera and species. Soil bacteria are important in geochemical and nutrient cycles and have been used in crop protection for decades due to their effects upon plant growth and health (Hayat et al., 2010).

Genera of beneficial bacteria can be split into two groups: those that exist symbiotically with the plant root, (this includes bacteria from the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, *Sinorhizobium*, *Devosia* and *Mesorhizobium*) (Hayat et al., 2010); and those that exist as free-living bacteria (this includes bacteria from the genera *Azospirillum*, *Enterobacter*, *Klebsiella* and *Pseudomonas*) (Hayat et al., 2010). Beneficial bacteria provide plant-health promoting functions in several ways: synthesising particular compounds that the plants may use directly; facilitating the uptake of certain nutrients from the soil; lessening or preventing the invasion and spread of pathogens in the plant (Hayat et al., 2010).

Of the bacterial genera named, here we will focus on two: *Pseudomonas* and *Devosia*. This thesis will use species from these genera to determine their effect upon plant health and mycorrhization. Bacteria belonging to the *Pseudomonas* genus are very diverse and are thus still undergoing refinement (Berendsen et al., 2015; Mulet et al., 2010). In the past 30 years, over 300 publications have described the biological mechanisms of three strains of *Pseudomonas* in their abilities in promoting plant growth and reducing the effects of plant pathogen diseases (Berendsen et al., 2015): *Pseudomonas simiae* WCS417 (hereby referred to as WCS417), *Pseudomonas capeferrum* WCS358 (hereby referred to as WCS358) and *Pseudomonas defensor* WCS374 (hereby referred to as WCS374). When describing all three strains, they will hereby be referred to as ‘the WCS strains’. ‘WCS’ refers to Willie Commelin Scholten, a Dutch biology student who passed away in 1893 (Scholten, 1957). Following his death, Willie’s parents funded the institute, ‘the Willie Commelin Scholten Phytopathological Laboratory’ at the universities of Utrecht and Amsterdam (Scholten, 1957). Thus, all strain

names collected from this laboratory receive the label “WCS” (Pieterse et al., 2020). WCS358 and WCS374 were isolated from the rhizosphere of potato (Geels & Schippers, 1983) and WCS417 from the rhizosphere of wheat (WCS417) (Lamers et al., 1988).

Bacteria from the genus *Devosia* are a group of motile, gram-negative bacteria. *Devosia* species have shown their ability in nitrogen fixation in the root nodules of the legume *Neptunia natans* (Rivas et al., 2002). A recent genomic study of 27 *Devosia* strains found the dominant role of *Devosia* species was in the uptake and utilisation of nutrients for growth and survival, followed by the detoxification and degradation of organic pollutants (Talwar et al., 2020). Several *Devosia* species show promise in the degradation of deoxynivalenol (DON) (Sato et al., 2012; Wang et al., 2019). DON is a mycotoxin produced by the fungal plant pathogen *Fusarium*, and causes great losses in livestock farming and severe safety risks to human health (Wang et al., 2019). Hypothetical proteins involved in protection, adhesion and biofilm formation have been deduced in *Devosia* which may assist in the colonisation of these strains in plant roots (Talwar et al., 2020). The top metabolic pathways constructed from this study also deduced the biosynthesis of antibiotics (Talwar et al., 2020), which may also play a role in pathogen suppression. Although little research has been done on the role of *Devosia* in soil community dynamics and plant health, there is promise that *Devosia* may enhance plant health, as well as its potential use as a biocontrol agent.

Beneficial bacteria may produce compounds that the plants can use directly or indirectly, such as in the activation or deactivation of certain genes or transcription factors that prime the plants immune or stress responses. For example, it was found that WCS417 plays a role in inducing the Fe-deficiency response in *Arabidopsis* via the upregulation of the plant genes MYB72 and IRT1 (Verbon et al., 2019), which is useful in Fe-deficient soils. WCS417 has also shown its potential as a PGPR and stimulates lateral root formation and increases shoot growth in *Arabidopsis thaliana* (Wintermans et al., 2016).

Beneficial bacteria may facilitate the uptake of nutrients by plants. For example, phosphorous is usually present as inorganic phosphate in the soil, bound to minerals as a metal complex (Varga et al., 2020). Phosphate solubilizing bacteria (PSB) can solubilise this inorganic phosphate complex into soluble ionic phosphate (Pi, HPO_4^{2-} , H_2PO_4^-), which can be easily taken up by the plant (Alori et al., 2017). Phosphate can also exist as organic phosphorous, which must also first be mineralised by bacteria before uptake into the plant (Alori et al., 2017). These processes may either occur in the root itself, by bacteria colonising the root (Varga et al., 2020), or in the soil. Please see the review by (Alori et al., 2017) for a more detailed overview of the theories and mechanisms of phosphate solubilisation and mineralisation.

Possibly the most well-known mechanism by which bacteria facilitate nutrient acquisition in plants is via nitrogen fixation. Here, rhizobacteria associated with plant root nodules of leguminous plants fix atmospheric nitrogen (N_2) into nitrate (NO_3^-) via the action of a nitrogenase enzyme (Peix et al., 2014). The host plant may now absorb the nitrate, and in return the plant supplies the rhizobacteria with reduced carbon in the form of carbohydrates (Schwember et al., 2019).

Bacteria and fungi may also facilitate nutrient uptake by increasing the bioavailability of other nutrients. This includes potassium (K) and iron (Fe) mobilisation through the production of organic acids and siderophores (Rashid et al., 2016).

Beneficial bacteria in the rhizo- and hyphosphere may protect the plant against pathogen infection either directly or indirectly. For example, WCS417 has been used as a biocontrol agent against the plant pathogen *Gaeumannomyces graminis* var. *tritici*, the causative agent of wheat take-all disease, when applied as a seed treatment prior to planting (JG et al., 1988). This can be done directly via competition for nutrients in the soil. For example, WCS417, WCS358 and WCS374 each have distinct mechanisms in which they chelate iron to create siderophores (Berendsen et al., 2015). This therefore lowers the concentration of accessible iron in the soil, thereby inhibiting the growth of pathogens.

Many bacterial species within the soil produce antimicrobial compounds which can kill or hinder the growth of pathogens. Precursor genes for antimicrobial compounds such as 2,4-diacetylphloroglucinol, phenazines, hydrogen cyanide, and pyrrolnitrin have been abundantly present in many soil *Pseudomonas* species (Gross & Loper, 2009; Loper et al., 2012), but not in the three WCS strains. In fact, no studies have been able to isolate antimicrobial compounds from WCS417, WCS358 and WCS374. However, a genomic study of the three strains indicate a potential for each of these three strains in the production of a small number of antimicrobial metabolites (Berendsen et al., 2015). Thus, it can be assumed that the three WCS strains benefit plant health via other mechanisms.

Beneficial microbes may also indirectly prevent or lessen disease in plants via the stimulation of the plants local or induced systemic response (ISR). The ISR is defined as “a state of enhanced capacity developed by a plant when appropriately stimulated” (Choudhary et al., 2007) and is most commonly induced by pathogens (De Kesel et al., 2021), but also by the plants own beneficial microbiota. One way by which beneficial bacteria stimulate the ISR response is by stimulating the jasmonic acid (JA) and ethylene (ET) pathways (Pieterse et al., 1998). The potential of WCS417 in stimulating ISR was first demonstrated 30 years ago. Here, the colonisation of the root system by WCS417 helped plants acquire an enhanced level of resistance against the fungal pathogen *Fusarium oxysporum* (Peer, 1991). For example, WCS417 treated plants produced more antimicrobial compounds at the site of infection, and thus WCS417 has the ability to protect the plant from pathogens distally (Peer, 1991).

0.4.5 Interactions of AMF with rhizobacteria

In order to properly utilise microorganisms as bioagents in agriculture, we must begin to understand the complex interactions within the soil. AMF, rhizobacteria and PGPR all contribute to plant health, and when used in combination produce stronger effects upon plant growth than by themselves (Raklami et al., 2019), which suggest that these microorganisms act synergistically to promote plant growth and health. AMF stimulate the growth and development of bacteria and play a decisive role in the bacterial community structure (Vestergård et al., 2008), and the exudates of AMF may play a more important role than plant exudates in selecting for beneficial bacteria in the mycorrhizosphere (Roesti et al., 2005). Ectomycorrhizal fungi have been shown to select for *Pseudomonas* species beneficial to symbiosis and plant health, whilst also decrease the abundance of bacteria detrimental to mycorrhizal development (Pascale Frey-Klett et al., 2005).

This is not a one way system, and Mycorrhiza Helper Bacteria (MHB) also promote the bacteria-AMF and AMF-plant symbioses by stimulating mycelial extension, increasing root-fungus contacts and colonisation and protecting the AMF from adverse environmental conditions (P Frey-Klett et al., 2007). This can be achieved via the production of growth

factors, via the detoxification of certain substances or via inhibition of competitors and antagonists (P Frey-Klett et al., 2007). MHB may also stimulate AMF germination by degrading chitin and chitosan of fungal spores (Turrini et al., 2018).

0.4.6 Thesis Overview

For ease of the reader, this thesis has been split into four chapters: Chapter 1 – The Effects of Arbuscular Mycorrhizal Hyphosphere Bacteria on *Prunella vulgaris*; Chapter 2 – Designing a qPCR Method for Quantification of AMF Colonisation in *P. vulgaris* Roots; Chapter 3 – Mycorrhizal fungal interactions with potential plant promoting *Pseudomonas* (WCS417, WCS374, WCS358) strains; Chapter 4 – Effects of WCS Strains Upon Immunity Against Pathogens in *Prunella vulgaris*.

The overall research goal that this thesis aims to elucidate, is in gaining a better understanding of the interactions that occur between plant host and the microorganisms that exist in its mycorrhizosphere (including beneficial bacteria and AMF). *Prunella vulgaris*, better known as ‘heal-all’, was used throughout the experiments in this thesis, as it has a high level of AMF colonisation, thus making it an ideal model to study mycorrhizal interactions.

In this thesis, we look at a range of soil bacteria, including recently elucidated hyphosphere bacteria and some well-studied *Pseudomonas* strains (WCS strains). We treated the soil with these various bacterial strains, either as a community or as a single strain, to determine the effect of these treatments upon *P. vulgaris* phenotypes, including plant above- and below-ground biomass and total root colonisation by AMF.

In Chapter 1, “The Effects of Arbuscular Mycorrhizal Hyphosphere Bacteria on *Prunella vulgaris*”, soil was treated with one or more bacterial strains, which had been previously isolated by Changfeng Zhang (PMI, UU) in the hyphosphere of *Prunella vulgaris*. Here, the goal was to determine the effect that these strains had upon: i) above- and below- ground biomass of *P. vulgaris*; and ii) the colonisation of *P. vulgaris* roots by AMF (mycorrhization).

Mycorrhization was measured using traditional microscopic methods, which is time consuming. Thus, chapter 2, “Designing a qPCR Method for Quantification of AMF Colonisation in *P. vulgaris* Roots” aims to design a qPCR method for quicker and more accurate mycorrhization data, to give higher throughput analysis.

Chapter 3, “Mycorrhizal fungal interactions with potential plant promoting *Pseudomonas* (WCS417, WCS374, WCS358) strains”, aims to elucidate the roles of the three plant-growth promoting strains of *Pseudomonas* (WCS417, WCS374, WCS358) upon *P. vulgaris*. These three *Pseudomonas* strains have been previously well studied, with over 300 publications demonstrating their beneficial roles towards plants (Berendsen et al., 2015). In previous experiments performed in PMI by Bich Nguyen Thi (Nguyen, 2021), WCS417-treated soil promoted root growth, whereas WCS358 and WCS374 did not; WCS417 and WCS374 promoted shoot growth, whereas WCS358 did not. WCS358 reduced both above and below ground biomass, which was not expected. Here, one hypothesis formed is that WCS358 activates *P. vulgaris*’ induced systemic resistance (ISR). The priming of the ISR can hinder plant growth in what is known as the plant growth-defense trade off (Huot et al., 2014), although ISR does not lead to a particularly strong growth-defense trade off. Another hypothesis is that because of the activation of the plants ISR by the beneficial bacteria,

colonisation of roots by fungi (including AMF) may be hindered. The experiments performed in Chapter 3 were designed to repeat the experiments performed by Bich Nguyen Thi, with different soil conditions, to test how the *Pseudomonas* strains affect *P. vulgaris* growth under different conditions, and to test its reproducibility.

And finally, Chapter 4, “Effects of WCS strains upon immunity against pathogens in *Prunella vulgaris*” aimed to test the hypothesis that the *Pseudomonas* strains from Chapter 3 activate the plants ISR response. Here, the *Pseudomonas* strains were added to the soil and leaves were infected with the air-borne fungal pathogen, *Botrytis cinerea*. Infection was measured in two ways, and in plants with lower levels of infection, it can be assumed that this is done via the activation of the ISR.

Chapter 1: The Effects of Arbuscular Mycorrhiza Hyphosphere Bacteria on *Prunella vulgaris*

1.1 Abstract

The purpose of these series of experiments was to determine the interactions between hyphosphere bacterial community, the AMF community and *Prunella vulgaris*, to determine how these interactions can affect mycorrhizal colonisation of plant roots, and if this will benefit plants, i.e. can this increase plant growth and yield. Two bacterial communities were created, the hyphosphere bacterial community (HB community) and the soil bacteria community (SB community) and used to inoculate natural or autoclaved soil. Germinated *P. vulgaris* seedlings were grown in the soil for a pre-determined length of time and harvested. Plant dry biomass was measured from both the above- and below-ground parts, and a section of root was stained to analyse mycorrhizal colonisation of the *P. vulgaris* roots. In all bacterial treatments, roots grew larger in autoclaved soil than when compared to the corresponding bacterial treatment in natural soil. In general, shoot weight also tended to be higher in autoclaved soil than when compared to the natural soil, with the exception of one treatment: the HB1 treatment, which grew larger shoots in natural soil than in autoclaved soil. The autoclaving of soil alters the composition of soil and thus it is hypothesised that the autoclaving of soil allows better access to nutrients for the plant. By autoclaving the soil, all AMF are removed. Thus, another hypothesis is that the AMF obtain part of *Prunella's* photosynthetically derived carbon for their own growth, thus slowing the rate of growth for *P. vulgaris*. Roots were also analysed for the mycorrhizal colonisation by checking for three fungal structures: hyphae, vesicles and arbuscules. The SB1 and HB1 treatments had significantly fewer arbuscules than compared with the other bacterial treatments. *P. vulgaris* in the HB1 with natural soil treatment also grew significantly larger shoots than the other treatments in natural soil. Since arbuscules are the site of carbon and nutrient transfer between plant host and symbiont, this supports the hypothesis that the presence of AMF hinders the growth of *P. vulgaris*.

1.2 Background

In a previous 5-compartment experiment (Supplementary Figure 1) designed by Changfeng Zhang and Bich Nguyen Thi (Nguyen, 2021), *P. vulgaris* was grown in compartment 3 (Supplementary Figure 1), and roots separated from the remainder of the soil using a 30 μ M mesh. This allowed hyphal growth through the mesh to compartments 2, 4 and 5, while preventing root growth. Thus, this 5-compartment system was designed to mimic the mycorrhizosphere of *P. vulgaris* and provided a clear separation between the rhizosphere and hyphosphere (Figure 1). Changfeng Zhang and Bich Nguyen Thi (Nguyen, 2021), following *P. vulgaris* growth, sequenced the soil from compartment 5, and found many bacterial species, identified via matching 16S rRNA genes. Of these species, eight were able to be cultivated, and are detailed in Table 1 and Table 2. Here, we see two different communities: the hyphosphere bacterial community (HB; bacteria were isolated from compartment 5 and were enriched in compartment 5 [i.e. the hyphosphere]; Table 1) and the soil bacteria community (SB; bacteria were isolated from compartment 5, but were found to be enriched in the bulk soil; Table 2).

In this chapter, the purpose of these series of experiments was to determine the interactions between hyphosphere bacterial community, AMF community and *Prunella vulgaris*, to determine how these interactions can affect mycorrhizal colonisation of plant roots, and if this will benefit plants, i.e. can this increase plant growth and yield. Two bacterial communities were created, the hyphosphere bacterial community (HB community) and the soil bacteria community (SB community). The HB community and SB community are two bacterial communities comprising of five and three bacterial strains isolated from the hyphosphere by Changfeng Zhang (PMI, UU) in previous experiments. Community is detailed in Table 1 and Table 2.

Table 1: The hyphosphere bacterial community isolated by Changfeng Zhang in a previous 5-compartment experiment. Isolated bacteria were sequenced using a 16S approach to confirm their identities. The hyphal bacterial samples were also sequenced using 16S amplicon sequencing using best hits, to study the whole bacterial community attached to the hyphae.

Best hits	Best match isolates	Species name	Abbreviation
C7511f6ef1f1bdb63c249524f83d76e9 (99%), aaa06e5aa8245ee7e8e508b7d1df7028 (99%)	ZB163	<i>Devosia sp.</i>	HB1
A06650cfd4367728f2cd52010014fa05 (99%)	ZB026	<i>Bosea thiooxidans</i>	HB2
07a7ea4961b8eec0ed939e5285a485bb (99%), dde56901f2d4d4008b554a55287be1d2 (100%)	ZB004	<i>Sphingopyxis italica</i>	HB3
63b419d0681cb1e6cc85e5a30570d5e6 (100%)	ZB019	<i>Achromobacter piechaudii</i>	HB4
86c0fbccd830c768f8e83b1671ec2569 (100%)	ZB113	<i>Microbacterium saccharophilum</i>	HB5

Table 2: The soil bacterial community isolated by Changfeng Zhang in a previous 5-compartment experiment. Isolated bacteria were sequenced using a 16S approach to confirm their identities. The hyphal bacterial samples were also sequenced using 16S amplicon sequencing using best hits, to study the whole bacterial community attached to the hyphae. No hits indicates that there were no match in the hyphal bacterial community

Best hits	Best match isolates	Species name	Abbreviation
c1d8f746065975c3dc82d29b9eee6f45	ZB074	<i>Arthrobacter globiformis</i>	SB1
245ff9d92575941429a5ce98fcc79130	ZB117	<i>Streptomyces zhihengii</i>	SB2
no hits	ZB042	<i>Pseudomonas migulae</i>	SB3

1.3 Materials and Methods

1.3.1 Seed preparation and germination

P. vulgaris seeds were surface sterilised by vapour from 3.2mL of HCl 37% and 100mL of bleach (NaOCl) mixture for 3.5 hours. Seeds were contained in a 1.5mL Eppendorf tube with the lid off, during sterilisation, which were contained in a sealed vessel. To remove the vapour, seeds were left in a flow bench for ten minutes in an Eppendorf with the cap off. Sterilised seeds were sown on ½ Murashige and Skoog agar-solidified medium MS agar (0.5 x MS medium) supplemented with 1% (w/v) sucrose at a density of 10 seeds per plate. Petri dishes were immediately transferred to a growth chamber under a long-day photoperiod (22°C, 16h of light, intensity 100µmol m⁻² s⁻¹) and positioned horizontally. Seeds were left to germinate for 10-14 days.

1.3.2 Soil sterilisation procedure

Eco2Box: River sand and potting soil were mixed in a ratio of 4:1 (w/w) and 150g of this mixture added to magenta boxes. The magenta box was added to the Eco2Box (www.eco2box.com; 90mm diameter; 120 mm height), and the Eco2Box was covered and seal with aluminium foil and elastic bands. The Eco2Boxes containing sand/soil mixture were autoclaved twice at 121°C for 50 minutes, and boxes assembled with lids. Dimensions of Eco2Boxes are as follows: cover 118mm diameter; base 90mm diameter; height 120mm. Eco2Boxes were fitted with the red XXL filter (<http://www.eco2box.com/>).

Natural soil: River sand and Agroscope Organic soil (Wittwer et al., 2021) were mixed thoroughly in a ratio of 4:1 w/w. For the autoclaved samples, the mixed soil was autoclaved twice for 50 minutes at 121°C. For the natural soil, this was not autoclaved. Following bacterial or control inoculation, 200g soil/sand mixture was added to pots.

1.3.3 Bacterial Inoculum preparation

Bacterial strains (Table 1; Table 2) were grown separately on ISP2 (4% w/v yeast extract, 10% w/v malt extract, 4% w/v dextrose, 15% w/v agar in distilled water, pH 7.2-7.4) plates at 28°C until considerable growth was seen (2-10 days, dependent on strain). Cells were then suspended in 10mM sterile MgSO₄ and re-plated on ISP2 plates at 28 plates at 28°C until considerable growth was seen. Cells were then suspended in 10mM sterile MgSO₄ and concentration measured spectrophotometrically ($\lambda = 660\text{nm}$, if $A = 1$, $\delta = 10^9$) to achieve a concentration of 10¹⁰-10¹¹ colony forming units/mL (cfu/mL).

Cells were added to the soil to achieve a final concentration of 10⁷ cfu/g (for the HB community, each bacterial strain was added to a concentration of 2 x 10⁶cfu/mL, and for the SB community, each bacterial strain was added to achieve a concentration of 3.33 x 10⁶cfu/mL, achieving final bacterial concentration of 10⁷cfu/mL) in 60mL 10mM sterile MgSO₄ and mixed thoroughly. For the Eco2Box, cells were mixed under sterile conditions. For the non-bacterial controls, equal volume of 10mM sterile MgSO₄ was added to the soil.

1.3.4 Mycorrhizal inoculum preparation

Rhizophagus irregularis MUCL43194 spores were obtained stored in Modified Strullu and Romand (MSR) medium (Declerck et al., 1998) from AgroScope. The MSR medium was dissolved in citrate buffer (18mM citric acid, 82mM sodium citrate, adjusted to pH 6.0 with 1M NaOH and sterilised by passing through a 0.2µM Acrodisc® filter). 50 spores were then counted using a stereomicroscope and added to the soil in the Eco2Box and mixed thoroughly under sterile conditions.

1.3.5 Plant growth conditions and harvesting

The Eco2boxes were transferred to the phytotron on long day settings (21°C, 16h light, 8h dark) in a closed system and plants were harvested after 68 days. Greenhouse experiments were transferred to the greenhouse on long day settings (21°C, 16h light, 8h dark). *P. vulgaris* plants were removed from Eco2Box or plant pot and soil shaken from roots. Roots were washed thoroughly using tap water to remove the remaining dirt, and root and shoot were separated from one another using a scalpel. Total wet root weight was measured, and roots were then cut into three sections as shown in Figure 3. The weight of each section was also measured. The sections for microscopy analysis were stored in 50% ethanol at 4°C, and section for qPCR analysis were stored in 50 % glycerol at -20°C. The plant shoot and section of plant root for weight analysis were dried at 70°C for a minimum of 48h and dry weight weighed. Total dry weight of roots was then calculated using the wet weights (percentage of weight) from the three root sections.

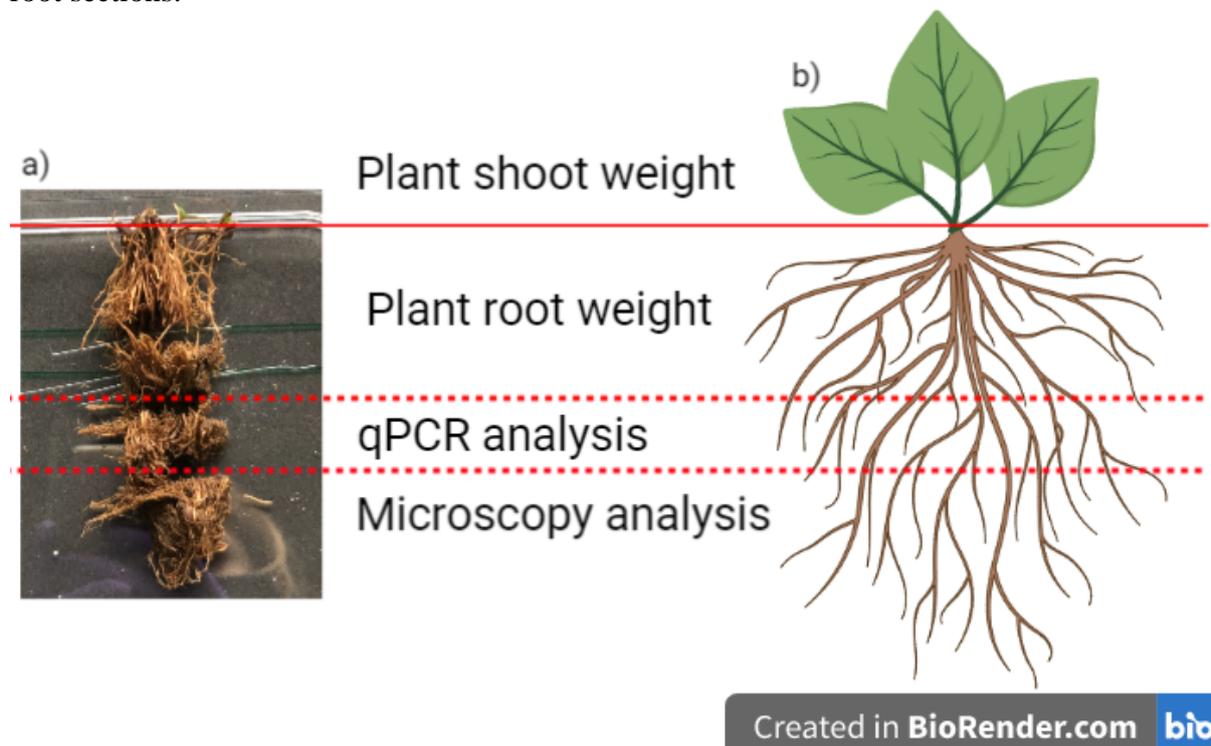


Figure 3: Method of root and shoot collection

1.3.6 Quantitative assessment of AMF colonisation

Root staining

AMF colonisation of roots was measured using microscopy using the magnified intersections method (McGONIGLE et al., 1990). Roots obtained from the microscopy analysis (Figure 3) were first processed and stained for visualisation of mycorrhizae and fungal structures using an ink-vinegar solution (Vierheilig et al., 1998). Washing steps require a minimum of 2-3 washes in deionised water. Roots stored in 50% ethanol were washed thoroughly with deionised water. To the roots, 10% w/v KOH were added to cover the roots and incubated at 80°C in a water bath for 40 minutes. Roots were washed thoroughly, and 5% ink vinegar solution added (5% acetic acid, 5% black ink from Parker Quink, in deionised water), and roots stained at 80°C in a water bath for 40 minutes. Roots were washed thoroughly and stored at -20°C in 50% glycerol until slide preparation.

Slide preparation

Root fragments were mounted onto microscope slides (Figure 4a) and covered with 40 x 22 mm coverslips. Roots were aligned parallel to the long axis of the slides (Figure 4) and observed at a 200x magnification using the magnified intersection method as according to (McGONIGLE et al., 1990), with slight modification during the positioning of the root, detailed in Figure 4c. The field view of the microscope was moved perpendicular to the slide (Figure 4c) and measurements taken when the crosshair passes through the centre of the root at x 200 magnification. Structures passing through the vertical crosshair perpendicular to the root were counted until 100 measurements per slide were taken. The following structures were counted: negative (no fungal material in root), hyphae, vesicles and arbuscules (Figure 4). In the case where hyphae and either arbuscule and/or vesicle pass through the crosshair, hyphae are not counted in the measurement as it is assumed that the presence of vesicle/arbuscule requires the presence of hyphae. In the case that both an arbuscule and vesicle pass through the vertical crosshair, this counts as one measurement and is classified under arbuscule & vesicle.

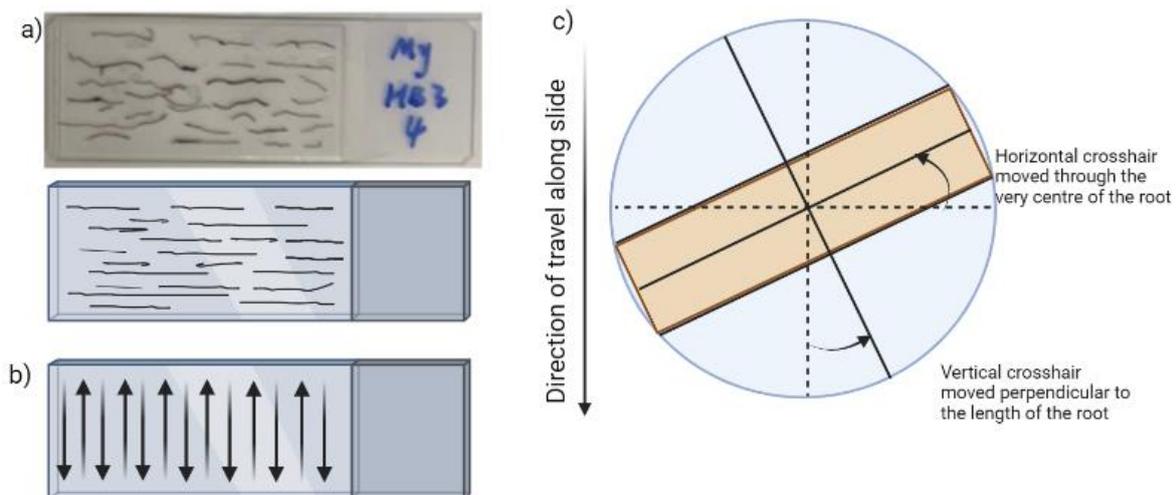


Figure 4: Microscopy method. a) Positioning of roots onto microscope slide. b) Direction of travel with the slides under the microscope. c) Correctly orienting the crosshair to align perpendicularly with the root.

1.4 Results and Discussion

1.4.1 The Biomass of *Prunella Vulgaris* Under Gnotobiotic Conditions using Eco2Boxes with Different Bacterial Treatments

For this experiment, it was necessary that conditions remained gnotobiotic. Prior to inoculation, sterility of the boxes was checked by plating onto ISP2 and R2A agar in a dilution series, and were confirmed to be sterile. Following inoculation with bacteria and mycorrhizal spores, sterility of the boxes was rechecked for infection by plating on ISP2 and R2A agar in a dilution series. Unknown colonies formed; thus, soil in most of the Eco2Boxes were contaminated. It is very difficult to fully sterilise soil via autoclave as it is a bad conductor of heat, and additionally there were many steps involved thus lots of opportunities for contamination to enter the soil. As soil is so enriched with nutrients, any bacteria that enter have a good medium for fast growth. Additionally, plants in the Eco2Boxes did not grow well. A closed system meant that many of the plants died and those that did not were in bad condition (small, browning and wilting). Plants could not be watered, and nutrients could not be added. The presence of lids may prevent some light from entering, and air flow is also poor in these boxes. The limited CO₂ in the box may have also prevented the AMF spores from germinating and colonising the plants.

While harvesting the *P. vulgaris* from the Eco2Box, roots stuck to the magenta box (due to lack of flexibility of the pots) and therefore many of the roots were broken up and lost, hence, root weight from this experiment was not measured.

For the reasons highlighted above, I do not recommend that this experiment is repeated. In addition, the conditions within the magenta box do not mimic a natural system well, so there may be poor translation into agriculture.

Results will be reported and briefly discussed, although they cannot be taken as reliable. Results are shown in Figure 5. Treatment with the soil bacterial community provided considerably larger shoots when compared to all other treatments (Tukey HSD t-test, $p < 0.05$). The SB mycorrhizal treatment also grew larger than the control and mycorrhizal control, however this is not significant (t-test, $p > 0.05$). Thus, this indicates that the SB treatment may encourage plant growth, when compared to the control and HB treatment, and that the presence of mycorrhiza hinders the HB bacterial community in the promotion of plant growth, when compared to non-mycorrhizal treatments.

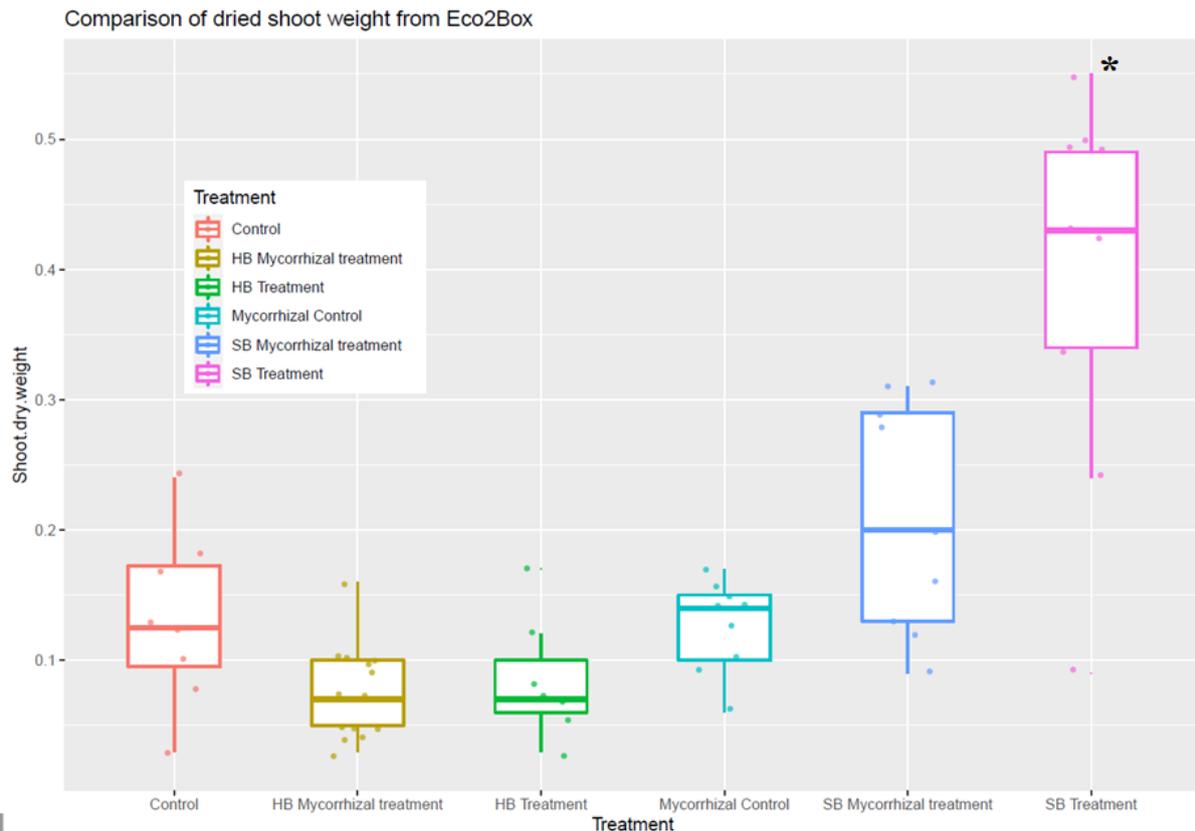


Figure 5: Comparison of dried shoot weights of *Prunella vulgaris* harvested from Eco2Boxes with different bacterial or mycorrhizal treatments. Treatments marked with (*) show significant value (t-test, $p < 0.05$)

1.4.2 The Biomass of *Prunella Vulgaris* in Greenhouse Conditions with Different Bacterial Treatments

The *P. vulgaris* plants in the greenhouse grew successfully and results of this is shown in Figure 7 and Figure 6.

Roots

In all cases, roots grew larger in autoclaved soil (Figure 6). In the autoclaved soil all HB treatments and SB3 appear to grow smaller than when compared to the AS control, although this produced no significant results (t-test, $p < 0.05$). The SB samples (SB Com, SB1, SB2) appear to grow slightly better than the control in the autoclaved soil, but this also produced no statistically significant results (t-test, $p < 0.05$), when compared to the control treatment. However, all bacterial treatments in the natural soil (NS) grew better than the NS control.

In nutrient limiting soil, or in soil where nutrients are not easily accessible to plants, bacteria play roles in oxidising and fixing these nutrients, and can also assist plants in the uptake of nutrients (White et al., 2018). Nitrogen fixation by bacteria in root nodules is perhaps the most well-known plant-microbe symbiosis, but bacteria have also been shown to solubilise bound phosphates in the soil, which allows for better phosphate uptake by plants (Shehata et al., 2017). By autoclaving the soil, this changes the structure of soil components including organic matter. In autoclaved soils, more dissolved organic matter is present (Berns et al., 2008), which may impact the effect of plants in their growth. I hypothesise that the autoclaving of the soil allows easier access to nutrients and organic matter for the plant. In this way, the role of the bacteria

is redundant. Additionally, by autoclaving the soil, this removes all AMF. There is no doubt that AMF play a beneficial role in supporting plant performance, in particular under stressful circumstances (Begum, Qin, Abass Ahanger, et al., 2019). However, AMF may also take up to 20% of the plants photosynthetically derived carbon (Thirkell et al., 2020). Since the *Prunella* grown in these experiments were not grown under stressful conditions (not limited by water, nutrients, light, and no pathogens present), I also hypothesise that the presence of AMF may have indeed hindered the growth of *P. vulgaris*.

As samples for microscopy and PCR analysis were taken from the roots (Figure 3) dry weight was calculated by first taking the whole wet weight of the root, then the wet weight of the root for dry weight analysis. Thus, a percentage of whole root weight could be taken, and total dry root weight calculated accordingly:

$$\text{Total dry root weight} = \text{Dry root weight} / \left(\frac{\text{Wet weight for dry root analysis}}{\text{Total weight of wet root}} \right)$$

This holds the assumption that water in the roots is distributed evenly over the root and between samples.

Shoots

The HB5-treated plants in the autoclaved soil had a significantly lower weight than the control plants (t-test, $p < 0.05$) when compared to the autoclaved control and appears to reduce the aboveground biomass. However, in the natural soil HB5 appears to have little role influencing shoot biomass when compared to the natural soil control. The HB1 treatment appears to be the most interesting when in natural soil. No statistically significant results were produced (t-test, $p < 0.05$), however, while the HB1 treatment decreases aboveground biomass of the plant in autoclaved soil, the opposite effect is seen in the natural soil (

Figure 7), when compared to the control. The HB1 treatment was therefore repeated under the same conditions, and results shown in Figure 9 and

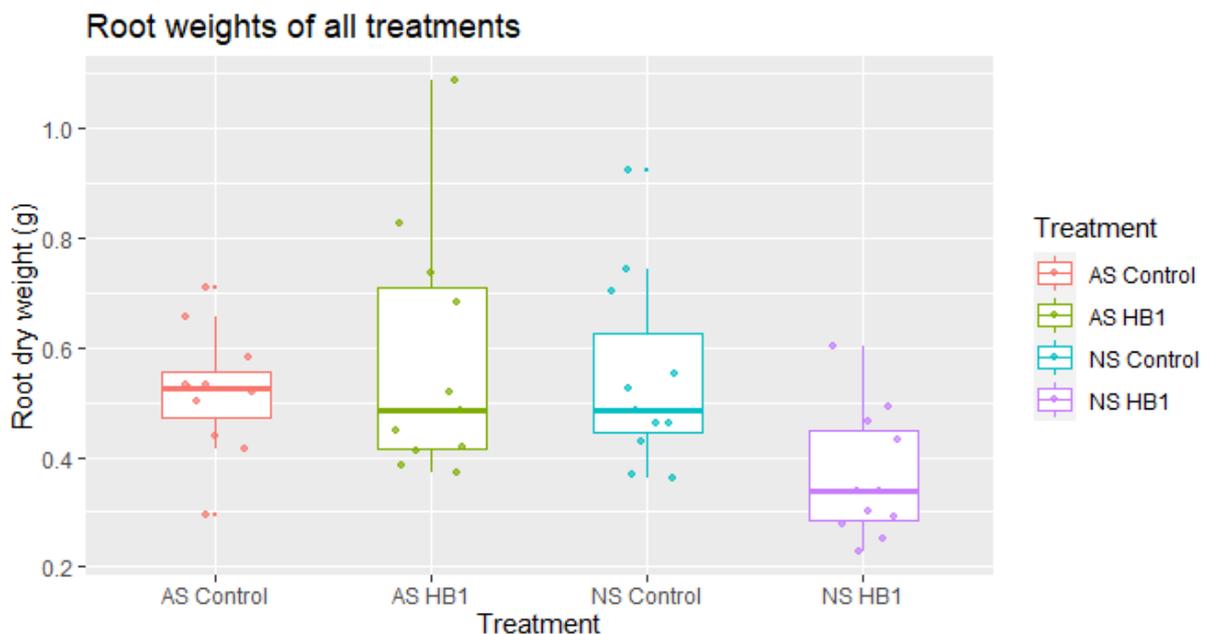


Figure 8. As with the first repeat, no significant results were produced (t-test, $p < 0.05$), but the same trend was observed for shoot weight (Figure 9).

This suggests that this HB1 Devosia species plays a key role in plant productivity and plant growth. I hypothesise that this can occur through one of several mechanisms:

1. Providing the plant with nutrients directly by converting the nutrients into a form in which plants can easily uptake into their roots (Alori et al., 2017) (Rashid et al., 2016).
2. Providing the plants with nutrients indirectly via the AMF: by playing a key role in the mycorrhization of plant roots, whereby then plants, through their association with AMF, can uptake more nutrients via hyphae and the formation of arbuscules (P Frey-Klett et al., 2007).
3. Producing signalling compounds that can modulate the plant response (Glick, 2005).

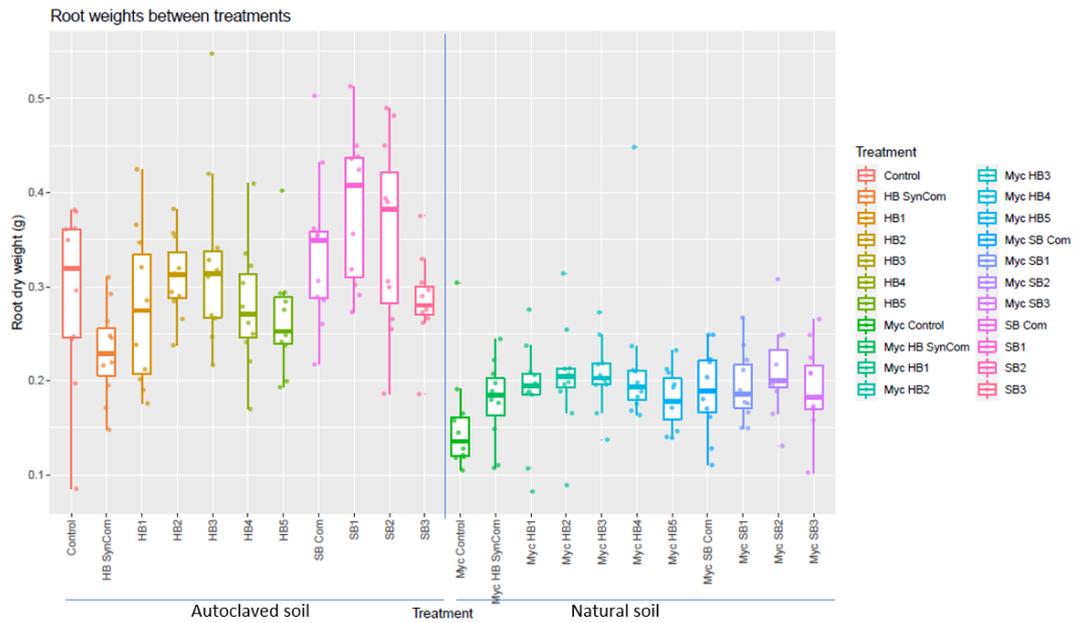


Figure 6: Dry root weights between treatments. No significant results were seen (t -test, $p < 0.05$).

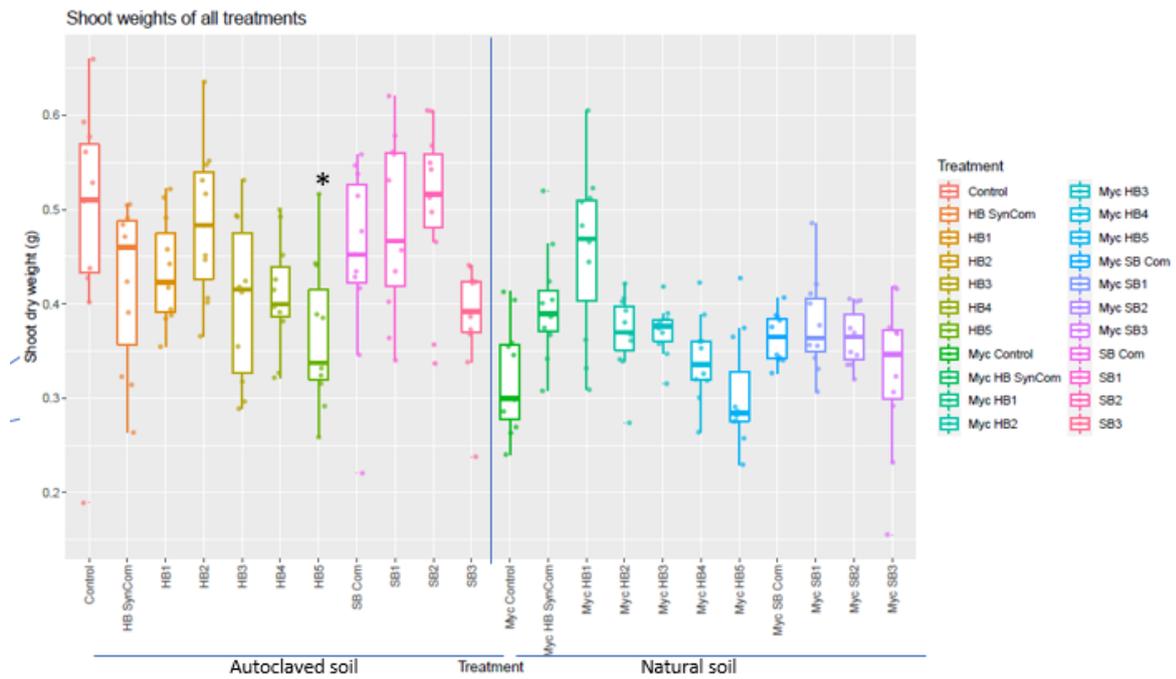


Figure 7: Dry shoot weights between treatments. Significant results are indicated with asterisks (t -test, $p < 0.05$).

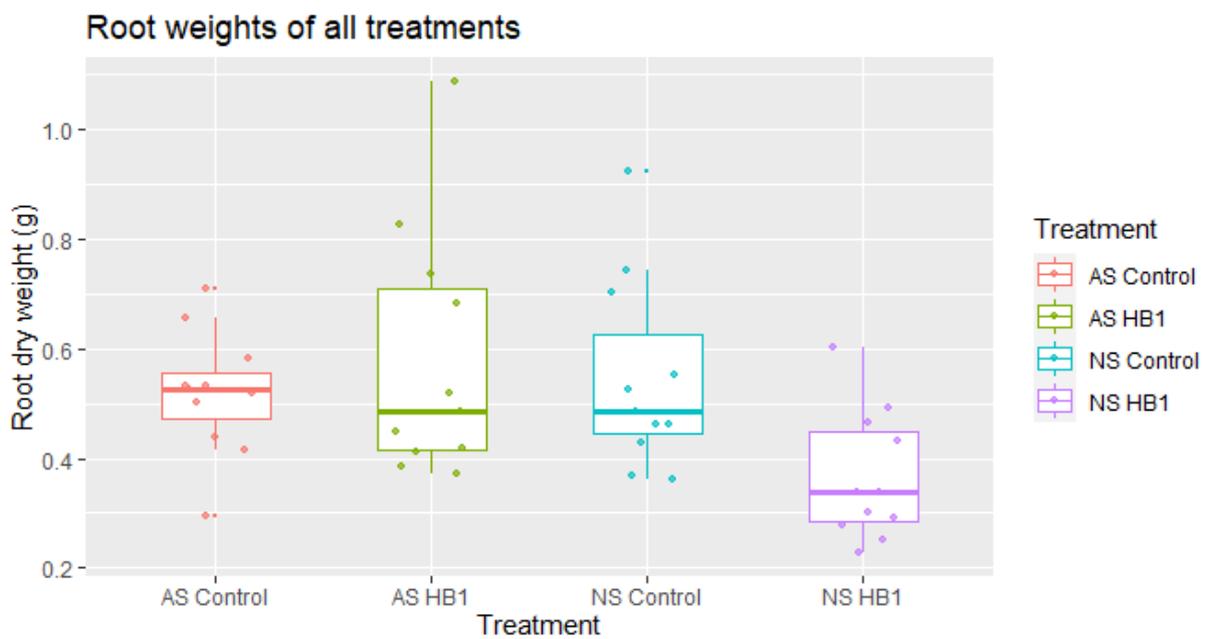


Figure 8: Root weights from the repeated greenhouse experiment. AS refers to autoclaved soil, NS refers to natural soil. No significant results were seen (t -test, $p < 0.05$)

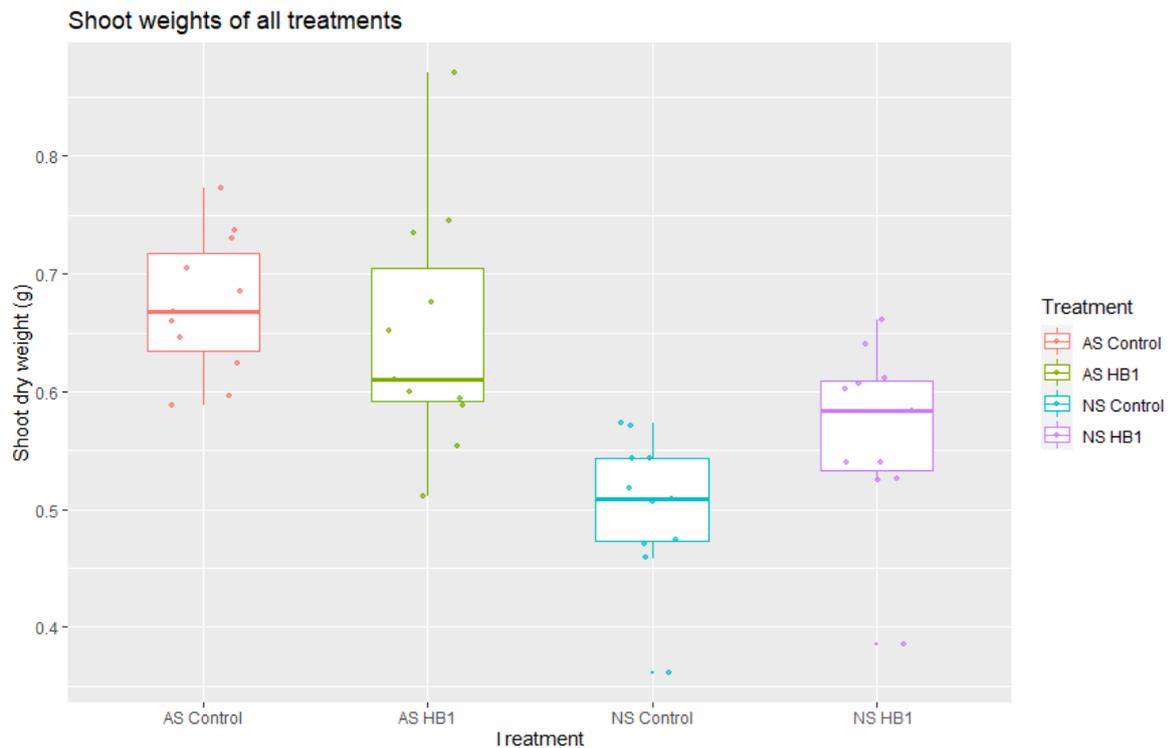


Figure 9: Shoot weights from the repeated greenhouse experiment. AS refers to autoclaved soil, NS refers to natural soil. No significant results were seen (t -test, $p < 0.05$)

1.4.3 Effect of bacterial treatment upon mycorrhization in greenhouse conditions

Roots were then stained and analysed microscopically (see methods). Prior to staining, several smaller samples of roots were taken and stained with 10% KOH and ink-vinegar solution for different periods of time and analysed microscopically to determine the optimal time for staining, as this differs between plant species, root diameter and age of the root. It was found that bleaching for 40 mins at 80°C in KOH and then staining for 40 minutes in ink-vinegar solution at 80°C was the optimal duration for roots to be bleached and fungal structures to be stained appropriately. This should be performed every time, as many factors can affect the staining, such as thickness and age of the roots and plant species.

All roots were stained, including the autoclaved soil controls, and analysed microscopically as described in Section 1.3.6. A section of a stained *P. vulgaris* root containing the fungal structures is shown in Figure 10.

Results are shown in Figure 11, Figure 12 and Figure 13. All autoclaved soil samples returned zero mycorrhizal structures, except one (HB1 sample 3). It is unsure why this sample shows mycorrhization, but it is assumed that there was a contamination here, or the wrong soil was weighed into the wrong pot. This sample has thus been removed from all analysis, including in plant root and shoot biomass analysis.

The different bacterial treatments appear to have little effect on the colonisation of plant roots by AMF, with no statistically significant results produced for hyphal or colonisation or vesicle formation. However, the HB1 and SB1 bacterial treatments reduced the formation of arbuscules (Figure 12) to a significant level (t-test, $p < 0.05$).

As previously discussed, this experiment shows that HB1 treatment promotes plant growth in natural soil. HB1 also prevents the formation of arbuscules but does not affect the total colonisation of hyphae in plant roots or formation of vesicles. Although not significant, the SB1 treatment slightly encourages plant shoot growth in natural soil, but not autoclaved soil, when compared to the control (

Figure 7). The correlation between arbuscule formation and the dry shoot biomass of *P. vulgaris* was calculated between all samples (Figure 14), and a slight negative correlation was calculated to be -0.0204, which indicates that there may be a link between arbuscule formation and reduced shoot biomass.

AMF typically improve plant performance under stressful conditions (Begum, Qin, Ahanger, et al., 2019), yet the conditions in these experiments did not put *Prunella vulgaris* under a significant amount of stress. Arbuscules are the site of nutrient transfer between host and symbiont, and AMF can obtain up to 20% of the recently fixed carbon from the through the arbuscules (Thirkell et al., 2020).

Thus, I hypothesise that while under these controlled conditions, AMF may reduce plant biomass formation, and that the reduced arbuscule formation in the HB1 treated soil means that the carbon obtained from photosynthesis may be used in plant biomass formation, rather than supporting the growth of AMF hyphae in the soil. However, this is speculative and there is little literature to support this. Further experiments must be designed to test this hypothesis.

Another hypothesis is that HB1 acts as a plant growth promoting rhizobacteria (PGPR) in *P. vulgaris*, alleviating certain plant stresses (Vocciante et al., 2022), and allowing the plant to focus its energy into growth.

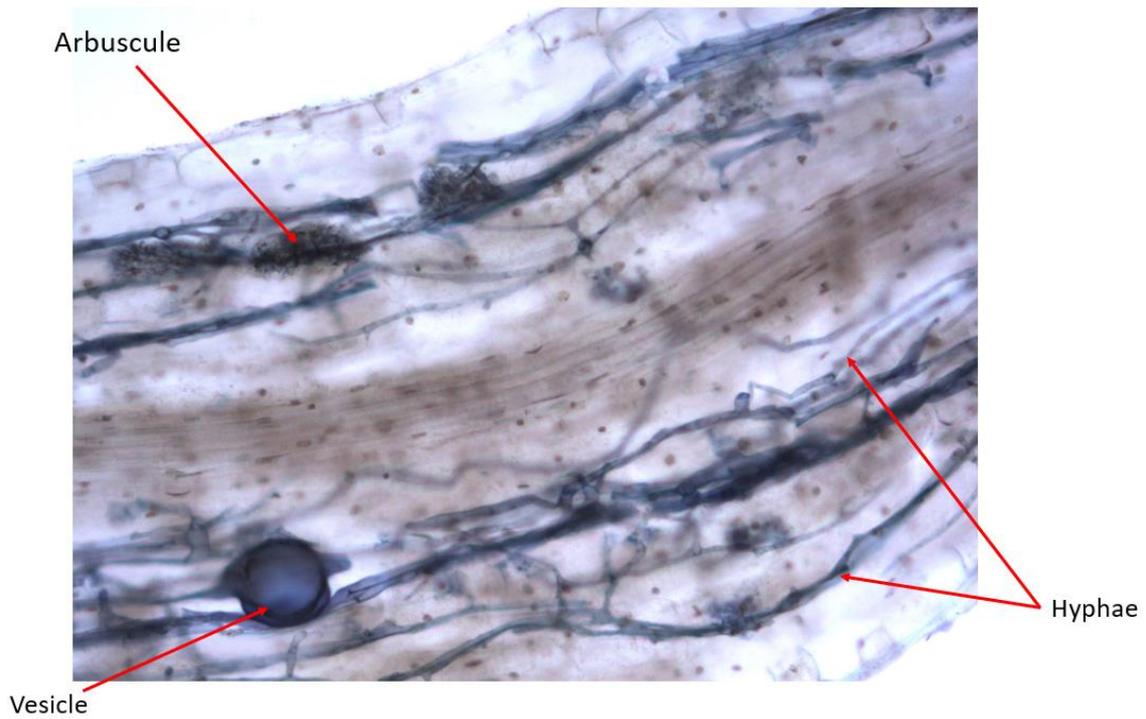


Figure 10: Light microscopy image of a section of *Prunella vulgaris* root, stained using the method described in section 1.3.6. Fungal structures are stained blue and the image shows arbuscules, hyphae and vesicles.

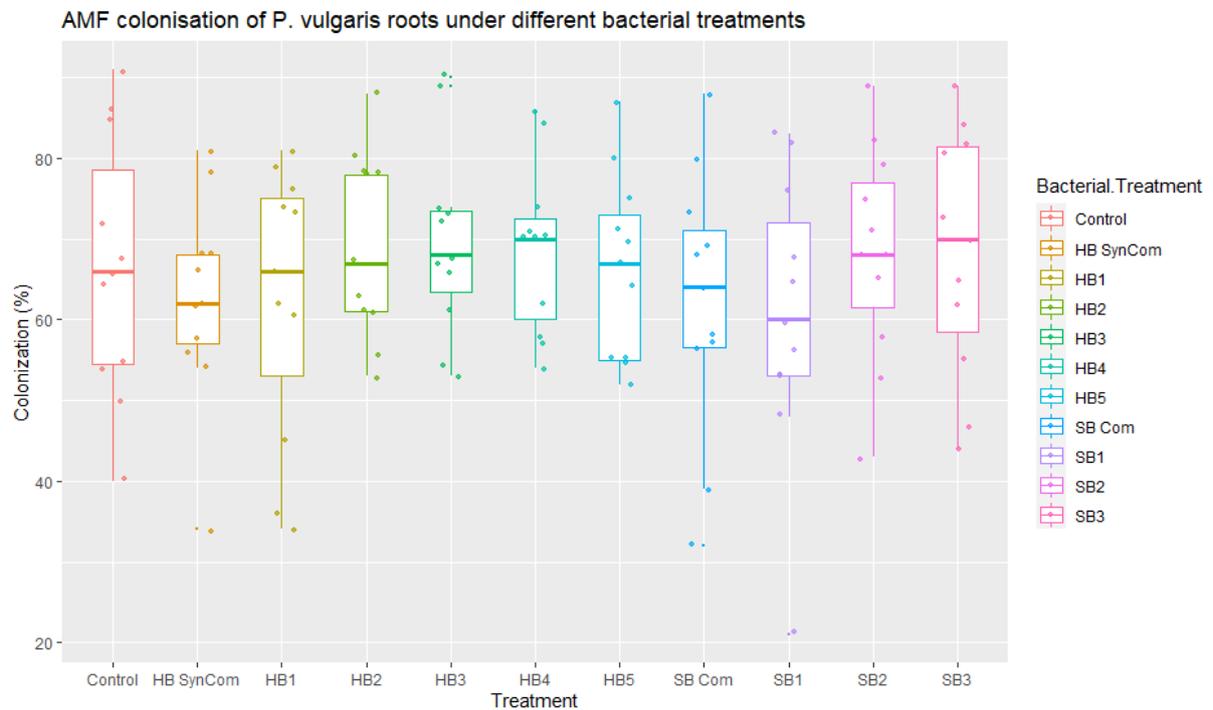


Figure 11: Total AMF colonisation of *Prunella vulgaris* roots using different bacterial treatments in natural soil. No significant results were seen (t -test, $p < 0.05$)

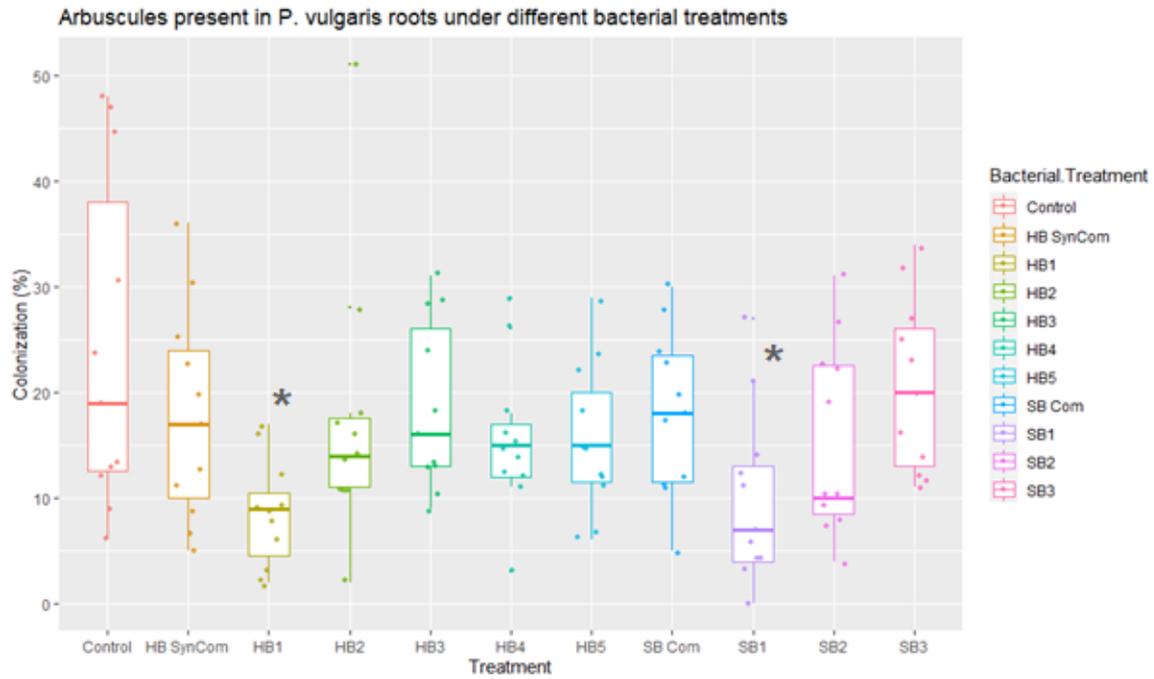


Figure 12: Total arbuscules present in *P. vulgaris* roots using different bacterial treatments in natural soil. Significant results are indicated with asterisks (*t*-test, $p < 0.05$).

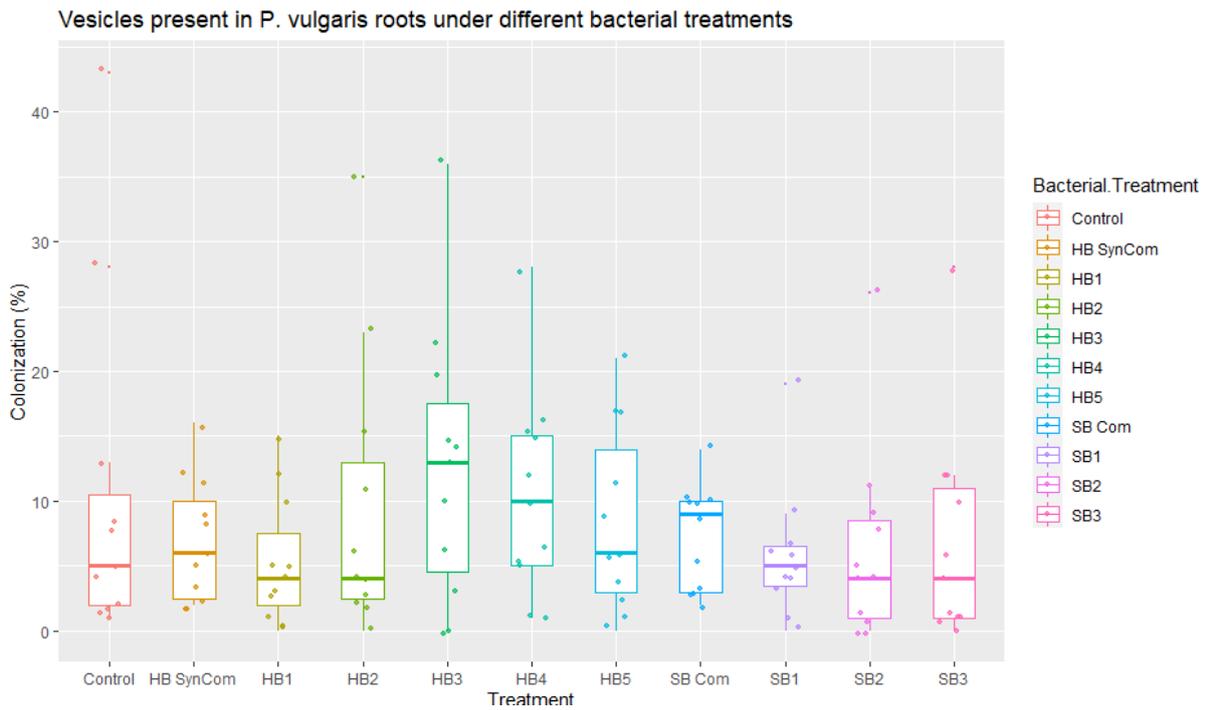


Figure 13: Total vesicles present in *P. vulgaris* roots using different bacterial treatments in natural soil. No significant results were seen (*t*-test, $p < 0.05$).

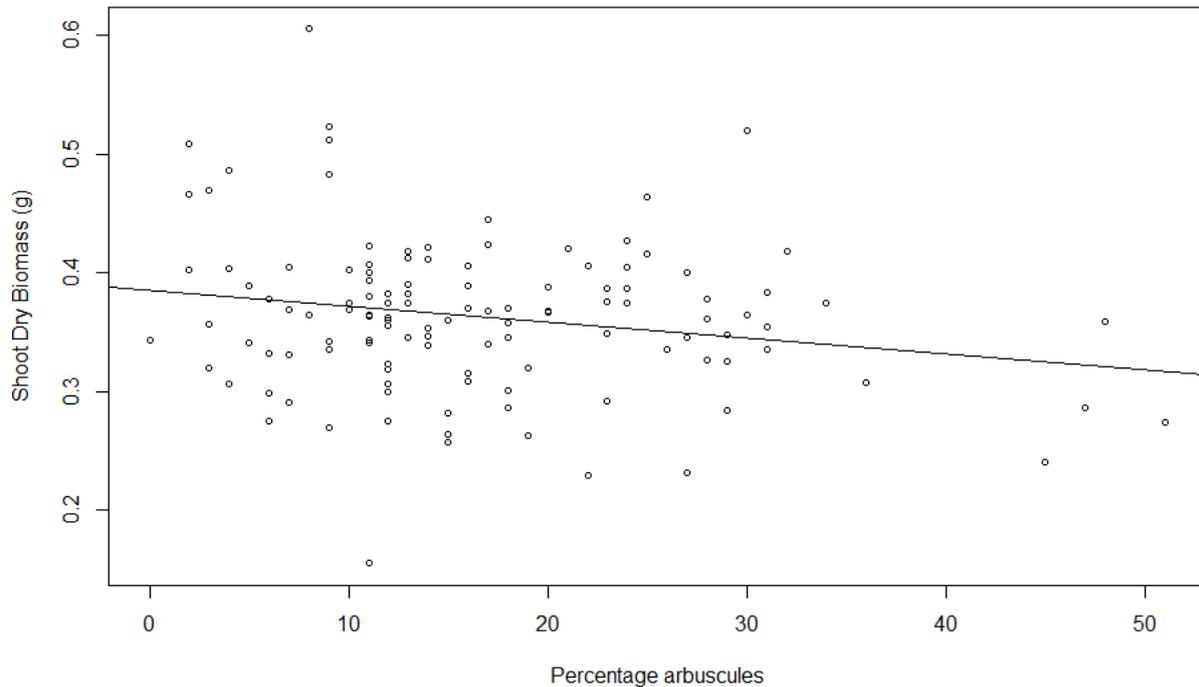


Figure 14: Correlation between percentage of arbuscule coverage and *Prunella vulgaris* shoot dry biomass. A slight negative correlation was observed, and correlation was calculated to be -0.204 .

1.4.4 Further steps

First, the experiment should be repeated to ensure the results are reproducible. The experiments can be performed in different soils and/or with different plants; this would give an indication of the efficacy of the bacteria in different conditions and may give some indication of the mode of action.

To test whether HB1 does or does not directly influence plant growth, *P. vulgaris* can be grown in a square petri dish containing agar-solidified Murashige and Skoog (MS) medium, treated with HB1, as described by Wintermans et al., (2016). Here, root growth, including root lateral formation and root length can be measured. If HB1 were to promote root growth in *P. vulgaris* directly, this would be demonstrated here. An indirect effect of HB1 upon *P. vulgaris* growth would not produce statistically significant results between control and HB1 treatment.

The hypothesis that increased arbuscule formation reduces above ground biomass via carbon uptake by AMF can be tested in several ways:

1. Measuring the soil organic matter (SOM) concentration in the soil (Roper et al., 2019). If the hypothesis is true, the presence of more arbuscules should increase the SOM content within the soil. More arbuscules should, in theory, be able to obtain more carbon from the plant host. It is known that AMF increase rhizodeposition and thus increase the carbon content within the soil (Zhou et al., 2020).
2. Calculating the abundance of extraradical mycorrhizal fungi in the soil as described by (Barceló Id et al., 2020). The presence of arbuscules should, in theory, allow the AMF to obtain more carbon, thus accelerating their extraradical hyphal growth within the soil.
3. Measuring the rate of photosynthesis. A higher level of photosynthesis should be associated with an increase in plant biomass, but if this is not the case, we may assume

that the carbon generated is being used by the AMF. There are several methods to measure the rate of photosynthesis and include gas exchange and remote and proximal sensing, detailed by (Siebers et al., 2021). This approach should be taken with caution, as not all carbon generated is used for biomass growth. For example, the activation of plant immune response uses a lot of energy, in what is known as the growth-defense trade off (Huot et al., 2014).

The other hypothesis that autoclaved soils release more nutrients into an accessible form for plants to uptake, thus increasing plant biomass can also be tested:

1. The Arnold method of soil sterilisation has been developed, that does not alter the humic components present in the soil (Varadachari et al., 1982). Here, steam (100°C) is applied without pressure for 30 minutes in three consecutive days. The experiment can be repeated using soil sterilised in this way.

What is often seen with successful laboratory experiments, is that the results are not reproducible in the field. This is due to a wide range of reasons. While laboratory conditions are controlled and reproducible, field conditions are not and are subject to constant changes (pH, nutrient stoichiometry, aridity, etc). This can make it difficult for the bacteria to colonise the soil effectively, and due to these constant changes, can make it difficult to take accurate measurements. When applied to the field, soil inoculants can become quickly consumed (for example by protists) or outcompeted by other soil microorganisms. Thus, an effective inoculant must be able to integrate itself into the microbiome and the food web (Wallenstein, 2017). Laboratory experiments can be performed under different conditions (such as water stress (drought or flooding), different soils, different light and temperature settings) to see how *Devosia* may impact plant performance under these different stresses. Further, we may test the *Devosia* ability to perform under conditions that mimic natural systems better. For example, the UU Biodiversity and Climate Variability Experiment (BioCliVE) uses a 352 container set up to mimic natural grassland conditions, to predict how biological diversity can provide us with natural insurance against climate change (*UU BioCliVE | Universiteit Utrecht*, n.d.). Here, the *Devosia* strains can be applied, and biomass analysed, as well as mycorrhizal colonisation.

Chapter 2: Designing a qPCR method for quantification of AMF colonisation in *P. vulgaris* roots

2.1 Abstract

This chapter aimed to design and validate a qPCR method for quantification of AMF colonisation in *Prunella vulgaris* roots. Plant primers to amplify *P. vulgaris* DNA were designed using the Primer-BLAST function from NCBI, and AMF primers were pre-designed based on existing methods. A qPCR efficacy test was performed to validate the primers to ensure specific binding and to test the efficiency of the primers. All primer pairs amplified the negative control DNA, and thus primers are non-specific. All primers had a poor efficiency and produced a poor ΔR_n value between the DNA in a dilution series. It is for these reasons that it was decided that the qPCR method development was to be discontinued.

2.2 Background

The aim of this chapter is to design and validate a qPCR method for quantification of AMF colonisation in *P. vulgaris* roots:

1. A qPCR efficacy test will be performed to validate AMF and *P. vulgaris* primers
2. Following a successful efficacy test, root samples collected from Section 1.3.5 will be subjected to qPCR analysis, and results will be compared to the results from the microscopic analysis of the same samples.

Traditionally within the PMI department at UU, AMF colonisation has been measured via microscopic methods, described in section 1.3.6. However, microscopy methods are tedious, time consuming (in root staining, preparation of slides and a large number of measurements taken under the microscope) and dependent on the observer, and beginners must be well trained for accurate identification of the different fungal structures (Bodenhausen et al., 2021). The microscopy method does not differentiate between highly colonised and low colonised roots. For example, the measurement of a root sample containing a high level of hyphae or AMF structures will be the same as a root sample containing only one hyphae or fungal structure. In roots highly colonised by hyphae, oftentimes it is difficult to visualise AMF structures, in particular arbuscules. Some arbuscules are more clearly defined than other arbuscules, and thus user must make a judgement about what can be classed as an arbuscule: some arbuscules may be newly forming or degrading. Thus, the dependency on the observer is important, and must remain constant throughout the analyses as observers tend to over- or underestimate colonisation levels (McGONIGLE et al., 1990). In this way, the margin of human error is quite high, and qPCR approaches may provide more accurate and standardised data, and results between observers can be compared with a higher degree of confidence.

Thus, qPCR analysis to determine AMF colonisation appears to be an attractive alternative to microscopic analysis. However, qPCR analysis does also provide its own limitations. Appropriate primers must be designed and validated via an efficacy test, and qPCR analysis

simply quantifies colonisation without distinguishing between fungal structures, i.e. hyphae, vesicles and arbuscules.

2.3 Materials and Methods

2.3.1 DNA Extraction

Plant DNA was collected from sterile roots following germination (see section 1.3.1) and from root samples from previous experiments (section 1.3.6).

Samples for DNA extraction were stored at -20°C until processing and were then freeze dried overnight to ensure more efficient lysing of the cells. The DNA was then extracted using the DNeasy PowerLyzer PowerSoil Kit (Qiagen) according to manufacturer's instructions, with a few deviations from the method. Samples were added to the PowerBead Tube and shaken for a minimum of five minutes in the TissueLyser at full speed, before addition of the PowerBead solution and solution C1. At step 17, 60 μL of solution C6 was added the white filter membrane in the MB spin column and left for a minimum of 5 minutes. This ensures binding of the DNA to solution C6 and increases the concentration of DNA in the final sample.

2.3.2 AMF primer design

The AMG1f (forward primer) (Hewins et al., 2015) and AM1 (reverse primer) (Helgason et al., 1998) were used to amplify the AMF DNA. These primers were validated by Bodenhausen et al (Bodenhausen et al., 2021).

2.3.3 Plant primer design

Plant primers were designed using the Primer-BLAST function from NCBI, and six primer pairs were designed from two accession numbers (KM053280 (Trócsányi et al., 2020); KJ010816 (Kim et al., 2014) to account for the possibility of the presence of intron coding regions. Requirements for the primer design are as follows: melting temperature (Melting temperature(T_m) $\sim 60^{\circ}\text{C}$; amplicon size 75-150bp). Six primer pairs were designed (Supplementary Table 1) and tested against the BLAST database, along with the AMF to ensure specificity to *Prunella vulgaris* or AMF, respectively. Primer pairs were then ordered through Integrated DNA Technologies (IDT), Belgium.

2.3.4 Testing primers

Sterile *Prunella* root DNA was then amplified using each of the primer pairs (including the AMF primers) using PCR. The reaction mix was 1X DreamTaq buffer, 5% DreamTaq polymerase, 0.2mM dNTPs, 0.25mM of each forward and reverse primer, and approximately 0.8ng/ μL of the DNA template in a 50 μL reaction mix.

The PCR programme consisted of an initial denaturation step of 2 minutes at 95°C , followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 1 minute. Following the completion of the cycling, samples were held at 72°C for 10 minutes. PCR products were then checked by running on 1.7% agarose gel.

2.3.5 qPCR efficacy test

Following confirmation of amplification of AMF and *P. vulgaris* DNA with the primers, a qPCR efficacy test was performed. DNA was extracted from the following samples: sterile *P. vulgaris* plant root (negative control); *P. vulgaris* root grown in soil (mycorrhizal control); *P. vulgaris* grown in soil treated with WCS417 (WCS417-Root); *P. vulgaris* grown in soil treated with WCS358 (WCS358-Root); *P. vulgaris* grown in soil treated with WCS374 (WCS374-Root); *P. vulgaris* grown in soil treated with Devosia ZB119 (Dev119-Root); *P. vulgaris* grown in soil treated with Devosia ZB006 (Dev119-Root). DNA from the bacterial strains WCS417, WCS358 and WCS374 were also extracted to be used as an additional control. Concentrations of DNA were checked using NanoDrop and QuBit and samples were normalised to a DNA concentration between 5-30ng/ μ L, as according to QuBit values.

To run the efficacy test, a ten-fold dilution series was created using the negative control and mycorrhizal control templates from $10^0 - 10^{-6}$. A no template control (NTC) was included. Efficacy tests for the negative control included all seven primer pairs, and efficacy test was also performed on the mycorrhizal control with the AMF primer pair.

The other DNA templates were run with each of the primer pairs, without the need for a dilution series.

2.3.6 qPCR Method

For the qPCR analysis, samples were run in duplicate, in 5 μ L reaction volumes containing 1xSYBR green (iTaq Universal SYBR Green Supermix, BioRad), 800nM primer, and 2 μ L of the DNA template. The qPCR programme consisted of a hold stage, a PCR stage and a melt curve stage. Rate of change between temperatures is 1.6 $^{\circ}$ C/s, unless otherwise specified. The initial hold stage consisted of a hold of 2 minutes at 50 $^{\circ}$ C, followed by a denaturation step of 3 minutes at 95 $^{\circ}$ C. The PCR stage consisted of 40 cycles of a 15 second denaturation step at 95 $^{\circ}$ C, followed by annealing at 60 $^{\circ}$ C for 1 minute. The final melt curve stage consisted of a denaturation step of 15 seconds at 95 $^{\circ}$ C, an annealing step for 1 minute at 60 $^{\circ}$ C, and finally a dissociation step increasing from 60 $^{\circ}$ C to 95 $^{\circ}$ C at an interval of 0.05 $^{\circ}$ C/s, and a hold of 15 seconds at 95 $^{\circ}$ C.

Samples were analysed using the QuantStudio Real-Time PCR Software v1.3.

2.4 Results and Discussion

2.4.1 Testing the designed *P. vulgaris* primer pairs

The genome of *Prunella vulgaris* has not yet been fully sequenced, thus several primers were designed based upon mRNA sequencing, based on accession numbers shown in Supplementary Table 1. Primers were designed using the Primer-BLAST function from NCBI, and several primer pairs were designed from two accession numbers, to account for the possibility of the presence of intron coding regions. Requirements for the primer design are as follows: melting temperature (Melting temperature(T_m) ~60 $^{\circ}$ C; amplicon size 75-150bp). Six primer pairs were designed (Supplementary Table 1) and tested against the BLAST database to ensure specificity

to *Prunella vulgaris*. Primer pairs were then ordered through IDT (Integrated DNA Technologies, Belgium).

The designed *P. vulgaris* primer pairs all successfully amplified sterile *P. vulgaris* DNA and the AMF primers successfully amplified plant root containing AMF colonisation (Figure 15). Although not all plant primers successfully amplified *P. vulgaris* with AMF DNA, (Figure 15C), they did successfully amplify all *P. vulgaris* samples in Figure 15B, and therefore all six primer pairs were chosen to use in the qPCR efficiency test, in order to decide the optimum primers in qPCR conditions.

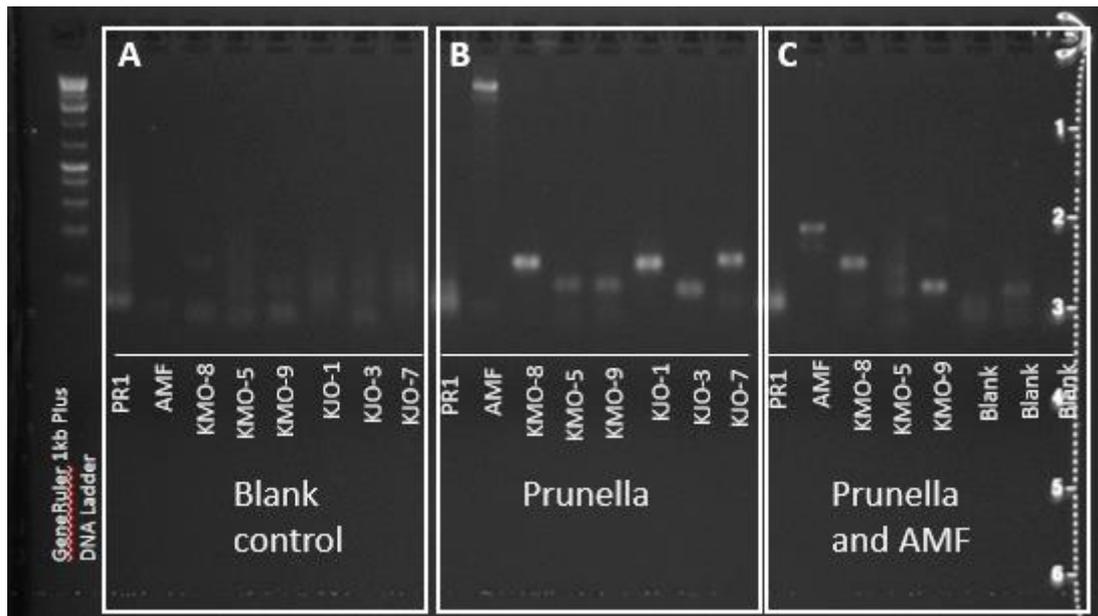


Figure 15: Amplification of *Prunella vulgaris* DNA using the AMF and *Prunella vulgaris* primers. PR1 primer will not be used. A) Blank control. B) DNA amplified from sterile *P. vulgaris* DNA. The designed primers all successfully amplified *Prunella vulgaris* DNA. The AMF primer also produces a band of ~10,000-20,000 bp in *P. vulgaris*. This is not expected to be a problem in the qPCR analysis, as this band size falls outside of the amplification limit. C) DNA amplified from *P. vulgaris* roots colonised with AMF. A clear band can be seen. KMO-5, KJO-1, KJO-3 and KJO-7 primers did not amplify *P. vulgaris* very successfully in this sample.

2.4.2 qPCR efficacy

Before performing the qPCR on the samples from Chapter 1, a qPCR efficacy and specificity test is performed. The purpose of the qPCR efficiency test is to:

1. Ensure **specific binding** to the area of interest; there should be one clear melt curve that is present in the same location for multiple replicates. This can also detect possible primer dimers and hairpin structure formations.
2. Testing the **efficiency** of primer pairs. A 10-fold dilution series of DNA should produce a ΔR_n of 3.33 between each dilution series.

The purpose of the efficiency test is to ensure that the primers are quality and stable, that the PCR conditions are appropriate, and may detect any inhibitors present in the DNA sample. From here, the best *Prunella vulgaris* primer pair can be determined for the final qPCR analysis.

2.4.3 Efficiency of plant primers

DNA was extracted from the following samples: sterile *P. vulgaris* plant root (negative control); *P. vulgaris* root grown in soil (mycorrhizal control); *P. vulgaris* grown in soil treated with WCS417 (WCS417-Root); *P. vulgaris* grown in soil treated with WCS358 (WCS358-Root); *P. vulgaris* grown in soil treated with WCS374 (WCS374-Root); *P. vulgaris* grown in soil treated with Devosia ZB119 (Dev119-Root); *P. vulgaris* grown in soil treated with Devosia ZB006 (Dev119-Root). DNA from the bacterial strains WCS417, WCS358 and WCS374 were also extracted to be used as an additional control. Concentrations of DNA were checked using NanoDrop and QuBit and samples were normalised to a DNA concentration between 5-30ng/ μ L, as according to QuBit values.

To run the efficacy test, a ten-fold dilution series was created using the negative control and mycorrhizal control templates from $10^0 - 10^{-6}$. A no template control (NTC) was included. Efficacy tests for the negative control included all seven primer pairs, and efficacy test was also performed on the mycorrhizal control with the AMF primer pair.

The other DNA templates were run with each of the primer pairs, without the need for a dilution series.

Figure 16 and Supplementary Table 2 shows the results from the qPCR efficiency test. These results show that the qPCR conditions are not optimal and primers are not efficient as the Δ Ct values deviate from 3.33 (Supplementary Table 2). Additionally, the standard deviation between some of the samples is greater than the cut-off of 0.5, indicating either a pipetting error or non-specific binding.

In addition to the efficacy test, primer specificity was tested on 10 different samples (summarised in Table 3). In these samples, DNA concentration was measured using both QuBit and NanoDrop and concentration standardised to 5 μ L - 30 μ L. The three WCS strains (Table 3: samples 8-10) were used in the soil treatments as additional controls (WCS417, WCS358, WCS374 DNA) to ensure specificity of the primers to the intended targets (i.e. *P. vulgaris* DNA or AMF DNA). The *Prunella vulgaris* primers should successfully amplify all root samples (sample 1-7; Table 3), but not the bacterial samples (Samples 8-10; Table 3). Amplification of samples 8-10 would indicate non-specific primer binding and amplification.

The results of the specificity test show that all primers amplify the bacterial strains better than *P. vulgaris* or AMF DNA (Supplementary Figures 2-8).

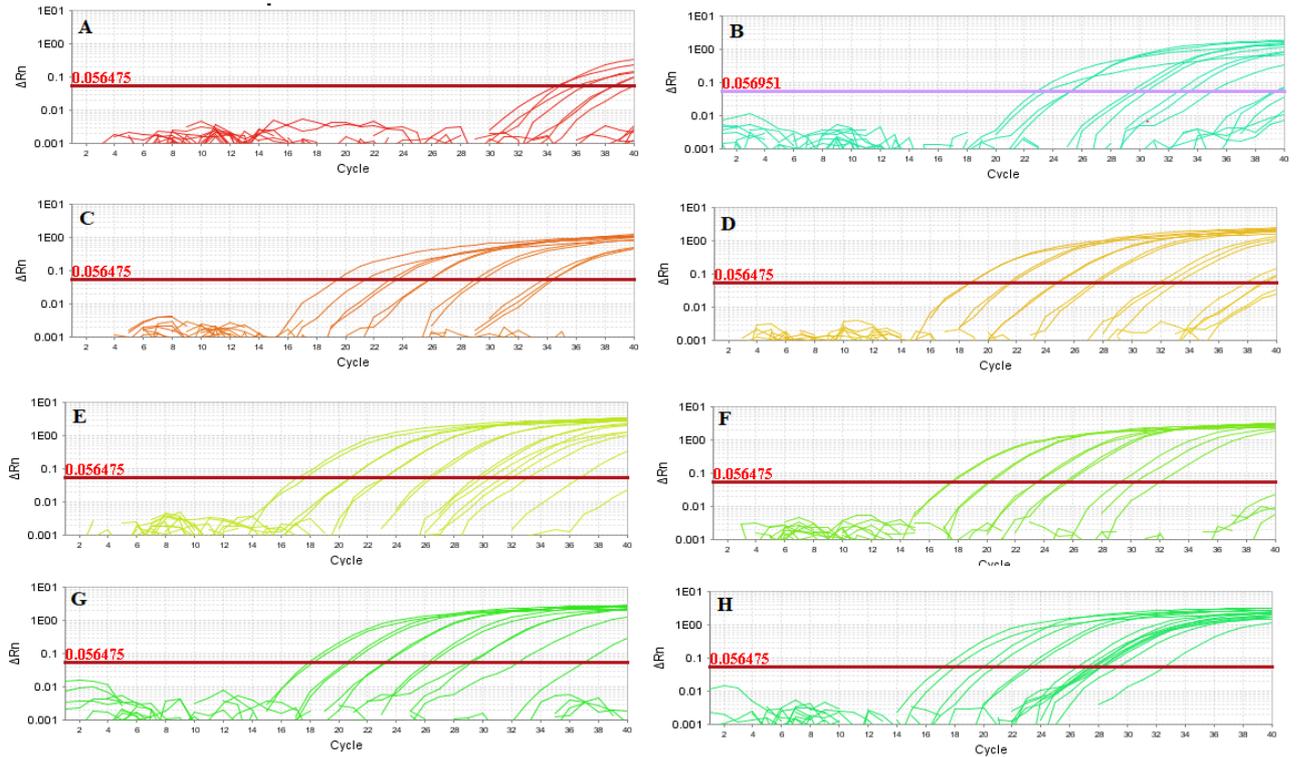


Figure 16: Plot of ΔRn vs cycle of various primer pairs. Straight red line indicates the threshold for detection.
 A) AMF primer pair with sterile *P. vulgaris* root. B) AMF primer pair with *P. vulgaris* root colonised with AMF. C) KMO-8 primer pair with sterile *P. vulgaris* root. D) KMO-5 primer pair with sterile *P. vulgaris* root. E) KMO-9 primer pair with sterile *P. vulgaris* root. F) KJO-1 primer pair with sterile *P. vulgaris* root. G) KJO-3 primer pair with sterile *P. vulgaris* root. H) KJO-7 primer pair with sterile *P. vulgaris* root.

Table 3: Overview of the samples used to test primer specificity. Samples 1-7 are root samples. Samples 8-10 are only bacterial samples, and were used to ensure zero primer specificity on the bacteria.

Sample	Root sample?	AMF present?	Bacterial treatment in soil	Isolated bacteria
1	✓	✗	✗	✗
2	✓	✓	✗	✗
3	✓	✓	WCS417	✗
4	✓	✓	WCS358	✗
5	✓	✓	WCS374	✗
6	✓	✓	Devosia ZB119	✗
7	✓	✓	Devosia ZB006	✗
8	✗	✗	✗	WCS417
9	✗	✗	✗	WCS358
10	✗	✗	✗	WCS374

Based on the results from the efficacy test and specificity test, it was decided that the qPCR method development will not continue:

- The efficacy test did not produce reliable standard deviation values between replicates, and the ΔC_t values produced varied too from 3.33 much between dilutions.
- The specificity test proved that the primers amplify bacterial DNA better than both root or AMF DNA, and thus this indicates non-specific binding of the primers to bacterial DNA.

Thus, root colonisation of AMF will be quantified via microscopic methods.

Future steps to ensure a successful qPCR analysis could include:

- Efficient washing of roots to ensure no bacterial DNA is present. The bacterial strains exist only in the soil and should not colonise the plant root. This can be confirmed by PCR.
- Altering the qPCR conditions for more efficient amplification.
- Altering the DNA extraction method to obtain more DNA.

Chapter 3: Mycorrhizal fungal interactions with potential plant promoting *Pseudomonas* (WCS417, WCS374, WCS358) strains

3.1 Abstract

In this chapter, two experiments were designed to follow on from the experiments performed by Bich Nguyen (Nguyen, 2021), the previous MSc student of Changfeng Zhang. Nguyen formed the hypothesis that the *Pseudomonas simiae* WCS417 stimulates *Prunella vulgaris* endogenous programs related to primary root and lateral root formation. To test this hypothesis, the experiments performed by Nguyen were repeated in soil comprising of a mixture of river sand, potting soil and conventional soil. Soil was either left as natural soil, comprising of the natural microbial communities formed, or autoclaved to remove the natural microbial communities, and treated with one of three *Pseudomonas* strains: *P. simiae* WCS417, *Pseudomonas capeferrum* WCS358, or *Pseudomonas defensor* WCS374. Following inoculation, germinated *P. vulgaris* seedlings were planted and harvested after 8-10 weeks. Here, WCS417 significantly increased root dry weight in autoclaved soil, but not in natural soil, which compliments the results generated by Nguyen. WCS358 appears to increase *P. vulgaris* biomass in both autoclaved and natural soil, significantly for the root dry weight, which contrasts with the results generated by Nguyen. I propose that WCS417 promotes growth more in autoclaved soil due to the lack of microorganisms. WCS417 can more easily establish itself in the autoclaved soil, as the presence of other microorganisms in the natural soil may provide a barrier for colonisation. To account for the differences between the results reported here and in the report by Nguyen, I hypothesise that in different soils, the *Pseudomonas* strains may impact the growth of *P. vulgaris* differently. By repeating these experiments under different conditions, for example in different soils or by applying different stresses, we may begin to better understand the interactions happening between plant, its symbiont, and the soil bacterial community.

3.2 Background

As part of this chapter, two experiments were performed to follow on from the master thesis of Bich Nguyen Thi (Nguyen, 2021), the master student of Changfeng Zhang. Three *Pseudomonas* strains (*P. simiae* WCS417, *P. capeferrum* WCS358, and *P. defensor* WCS374) were used to inoculate soil to analyse the effects of these strains upon the growth of *P. vulgaris*. Over 300 publications have demonstrated the beneficial roles of these three strains, for example in promoting plant growth and in providing protection from pathogens (Berendsen et al., 2015). Bich found that the two strains WCS374 and WCS417 promoted shoot weight in *P. vulgaris*, but not root weight, and that WCS358 appeared to reduce both shoot and root weight. This contrasts with the results generated here, where all *Pseudomonas* strains increased overall biomass. In particular, the main difference seen was that WCS358 increased root weight to a statistically significant amount. The main difference between the experiments designed here and by Bich (Nguyen, 2021), was the soil used, which may impact the role that the *Pseudomonas* strains play in plant growth promotion.

3.3 Materials and Methods

3.3.1 Seed preparation and germination

P. vulgaris seeds were surface sterilised by vapour from 3.2mL of HCl 37% and 100mL of bleach (NaOCl) mixture for 3.5 hours. To remove the vapour, seeds were left in a flow bench for ten minutes in an Eppendorf with the cap off. Sterilised seeds were sown on Murashige and Skoog agar-solidified medium MS agar (0.5 x MS medium) supplemented with 1% (w/v) sucrose at a density of 10 seeds per plate. Petri dishes were immediately transferred to a growth chamber under a long-day photoperiod (22°C, 16h of light, intensity 100 μ mol m⁻² s⁻¹) and positioned horizontally. Seeds were left to germinate for 10-14 days.

3.3.2 Bacterial inoculum preparation

Bacterial strains were grown separately (Supplementary Table 3) were grown separately from glycerol stock solutions on King's B Agar (KBA) plates (King ED, Ward MK, 1954) at 28°C. After approximately 1-10 days of growth (Supplementary Table 3), after considerable growth was seen, cells were then suspended in 10mM sterile MgSO₄ and re-plated on KBA plates under the same conditions. Following considerable growth, cells were then suspended in 10mM sterile MgSO₄ and concentration measured spectrophotometrically ($\lambda = 660\text{nm}$, if $A = 1$, $\delta = 10^9$). Cells were added to the soil to achieve a final concentration of 10⁸ cfu/g. For the non-bacteria controls, an equivalent volume of 10mM MgSO₄ was added to the soil.

3.3.3 Soil preparation

For the soil samples, river sand, potting soil and conventional soil were mixed thoroughly in a ratio of 12:4:3 w/w/w. The autoclaved soil contains autoclaved river sand (ARS), autoclaved potting soil (APS) and autoclaved conventional soil (ACS). The natural soil contains ARS, APS and non-autoclaved conventional soil (CS). Autoclaved soil was autoclaved twice at 121°C for 50 minutes. Of this mixture 150g was added to each pot, following inoculation with bacteria or control.

3.3.4 Plant growth conditions and harvesting

Plant pots were transferred to the greenhouse on long day settings (21°C, 16h light, 8h dark) and plants were harvested after 8-10 weeks. *P. vulgaris* plants were removed from plant pot and soil shaken from roots. Roots were washed thoroughly using tap water to remove the remaining dirt, and root and shoot were separated from one another using a scalpel. Total wet root weight was measured, and roots were then cut into three sections as shown in Figure 3. The weight of each section was also measured. The sections for microscopy analysis were stored in 50% ethanol at 4°C, and section for qPCR analysis were stored in 50% glycerol at -20°C. The plant shoot and section of plant root for weight analysis were dried at 70°C for a minimum of 48h and dry weight weighed. Total dry weight of roots was then calculated using the wet weights (percentage of weight) from the three root sections.

3.4 Results

3.4.1 Mycorrhizal interactions with *Pseudomonas* (WCS417, WCS374, WCS358) strains

Roots

In contrast with the results generated in Chapter 1, roots from the autoclaved soil did not grow significantly larger in the autoclaved soil when comparing the two controls (Figure 17). In the autoclaved soil, WCS417 showed a significant increase in root dry weight when compared to the autoclave control (Figure 17A) (t-test, $p < 0.05$). However, the WCS417 treatment in the natural soil showed no significant increase when compared to the natural soil control (Figure 17B), and showed a significant reduction in dry weight when compared to the WCS417 treatment in autoclaved soil (Figure 17). In both autoclaved and natural soil treatments, WCS358 shows a significant increase in root dry weight when compared to its corresponding control (Figure 17).

WCS374 also appears to increase root dry weight in both autoclaved and natural soil, although this is not statistically significant (t-test, $p < 0.05$).

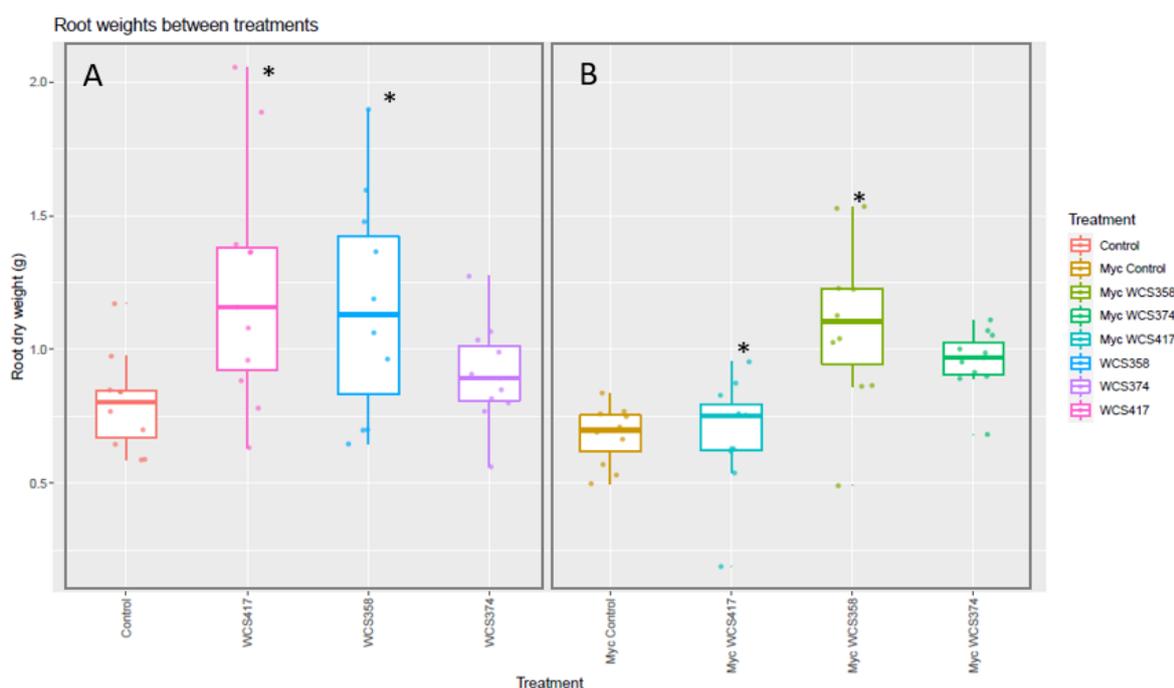


Figure 17: Dry root weights between treatments. A) In autoclaved soil; B) In natural soil. Treatments marked with (*) show significant value (t-test, $p < 0.05$).

Shoots

No statistically significant results were seen for shoot dry weight (Figure 18) between any of the treatments, and there is no observable difference in shoot dry weight between autoclaved and natural soil. However, all the bacterial treatments increase dry shoot weight slightly in the autoclaved soil when compared to the control, which is consistent with the results generated

from Chapter 1. WCS417 slightly decreases shoot dry weight, and WCS358 and WCS374 increase the shoot dry weight in the natural soil.

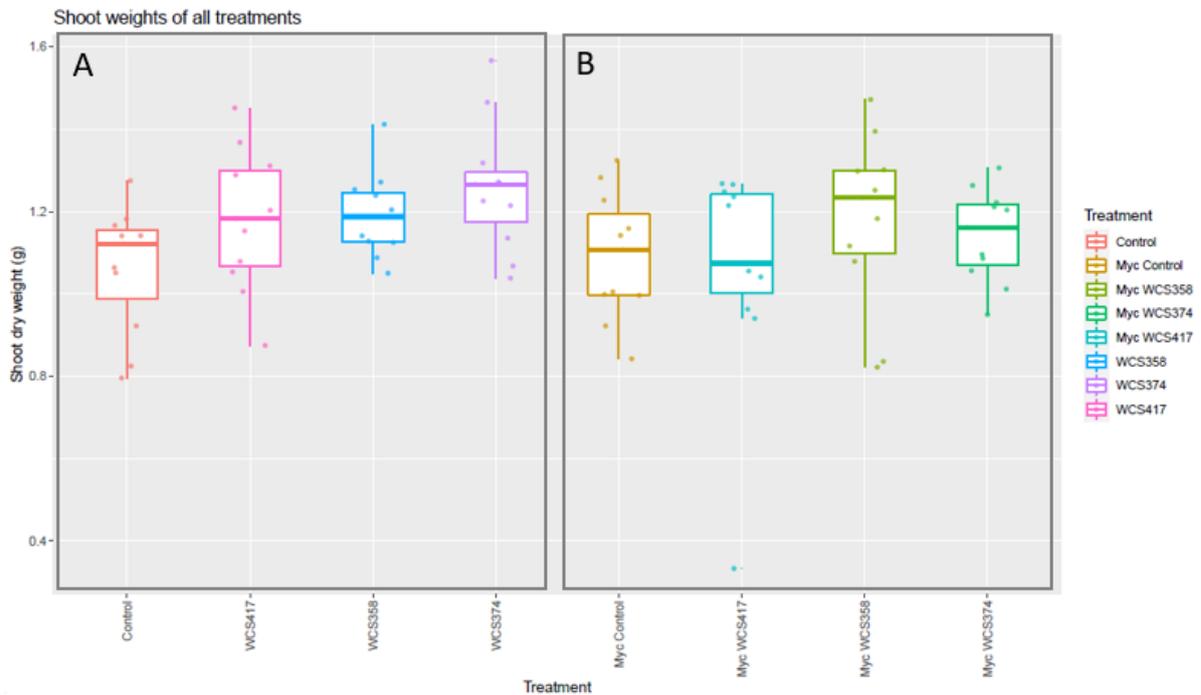


Figure 18: Shoot weights between treatments. A) In autoclaved soil; B) In natural soil. No statistically significant results were found (t -test, $p < 0.05$)

3.4.2 Effect of bacterial treatment upon mycorrhization

Due to an error with the preparation of the roots during staining as described in section 1.3.6, quantitative assessment of the colonisation of *P. vulgaris* roots could not be performed, and the effects of the three *Pseudomonas* strains upon AMF colonisation in *P. vulgaris* could not be analysed. Roots were left too compacted, and thus the staining solutions could not penetrate through the core of the root ‘ball’ effectively. Thus, this analysis could not be performed.

3.5 Discussion

The first of these experiments produced unreliable results, due to quite severe leaf browning (Figure 19). The cause of this was unclear. In general, plants most often encounter leaf browning from under- or over-watering, and *Prunella vulgaris* prefers to have moist soil. As the experiment progressed and *Prunella vulgaris* grew larger, more water was taken up by the plant and soil became dryer over time. (Chen et al., 2016) observed that water stress (drought) decreased the photosynthetic pigment content in *Prunella vulgaris*, thus inhibiting photosynthetic efficiency and inducing photodamage in photosystem 2 (PS2). They also observed that photosynthesis can be improved by the addition of NPK fertiliser even under

water deficit. *Prunella vulgaris* is also prone sensitive to photo-oxidative damage (Chen et al., 2016).

Therefore, in order to minimise leaf browning in the second of these experiments, water addition was increased appropriately as the experiment progressed, and water moisture tested by hand. After 30 days, plants were supplemented with 20mL Hoogland solution once a week to provide *Prunella vulgaris* with appropriate NPK nutrients. Following the harvest of the second experiment, little to no leaf browning was seen in the plants.



Figure 19: Example of leaf browning as seen in the first mycorrhizal interactions with *Pseudomonas* experiment.

In the experiment performed by Bich Nguyen Thi (2021), root biomass in the negative control (autoclaved soil) was measured in a biased manner and was therefore omitted from the analysis. Hence, autoclaved samples cannot be compared between experiments.

The results reported in this thesis indicate that WCS358 increases *P. vulgaris* biomass in both autoclaved and natural soil: significantly for root weight (Figure 17; t-test, $p < 0.05$), but not significantly for shoot weight (Figure 18; t-test, $p \leq 0.05$). The results reported by Bich Nguyen Thi (2021) show the opposite result (Figure 20): in her results, WCS358 decreases shoot dry weight significantly (Figure 20A) (when compared to the positive control; SNK test $P \leq 0.05$), and a decrease is also seen with root dry weight (Figure 20B), albeit non-significant.

This report shows that in autoclaved soil, WCS417 significantly increases root dry weight when compared to the control, but not in the natural soil (Figure 17), although a slight increase in weight is observed. The experiment performed by N. T. Bich (2021) shows a similar result in natural soil. Here, I propose that WCS417 promotes growth more in autoclaved soil due to the lack of microorganisms. WCS417 can more easily establish itself in the autoclaved soil, as the presence of other microorganisms in the natural soil may provide a barrier for colonisation, through competition or predation, from protists for example (Gao et al., 2019). WCS417 presence in the hyphosphere can be confirmed in future experiments through PCR or sequencing of the soil collected during harvest. Both the results reported in this report and by Bich Nguyen Thi (2021), showed no significant effect of WCS417 upon shoot weight, although a similar trend is observed as with the root weights.

No significant results are reported for shoot or root weight in WCS374 treated soil in both this report and the report from N. T. Bich (2021), although in general WCS374 treated soil increases biomass in both experiments. What is promising with these results, is that in both experiments, WCS374 increases shoot weight in both autoclaved and natural soil. Although not significant in this thesis, plant shoot weight is still increased, and the results generated by Bich Nguyen Thi (2021) show significance. Thus, it appears that WCS374 may play an important role in promoting above ground biomass in *Prunella vulgaris*.

Bich Nguyen Thi (2021) did find more significant differences between treatments in fresh root weight, however I do not feel that root fresh weight provides a precise measurement and thus these results will be omitted. Roots are first washed in water before fresh weight can be taken to remove all soil. Any slight differences in root architecture or structure can alter the volume of water absorbed in the root system. Human error may also affect the results of the fresh weight: roots are first patted dry with tissue paper to remove excess moisture and thus the level of water removed may differ between samples; additionally roots left for different lengths of time will dry out to varying degrees. It is for this reason that shoot and root fresh weights were chosen not to be reported here, and why the fresh weight results from Bich Nguyen Thi (2021) will also not be used here. Dry weight provides a much more accurate representation of the true biomass of *P. vulgaris*.

To explain the differences between the results generated by Bich Nguyen Thi (2021) and in this thesis, I have formed a hypothesis: the major difference between the two experiments are the soils used. The soil used in this thesis was a mixture of potting soil and river sand (Section 3.3.3). The soil used by Bich Nguyen Thi (2021) was soil collected from a conventional farm in Zurich, Switzerland. Here, many biotic and abiotic factors within the soil may influence the outcome of the results. For example, a different microbial community may prevent the establishment of then WCS strains in the soil, or vice-versa, the establishment of the WCS strains in the soil may outcompete harmful or beneficial microorganisms in the soil, that may be present in only one of the soils. Abiotic factors such as pH, nutrient status and moisture levels may also influence the ability of WCS strains as a beneficial bacteria. To understand the true effect of WCS358 upon plant growth, the experiment should be repeated using both soils, as well as repeating under different conditions, such as different soils, different water and humidity levels, different temperature etc. This will also help us to better understand the conditions in which the WCS strains survives better in the soil. In order to confirm the establishment of such bacterial communities, the presence of each of the WCS strains should be checked in the hyphosphere using PCR or sequencing methods. This is also helpful to ensure soils have not been cross-contaminated.

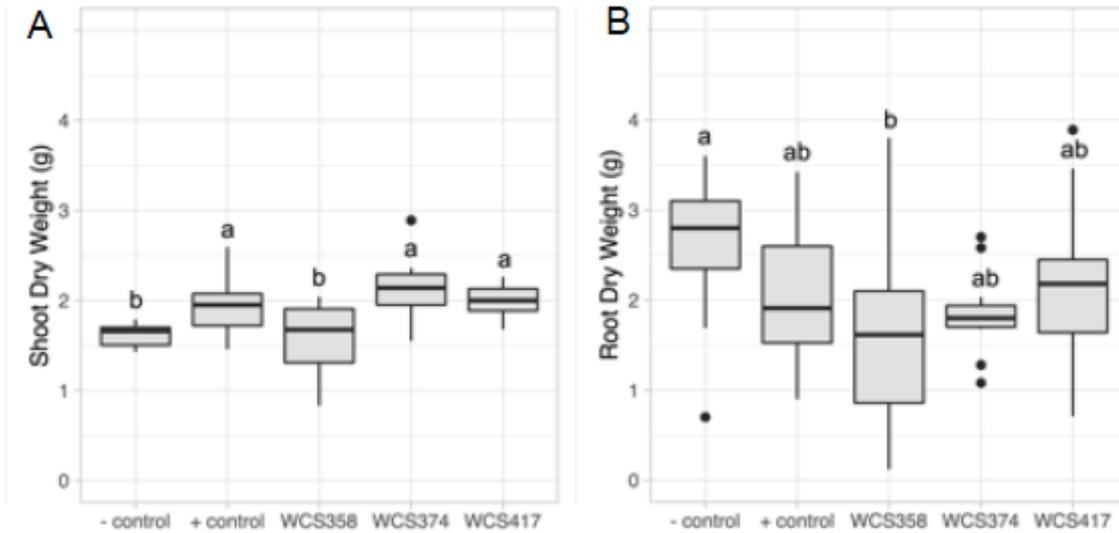


Figure 20: Effects of the different *Pseudomonas* strains upon shoot (A) and root (B) dry weight in greenhouse conditions. The negative control here is autoclaved soil, the rest of the treatments are performed with natural soil, without (+ control) and with the three bacterial strains (WCS358, WCS374, WCS417). The data was collected after 2 months of transplanting and bacterial inoculation. Due to a biased harvesting procedure with the dry root negative control, this is emitted from analysis. Band inside the box represents the median. Bottom and top of the box are the first and third quartiles. Different letters above box plot indicate a significant difference ($P \leq 0.05$) according to SNK test. (Nguyen, 2021)

Chapter 4: Effects of WCS strains upon immunity against pathogens in *Prunella vulgaris*

4.1 Abstract

This chapter aims to elucidate the beneficial roles three *Pseudomonas* strains (*P. simiae* WCS417, *P. capeferrum* WCS358, and *P. defensor* WCS374), may have upon protecting the plant *Prunella vulgaris* against the pathogen *Botrytis cinerea*. *P. vulgaris* seedlings were planted in soil inoculated with one of the three *Pseudomonas* strains. After three weeks of growth, *P. vulgaris* leaves were inoculated with spores of *B. cinerea* isolate B0510. Infection was measured in two ways: by lesion size, and by via an in-house scoring system. The results generated indicate that the strains WCS417 and WCS374 may play a role in inducing the induced systemic resistance (ISR) of *P. vulgaris*, although further work must be done to confirm this.

4.2 Background

Over 300 publications have demonstrated the beneficial roles of three *Pseudomonas* species (*P. simiae* WCS417, *P. capeferrum* WCS358, and *P. defensor* WCS374), for example in promoting plant growth and in providing protection from pathogens (Berendsen et al., 2015). This chapter aims to see how these three roles may protect *Prunella vulgaris* against the necrotrophic fungus *Botrytis cinerea*, the causative agent of grey mould disease. *B. cinerea* can infect over 200 plant species, and causes agricultural losses of \$10-100 billion dollars per year (Boddy, 2016). *B. cinerea* is an air-borne pathogen and forms lesions on the leaves of its host. Since the *Pseudomonas* strains exist in the soil, any protective effects will occur distally, and it can be assumed that this is done via activating the induced systemic response (ISR) of *P. vulgaris*.

4.3 Materials and Methods

The preparation of the inoculum and subsequent inoculation of *P. vulgaris* is based on the method detailed by (Courbier et al., 2020) and adjusted based on pre-trial experiments and advice from Hans van Pelt (PMI, UU).

4.3.1 Seed preparation and germination, soil sterilisation and bacterial inoculum preparation

Seed preparation and germination was prepared as described in section 1.3.1. River sand and potting soil were mixed in a ratio of 4:1 (w/w), and for the autoclaved samples, the mixed soil was autoclaved twice for 50 minutes at 121°C. 200g of this mixture was added to pots, and inoculated with the three WCS strains as in section 3.3.1. Pots were transferred to the greenhouse under long day settings (21°C, 16h light, 8h dark) for 3 weeks. After three weeks, leaves were inoculated with *Botrytis cinerea*.

4.3.2 Preparation of *Botrytis cinerea* inoculum

B. cinerea isolate B0510 (stored in 25% glycerol at -80°C and isolated from *P. vulgaris* leaves) was inoculated on half strength PDA plates (potato dextrose agar) supplemented with 100ppm penicillin and 200ppm streptomycin for two weeks at room temperature and pressure until spores formed. Conidia was harvested by scraping plates in PDB (potato dextrose broth) and filtered through glass wool. Conidia density was measured in a hemocytometer with a light microscope and diluted to a concentration of 10⁴ spores/mL.

4.3.3 Inoculation of *Prunella vulgaris* with *Botrytis cinerea*

To the four largest leaves on each *Prunella vulgaris* plant, 5µL of *B. cinerea* inoculum was added (approx. 50 spores). Plants were kept under humid conditions by placing in a tray and sealing the lid with parafilm. After three days inoculation, plants were harvested, and infection measured.

4.4 Results

The summary of results is shown in Table 4. A total of 236 leaves were measured for infection, and of these, 85 leaves showed infection: a mean infection rate of 36%. Plants from the WCS358 treated soil showed the highest level of infected leaves at 46.4%, followed by the control treatment (45% infection) and then the WCS417 and WCS374 treated soils (both 26.7% infection). Please note that one of the plants from the WCS358 treated soil days shortly after planting, and thus is not included in the analysis.

Table 4: Summary of results of *Botrytis cinerea* infection in *Prunella vulgaris* leaves. The average scores are the mean score from the scoring system described in Section 4.4.2...? The mean lesion area is also calculated.

Treatment	Number of plants	Number of leaves	Infected leaves (%)	Mean score (all leaves)	Mean score (infected leaves only)	Mean lesion area (all leaves)	Mean lesion area (infected leaves only)
CONTROL	15	60	45.0	0.7	1.6	6.8	12.9
WCS417	15	60	26.7	0.5	1.9	4.1	12.3
WCS374	15	60	26.7	0.4	1.6	4.0	11.7
WCS358	14	56	46.4	0.8	1.6	5.6	10.2

4.4.1 Lesion size

Figure 21 shows the lesion sizes of *B. cinerea* infected between the different microbial treatments. 64% of leaves showed zero signs of infection. Figure 21A shows the lesion sizes of all leaves, including those with no infection (and thus measured as zero). Here, the median lesion size for all microbial treatments is zero, since most leaves showed zero signs of infection. However, it appears that WCS417 and WCS374 may have a better protective effect against *B. cinerea* infection in *Prunella vulgaris*, since the upper quartile is shifted much lower than compared to the control and WCS358 treatment (Figure 21A). Additionally, the mean lesion

area is also lower in the WCS417, WCS374 and WCS358 treated soil (Table 4) when compared to the control treatment. However, no significant results were generated here, using the Tukey multiple comparison of means test, significance: $p \leq 0.05$.

Next, the lesion sizes were compared only in those leaves that showed signs of infection, and results shown in Figure 21B. Here, all soil treatments show a lower median score (Figure 21B) and mean score (Table 4) than the control, although no significant values were generated (TukeyHSD, $p \leq 0.05$). This may indicate that when *B. cinerea* does successfully infect *P. vulgaris* leaves, the WCS strains may slow the spread of lesions.

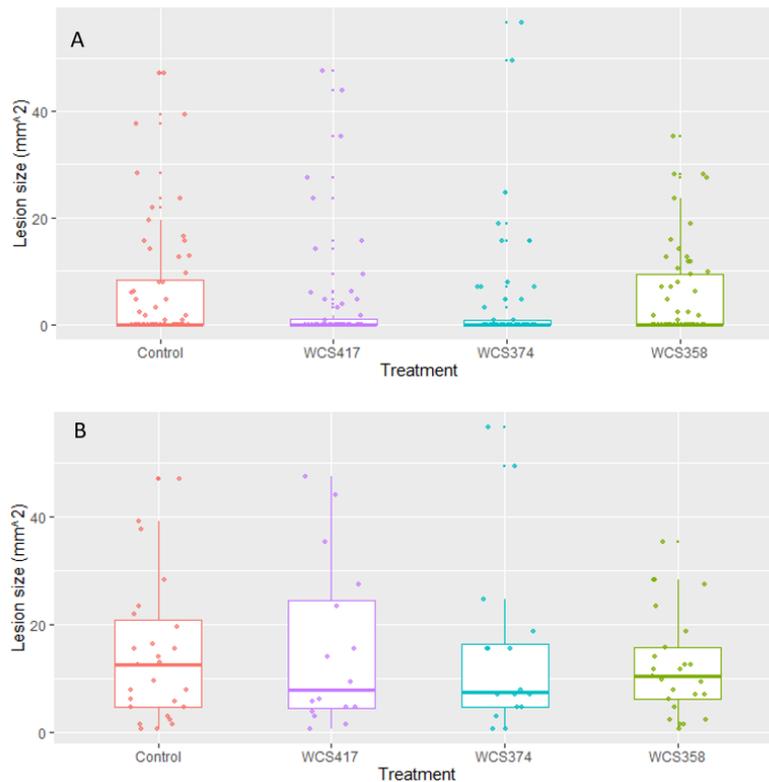


Figure 21: Lesion sizes of *Botrytis cinerea* infection in *Prunella vulgaris*. A) account for all leaves measured, including scores of zero. B) accounts for only leaves containing infection, with zero scores removed.

4.4.2 Scoring system

As well as measuring lesion size, a scoring system was developed to mark the severity of disease (Figure 22). Here, a score of zero was given to leaves containing no visible signs of infection, a score of 1 was given to leaves containing small signs of infection (<10 spots of *B. cinerea* infection), a score of 2 was given to leaves containing a medium level of infection (<50 spots of *B. cinerea* infection) and a score of 3 was given to leaves showing the most severe level of infection (indistinct spots/spreading lesion). A summary of the raw counts is summarised in Table 5, and visualised in Figure 23.

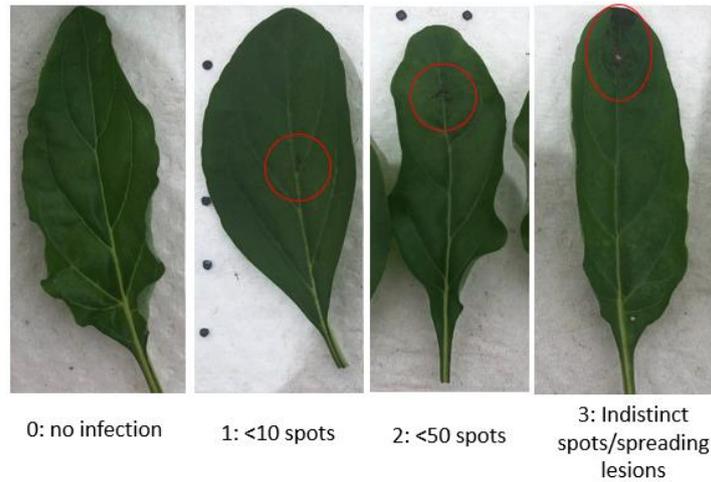


Figure 22: Scoring system for *Botrytis cinerea* infection in *P. vulgaris*. Scoring is observation dependent and is based on the number of spots visible. A score of 0 represents no infection, a score of 1 represents a lesion containing less than 10 spots of *B. cinerea*, a score of 2 represents a lesion containing less than 50 spots of *B. cinerea*, a score of 3 represents an infection with indistinct spots and spreading lesions. The red circles indicate the area of infection.

Table 5: Summary of infection levels in *Prunella vulgaris* leaves by *Botrytis cinerea*. Scoring system is described in Figure 22. Numbers indicate the number of counts of each score per treatment, with percentage in brackets ().

Bacterial Treatment	Counts of score 0 in leaves	Counts of score 1 in leaves	Counts of score 2 in leaves	Counts of score 3 in leaves
Control	33 (55%)	12 (20%)	13 (22%)	2 (3%)
WCS417	44 (73%)	6 (10%)	5 (8%)	5 (8%)
WCS374	44 (73%)	7 (12%)	9 (15%)	0 (0%)
WCS358	34 (61%)	12 (21%)	12 (21%)	2 (4%)

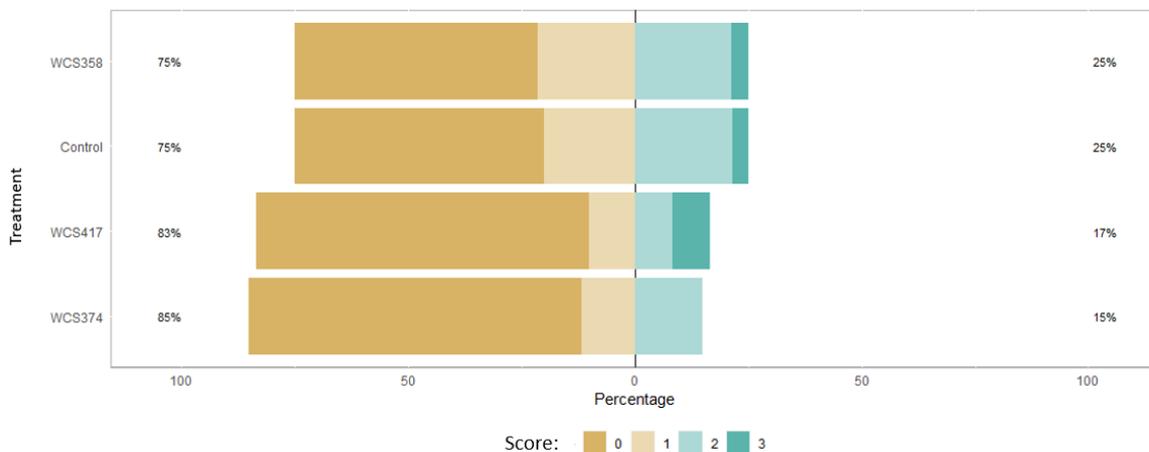


Figure 23: Bar graph showing the severity of *Botrytis cinerea* infection in *Prunella vulgaris* using the scoring system detailed in Figure 22.

A chi-squared test was performed on the data in Table 5, showing the summary of scores for each treatment. When compared to the control data, the WCS417, WCS374 and WCS358 treatments generated p-values of 0.038, 0.132, and 0.997, respectively (significance value $p < 0.05$).

A Tukey multiple comparison of means test was also performed using the raw data counts and when compared to the control data the WCS417, WCS374 and WCS358 treatments generated p-values of 0.54, 0.21, and 1.00 respectively (significant value $p < 0.05$).

A Friedman test was also performed. As WCS358 had only 56 measurements rather than 60 as the three other treatments, this was excluded from the analysis. From the other statistical analyses and Figure 23, it is assumed that the WCS358 treatment will not be significant. The Friedman test generated p-values of 0.273 and 0.041 for the WCS417 and WCS374 treatments respectively, when compared to the control (significance value $p < 0.05$). The WCS374 treatment therefore generates a significant result, and thus may play a role in protecting plants from pathogen invasion.

4.5 Discussion

4.5.1 Pre-trial experiment

A pre-trial experiment was performed to determine i) that *B. cinerea* can successfully infect *P. vulgaris*; ii) the best concentration of *B. cinerea* to add to the leaves. Here, 5 μ L of 10^4 , 10^5 and 10^6 spores/mL were used to inoculate several *P. vulgaris* plants as described in section 4.3.3. In most cases, the 10^4 dilution successfully infected plants. Thus, this concentration of *B. cinerea* was chosen to infect plants within the main experiment, based on the idea that if the bacterial treatments had any protective effect, the effect would be better visualised at lower concentrations (i.e. if the concentration of *B. cinerea* is too high, they may dominate the leaf and may leave no space for the bacterial treatments to work).

4.5.2 Development of the scoring system

Lesion sizes are a good indication of the spread of *Botrytis cinerea*, however during observation it appeared that lesion size does not necessarily indicate the severity of the disease. For example, there were some highly concentrated lesions that had a small area, and some lesions that were spread out where infection did not appear as severe. Thus, the scoring system was generated to indicate severity of the disease (Figure 22).

4.5.3 Do the *Pseudomonas* strains protect *P. vulgaris* against *B. cinerea* infection?

The only significant result for this chapter was generated when performing the chi-squared test, when comparing the WCS417 treatment with the control treatment. As WCS417 shows much lower signs of infection than the control, it may appear that WCS417 significantly protects plant against pathogen infection. However, when looking at the raw data (Table 5), it would appear that WCS374 protects *P. vulgaris* to a higher extent than all other treatments, although this generated no significant result. The chi-squared test calculates differences between the expected (control) results and observed (bacterial treatment) results. Both WCS374 and

WCS417 treatments score the same number of zero counts, but the WCS417 treatment also receives higher infection levels of score 3 when compared to all other treatments, thus the observed results differ more than the expected results. The chi-squared test successfully calculated the difference in scoring between the treatments, however this does not necessarily indicate successful protection from pathogens.

The chi-squared test can therefore be taken as an indication. As no other results were significant, we cannot assume that the bacterial soil treatments successfully protect *Prunella vulgaris* against *B. cinerea* infection. However, the WCS417 and WCS374 treatments showed almost half the level of leaf infection, when compared to the control and WCS358 treatments (Table 4). It also appears that in cases where infection does take hold, the bacterial treatments may also play a role in preventing the spread of disease when looking at lesion area (Table 4 and Figure 21). Additionally, the WCS374 treatment was the only treatment that received no counts of score 3.

From these results, the WCS417 and WCS374 treatments may play a role in protecting *P. vulgaris* from *B. cinerea* infection. Three statistical tests were performed, which generated two significant values. The chi-squared test generated a significant value for the WCS417, which as already discussed may not be reliable. The other two statistical tests generated no significant value for the WCS417 treatment. The Freidman test generated a significant value for the WCS374 treated soil, but the other statistical tests did not. Thus, further analyses must be performed in order to draw a conclusion. It appears likely that WCS358 plays no role in *P. vulgaris* protection against *B. cinerea*.

As the WCS strains are present in the soil, and *B. cinerea* is an air borne pathogen infecting leaves, WCS417 and WCS374 may provide protection against *B. cinerea* by stimulating the induced systemic resistance of *P. vulgaris*.

4.5.4 Further work

It has already been demonstrated that WCS417 can induce the ISR of radish (Hoffland et al., 1995) and *Arabidopsis thaliana* (Pieterse et al., 1996) against *F. oxysporum*. In *A. thaliana*, this is mediated by WCS417 via a salicylic acid (SA) mediated pathway (Pieterse et al., 1996). This SA-mediated pathway is further regulated through the action of jasmonic acid (JA) and ethylene (ET) signalling (Pieterse et al., 2014). It has also been demonstrated that WCS417 induces the expression of the root transcription factor *MYB72* (Zamioudis et al., 2015), a central regulator in the induction of plant growth and immunity (Stringlis et al., 2018). Since the genome of *P. vulgaris* has not yet been sequenced, knockout mutants of these processes cannot be created, and thus this limits the work that can be done to elucidate the potential mechanisms of WCS induced systemic resistance in *P. vulgaris*.

Thus, a more practical approach can be used. To confirm whether WCS417 and WCS374 does indeed stimulate the systemic immunity of *P. vulgaris*, this experiment must be repeated. Additional controls and concentrations of *B. cinerea* will be used:

- 5µL of PDB containing no spores of *B. cinerea* will be added to additional *P. vulgaris* plants as an additional control.

- 5µL droplets of 10^4 , 10^5 and 10^6 spores/mL should also be added. From the pre-trial experiment, it appeared that 10^4 spores/mL effectively infected *Prunella vulgaris*. However in the final experiment, the infection rate was very low (Table 4).

As well as repeating the experiment with additional controls and concentrations, different pathogens can be used. Air-borne pathogens such as *Pseudomonas syringae* and powdery mildews and soil-borne pathogens such as *Fusarium oxysporum*, *Trichoderma* spp. and *Piriformospora indica* can also be used to investigate the protective effects of the three WCS strains in *Prunella vulgaris*. By using both air-borne and soil borne pathogens, a better understanding of the mechanisms of protection by the three WCS strains can be understood, and we can determine if these bacterial strains induce a systemic or local resistance in *P. vulgaris*. With the soil-borne pathogens, a split root experiment can also be designed as described by (Saiz-Fernández et al., 2021) (Figure 24). Disease severity can then be measured by one of the methods described by (Bock et al., 2020).

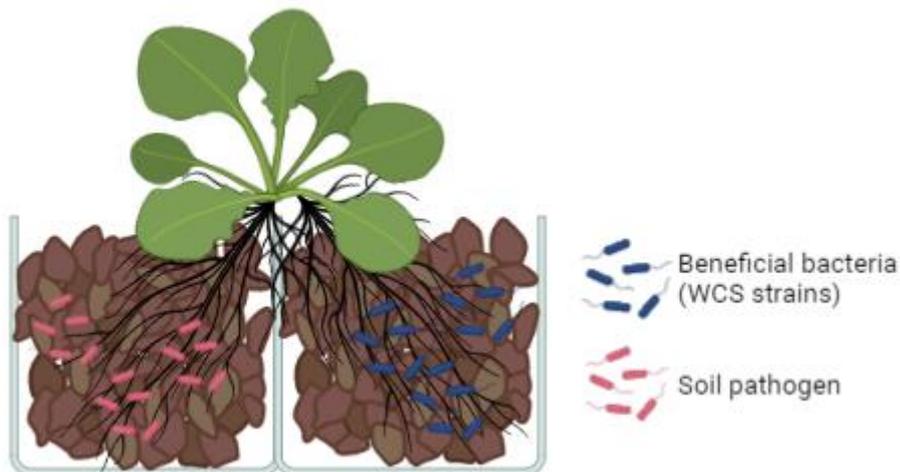


Figure 24: Split root design. Roots will be split into two portions and grown in two separate pots. In one pot, one or more of the *Pseudomonas* strains (WCS417, WCS374, WCS358) will be added. Into the other pot, a soil borne pathogen will be added.

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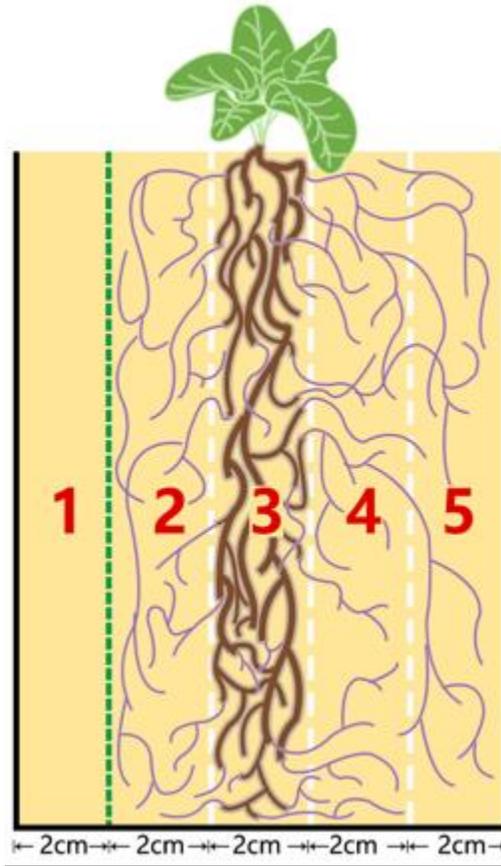
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Supplementary Data



Supplementary Figure 1: Design of 5 compartment microcosm from the experiments designed by Changfeng Zhang and Bich Nguyen Thi (Nguyen, 2021). COM 1,2,4,5 was filled with autoclaved 8% soil, mixed with Oil-Dri and sand mixture. Compartment 3 was filled with 30% field soil, mixed with Oil Dri and sand mixture. The green dash line separating COM1 and COM2 representing 1 μ m mesh. The white dash line separating the rest of compartments representing 30 μ m mesh.

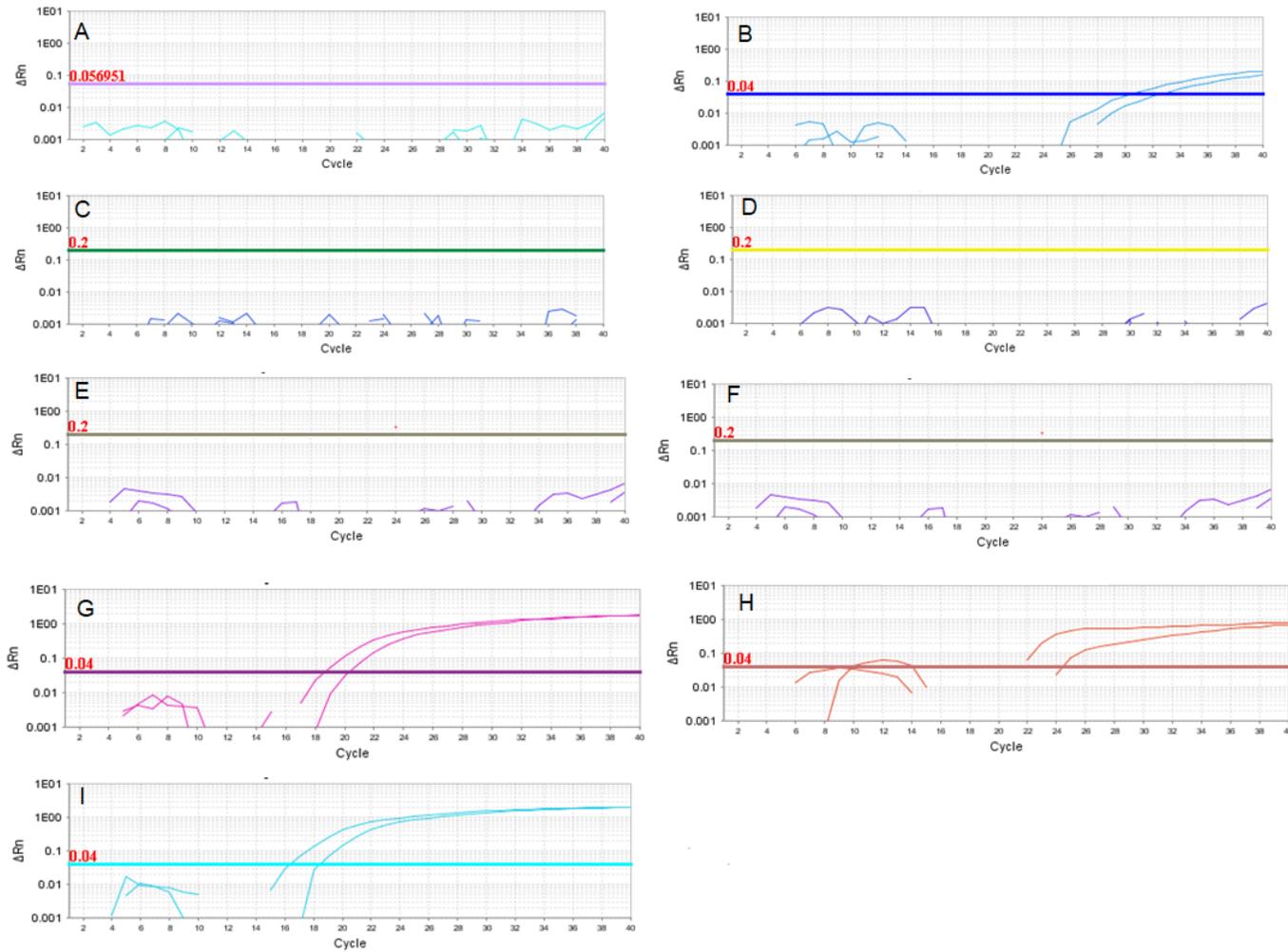
Accession Number	Primer pair name	Primer sequence	Amplicon size	Forward/reverse (F/R)
KM053280 (Trócsányi et al., 2020)	KMO5	GAAACTGCACATCCCGTTCG	85	F
		CGGCGTTGCGAAGAATATCG		R
	KMO8	TGTGGGTGATAAGCTGGGTG	130	F
		TCGTTGTCGGGATTGGGAAG		R
	KMO9	CGGAGTAGGGTTAGGCGTTG	83	F
		GGTGGCACCATGTGTTGATG		R
KJ010816 (Kim et al., 2014)	KJO1	GTACCTTCCCTTCGGTGTGG	131	F
		CAATCTTCTTCTGCCCCGGT		R
	KJO3	ACCCTGCCCAATGGAAGAAG	75	F
		GTTGGCCTCGACTTTAGCCT		R
	KJO7	CAGGTCGGCGATGATCTGAA	138	F
		CGTGTGCAGGACCTCCTTAG		R

Supplementary Table 1: Designed *Prunella vulgaris* primer pairs. Primers were designed using the Primer-BLAST function from NCBI. Six primer pairs were designed using the two accession numbers

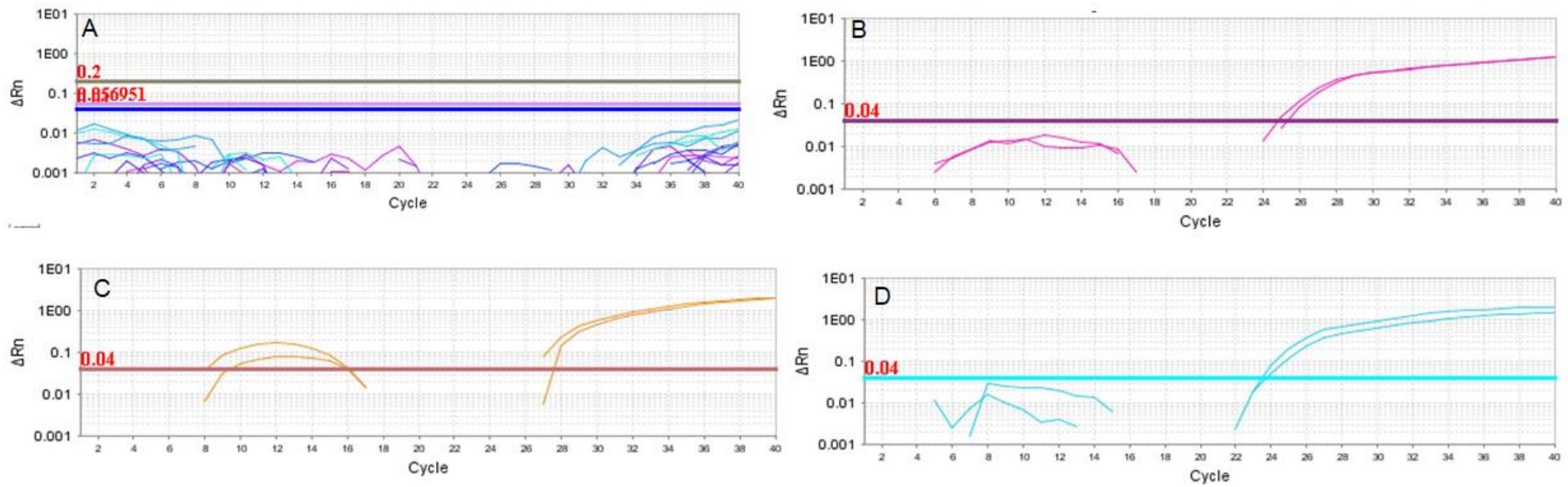
Sample / Template dilution	AMF primers on sterile root		AMF primers on root colonised by AMF		KMO-8 primers on sterile root		KMO-5 primers on sterile root	
	Standard deviation between replicates	Δ Ct						
No dilution	N/A	N/A	N/A	N/A	1.33	3.06	0.04	2.90
1/10	N/A	2.08	0.36	2.03	0.27	2.50	0.20	3.28
1/100	N/A	1.88	0.05	4.47	0.02	3.38	0.05	2.60
1/1000	N/A	N/A	0.40	0.86	0.31	4.73	0.19	5.07
1/10.000	N/A	N/A	N/A	3.27	0.46	0.45	0.87	3.15
1/100.000	N/A	1.55	1.60	5.52	N/A	N/A	4.58	3.15
1/1000000	3.43	1.11	N/A	-3.06	N/A	N/A	N/A	-1.06

Sample / Template dilution	KMO-9 primers on sterile root		KJO-1 primers on sterile root		KJO-3 primers on sterile root		KJO-7 primers on sterile root	
	Standard deviation between replicates	Δ Ct						
No dilution	0.99	1.92	0.06	2.60	0.17	2.58	0.56	2.81
1/10	N/A	0.87	0.12	3.43	0.16	2.59	0.59	2.84
1/100	3.78	-4.23	0.06	1.97	0.07	3.13	0.20	3.65
1/1000	0.21	-3.36	0.18	4.25	0.24	2.69	0.40	0.63
1/10.000	0.26	-3.23	0.72	2.20	0.27	3.25	0.13	1.19
1/100.000	0.07	-2.39	N/A	N/A	N/A	4.64	0.79	1.62
1/1000000	0.02	-3.37	N/A	N/A	N/A	N/A	3.03	-2.47

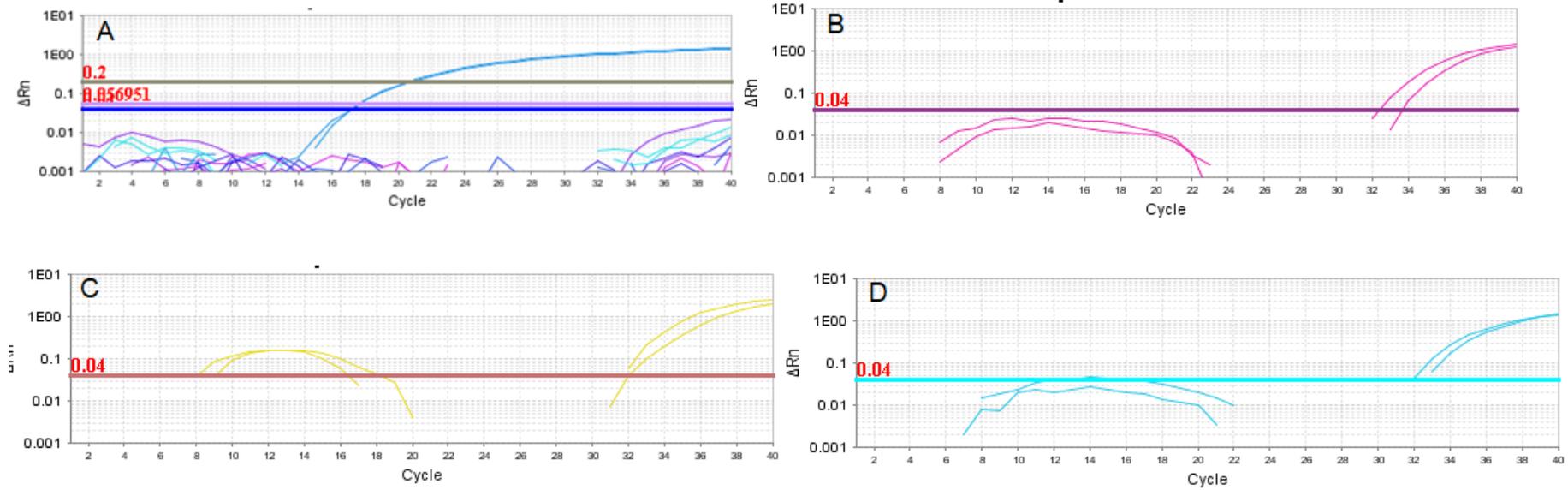
Supplementary Table 2: Standard deviation and Δ Ct values from primer efficiency test. N/A values indicate that for at least one of the replicates within a duplicate was undetected by the qPCR system.



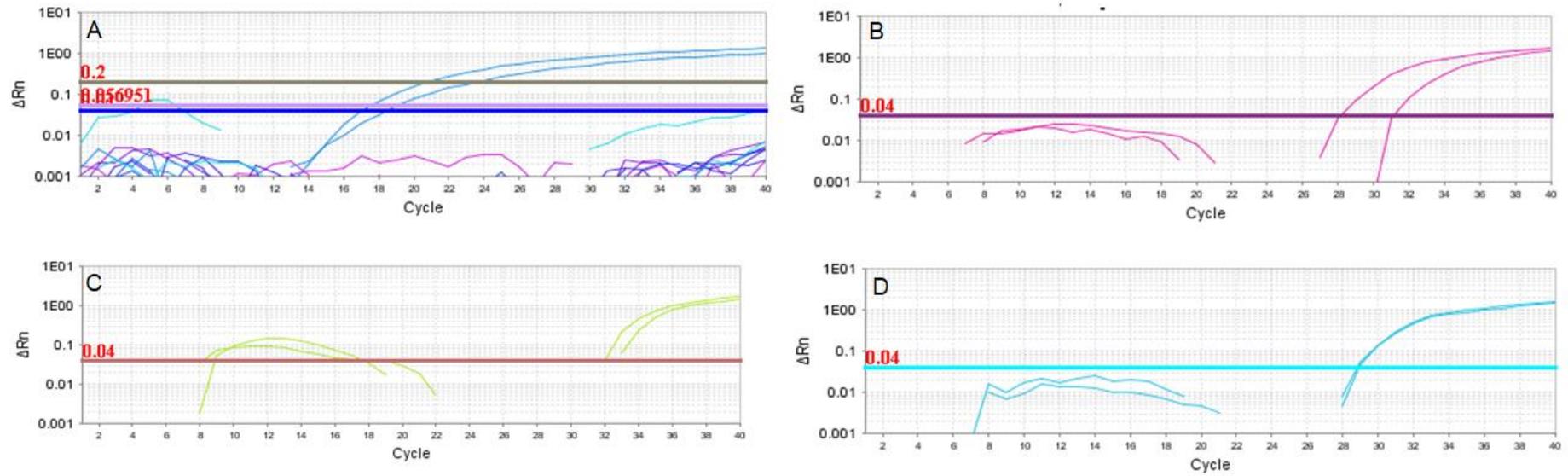
Supplementary Figure 2: Amplification plot of AMF primers with different samples to test for primer specificity. A-F: AMF primers tested on different root samples colonised with AMF, with soil treated with A) no bacteria; B) WCS417; C) WCS358; D) WCS374; E) ZB199; F) Devosia ZB006. G-I: AMF primers tested on bacteria only. G) WCS417; H) WCS358; I) WCS374



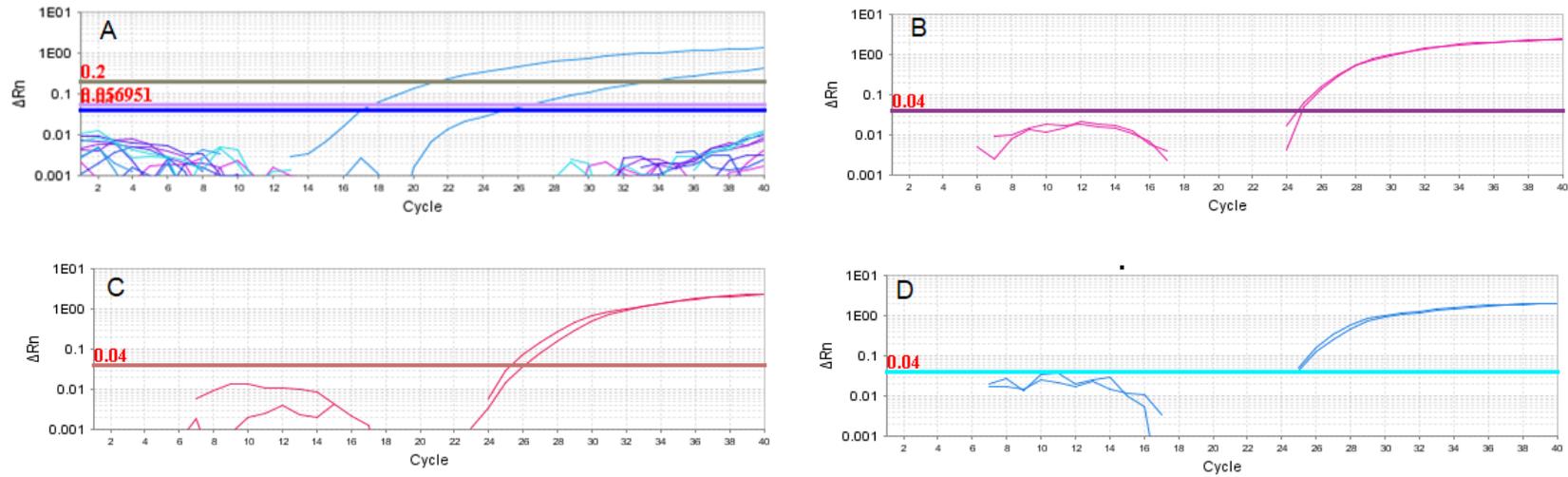
Supplementary Figure 3: Amplification plot of KMO-8 primer with different samples to test for primer specificity. A: KMO-8 primers tested on different root samples colonised with AMF, with different soil treatments. B-D: KMO-8 primers tested on bacteria only. B) WCS417; C) WCS358; D) WCS374



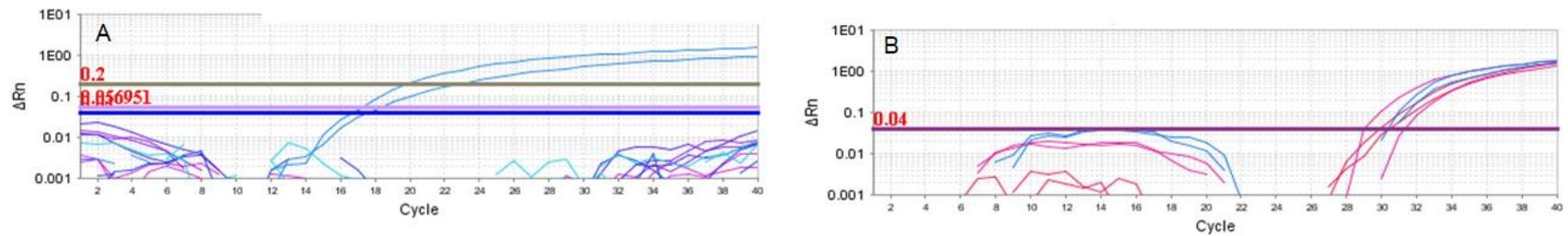
Supplementary Figure 4: Amplification plot of KMO-5 primer with different samples to test for primer specificity. A: KMO-5 primers tested on different root samples colonised with AMF, with different soil treatments. B-D: KMO-5 primers tested on bacteria only. B) WCS417; C) WCS358; D) WCS374



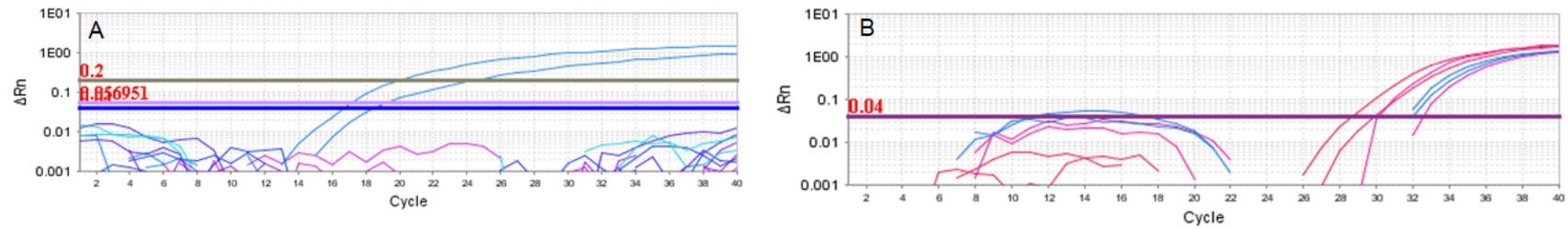
Supplementary Figure 5: Amplification plot of KMO-9 primer with different samples to test for primer specificity. A: KMO-9 primers tested on different root samples colonised with AMF, with different soil treatments. B-D: KMO-9 primers tested on bacteria only. B) WCS417; C) WCS358; D) WCS374



Supplementary Figure 6: Amplification plot of KJO-1 primer with different samples to test for primer specificity. A: KJO-1 primers tested on different root samples colonised with AMF, with different soil treatments. B-D: KJO-1 primers tested on bacteria only. B) WCS417; C) WCS358; D) WCS374



Supplementary Figure 7: Amplification plot of KJO-3 primer with different samples to test for primer specificity. A: KJO-3 primers tested on different root samples colonised with AMF, with different soil treatments. B: KJO-3 primers tested on bacteria only (WCS417; WCS358; WCS374)



Supplementary Figure 8: Amplification plot of KJO-7 primer with different samples to test for primer specificity. A: KJO-7 primers tested on different root samples colonised with AMF, with different soil treatments. B: KJO-7 primers tested on bacteria only (WCS417; WCS358; WCS374)

Strain	Used in experiment harvest 09/04/21	Used in experiment harvest 01/06/21	Duration of growth on KBA
WCS417	✓	✓	24-48 hours
WCS374	✓	✓	24-48 hours
WCS358	✓	✓	24-48 hours
ZB119	✓	✗	8-10 days
ZB006	✓	✗	8-10 days

Supplementary Table 3: Bacterial strains used from the experiments in Chapter 3, indicates the approximate duration of growth on KBA agar and which experiment the strain is used in.